INDUCTION OF MACROPHAGE PLASMINOGEN ACTIVATOR BY ENDOTOXIN STIMULATION AND PHAGOCYTOSIS

Evidence for A Two-Stage Process*

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The macrophage plays an important role in host defense against infection, in the course of which it may become activated and gain an enhanced capacity to kill microorganisms and possibly tumor cells (1). Very little is known about the cellular properties which are associated with macrophage activation and the absence of a suitable biochemical marker has been a particular handicap in the study of this process.

Mouse macrophages obtained after intraperitoneal stimulation with thioglycollate medium accumulate and secrete high levels of plasminogen activator in culture, whereas macrophages obtained from unstimulated mice do not (2). The major plasminogen activator is a serine protease of apparent mol wt 48,000 with trypsinlike specificity, which resembles the enzyme released by mouse embryo fibroblasts after transformation by mouse sarcoma virus. In view of the striking difference in plasminogen activator production by thioglycollate-stimulated and unstimulated macrophages and since the generation of plasmin could itself play a direct part in the function of the activated macrophage, further study of the regulation of plasminogen activator levels in the macrophage was undertaken.

In this paper we show that macrophages harvested from mice after intraperitoneal injection of endotoxin contain and release little plasminogen activator, but these primed cells can be triggered to secrete high levels of the enzyme by subsequent phagocytosis of latex or bacteria. Secretion of plasminogen activator stops soon after phagocytosis of a digestible particle, such as *Micrococcus lysodeikticus*, but it is prolonged after uptake of a nondigestible particle like latex.

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Materials and Methods

Reagents were obtained and prepared as follows: Dulbecco's medium (H-21) and fetal calf serum (FCS)[1] (Grand Island Biological Co., Grand Island, N. Y.). Acid-treated FCS (AT-FCS) was prepared by adjusting the pH to 3.2 with 1 N HCl; after 2 h at room temperature the pH was adjusted to 7.4 with 1 N NaOH, and the serum was filtered; Brewer's thioglycollate medium, prepared according to the manufacturer's instructions (Difco Laboratories, Detroit, Mich.); lactalbumin hydrolysate (Nutritional Biochemical Corp., Cleveland, Ohio); endotoxin and lipid A were generously provided by Dr. O. Westphal, Max Planck Institute for Immunobiology, Freiburg, Germany. The endotoxin, batch AE 3390-S_3, was purified from *Salmonella abortus equi* and consisted of an ultracentrifuged "high molecular weight" preparation. The endotoxin was suspended in sterile saline, 1 mg/ml, briefly sonicated, and stored at −20°C until use. Lipid A: BSA complex, 1:1, was dissolved in sterile saline and stored at −20°C. BCG (Aronson), lot 581, a water-washed, lyophilized preparation, was suspended in sterile saline, 1 mg/ml, sonicated, and stored at −20°C. Listeria antigen was obtained from Dr. D. McGregor. Trudeau Institute, Inc., Saranac Lake, N. Y.; mineral oil (Nujol, Plough, Inc., Memphis, Tenn.). Polystyrene latex particles, 1.01 μ (Dow Diagnostics, Indianapolis, Ind.); *M. lysodeikticus*, spray dried, and soybean trypsin inhibitor, fraction VI, (Miles Chemical Co., Kankakee, Ill.). All other reagents were purest grade available from standard commercial suppliers.

Cell culture. Female NCSR mice weighing 25–30 g were obtained from the pathogen-free colony maintained at The Rockefeller University. Peritoneal macrophages were harvested from unstimulated control animals or from mice injected intraperitoneally with 0.75 ml thioglycollate medium or 30 μg endotoxin 4 days previously. The cell yield and conditions for cultivation of control and thioglycollate-stimulated macrophages have been described previously (2, 3). The endotoxin-treated animals yielded 1.0–2.2 × 10^7 cells of which 40–55% were macrophages, 0–8% were polymorphonuclear leukocytes (PMN), and the remainder lymphocytes. Endotoxin-stimulated macrophages were cultivated at a density of 5 × 10^5 cells/cm^2.

Human blood monocytes and PMN were purified as described (3), using EDTA, 1.5 mM final concentration, as anticoagulant. Monocyte populations were more than 98% pure and contained 0–2% PMN when centrifuged preparations were examined.

Cells were plated on 35 mm [^{125}I]fibrin-coated dishes or in uncoated plastic tissue culture dishes, as described (2). All cells were cultivated in Dulbecco's medium supplemented with 5–10% (FCS), as specified. Soybean trypsin inhibitor (STI), 60 μg/ml, was added to media to prevent fibrinolysis, and had no effect on cell viability or subsequent plasminogen activator release. Fibrinolytic assays on radioactive fibrin plates were initiated by washing the cells three times with Hanks' balanced salt solution (HBSS) followed by further incubation in Dulbecco's medium containing 5% AT-FCS (2). The use of AT-FCS amplified detection of fibrinolysis since serum inhibitors of plasmin such as α_2-macroglobulin were destroyed by acid treatment. Macrophages cultivated in medium with AT-FCS were particularly well spread and remained healthy as judged by their ability to secrete lysozyme, a major secretory product of these cells (3). Aliquots of medium were withdrawn and assayed for release of radioactivity in a Packard gamma counter. Replicate cultures were used for all experiments and showed less than 15% variation.

Conditioned Media (CM) and Cell Lysates. CM and cell lysates were prepared (2) from macrophages cultivated in uncoated dishes. Cells were incubated for up to 48 h in medium supplemented with lactalbumin hydrolysate (LH), 0.05% wt/vol, without deleterious effect on cell viability or lysozyme secretion. Triton X-100, 0.2% wt/vol, was present in cell lysates, but was not added to CM.

Plasminogen activator in cell lysates and CM was assayed on [^{125}I]fibrin-coated dishes (2). Plasminogen-dependent fibrinolysis was defined as the difference in the amount of radioactivity released in the presence and absence of plasminogen; 1 U of plasminogen activator released 10% of total radioactivity in 4 h. Appropriate controls were included in all assays. The background of the

[1] *Abbreviations used in this paper:* AT-FCS, acid-treated fetal calf serum; BSA, bovine serum albumin; CM, conditioned medium; HBSS, Hank's balanced salt solution; HRP, horseradish peroxidase; LH, lactalbumin hydrolysate; PBS, phosphate-buffered saline; PMN, polymorphonuclear leukocyte; STI, soybean trypsin inhibitor.

assay conducted in the presence or absence of plasminogen was the same, and less than 1% of the total radioactivity on the dishes.

Phagocytosis. Particles were prepared for phagocytosis experiments as follows. Latex particles and formaldehyde-treated sheep erythrocytes (SRBC) were washed seven times and resuspended in Dulbecco's medium. Stock suspensions containing 5×10^9 particles/ml were irradiated with ultraviolet light and kept at 4°C until use. *M. lysodeikticus* was suspended in Dulbecco's medium, 2.5 mg/ml, autoclaved, and washed three times before use. Purified heat-aggregated human γ-globulin was provided by Dr. R. Winchester (The Rockefeller University). The preparation contained soluble aggregates, most of which sedimented in the range of 30–150S. The aggregates were washed by centrifugation at 100,000 *g* for 60 min and resuspended before use. Immune complexes of horseradish peroxidase (HRP)-rabbit anti-HRP were obtained from Dr. R. Steinman (The Rockefeller University). 30 μg HRP were reacted at equivalence with antiserum for 30 min at 37°C, followed by 2 days at 4°C. The complexes were then washed with phosphate-buffered saline (PBS) before use.

Phagocytosis experiments were performed in macrophages that had been cultivated for 24 h. The monolayers were washed twice and incubated in medium containing 5% FCS. Soybean trypsin inhibitor (60 μg/ml) was included in the medium during phagocytosis in all experiments on radioactive plates. Particulate preparations were resuspended in serum-free medium by passage through a syringe and 26G needle and carefully distributed in the culture medium. Phagocytosis was observed by phase-contrast microscopy and stopped when >95% of cells had ingested a large number of particles, as follows: latex 20–50 particles, SRBC 5–10 cells and *M. lysodeikticus*, more than 50 bacteria per cell. The medium was removed and monitored for release of radioactivity as indicated. The cells were then washed three times and placed in fresh medium. Cultures on radioactive plates were placed in Dulbecco's medium containing either 10% FCS and 60 μg/ml STI, to block fibrinolysis, or 5% AT-FCS for immediate assay. Alternatively, cultures in regular nonradioactive dishes were incubated in Dulbecco's medium containing 10% FCS. At the desired time after phagocytosis the cultures were washed three times with HBSS to remove serum and placed in Dulbecco's medium containing 0.05% wt/vol LH for 24–48 h. Conditioned medium and cell lysates were then collected for assay.

Lysozyme was assayed as described (3) and its activity expressed in terms of a rat enzyme standard. *N*-Acetyl-β-D-glucosaminidase and cathepsin D were assayed as described (3).

Protein was measured by the method of Lowry et al. (4), using egg lysozyme as standard.

Results

Fibrinolysis by Macrophages. Unlike macrophages obtained from control, unstimulated animals, the thioglycollate-stimulated macrophages cultured on radioactive fibrin dishes displayed active plasminogen-dependent fibrinolysis (2). The stimulated cells contained and secreted at least a 100-fold more plasminogen activator than unstimulated cells. In order to study the regulation of plasminogen activator levels in the macrophage, it was necessary to define the stability of fibrinolysis by the cells as a function of time in culture. Fig. 1 shows an experiment in which thioglycollate-stimulated and unstimulated macrophages were cultivated on radioactive fibrin dishes for different periods of time before assay of fibrinolytic activity in situ. It was found that the difference in rates of fibrinolysis by the stimulated and unstimulated macrophages remained unaltered during at least 4 days of cultivation. As a rule cells could not be assayed directly on radioactive fibrin dishes after cultivation for more than 4 days because of gradual solubilization of the radioactive fibrin, even in the presence of STI. The use of an increased concentration of FCS, up to 40% vol/vol, or of horse and newborn calf serum did not significantly increase fibrinolysis by the unstimulated macrophages. On occasion cells prepared from different groups of unstimulated animals did show some fibrinolysis after prolonged cultivation,

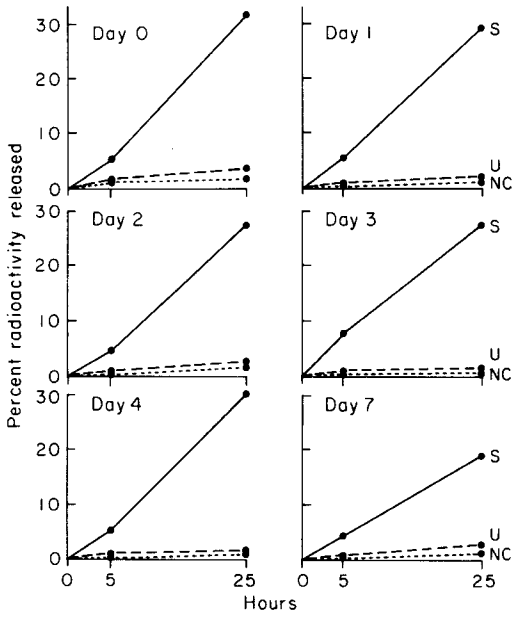

FIG. 1. Fibrinolysis by macrophages after cultivation for different periods of time. 4×10^6 unstimulated (U) or 1×10^6 thioglycollate-stimulated (S) macrophages were cultured on ^{125}I-labeled fibrin plates in the presence of 60 μg/ml STI. After 0–7 days the cultures were washed and placed in AT-FCS for assay. (NC, no cells.)

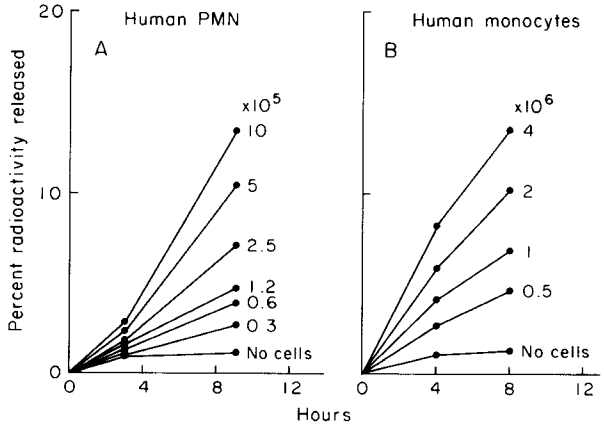

FIG. 2. Fibrinolysis by purified human PMN (A) and monocytes (B). Serial dilutions of human PMN were assayed immediately; human monocytes were assayed after 1 day in culture.

but this represented less than 5% of the activity of stimulated macrophages and was not necessarily dependent on plasminogen (2).

Fibrinolysis by PMN and Monocytes. Since macrophage populations harvested after injection of an intraperitoneal irritant such as thioglycollate medium could contain macrophage precursors as well as a small number of contaminant PMN, it was of interest to evaluate the fibrinolytic activity of purified monocytes

and PMN under similar conditions of culture and assay. We used human monocytes and PMN for these studies because they can be readily purified in sufficient numbers. When purified human PMN were cultivated in AT-FCS on radioactive fibrin plates, they displayed extremely active fibrinolysis in proportion to cell number (Fig. 2 A). However, the PMN died within 6–12 h of cultivation in vitro and no fibrinolysis was detected when similar dishes with 1×10^5 PMN were preincubated in 5% FCS and STI for 1 day before assay. In order to avoid fibrinolysis by contaminant PMN, macrophage preparations were therefore cultivated in STI for at least 1 day before assay in all subsequent experiments. Purified human monocytes still showed fibrinolytic activity proportional to cell number when assayed after one day in the presence of STI (Fig. 2 B). Fibrinolytic activity by such monocytes was 18% of that of freshly explanted PMN and, in contrast to the PMN, the monocytes contained to display fibrinolytic activity after 3 days in culture.

Macrophage Stimulation and Fibrinolysis. Because of the increase in plasminogen activator activity observed after injection of thioglycollate medium, we tested a variety of other intraperitoneal irritants as potential inducers of macrophage fibrinolytic activity. Peritoneal cells were harvested from groups of three animals 4 days after injection of different materials, and dilutions of cells were cultivated in radioactive fibrin dishes for 24 h before assay. Fibrinolytic activity was calculated from the linear region of a dose-response curve for each type of macrophage.

Table I shows that intraperitoneal injection of thioglycollate medium provided the most potent stimulus for the induction of macrophage fibrinolysis. Injection of mineral oil or endotoxin induced macrophage fibrinolysis to a more limited extent, 10% and 5% respectively, of thioglycollate-stimulated cells. Preparations of BCG and Listeria antigen also yielded macrophage-rich peritoneal exudates, but such cells showed no fibrinolytic activity under these conditions. Rapid spreading after cell attachment was also not necessarily associated with high

TABLE I

*Macrophage Stimulation and Fibrinolysis**

Intraperitoneal treatment	Macrophage cell no. $\times 10^6$/mouse	Spreading	Fibrinolysis: % radioactivity released/4 h/ 1×10^6 macrophages
Nil	2	–	<0.01
Thioglycollate medium (0.75 ml)	14	++	20
Mineral oil (0.75 ml)	6	+	2
Endotoxin (10 μg)	9	++	1
BCG antigen (50 μg)	6	+	<0.01
Listeria antigen (50 μg)	5	+	<0.01
FCS (1 ml)	3	–	<0.01

* Serial dilutions of peritoneal cells were plated on [^{125}I]fibrin dishes in Dulbecco's medium + 5% FCS + 60 μg/ml STI. Percent macrophages spread after 1 h: –, <10%, +, 10–50%, ++ >50%. After 1 day the macrophages were washed and placed in AT-FCS for assay of fibrinolysis.

levels of enzyme activity. These findings made it clear that the agent used to produce an inflammatory exudate in the peritoneal cavity had an important influence on the ability to induce macrophage fibrinolysis within 4 days of exposure.

Thioglycollate-stimulated macrophages contain residual undegraded constituents derived from the thioglycollate medium (5). Agar could represent one such constituent, but intraperitoneal injection of agar at a concentration comparable with that present in thioglycollate medium failed to induce fibrinolysis. The addition of thioglycollate medium (50% vol/vol) to the culture medium of unstimulated macrophages also failed to induce macrophage fibrinolysis in vitro.

Phagocytosis and Fibrinolysis by Unstimulated Macrophages. Since thioglycollate-stimulated macrophages contained inclusions of apparently undigested materials, probably the result of intense phagocytic activity in the peritoneal cavity, it was possible that phagocytosis played a role in the induction of plasminogen activator. This hypothesis was examined in unstimulated macrophages which had been fed a defined phagocytic load in vitro. Fig. 3 illustrates an experiment in which unstimulated macrophages were fed latex, a nondigestible particle, or formaldehyde-treated SRBC which were digested within 24–36 h of uptake. Fibrinolysis was stimulated three- to fivefold after phagocytosis of both types of particles, but this represented less than 10% of the activity of thioglycollate-stimulated cells. Analysis of the kinetics of the response to phagocytosis showed a lag period of 1 day preceding stimulation of fibrinolysis

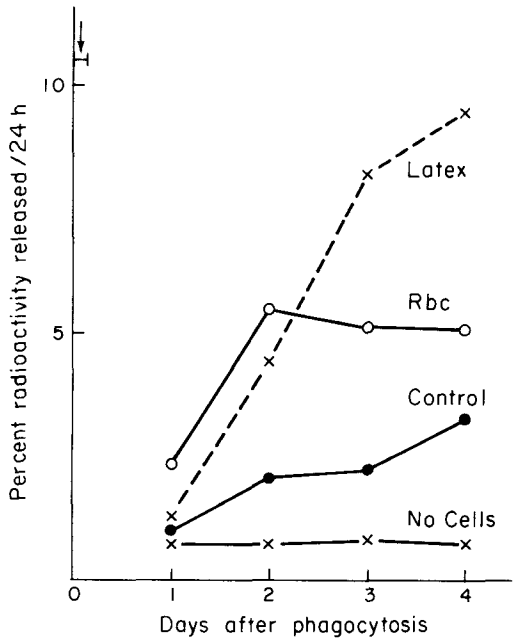

FIG. 3. The effect of phagocytosis on fibrinolysis by unstimulated macrophages. 4×10^6 macrophages were cultivated on ^{125}I-labeled fibrin plates for 1 day before phagocytosis of latex or RBC (arrow). STI was present at all times before transfer of cells to AT-FCS for fibrinolytic assay on consecutive days. (×—×), no cells; (●—●), control cells; (O—O), fed RBC; (×---×), fed latex.

by both particles and a different time-course on subsequent days. Fibrinolysis was maximal 2 days after uptake of RBC and showed no further increase relative to control macrophages, whereas fibrinolysis after latex ingestion continued to increase on consecutive days.

Combined Macrophage Stimulation and Phagocytosis

IN SITU FIBRINOLYSIS. The experiments described above indicated that endotoxin stimulation and phagocytosis of latex each induced only a small fraction of the fibrinolytic activity of thioglycollate-stimulated cells. To test whether a combination of these treatments might have an additive or synergistic effect on fibrinolysis, mice were injected intraperitoneally with 30 μg endotoxin, the cells harvested after 4 days and the macrophages cultivated on radioactive fibrin plates for 1 day before phagocytosis of latex. Fibrinolytic activity was then measured in situ on consecutive days. As shown in Fig. 4, combined treatment

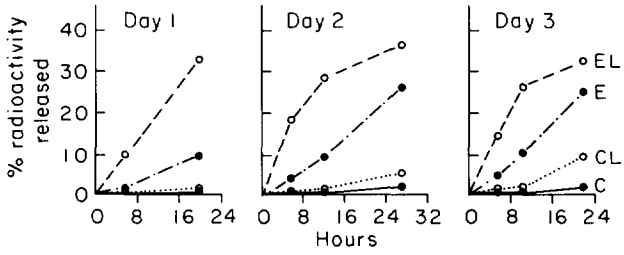

FIG. 4. The effect of endotoxin stimulation and latex phagocytosis on macrophage fibrinolysis. 4 × 10^6 macrophages were cultivated on ^{125}I-labeled fibrin plates for 24 h before phagocytosis. Cells were maintained in STI and placed in AT-FCS for fibrinolytic assay on consecutive days. C, control unstimulated; E, endotoxin-stimulated macrophages; L, latex-fed. (●—●), control unstimulated; (O···O), control latex; (●-·-·-●), endotoxin; (O---O), endotoxin latex.

with endotoxin and latex had a potent stimulatory effect on macrophage fibrinolysis whereas each treatment alone could account for only part of this response. As in the previous experiments, unstimulated control cells lacked significant fibrinolytic activity and phagocytosis of latex by these cells resulted in a three- to fivefold increase in fibrinolysis after 2 to 3 days, respectively. Endotoxin-stimulated macrophages showed 5 to 13 times more fibrinolytic activity than control cells, depending on the time in culture. Phagocytosis of latex by endotoxin-stimulated macrophages resulted in a further four- to fivefold increase in fibrinolytic activity.

PLASMINOGEN ACTIVATOR. The experiment described above demonstrated that combined endotoxin stimulation and phagocytosis induced high levels of fibrinolysis by macrophage cultures in radioactive fibrin dishes. In order to show that fibrinolysis after combined treatment was also plasminogen-dependent and to measure plasminogen activator levels in macrophage lysates and secretion products, similar cultures were prepared in ordinary culture dishes and incubated in serum-free medium for 24 h before assay. As shown in Fig. 5, in vivo endotoxin stimulation followed by in vitro phagocytosis of latex resulted in a striking increase of plasminogen activator in cell lysates (5 A) as well as in

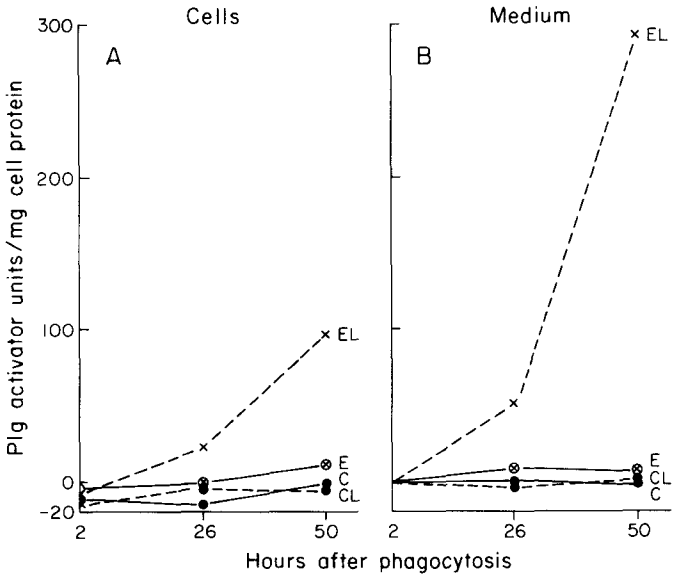

FIG. 5. The effect of endotoxin stimulation and latex phagocytosis on macrophage plasminogen activator in cells (A) and conditioned medium (B). 4×10^6 macrophages were cultivated in Dulbecco's medium and 10% FCS for 1 day. The cells were then fed latex and placed in Dulbecco's medium + 0.05% LH either immediately or 26 h after phagocytosis. Plasminogen-dependent fibrinolysis was defined as the difference in radioactivity released in the presence and absence of plasminogen. Results for plasminogen activator in medium show secretion between 2–26 and 26–50 h respectively. (●—●), control; (●---●), control-fed latex; (⊗—⊗), endotoxin; (×---×), endotoxin-fed latex.

conditioned medium (5 B). Low levels of fibrinolysis were observed in unstimulated cell lysates in the absence of plasminogen and represent the effect of proteases with different specificity (2). Stimulation after combined treatment with endotoxin and latex was apparent within 1 day following phagocytosis; plasminogen activator levels increased fivefold in both the cell lysate and medium during further cultivation for 1 day. Such cells secreted 70–85% of their total content of plasminogen activator produced per day. Though endotoxin-stimulated macrophages show a much higher rate of fibrinolysis on [^{125}I]fibrin dishes than unstimulated macrophages (Figs. 4 and 7), significant secretion of plasminogen activator was seen only by endotoxin-stimulated macrophages following phagocytosis of latex or by thioglycollate-stimulated cells.

SELECTIVITY OF PRODUCTION AND RELEASE OF MACROPHAGE ENZYMES. Evidence for the selective induction of plasminogen activator was obtained by comparing the effect of endotoxin, latex, and thioglycollate treatment on plasminogen activator, lysozyme, and two intracellular acid hydrolases, cathepsin D and *N*-acetyl-β-D-glucosaminidase. Table II shows that stimulation by thioglycollate and endotoxin followed by latex phagocytosis selectively induced production and secretion of the plasminogen activator. Unstimulated macrophages displayed little fibrinolytic activity which was plasminogen dependent. Latex or endotoxin alone stimulated a small fraction, 2 and 7% of thioglycollate cell activity respectively, compared with 31% after combined treatment; 83–100% of the induced enzyme

TABLE II

*Effect of Stimulation and Phagocytosis on Production and Release of Enzymes by Macrophages**

Macrophages	Cell protein/ 1×10^7 macrophages	Plasminogen activator		Lysozyme		Cathepsin D		*N*-acetyl-β-D-glucosaminidase	
		U‡/mg cell protein	%§ *secreted*	*μg/mg cell protein*	% *secreted*	*U‖/mg cell protein*	% *secreted*	*μm/min/mg cell protein*	% *secreted*
Unstimulated	336	−3.6	—	56	95	0.38	<20	0.15	8
Unstimulated + latex	738	15	—	31	92	0.44	11	0.14	6
Endotoxin	246	55	100	80	98	0.39	<20	0.095	16
Endotoxin + latex	594	250	87	39	95	0.43	11	0.13	5
Thioglycollate	864	800	83	37	94	0.52	25	0.19	29

* 2×10^7 Macrophages were cultivated in Dulbecco's medium containing 10% FCS. After 24 h the cells were fed latex and placed in Dulbecco's medium with 0.05% LH for 48 h.

‡ Cell lysate + medium.

§ Medium/Cell lysate + medium × 100.

‖ Units in chromogenic equivalents of 1 mg/ml egg lysozyme released per min.

activity was secreted into the medium. In contrast, all groups of macrophages produced comparable levels of lysozyme, predominantly as secretion, and of cathepsin D and *N*-acetyl-β-D-glucosaminidase, which remained largely intracellular.

The Macrophage Priming Reaction. These experiments showed that the induction of macrophage plasminogen activator could be achieved by two steps consisting of a priming reaction which resulted in partial stimulation and a phagocytic trigger which further stimulated production and secretion. Further experiments were designed to study each stage independently.

The relation between intraperitoneal dose of endotoxin and macrophage fibrinolysis was studied as follows. Groups of three mice were injected intraperitoneally with 0, 3, 10, 30, and 100 μg endotoxin and the peritoneal cells were harvested after 4 days. Cell yield: control 8×10^6/mouse, 37% macrophages, 0% PMN; endotoxin stimulated $1.7–2.2 \times 10^7$/mouse, 35–41% macrophages, 6–11% PMN. The cells were plated on radioactive fibrin dishes for 24 h and fed latex for 60 min. Fibrinolysis was measured 1 day after phagocytosis.

Maximum fibrinolysis was observed after injection of 30 μg endotoxin, whereas a lower or higher dose yielded less active macrophages (Fig.6). The morphologic appearance of the macrophages and the rate of latex uptake were correlated with subsequent fibrinolytic activity. Optimally primed macrophages spread rapidly on the fibrin, showed active membrane ruffling and contained prominent cytoplasmic granules.

Since lipid A contains the chemical moiety which is responsible for several of

FIG. 6. The relation between intraperitoneal dose of endotoxin and macrophage fibrinolysis. 4 $\times$ 10^6 macrophages were cultured on radioactive fibrin plates for 24 h in the presence of STI. After latex phagocytosis the cells were placed in Dulbecco's medium and 10% FCS + 60 μg/ml STI for 24 h before assay in AT-FCS.

the biological activities of endotoxin (6), a similar dose-response experiment was performed with 0–200 μg of a 1:1 lipid A: BSA complex. The yield of peritoneal cells was stimulated two to threefold, depending on dose, and consisted of 42–55% macrophages and 2–5% PMN. Early macrophage spreading was enhanced. Lipid A pretreatment also induced macrophage fibrinolysis after latex phagocytosis, but since 200 μg of the complex was required to achieve the same level of priming as 30 μg endotoxin, its efficacy, weight for weight, was less.

The ability of other intraperitoneal irritants to prime macrophages was next examined. Peritoneal cells were harvested from groups of four mice 4 days after intraperitoneal injection of a variety of substances. Cell suspensions were plated on 35-mm radioactive fibrin dishes or in uncoated 15-mm Linbro wells for assay of in situ fibrinolysis and of plasminogen activator secretion, respectively. Macrophages were fed latex after 24 h cultivation, washed, and placed in AT-FCS or LH medium for assay.

The results of such an experiment are shown in Fig. 7. In situ fibrinolysis by the macrophages varied over a wide range depending on the intraperitoneal treatment, but in all cases, except for thioglycollate-stimulated cells, latex ingestion enhanced fibrinolysis three- to sixfold. BCG stimulation, after a prolonged lag period, resulted in substantial fibrinolysis; mineral oil showed a much shorter lag period. Thioglycollate-stimulated cells were most active; endotoxin, mineral oil and, to a lesser extent, BCG, provided an intermediate stimulus, whereas FCS- and saline-treated cells approached control cells in activity. The response to latex was accelerated in the endotoxin, mineral oil, and BCG-treated cells.

The cells tested for fibrinolysis in Fig. 7 were also tested for secretion of plasminogen activator. Thioglycollate-stimulated cells without and with ingested latex secreted 163 and 130 U/mg cell protein respectively in 48 h. Endotoxin-primed cells secreted no detectable activator; following latex ingestion they secreted 41 U/mg cell protein, 25% that of the thioglycollate-stimulated cells. No secreted plasminogen activator was detectable from any other macro-

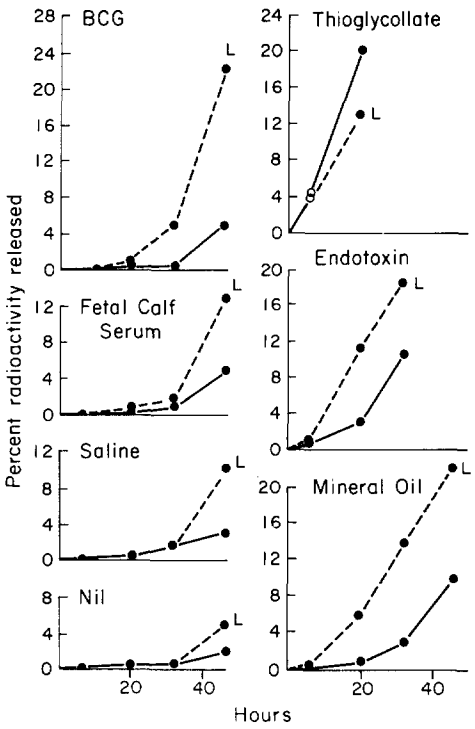

FIG. 7. Macrophage priming and fibrinolysis in situ. For details see text. Number of macrophages plated: thioglycollate 6 × 10^5, others 4–6 × 10^6. (●—●), no latex; (●---●), fed latex.

phages tested. Although a number of agents stimulated fibrinolysis, endotoxin was the most effective priming stimulus. This experiment also confirmed earlier observations that the in situ fibrinolytic assays may show considerable activity under conditions where no secretion of the plasminogen activator is seen. This discrepancy may be due to the intimate contact of the cells with the fibrin, which results in fibrinolysis mediated by an undetectable amount of secreted enzyme.

Role of Phagocytosis as Trigger. Earlier experiments had shown that phagocytosis of numerous latex particles stimulated macrophage fibrinolysis up to fivefold, triggering the rapid and sustained release of high levels of plasminogen activator by endotoxin-primed cells, in comparison with the delayed and lesser effect on unprimed, control macrophages. Further experiments were undertaken to study the role of the type of particles and phagocytic load in triggering endotoxin-primed macrophages. Table III illustrates that a variety of other particles could stimulate fibrinolysis as effectively as latex, including *M. lysodeikticus*, heat-aggregated γ-globulin and immune complexes of HRP-anti-HRP. These materials were all rapidly ingested by the endotoxin-primed macrophages and the degree of stimulation of fibrinolysis, two to sixfold, was proportional to particle dose. We therefore concluded that the triggering reaction was associated with phagocytosis in general.

The data presented in Fig. 3 suggested that the digestibility of the particle did,

TABLE III

*Phagocytosis and Fibrinolysis by Endotoxin-Stimulated Macrophages**

Substance phagocytized	Load	Radioactivity released	Percent control
		%/12 h	
Nil	—	5.3	100
Latex	5×10^8 particles	21	400
	1.5×10^8 particles	17	330
	0.5×10^8 particles	12	250
M. lysodeikticus	0.5 mg	16	310
	0.17 mg	11	210
	0.06 mg	5.6	130
Heat aggregated γ-globulin	150 μg	34	650
	15 μg	18	350
Peroxidase antibody complexes	30 μg	28	540
	3 μg	12	240

* 3×10^6 macrophages were cultivated for 24 h on 35 mm [^{125}I]fibrin plates in the presence of STI. Cultures were washed and fed particulates for 60 min in a vol of 2.5 ml of medium containing STI. The cells were then washed and placed in AT-FCS for assay.

however, determine the duration of induced fibrinolysis. To test this hypothesis, endotoxin-primed macrophages plated on [^{125}I]fibrin plates were fed latex or *M. lysodeikticus* and assayed for fibrinolysis on consecutive days. *M. lysodeikticus*, an effective trigger, was chosen since macrophages digested large numbers of these bacteria within 24 h. The results of such an experiment, illustrated in Fig. 8, show that both particles triggered fibrinolysis within 8 h of phagocytosis but that the subsequent time-course of induced fibrinolysis was quite different. As in previous experiments fibrinolysis after latex ingestion increased and persisted, whereas the activity of cells fed *M. lysodeikticus* diminished within a day and approached that of unfed cells after further cultivation. Similar experiments that were performed on nonradioactive plates confirmed that plasminogen activator was secreted only transiently by endotoxin-stimulated macrophages after ingestion of *M. lysodeikticus*, whereas the latex-laden macrophages continued to release high levels of enzyme. Endotoxin-primed macrophages after the phagocytosis of latex continued to accumulate and secrete large amounts of plasminogen activator for at least 9 days.

Discussion

Our studies have shown that the hydrolases of cultured macrophages can be differentiated into at least three classes which differ in their control mechanisms. The plasminogen activator is an inducible enzyme associated with macrophage stimulation and is markedly influenced by phagocytosis, whereas lysozyme secretion is a continuous process, independent of cell stimulation and unaffected

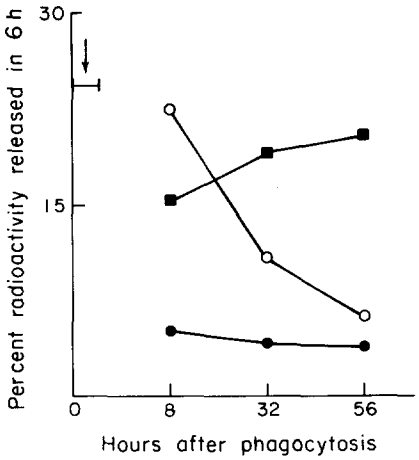

FIG. 8. Fibrinolysis by endotoxin-primed macrophages after phagocytosis of latex or *M. lysodeikticus*. 5×10^6 macrophages were cultured on [^{125}I]labeled fibrin dishes for 24 h in the presence of STI. After phagocytosis (arrow) the cultures were maintained in Dulbecco's medium containing 10% FCS and STI until assay in AT-FCS at 2, 26, and 50 h. (●—●) endotoxin-primed macrophages; (○—○) fed *M. lysodeikticus;* (■—■) fed latex.

by phagocytosis (3). The secretion of plasminogen activator and lysozyme is unaffected under conditions where there are considerable increases in intracellular acid hydrolases (7). These differences in enzyme regulation suggest distinct functions for each class of enzymes.

High levels of plasminogen activator can be induced by a single intraperitoneal injection of thioglycollate medium or, in two stages, namely, by endotoxin in vivo followed by latex phagocytosis in vitro. Thioglycollate treatment induces higher levels of activity and latex phagocytosis does not further enhance secretion or fibrinolysis. A likely explanation for this difference is that phagocytosis of thioglycollate medium takes place in the peritoneal cavity and that some of the constituents may be undegradable and persist in the macrophages. In the case of thioglycollate treatment both stimuli are thus delivered simultaneously and result in a maximal effect.

The endotoxin-primed macrophage is characterized by a relatively low level of fibrinolytic activity, but it has acquired the capacity to respond to phagocytosis with a high output of plasminogen activator. The mechanism for altering the properties of the peritoneal macrophage population could be complex, since both endotoxin and thioglycollate injection produce a vigorous inflammation in the peritoneal cavity, with all its attendant humoral and cellular sequelae. Endotoxin, for example, is known to have potent effects on PMN (8) and lymphocytes (9) as well as activating humoral factors, like complement (10), all of which could influence macrophage function indirectly. Endotoxin could also exert a direct effect on macrophage stem cells (11) and differentiation, as well as acting on mature peripheral cells (12). Macrophages take up and detoxify endotoxin (13), and endotoxin stimulates the transient release of colony-stimulating factor (14). Much remains to be learned about the interaction between endotoxin and defined populations of macrophages.

Secretion of the plasminogen activator may play an important role in macrophage function, since it results in the formation of plasmin, a potent protease. Plasmin has been implicated in complement activation (15), as well as the activation of prekallikrein and subsequent kinin formation (16). In addition plasmin may affect the properties of other cells in the immediate environment, which has been demonstrated in tissue culture (17, 18).

Continued production and secretion of plasminogen activator is associated with intracellular persistence of the phagocytosed particles. Although only a limited number of particles have been examined, prolonged secretion of plasminogen activator is probably related to nondigestibility of the particle, in striking contrast with induction of intracellular acid hydrolases solely by digestible particles (19). The long-term accumulation and secretion of plasminogen activator provides some evidence for synthesis of the enzyme, rather than conversion of an inactive intracellular precursor as a result of phagocytosis. These findings raise many interesting questions concerning the intracellular localization of plasminogen activator, the mechanism for increased production and the secretory pathway of the enzyme.

The concept of macrophage activation was developed to explain the nonspecific enhancement of macrophage bactericidal capacity observed after infection by BCG and other agents (20). Activated macrophages spread rapidly, have a high basal rate of metabolism, and show a burst of respiratory activity during phagocytosis (21), but the biochemical basis for cellular activation is unknown. It has been reported that endotoxin treatment is able to enhance phagocytosis and killing of bacteria by macrophages (22) and to promote their cytocidal and cytostatic capacity for tumor cells (23, 24). Our studies show that induction of plasminogen activator accompanies these parameters of macrophage activation and suggest that it could play a role in some of the effector functions of the activated macrophage (25). The requirement of phagocytosis for secretion of the plasminogen activator and the fall in fibrinolytic activity and secretion after digestion of the particles may serve to regulate closely the activation of plasminogen to plasmin by macrophages.

Summary

The injection of thioglycollate medium into the peritoneal cavity of the mouse induces high levels of macrophage fibrinolytic activity due to the production and secretion of a plasminogen activator, a trypsinlike serine protease, which is absent in unstimulated macrophages. Intraperitoneal injection of endotoxin or mineral oil can stimulate only a fraction ($<10\%$) of the fibrinolytic activity of thioglycollate cells, similar to the partial stimulation ($<10\%$) seen 1–2 days after phagocytosis of latex or SRBC by unstimulated macrophages. The endotoxin-stimulated macrophages contain and release relatively low levels of plasminogen activator, but these primed cells can be triggered to produce and secrete high levels of enzyme, by phagocytosis of latex. Under conditions where the plasminogen activator is induced and secreted, there are no effects on the production and/or release of lysozyme or intracellular acid hydrolases.

Discovery of a two-stage procedure for inducing macrophage plasminogen

activator made it possible to study the role of cell priming and phagocytosis separately. Endotoxin was a more effective priming agent, weight for weight, than lipid A:BSA complex. Secretion of the plasminogen activator was induced only by thioglycollate, or endotoxin and latex. In situ fibrinolysis was induced by these agents and mineral oil, BCG, and fetal calf serum, in decreasing order of effectiveness. Phagocytosis of latex in all cases except thioglycollate stimulation, increased fibrinolytic activity from three- to sixfold.

Latex and a variety of other particles such as *M. lysodeikticus*, aggregated γ-globulin and immune complexes showed dose-dependent stimulation of fibrinolysis by endotoxin-primed macrophages. Although the initial phagocytic trigger was not specific for the substance employed, the ability to induce a sustained response depended on the persistence of the phagocytized particle within the cell. Fibrinolysis and secretion of plasminogen activator continued at high levels for at least 9 days after uptake of latex, a nondigestible particle, whereas plasminogen activator was secreted only transiently after ingestion of rapidly digested *M. lysodeikticus*. The induction of plasminogen activator secretion provides a mechanism by which the activated macrophage can exert a selective effect on its extracellular environment.

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