

Biochemical Components of Bronchoalveolar Lavage in Early Experimental Asbestosis of the Sheep: Phospholipase A₂ Activity, Prostaglandin E₂ and Proteins

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Biochemical analyses of bronchoalveolar lavage (BAL) supernatants of sheep treated with weekly intratracheal instillations (for 6 months) of saline, 2 mg, or 128 mg of asbestos (chrysotile B; UICC) were performed. Our results showed that proteins (either total or its various components) and phospholipase A₂ activity were unchanged in the low exposure group as compared to controls. However, in the high exposure group, with histopathological evidence of early asbestosis, there were significant increases in total proteins, albumin, α_2 -globulin, β - and γ -globulins as well as phospholipase A₂ activity of BAL fluid. Prostaglandin E₂ activity was significantly increased in both low and high dose groups. These changes in protein and lipid components of BAL following asbestos exposure constitute early indices of lung inflammatory reactions which may contribute to the development of asbestosis.

Introduction

Exposure to asbestos fibers produces various biological reactions in the lungs and could lead to asbestosis. The basic mechanisms at molecular and biochemical levels underlying the development of this disease remain unknown. The recent use of bronchoalveolar lavage (BAL) and its application to sampling both cellular and humoral elements represents a simple technique to have access to the alveolar milieu and to obtain major information for asbestosis diagnosis. Indeed, during the development of this disease, certain lung cells, especially the macrophages, ingest asbestos fibers and release, as a consequence of the resultant inflammatory reactions, a series of substances such as proteins and en-

zymes, components of complement, and mediators as well as modulators of various cell functions (1). Changes in the content of some of these factors in BAL fluid have been noted by many investigators. For instance, Bignon et al. (2) have found increases of albumin and immunoglobulins in BAL fluids in asbestosis, whereas other studies failed to demonstrate significant changes (3, 4). Enhancements of protein contents into the alveolar space were also reported by Alpert et al. (5), Gardiner and Shanker (6), Henderson et al. (7) and Gross (8) in lung inflammatory reactions.

In previous experiments (9, 10), we have studied the sequence of lung events in sheep exposed to monthly low doses of asbestos and found that during the initial period of 6 months, asbestos did not modify normal pulmonary histology and physiology but induced changes in cell populations and in protein contents of BAL fluid. The present study was designed to analyze lipid factors (phospholipase A₂

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and prostaglandin E_2) and proteins (total proteins and components) in longer term experiments when animals were given high doses of asbestos fibers.

Material and Methods

Induction of Asbestosis

Male sheep weighing 25-50 kg each were used in this investigation. One group was given intratracheal monthly instillations of sterile saline (50 mL) and served as controls. Other sheep were divided into small groups and received monthly instillations 1, 2, 4, 8 and 16 mg of Canadian chrysotile B (UICC standard) (11) in 50 mL of saline for 3 months followed by doses between 2 and 128 mg for an additional 3 months. Since the low-dose exposures over a 6-month period failed to produce histopathological or functional changes, the treated groups were redivided into two groups of six animals: one receiving weekly instillations of 2 mg and the other, 128 mg. The control group received weekly intratracheal saline.

Bronchoalveolar Lavage

Monthly bronchoalveolar lavages were performed as described previously (12) in all sheep in random order. In brief, 3×50 mL of phosphate-buffered saline were infused through the work channel of the bronchoscope in randomly selected subsegmental bronchi and effluent was withdrawn the same way. The BAL effluents (approximately 105 mL) were filtered with cheese cloth and centrifuged to remove the cell pellets.

Phospholipase A_2 Activity

Phospholipase A_2 activity of BAL effluent was performed as described by Sirois et al. (13). In brief, 1 μ g of cold dioleoyl phosphatidylcholine and 25 nCi of 14 C-dioleoyl phosphatidylcholine were added to 1 mL of BAL supernates. Following 3 hr of incubation, each sample was mixed with 4 mL of a solution of chloroform:methanol:1 M HCl:butylated hydroxytoluene 0.5% (60:30:0.5:0.1) kept at 4°C for at least 1 hr and centrifuged (10 min; 1000g). The aqueous phase was removed and the organic phase was washed and spun (10 min, 1000g) twice with 1.4 mL of a solution of chloroform:methanol:0.58% NaCl (3:48:47) and evaporated under vacuum (EvapoMix Buchler). Lipid extracts were resuspended in 0.5 mL of chloroform:methanol (2:1) and aliquots (100 μ L) were spotted on dried Silica Gel HP-TLC plates (10 \times 10 cm) (BDH Chem.). The chromatography was run in chloroform:methanol:acetic acid:water

(25:15:4:2) for 40 min followed by drying of the plates and exposition to iodine vapor to reveal the lipid spots. Plates were divided according to the spots, the silica gel was scraped off, transferred to scintillation vials and counted. Phospholipase A_2 activity was expressed as the percent amount of labeled fatty acids formed as compared to the total 14 C-phosphatidylcholine added.

Prostaglandin Determination

Prostaglandin E_2 -like activity was measured as described by Sirois and Gagnon (14). Aliquots (5 mL) of BAL supernatant were acidified to pH 4.5 with citric acid, extracted twice with two volumes of ethyl acetate and dried under vacuum. Extracts were reconstituted in 0.5 mL of Krebs solution and bioassayed on superfused, inhibitors-treated rat stomach strips. Results were expressed in nanograms of prostaglandin E_2 (PGE $_2$) per milliliter of BAL supernates.

Proteins Determination

A 50 μ L portion of unconcentrated BAL fluid was used to measure total protein contents according to the method of Pesce and Strande (15). Albumin, α_1 -globulins, α_2 -globulins, β and γ -globulins were quantitated in concentrated aliquots of BAL fluid (100-fold; Amicon B-15 filters) by electrophoresis on cellulose acetate support with the model R-101 Microzone Electrophoresis Cell (Beckman Instruments). Values were expressed in μ g/mL of BAL supernates.

Drugs Used

The following drugs were used: atropine sulfate, L- α -phosphatidylcholine dioleoyl and butylated hydroxytoluene (Sigma Chemical Co.); L- α -dioleoyl-1- 14 C phosphatidylcholine (Applied Science Lab.); the following drugs were supplied as generous gifts: methysergide hydrogen maleate (Sandoz Ltd); propranolol hydrochloride (Dr. L. Smith of Ayerst Lab.); phenoxybenzamine hydrochloride (Dr. H. A. Sheppard of Smith, Kline & French); diphenhydramine hydrochloride (Dr. E. McMullen of Parke, Davis & Co.); prostaglandin E_2 (Dr. J. E. Pike of Upjohn Co.). Canadian chrysotile B was obtained from Union Internationale Contre le Cancer (UICC).

Statistical Analysis

All data were analyzed for statistical significance using Student's *t*-test.

Results

BAL Lipid Components

Evidence supports the role of prostaglandins in various inflammatory reactions. Among the enzymes involved in prostaglandin synthesis, phospholipase A_2 , which hydrolyzes phospholipids and releases fatty acids such as arachidonic acid, is believed to be the rate-limiting step in their formation and was measured in the first series of experiments. As illustrated on Figure 1, bronchoalveolar fluids of sheep which never received intratracheal asbestos administration contained some phospholipase A_2 activity, as shown by the $1.16 \pm 0.25\%$ fatty acids formed when the BAL fluids were incubated with labeled phosphatidylcholine. The chronic administration (2 mg weekly for more than 6 months) of Canadian chrysotile B did not alter the activity of the enzyme in BAL supernatants ($1.22 \pm 0.05\%$). However, the weekly administration of 128 mg of asbestos strongly stimulated the activity of phospholipase A_2 . In these conditions, the percent formation of fatty acids from the labeled precursor increased to $3.23 \pm 0.53\%$. This threefold enhancement which represents either the secretion of new enzyme by the lung cells or its activation was significant. Phospholipase activity remained elevated in

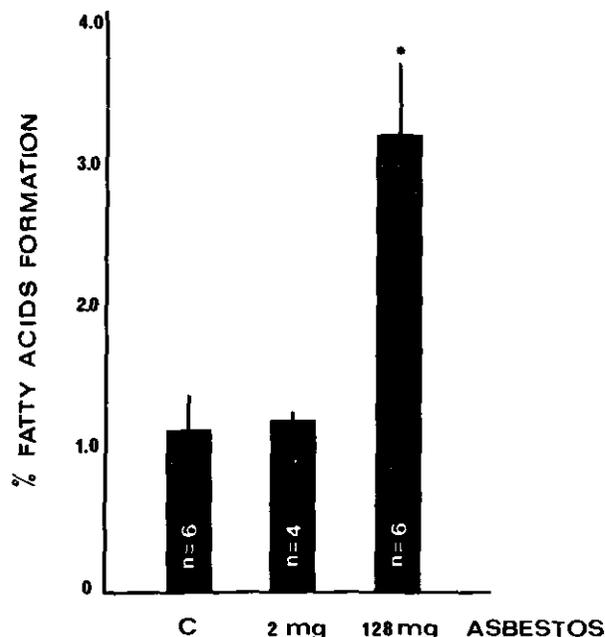


FIGURE 1. Effects of weekly (6 months) intratracheal instillations of saline (C), 2 mg and 128 mg of asbestos on phospholipase A_2 activity of sheep bronchoalveolar lavage supernatants. The activity of the enzyme is expressed as a percentage of formation of radiolabeled fatty acids \pm SEM. n is the number of sheep in each group. Statistical significance ($p < 0.05$) is shown with an asterisk.

BAL fluids of the high exposure group in subsequent months.

Figure 2 shows the effects of asbestos exposure on the prostaglandin contents of BAL supernatants. In BAL fluids of control animals, prostaglandin E_2 -like activity, as determined on the rat stomach strip, averaged 0.26 ± 0.07 ng/mL. Administration of low quantities of asbestos (2 mg) to the sheep doubled the amount of prostaglandins released in the BAL fluid (0.5 ± 0.04 ng/mL). Higher asbestos exposure further increased the amount of prostaglandin material in the BAL supernatants (0.66 ± 0.19 ng/mL), which suggested the dose dependency of the inflammatory reactions induced by the fibers.

BAL Protein Components

Total proteins, albumin and globulins were determined in aliquots of sheep BAL supernatants. As shown on the upper panel of Figure 3, the mean concentration of proteins of BAL liquid phase of normal sheep was around 200 μ g/mL. Although weekly treatment of the animals with 2 mg of asbestos for a relatively long period of time did not modify the total protein concentrations of BAL fluid, the treatment with larger doses of asbestos (128 mg)

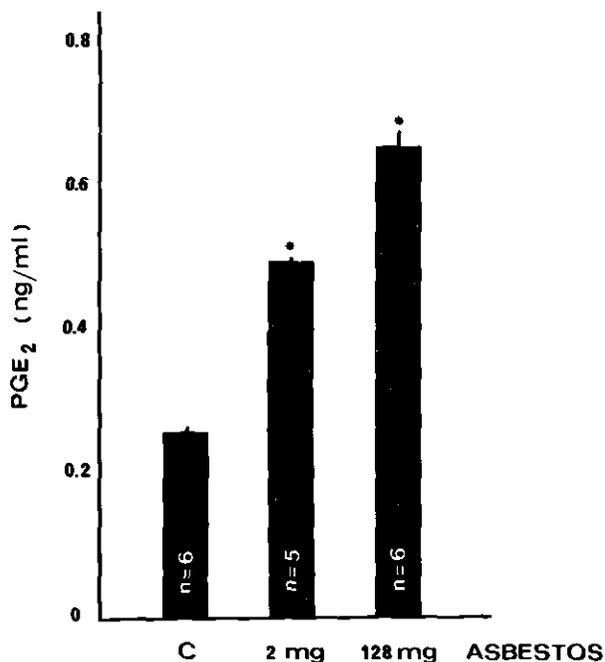


FIGURE 2. Prostaglandin E_2 -like activity (PGE₂) in bronchoalveolar lavage supernatants of sheep following weekly exposure (6 months) to low (2 mg) or high (128 mg) doses of asbestos as compared to exposure to saline (C). Vertical bars are means \pm SEM. n represents the number of sheep in each group. Statistical significance ($p < 0.05$) is shown with an asterisk.

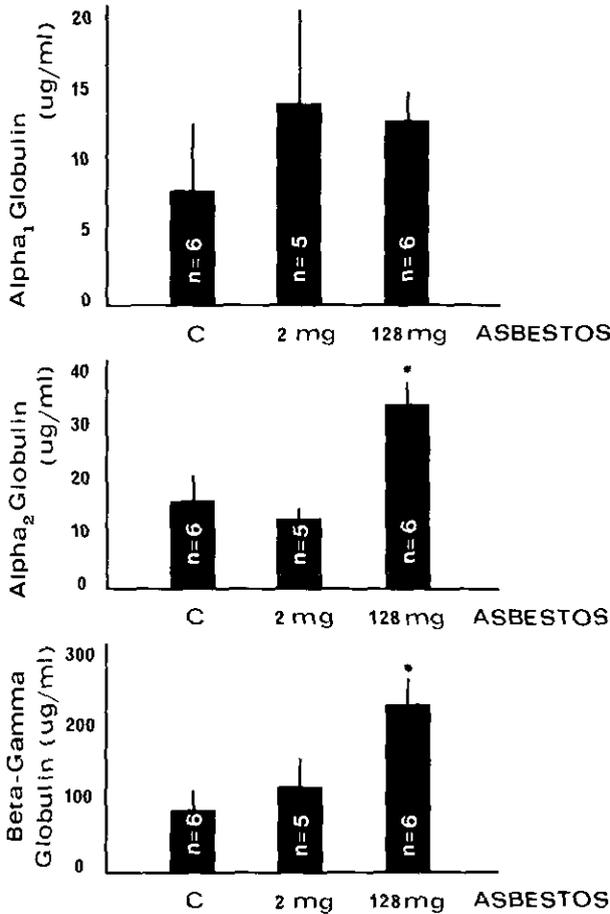


FIGURE 3. Effects of weekly (6 months) intratracheal instillations of saline (C), 2 mg and 128 mg of asbestos on total proteins (upper panel) and albumin (lower panel) in sheep bronchoalveolar lavage supernatants. Vertical bars are means \pm SEM. n represents the number of sheep in each group. Statistical significance ($p < 0.05$) is shown with an asterisk.

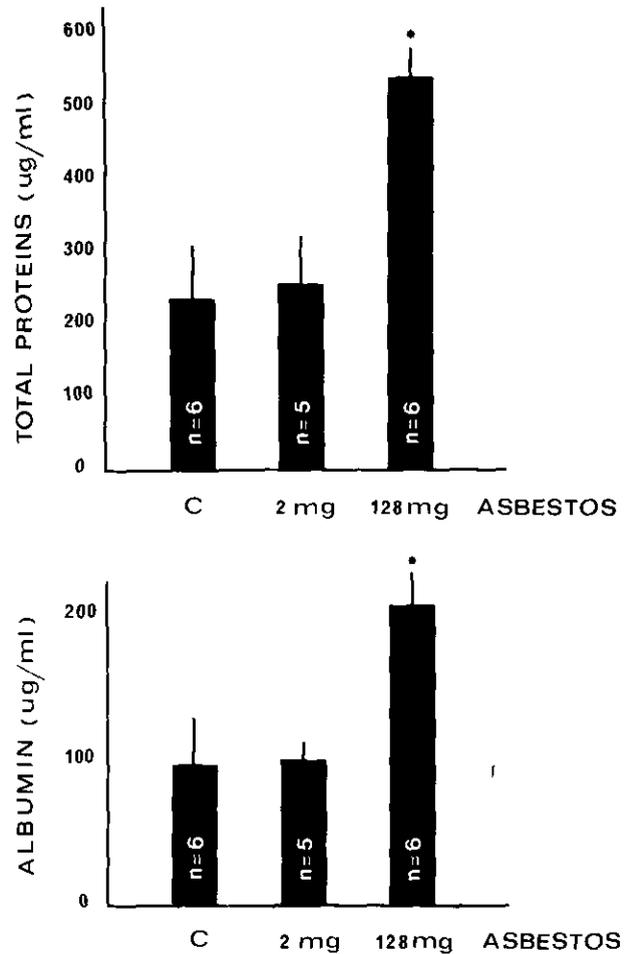


FIGURE 4. Effects of weekly (6 months) intratracheal instillations of saline (C), 2 mg and 128 mg of asbestos on α_1 -globulin, α_2 -globulin and β - and γ -globulins in sheep bronchoalveolar lavage supernatants. Vertical bars are means \pm SEM. n represents the number of sheep in each group. Statistical significance ($p < 0.05$) is shown with an asterisk.

produced more than 2-fold enhancements of the proteins in the lavage fluids.

Similar results were obtained with the albumin concentration as measured by electrophoresis on cellulose acetate. As illustrated on the lower panel of Figure 3, the concentration of albumin averaged 100 $\mu\text{g}/\text{mL}$ both in controls and in low exposure animals. However, the values increased to approximately 200 $\mu\text{g}/\text{mL}$ in BAL supernates of sheep which were exposed to 128 mg of asbestos weekly.

Globulin fractions of BAL fluids were also estimated in controls and asbestos-treated animals. α_1 -Globulins were quite variable from one animal to the other and were not altered significantly by either low or high doses of asbestos (see the upper panel of Fig. 4). α_2 -Globulin values, on the other

hand, were around 10 $\mu\text{g}/\text{mL}$ in control, and low exposure groups and increased significantly in the highly exposed animals (approximately 30 $\mu\text{g}/\text{mL}$). Finally asbestos exposure produced changes of the BAL contents of β - and γ -globulins. Again the values for control and low exposure groups were similar (approximately 100 $\mu\text{g}/\text{mL}$), while they increased significantly to approximately 200 $\mu\text{g}/\text{mL}$ in animals treated with weekly intratracheal instillations of 128 mg of asbestos.

Subsequent determinations of phospholipase A_2 activity, prostaglandins, total proteins and protein components in the same groups of sheep successively 1 and 2 months after these series of experiments gave results very similar to those presented herein.

Discussion

In the present study, we have analyzed early biochemical alterations, namely, lipid and protein components, in the sheep lungs during the development of experimental asbestosis. Our results have shown that repeated weekly instillations of large doses of asbestos (128 mg) for a few months can increase phospholipase A₂ activity in the BAL fluid, whereas low amounts of asbestos (2 mg weekly) appeared to have very little effect. Phospholipase A₂, an enzyme involved in the control of surfactant levels and of inflammatory reactions, hydrolyzes phospholipids and releases fatty acids which can immediately be converted to various oxidation products such as the hydroxy acids or the very potent prostaglandins and leukotrienes. The presence of this enzyme was also noted by Sahu and Lynn (16) in human and by Masliah et al. (17) in rat BAL fluids. Its activity was also shown to increase in cells during the inflammatory reactions induced by exposure to toxicants such as silica or asbestos (13, 18). However, many questions remain unresolved regarding this enzyme. For example, it appears that phospholipase A₂ activity exists in various cell pools and that selected stimuli could influence specific pools. Whether or not asbestos could stimulate one or more enzyme pools remains speculative. Furthermore, from our results, it is impossible to know if the increased formation of fatty acids by BAL fluids is due to the activation of the enzyme, to stimulation of its active formation by lung cells, to cell disruption by asbestos or to leakage from the vascular compartment.

Our results showed that asbestos exposure could increase the BAL levels of prostaglandin E₂. These substances are potent modulators of the inflammatory reactions and are probably the results of un-specific lung insults. Since both the prostaglandin formation and the phospholipase activation were previously shown to take place in macrophages incubated with asbestos (13), it is likely that asbestos has evoked similar reactions *in vivo* and that the prostaglandins were released by macrophages.

Because phospholipase A₂ activation is believed to be one of the first steps in the onset of inflammatory reactions and because its increased activity leads to production of inflammatory mediators having potent effects on vascular permeability, enhancements of its activity could also induce the protein accumulation. Weekly exposure to large doses of asbestos (128 mg) has indeed altered the concentration of specific proteins in BAL fluids, whereas exposure to low doses (2 mg) did not change BAL protein contents as compared to controls. Our results showed that, among the total proteins, specific entities such as albumin, α_2 -globulins and β - and γ -globulins were the most affected by asbestos treat-

ment. With regards to albumin, a protein which is not supposed to be formed in the bronchoalveolar milieu, our results support the hypothesis that asbestos exposure could modify vascular permeability and lead to leakage from the circulation into the alveolar space.

The increases in the BAL contents of α_2 -, β - and γ -globulins by asbestos exposure are also probably the result of vascular leakage as it is already well established that asbestos may cause immunological stimulations (19) and increases in circulating globulins (2) prior to the development of the well-established asbestosis. However, our results showed that α_1 -globulins were not significantly changed by asbestos exposure, suggesting the possibility of selective protein leakage or of local production by the epithelium cells lining the airways of the lungs.

In conclusion, we have shown that repeated intratracheal instillations of asbestos into sheep lungs produced, in a few months, alterations in phospholipase A₂ activity, in prostaglandin E₂ levels, in total proteins, albumin, α_2 -, β - and γ -globulins in BAL fluids. Whether these lipid and protein changes precede and induce the inflammatory reaction or are the results of it, remains an open question. These changes, however, are clearly related to the onset of the disease and could constitute useful indices for understanding its development and could possibly be useful markers of early lung injury.

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