Suppression of the Neutral Protease Activity of Macrophages Treated with Asbestos in Vitro

by John A. Hamilton*

Macrophages are often conspicuous in asbestos-induced inflammatory lesions. Chrysotile type B elicits macrophages into the peritoneal cavity of mice which produce high levels of the neutral protease, plasminogen activator; in vitro addition of these same fibers to mouse peritoneal macrophages stimulates enzyme production. It is reported here that, for endotoxin-elicited mouse peritoneal macrophages fed chrysotile type B in vitro, the increased plasminogen activator activity is suppressed by low concentrations of anti-inflammatory steroids. Other active drugs include colchicine and vinblastine. These studies are considered important, as they suggest an approach to controlling the levels of a potentially deleterious enzyme system (PA-plasmin) from macrophages treated with asbestos fibers.

Introduction

Asbestosis is characterized by inflammation and fibrosis surrounding the respiratory bronchioles and alveoli and leads to permanent impairment of lung function (1). Macrophages are found in normal alveoli and are the prominent cell type found in chronic inflammatory lesions, including those induced by asbestos (2). Macrophages are able to release in vitro a number of molecules which are probably important in the initiation and maintenance of an inflammatory reaction, for example, neutral proteases, complement components, endogenous pyrogens, oxygen metabolites, prostaglandins and analogs, proliferation factors for neighboring cells, etc. (9).

For monocyte macrophages in vitro, the activity of the neutral protease, plasminogen activator (PA), shows positive correlations with stimuli [for example, asbestos (4), endotoxin (5), lymphokines (6), and phorbol esters (7,8)] and suppressive drugs [for example, glucocorticoids (9)] associated with an inflammatory response. A role for macrophage PA activity in cell migration from blood to tissue and in the processes of tissue destruction and activation of several inflammatory enzyme cascades has previously been proposed (9). We have been able to show that IARC chrysotile type B elicits macrophages into the peritoneal cavity of mice which produce high levels of PA (4,10); in vitro addition of these fibers to mouse peritoneal macrophages stimulates enzyme production.

The present studies indicate that the in vitro stimulation of macrophage PA activity can be suppressed by anti-inflammatory glucocorticoids and, under certain culture conditions, by colchicine and vinblastine.

Materials and Methods

Reagents

Reagents were obtained commercially as follows: fetal bovine serum (Flow Laboratories, Hamden, CT); asbestos, IARC chrysotile type B, a gift from Johns-Manville Corp.; soybean trypsin inhibitor and bovine fibrinogen; fraction I (Miles Laboratories Inc., Kankakee, IL) lactalbumin hydrolyzate (Nutritional Biochemicals Corp., Cleveland, OH); polystyrene latex particles, 1.01 μm diameter (Dow Diagnostics, Indianapolis, IN); steroids and colchicine (Sigma Chemical Co., St. Louis, MO); vinblastine (Eli Lilly and Co., Indianapolis, IN).

All steroids were dissolved at 10⁻³ M in ethanol and stored at 4°C. Controls for the effect of solvent never differed from those of other untreated drug-
free cultures. Plasminogen-free fibrinogen for the fibrin plates was prepared as before (9). Endotoxin, *S. minnesota* lipopolysaccharide, mR595 (S418), was a gift from Dr. O. Ludewitz, Max Planck Institut fur Immunobiologie, Freiburg, West Germany. Human lysozyme was a gift from Dr. E. Osseman (College of Physicians and Surgeons of Columbia University, New York).

Stock suspensions of asbestos in PBS and latex particles in Dulbecco's medium (DB) at 5 × 10⁴ particles/mL were irradiated with ultraviolet light and kept at 4°C until use (4).

**Peritoneal Cells**

Peritoneal cells were obtained from NCS (Rockefeller) mice (25-30 g) or BDF, mice, 8-12 weeks old (Cumberland Farms), that had been injected intraperitoneally 4 days previously with phosphate-buffered saline (PBS), pH 7.4, or with 30 μg endotoxin (4).

**Fibrinolysis Measurement**

The peritoneal cells (2 × 10⁶) were plated on ¹²⁵I-fibrin-coated wells (Linbro Disposo Trays, FB 16-24 TC) (9) for 24 h in DB supplemented with 5% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS). At this stage, soybean trypsin inhibitor (STI) (100 μg/mL) was added to block any fibrinolysis. The cells were washed twice with PBS and placed again in DB + 5% FBS + STI. The particulate preparations were resuspended by passage through a syringe and 26G needle and carefully distributed in the culture medium. The particles were generally added for 2 hr, after which time >95% of the cells contained 20-50 latex particles. The macrophages were then washed three times with PBS and placed in DB + 5% acid-treated FBS (ATFBS) for assay. The acid treatment removes interfering protease inhibitors (9). Fibrinolysis was estimated by withdrawing aliquots of medium and counting the radioactivity in a Packard Autogamma scintillation spectrometer. The plasminogen dependence of the fibrinolytic activity can be shown by assaying in the presence of ATFBS from which the plasminogen has been removed (9).

**Measurement of Secreted Plasminogen Activator**

Peritoneal cells (2 × 10⁶) were plated in DB + 5% FBS on regular linbro trays. At 24 hr after plating, the cells were washed twice with PBS and placed again in DB + 5% FBS but also in the presence of the particles for an additional 24 hr. After this period, the cultures were washed twice with PBS and placed in DB containing 0.05% lactalbumin hydrolyzate (LH) (9). After 24 hr, the serum-free conditioned media (CM) were collected; also, the cells were washed twice with PBS, lysed by the addition of Triton X-100 (0.2% v/v in water) and scraped from the surface of the dish with a plastic policeman for protein determination.

Serum-free CM were kept frozen at -20°C until assay for PA activity. For this purpose, ¹²⁵I-fibrin-coated wells of Linbro Disposo Trays were used, and the assay mixture contained 2 μg human plasminogen (purified from outdated human plasma) and 10 μL CM in 250 μL 0.1 M Tris chloride, pH 8.1 (9).

All experiments included appropriate controls for all reagents and media. No plasminogen-independent fibrinolysis could be detected when plasminogen was not included in the assay mixture. One unit of PA is defined as the amount that stimulates the release of 10% of the initial radioactivity in 4 hr (9).

**Lysozyme**

Lysozyme in serum-free CM was assayed by measuring the initial rate of lysis of a suspension of *Micrococcus luteus*, as described by Gordon et al. (11). Human lysozyme diluted in DB + 0.05 % LH was used as standard.

**Lysosomal Enzymes**

*N*-Acetyl-β-D-glucosaminidase and β-galactosidase were assayed as described by Bowers et al. (12).

**Protein**

Protein was measured by the method of Lowry et al. (13) with bovine serum albumin as standard.

**Results**

**Enhanced Macrophage PA Activity after Asbestos Exposure in Vitro**

Phagocytosis of digestible and nondigestible materials enhances the PA activity of mouse peritoneal macrophages (4, 5). Figure 1 demonstrates that asbestos fibers can increase the PA activity of peritoneal macrophages obtained from endotoxin-injected mice and also PBS-injected mice as a control population. Comparable results were obtained whether the asbestos addition was allowed for 2 hr or for 24 hr prior to the PA measurement. For asbestos doses less than 100 μg, there did not appear to be any gross alterations in cell morphology, but with 100 μg fiber there was some cell rounding and detachment after 3-4 days. The control, unstimulated peritoneal macrophages released PA activity in the presence of the asbestos, but there was a longer lag period than for
and hence PA activity increased owing to asbestos-stimulated plasminogen-dependent fibrinolytic activity. The gluco- corticoids, dexamethasone and prednisolone, are effective whereas progesterone is not. The data using latex feeding to cells are included for comparison. This inhibition of the increased fibrinolysis persists for at least 72 hr, the maximum assay time.

Table 2 lists the relative efficacies of different steroids. Also included for comparison are colchicine and vinblastine, drugs implicated in the control of secretory processes. Certain glucocorticoids are again effective. It is interesting that the activity profile for the steroids is similar to that found for their suppression of the PA activity of thioglycollate-induced macrophages (9). Colchicine and vinblastine had little influence on the cells if plated on 125I-fibrin.

**Inhibition of in Vitro-Stimulated Fibrinolytic Activity with Drugs**

If the assumption is correct that the generation of excessive PA activity around macrophages interacting with asbestos fibers would be deleterious, then it would be desirable to find drugs which might suppress this local proteolysis. In Table 1, a comparison is made of three steroids for their effect on the asbestos-stimulated plasminogen-dependent fibrinolytic activity. The glucocorticoids, dexamethasone and prednisolone, are effective whereas progesterone is not. The data using latex feeding to cells are included for comparison. This inhibition of the increased fibrinolysis persists for at least 72 hr, the maximum assay time.

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(Table 2) but were quite effective if harvest fluid is assayed for the PA activity from asbestos-treated cells (see below). It can be noted that the PA activity of the endotoxin-primed cells themselves was also inhibited. From Table 1 and 2, it can be observed that

**Figure 1.** Effect of asbestos feeding in vitro on macrophage fibrinolysis. Before the asbestos feeding, 2 x 10^6 peritoneal cells were cultured for 24 hr on 125I-fibrin-coated Linbro wells. The cells were then washed once with PBS, then various quantities of the solid particles added in 1 mL DB containing 5% FBS and 100 µg/mL STI for 2 hr at 37°C, the excess particles removed by washing twice with PBS and then 2 mL of DB containing 5% ATBFS added. After washing, the solid particles were added in DB + 5% FBS and 100 µg/mL STI for 2 hr at 37°C. After washing to remove excess particles, DB + 5% ATBFS was added in presence or absence of drug. Samples assayed as in Fig. 1.
there is a comparable effect of the drugs for cells fed latex particles.

The most potent glucocorticoid tested was dexamethasone and, as can be seen from Table 3, it is effective even at doses as low as $10^{-9} M$. This low dose was also found to be effective on thioglycollate-induced macrophages (9).

### Enzyme Secretion after Asbestos Exposure in Vitro

The above experiments were all carried out with cells plated directly on the fibrin substrate. Macrophages obtained from endotoxin-injected mice can also secrete elevated PA levels after exposure to asbestos (or latex) in vitro (Table 4); the increase is predominantly extracellular (data not shown), indicating that enzyme synthesis and secretion were tightly coupled (9). There was no effect of asbestos (or latex) on the secretion either of lysozyme or of two lysosomal enzymes, N-acetyl-β-D-glucosaminidase and β-

galactosidase (Table 4). Further evidence in support of the concept of differential secretion control for PA and for lysozyme and lysosomal enzymes is the fact that dexamethasone and colchicine, drugs which have a dramatic effect on the extracellular PA levels, fail to alter the levels of lysozyme and lysosomal enzymes from macrophages when they are treated with asbestos or latex particles.

### Discussion

Previous studies (4, 10), have indicated that intraperitoneal injection of IARC chrysotile type B into mice induces an inflammatory exudate and that macrophages cultured from such exudates have elevated levels of PA. It was also shown that peritoneal macrophages when treated with the same asbestos fibers in vitro are stimulated to produce more PA activity. The possible significance of macrophage PA activity in inflammation has been outlined (9, 10). It has been shown above that as little as 0.1 μg of the asbestos fibers can raise the PA activity of endotoxin-primed mouse peritoneal macrophages. It is tempting to speculate that, since the in vitro responses resemble those found with latex particles, it is phagocytosis of the asbestos fibers which results in the heightened protease activity. However, until a quantitative analysis of fiber uptake is made, such a conclusion is unwarranted, particularly as other agents, such as phorbol esters (7, 8), concanavalin A (7) lymphokines (6) and colony-stimulating factors (14), which are presumably not acting via a phagocytic pathway, can all stimulate macrophage PA activity.

The enhanced PA activity is suppressed by anti-inflammatory glucocorticoids. It might be profitable
Table 4. Effect of drugs on secretion of plasminogen activator, lysozyme and lysozomal enzymes after adding asbestos and latex in vitro.

| Macrophages (2 × 10^6) | Drug added (10^-7 M) | Cell protein/dish, μg | Secreted plasminogen activator units/mg cell protein | Lysozyme secreted, μg/mg cell protein | N-Acetylβ-D-glucosaminidase secreted μmole/min/mg cell protein (× 10^6) | β-Galactosidase secreted μmole/min/mg cell protein (× 10^6) |
|-------------------------|----------------------|----------------------|-----------------------------------------------------|----------------------------------------|--------------------------------------------------------------------------|-----------------------------------------------------------------|
| Endotoxin               | —                    | 130                  | 620                                                 | 17.6                                   | 6.0                                                                      | 40                                                             |
| Endotoxin + asbestos (100 μg) | —            | 190                  | 2380                                                | 17.4                                   | 8.1                                                                      | 47                                                             |
| Endotoxin + latex       | —                    | 250                  | 2430                                                | 15.8                                   | 4.4                                                                      | 36                                                             |
| Endotoxin               | Dexamethasone        | 130                  | 240                                                 | 18.7                                   | 8.0                                                                      | 58                                                             |
| Endotoxin + asbestos (100 μg) | Dexamethasone | 210                  | 110                                                 | 16.3                                   | 7.3                                                                      | 59                                                             |
| Endotoxin + latex       | Dexamethasone        | 250                  | 280                                                 | 11.5                                   | 3.6                                                                      | 29                                                             |
| Endotoxin               | Colchicine           | 90                   | 140                                                 | 14.6                                   | 10.0                                                                     | 56                                                             |
| Endotoxin + asbestos (100 μg) | Colchicine | 100                  | 210                                                 | 14.6                                   | 11.1                                                                     | 75                                                             |
| Endotoxin + asbestos    | Colchicine           | 150                  | 310                                                 | 10.2                                   | 8.3                                                                      | 60                                                             |

* Peritoneal cells were cultured in DB containing 5% FBS for 24 hr. The cells were then fed asbestos or latex for 2 hr at 37°C and then, after washing, placed in DB containing 0.05% LH in the presence or absence of the drug. CM and cell lysates were collected after 48 hr.

if more were known about how glucocorticoids are working in this context; perhaps these drugs are inducing a "macrocortin-like" molecule which has been proposed to account for the manner in which they suppress cellular prostaglandin production (8, 15). It is again worth noting that, for macrophages, lysozyme and two lysosomal enzymes activities appear to be under different regulatory control to the PA activity.

Studies of carcinogenesis by various forms of asbestos, with and without hydrocarbon carcinogens, have shown that chrysotile can augment considerably the number of malignant tumors produced by benzo[a]pyrene (16, 17); these studies and others have suggested that asbestos fibers can function as cocarcinogens (promoters). Interestingly, we have shown that the tumor promoter in skin, namely, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), and asbestos fibers, elicit similar acute inflammatory responses in rabbit skin (18) and, as mentioned above, can both increase the PA activity of macrophages. We have suggested that vascular alterations due to an inflammatory reaction to both asbestos fibers and to TPA ought to be considered in any analysis of the development of neoplasms resulting from their action (8, 18).

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