

**MACROPHAGE PLASMINOGEN ACTIVATOR:
INDUCTION BY ASBESTOS IS BLOCKED BY
ANTI-INFLAMMATORY STEROIDS***

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Prolonged exposure to asbestos dust is the cause of asbestosis, one of the most severe pneumoconioses. This disease is characterized by inflammation and fibrosis surrounding the respiratory bronchioles and alveoli and leads to permanent impairment of lung function. Asbestosis is also associated with a high incidence of pleural and peritoneal mesotheliomas (1) and bronchogenic carcinomas (2). Macrophages, a cell type present both in normal alveoli and in chronic inflammatory reactions, are often conspicuous in asbestos-induced lesions (3), and investigation of the effects of asbestos particles on these cells might provide insights about the pathogenesis of asbestosis.

Peritoneal macrophages obtained from mice that have received inflammatory stimuli such as thioglycollate, endotoxin, mineral oil, etc., produce plasminogen activator (4, 5), whereas the resident peritoneal macrophages from unstimulated animals do not; hence a correlation between the production of this enzyme and the inflammatory response can be established. This correlation is further supported by the fact that low concentrations of anti-inflammatory steroids suppress the production of macrophage plasminogen activator (6).

This paper shows that asbestos, *in vivo* and *in vitro*, can induce plasminogen activator production by peritoneal macrophages, and that this can be suppressed by anti-inflammatory steroids.

Materials and Methods

Except when otherwise stated, all reagents and procedures were as previously described (4, 6).

Cell Culture. Cells were obtained from NCS (The Rockefeller University) mice (25–30 g) that had been injected intraperitoneally 4 days previously with one of the following: 1 ml of phosphate-buffered saline (0.01 M phosphate buffer, pH 7.4, 0.15 M NaCl) (PBS); a suspension of asbestos or of latex particles in 1 ml PBS; 30 μ g endotoxin (Salmonella minnesota LPS MR595 S418) in 1 ml PBS; 1 ml of Brewer's thioglycollate medium. After centrifugation, the cells were counted in Turk's solution; cells were also collected by centrifugation onto glass coverslips, fixed with methanol, and stained with Giemsa for differential counting. Cells from PBS, latex, asbestos, or endotoxin-injected mice were cultured at a density of 1×10^6 per cm^2 and cells from thioglycollate-injected mice at 3×10^6 per cm^2 .

Addition of Asbestos and Latex to Cultures. Asbestos and latex were added for 2 h before the cultures were washed and assayed for plasminogen activator secretion, as described previously for latex (6). In some experiments, cultures were exposed to asbestos for 24 h before washing.

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Lysosomal Enzymes. *N*-acetyl- β -D-glucosaminidase and β -galactosidase were assayed as described by Bowers et al. (7).

Materials. Asbestos (U.I.C.C. Chrysotile "B", Canadian sample) was obtained from Johns-Manville, Lapoc, Calif.; fibres were resuspended in PBS, washed seven times by centrifugation, and autoclaved.

Results

Effects of Intraperitoneal Injection of Asbestos

PERITONEAL CELL POPULATION. Intraperitoneal injection of asbestos increased the number of cells obtained by peritoneal washing and also affected the relative proportions of the different cell types present. In contrast, injection of latex, another nondigestible particle, caused only minor changes in the cell population. The total cell numbers and their relative proportions 4 days after injecting 1 ml of various materials were as follows: PBS, $3-5 \times 10^6$ cells (30-40% macrophages, 60-70% lymphocytes); asbestos, $9-20 \times 10^6$ cells, increasing with dose from 10 to 100 μ g (40-50% macrophages, 30-40% polymorphonuclear leukocytes (PMN), 10-20% lymphocytes, at 100 μ g asbestos); latex (500 μ g), $6-7 \times 10^6$ cells (30-40% macrophages, 5-10% PMN's, 50-60% lymphocytes); thioglycollate, $15-20 \times 10^6$ cells (80-90% macrophages, 10-20% lymphocytes).

The asbestos-induced macrophages were larger and more vacuolated than those from PBS-injected mice, but somewhat less so than those in the thioglycollate-induced population. Repeated administration of tritiated thymidine ($[^3\text{H}]\text{TdR}$) during the 4-day period of exudate formation after injection of 100 μ g asbestos resulted in autoradiographically detectable labeling of 70% of macrophage nuclei; the fraction of macrophages having replicated their DNA within the preceding 4-day period was therefore the same as that found in thioglycollate-induced exudates (6).

In view of the relatively large proportion of PMN in the asbestos-stimulated exudates, these cell populations were preincubated for 24 h during which all of the PMNs died; after washing, at least 95% of the remaining cells were identifiable as macrophages by Giemsa staining and light microscopy.

FIBRINOLYTIC ACTIVITY OF ASBESTOS-INDUCED PERITONEAL EXUDATE MACROPHAGES. Peritoneal macrophages cultured from asbestos-induced exudates produced significant plasminogen-dependent fibrinolytic activity indicating secretion of plasminogen activator. The rate of fibrinolysis by these cultures was a function of the amount of injected asbestos (Fig. 1) and was much greater than that in macrophage cultures prepared from PBS- or latex-injected animals.

ENZYME SECRETION BY ASBESTOS-INDUCED MACROPHAGES. Plasminogen activator secretion by asbestos-induced macrophages was at least 20-fold higher than by controls exposed to PBS alone (Table I), whereas the amounts of lysozyme secreted by the two types of cultures was the same. Similar results have been reported previously for the comparison of thioglycollate and control macrophages (4). Likewise, no difference was observed between asbestos-stimulated and control macrophages in the secretion of two lysosomal enzymes, *N*-acetyl- β -D-glucosaminidase and β -galactosidase. Hence the secretion of plasminogen activator by asbestos-induced cells is a relatively specific response and is not part of a general increase in lytic enzyme synthesis.

The apparent molecular weight and electrophoretic characteristics of the

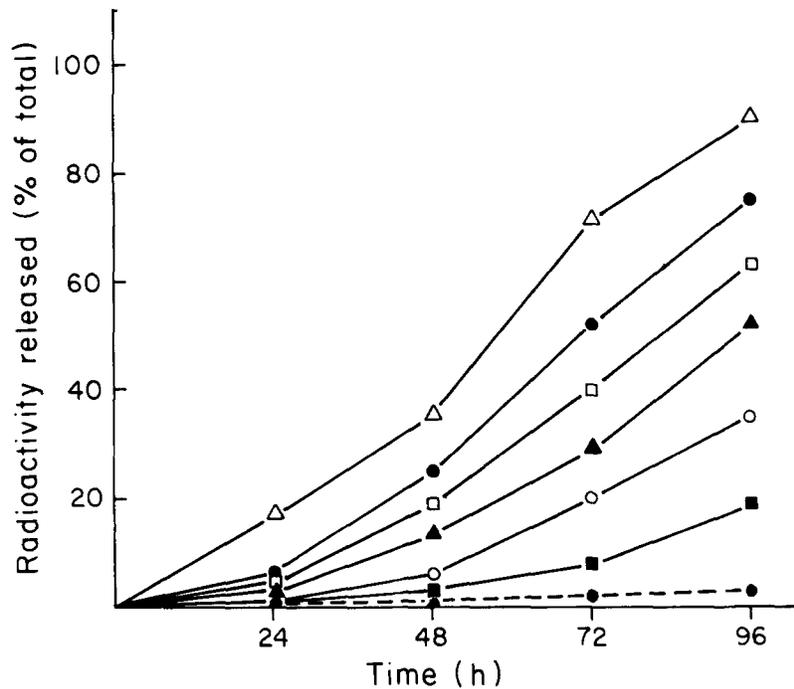


FIG. 1. Fibrinolysis by macrophages from asbestos, latex, PBS, and thioglycollate-injected mice. 2×10^6 asbestos-, latex-, or PBS-induced and 8×10^5 thioglycollate-injected peritoneal cells were plated on ^{125}I fibrin-coated Linbro wells in Dulbecco's minimum essential medium (MEM) containing 5% heat-inactivated fetal bovine serum and 100 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor and incubated for 24 h. After washing, Dulbecco's MEM containing 5% acid-treated, heat-inactivated fetal bovine serum (ATHIFBS) (—) or 5% of plasminogen-depleted ATHIFBS (---) (total vol 2 ml) was added, and 0.2-ml samples were removed as indicated. Cells were obtained from mice injected with (Δ) 1 ml thioglycollate medium; (\bullet) 300 μg , (\square) 100 μg , (\blacktriangle) 10 μg , (\circ) 1 μg asbestos; (\blacksquare) 1 ml PBS or 500 μg latex particles in 1 ml PBS. Each well contained 10 μg fibrin per cm^2 , total radioactivity 10^5 cpm.

TABLE I
Secretion of Plasminogen Activator, Lysozyme, and Lysosomal Enzymes by Asbestos-Induced Macrophages

Macrophages*	Time h	Plasminogen activator†		Lysozyme secreted $\mu\text{g}/\text{mg}$ cell protein	N-acetyl- β -glucosaminidase		β -galactosidase	
		Extracellular U/mg cell protein	Cell associated		Extracellular $\mu\text{mol}/\text{min}/\text{mg}$ cell protein ($\times 10^3$)	Cell associated	Extracellular $\mu\text{mol}/\text{min}/\text{mg}$ cell protein ($\times 10^3$)	Cell associated
Asbestos	24	1,710	205	15.5	2.6	11.8	3.5	14.9
	48	2,670	580	28.6	4.4	13.9	4.4	12.5
	72	3,430	660	45.0	6.5	13.2	7.3	11.9
PBS	24	82	15	9.4	2.2	7.4	5.0	15.8
	48	108	27	21.4	4.2	10.8	7.2	14.6
	72	136	36	40.9	8.0	11.0	10.1	12.3

* Peritoneal cells were obtained from mice injected with asbestos (100 μg) or PBS. 24 h after plating in Dulbecco's MEM + 5% HIFBS, the macrophages were washed and placed in Dulbecco's MEM + 0.05% lactalbumin hydrolysate. After 24, 48, or 72 h the serum-free conditioned medium was collected, the cultures washed two times with PBS, the cells lysed by the addition of Triton X-100 (0.2% vol/vol in water) and scraped from the surface of the dish with a plastic policeman.

† No plasminogen-independent fibrinolysis could be detected when plasminogen was omitted from the assay mixture.

plasminogen activator secreted by asbestos-induced macrophages were the same as those previously found for the enzyme released by thioglycollate-induced macrophages (4).

Dexamethasone inhibits plasminogen activator production by thioglycollate-induced macrophages (6); under the same conditions, production of plasminogen activator by asbestos-induced macrophages was inhibited by more than 95% at 10^{-7} M dexamethasone.

Effects of Asbestos on Cultured Macrophages

FIBRINOLYTIC ACTIVITY AFTER ASBESTOS EXPOSURE IN VITRO. Peritoneal macrophages obtained from endotoxin-injected mice produce increased amounts of plasminogen activator after phagocytosis of both digestible and nondigestible materials (5). Exposure of such cells to asbestos (1–100 μ g) for 2 h in vitro led to a two to fivefold increase in fibrinolytic activity that persisted for at least 4 days. Cultures exposed to 100 μ g asbestos appeared somewhat altered after 3–4 days, as shown by some rounding up and cell detachment, whereas cultures exposed to the lower doses of asbestos appeared not to be affected. The fibrinolytic activity of peritoneal macrophages from PBS-injected mice was also increased by asbestos but only after a lag period of 2–3 days; comparable results have been reported after phagocytosis of latex beads (5). All of the fibrinolytic activity induced by addition of asbestos in vitro was plasminogen dependent since none was detected when the experiments were performed using plasminogen-depleted sera.

ENZYME SECRETION AFTER ASBESTOS EXPOSURE IN VITRO. Macrophages obtained from endotoxin-injected mice secreted elevated levels of plasminogen activator after exposure to asbestos in vitro; this increased enzyme production was not associated with any increased intracellular content, indicating that plasminogen activator synthesis was tightly coupled to secretion (Table II). In contrast to the enhancement of plasminogen activator secretion, there was no effect of asbestos on the secretion either of lysozyme or of two lysosomal enzymes, *N*-acetyl- β -D-glucosaminidase and β -galactosidase (Table II). The same results were obtained when conditioned media and cell lysates were assayed 24, 48, 72, or 96 h after addition of asbestos to the cultures and irrespective of the duration of exposure to asbestos (i.e., 2 or 24 h) before placing the cells in serum-free medium.

The effect of asbestos on plasminogen activator secretion by endotoxin-primed macrophages was inhibited more than 95% by dexamethasone (10^{-8} M) and more than 90% by colchicine (10^{-6} M), and the inhibitory action of both drugs persisted for at least 48 h. There was no detectable effect of dexamethasone if this compound was only present during the 2 h of exposure to asbestos and then removed before incubation of the cells in serum-free medium. Other steroids were also tested; the order of relative potencies reflected both the anti-inflammatory effect in intact animals and the inhibitory effect on plasminogen activator production by thioglycollate-induced macrophages (6), namely, dexamethasone > fluorocortisone > prednisolone > hydrocortisone > corticosterone.

Discussion

That chronic inflammation can be initiated by asbestos has been known for many years, and the results described here can be interpreted in this context.

TABLE II
Effect of Asbestos Exposure In Vitro on the Secretion of Plasminogen Activator, Lysozyme, and Lysosomal Enzymes

Macrophages* (2 × 10 ⁶)	Plasminogen activator†		Lysozyme secreted	N-acetyl-β-D-glucosaminidase		β-galactosidase	
	Extracellu- lar	Cell associ- ated		Extracellu- lar	Cell associ- ated	Extracellu- lar	Cell associ- ated
	U/mg cell protein			μmol/min/mg cell protein (× 10 ³)		μmol/min/mg cell protein (× 10 ³)	
Endotoxin	280	150	17.6	6.0	9.0	3.8	4.5
Endotoxin + 1 μg as- bestos	330	160	27.2	8.0	8.3	4.1	5.7
Endotoxin + 10 μg asbestos	730	160	17.1	8.6	9.3	4.0	4.0
Endotoxin + 30 μg asbestos	1,560	210	18.4	9.7	9.3	4.4	4.8
Endotoxin + 100 μg asbestos	1,480	220	17.4	8.1	9.1	4.1	5.2

* Peritoneal cells from endotoxin injected mice were cultured in Dulbecco's MEM containing 5% HIFBS for 24 h. The cells were then washed and the asbestos fibres added in 1 ml Dulbecco's MEM containing 5% HIFBS for 2 h at 37°C; the excess particles were then removed by washing two times with PBS, and the cells placed in Dulbecco's MEM + 0.05% lactalbumin hydrolysate conditioned medium and cell lysates were collected 48 h later as for Table I.

† No plasminogen-independent fibrinolysis could be detected when plasminogen was omitted from the assay mixture.

Intraperitoneal injection of asbestos fibres induced inflammatory exudates, containing mainly PMN's and macrophages, and macrophages cultured from such exudates produced elevated levels of plasminogen activator, in contrast to those prepared from PBS-injected mice. Since approximately 70% of the asbestos-induced macrophages had incorporated [³H]TdR in vivo during the 4-day period of exudate formation, it appears that most of these were "young" cells, having recently divided and migrated, presumably in response to asbestos, from the bone marrow to the peritoneal cavity via the blood stream. These effects of asbestos are therefore comparable to those observed after intraperitoneal injection of thioglycollate medium (6). The mechanism by which asbestos induces such an exudate is not yet known. Since injection of latex beads produces no comparable exudate, the effects of asbestos are probably not due to its indigestibility alone; it seems reasonable to assume that the recruitment by asbestos of macrophages and PMN's reflects, at least in part, the ability of this material to provoke chronic inflammatory reactions.

The action of asbestos in enhancing macrophage plasminogen activator production was not limited to responses in vivo, since macrophages from endotoxin-treated mice responded similarly, and for a considerable period (4 days) in vitro. These in vitro responses to asbestos resemble those of macrophages that have phagocytically ingested latex beads; however, the heterogeneity in size of asbestos fibers, and the absence of experimental measurements of asbestos uptake, preclude a proven correlation between phagocytosis and stimulation of plasminogen activator secretion.

The effect of asbestos on the secretion of various enzymes by macrophages in culture was not uniform and resembled that previously found for latex (5); in particular, the release of lysozyme and lysosomal enzymes by these cells was not

increased after exposure to asbestos. This result differs from that obtained by Davies et al. (8) who noted an increase in lysosomal enzyme release from cultured mouse macrophages in the presence of asbestos. Differences in the experimental procedures, and especially in asbestos exposure times, can perhaps account for these conflicting observations.

The production of plasminogen activator by stimulated macrophages and the inhibition of enzyme formation by anti-inflammatory steroids establish this as the only known enzyme whose rate of synthesis, in contrast to that of lysozyme or lysosomal enzymes, can be correlated with varying degrees of inflammation. These facts also suggest some specific role for plasminogen activator in inflammation, a possibility that is reinforced by both the asbestos-induced stimulation of enzyme synthesis and its inhibition by glucocorticoids. Anti-inflammatory steroids may be of value in controlling the chronic inflammation associated with asbestosis and in exploring any possible relationship between the inflammatory, fibrotic, and proliferative components of the disease.

Summary

Intraperitoneal injection of asbestos fibres into mice induces the formation of exudates containing macrophages that produce plasminogen activator. Likewise, *in vitro* addition of asbestos to macrophage cultures stimulates plasminogen activator secretion; the synthesis and secretion of lysozyme and lysosomal enzymes are not changed under these conditions. The enhanced secretion of plasminogen activator by macrophages exposed to asbestos is suppressed by low concentrations of anti-inflammatory steroids.

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