

Biochemical Studies on the Toxicity of Slate Mine Dust

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As part of a detailed experimental study of the pathogenicity of disease of slate dust workers, the early biochemical changes in rat lung from 1 to 90 days after intratracheal inoculation of slate dust of particle size below $5\ \mu\text{m}$ were investigated. A severalfold increase in free cell population (initially macrophages) was elicited by the dust. The free activity of acid phosphatase tended to increase along with a break of lysosomal latency with increasing exposure period. However, actual release of enzyme activity into the acellular fraction was low. The phospholipid content varied both in cellular and acellular fractions, indicating altered turnover of membrane lipids and surfactants. At advanced periods of the study, sialic acid was found to be released into the acellular fraction, indicating membrane damage. Considerable decrease in glucose-6-phosphate dehydrogenase activity and free sulfhydryl content and enhanced osmotic fragility of erythrocytes were also recorded. These results indicate the potential toxicity of slate mine dust.

Introduction

The wandering cells of the lung lavage, the alveolar macrophages, are the first line of the body's defense against foreign materials and are often the first casualties of the biological effects of dusts (1, 2). Their plasma membranes are damaged by the cytotoxic dusts *in vitro* and *in vivo* (3-5). Biochemical studies have also revealed that these cytotoxic dusts cause an enhanced turnover of pulmonary surfactant (6, 7). The cytotoxic effects of slate dust (from Mandasaur, India) with the use of erythrocytes, in an *in vitro* model system, have already been described (8). In the present report, we present the cytotoxic effects and early biochemical changes *in vivo* in the lung lavage and in the blood of albino rats exposed to slate dust.

Materials and Methods

Dust

Slate dust collected from the affected areas of Mandasaur was a gift of Mr. M. M. Lal and Dr. S. K. Bhargava of the Industrial Toxicology Research

Center (ITRC). Dust with particle size below $5\ \mu\text{m}$ was prepared according to the procedure described by Zaidi et al. (9).

Animal Experimentation

Female albino rats from the ITRC colony, weighing 150-200 g, were used. The dust sample and 0.15 M NaCl were sterilized separately by autoclaving at 15 lb for 15 min. Seventy adult albino rats were administered intratracheally 50 mg of dust suspended in 1 mL of 0.15 M NaCl (9). Another 70 rats received only 1 mL of 0.15 M NaCl and served as controls. The animals were maintained on a commercial pellet diet supplied by Hindustan Lever Ltd., India, and were sacrificed 1, 2, 4, 8, 16, 30 and 90 days after treatment. Blood was collected from the jugular vein into heparinized centrifuged tubes. The lung lavage was collected according to Brain (10).

Treatment of the Lavage

Washings of lungs of each rat were pooled. An aliquot was taken for counting the free cell population. For separating cells from the medium, the lavage was centrifuged at 300g for 20 min in cold. The pellet was resuspended in 2.5 mL of 0.15 M NaCl. The supernatant and washings constituted the acellular fraction while the suspension of the sediment formed the cellular fraction.

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Estimations

Acid Phosphatase. The assay system (11) contained 2.0 mL substrate mixture (0.5 g sodium β -glycerophosphate and 0.4 g sodiumdiethylbarbiturate dissolved in water, pH adjusted to 5.0 with 1N acetic acid and made up to 100 mL), 0.2 mL cell suspension and 0.3 mL water. Incubation was carried out at 37°C for 30 min and reaction stopped by the addition of 1.5 mL 10% trichloroacetic acid (TCA). After centrifugation, aliquots from supernatant were taken for orthophosphate determination (12). The latency of the acid phosphatase in lysosomes was abolished by preincubation with 0.5% Triton X-100.

Silicic Acid. The procedure of King et al. (13) was followed for the estimation of silicic acid.

Sialic Acid. Sialic acid contents were determined by the method of Krantz and Lee (14).

Phospholipids. Phospholipids were extracted according to Folch et al. (15) and determined by the method of Wagner et al (16).

Studies on Blood

The heparinized blood was centrifuged and the plasma separated. The cells were washed three times with 0.15 M NaCl and suspended in the same medium to requisite concentration.

Glucose-6-phosphate (Glu-6-P) Dehydrogenase. The enzyme was assayed according to the procedure of Zaheer et al. (17). The reaction mixture contained, in a final volume of 3.0 mL, 0.25 mL of 0.1 M MgCl₂, 0.5 mL of 0.2 M Tris-HCl buffer, pH 7.6, 0.05 mL nicotinamide adenine dinucleotide phosphate (50 mg/mL), 0.05 mL of 0.05 M glucose-6-phosphate Glu-6-P and 0.2 mL of 1% packed red blood cells (RBC). After deproteinization, the contents were centrifuged at 300g for 10 min the optical density (OD) was read at 340 nm in a Unicam SP 500 spectrophotometer. Incubation period was 5 min at 30°C.

Glutathione (Reduced). Estimation of reduced glutathione was done according to Sedlak and Ludsay (18).

Protein. Protein content was estimated by the method of Lowry et al. (19) with bovine serum albumin (Sigma Type V) as standard.

Chemicals. All the chemicals used were either BDH, AnalaR or E. Merck, extra pure.

Statistics

All values are the result of at least six determinations per point. Standard deviations were less than 10% of the mean value.

Results and Discussion

Free Cell Population

Changes in the number of free cells in the lung are shown in Figure 1. Untreated animals had between 6.017 and 9.723 $\times 10^6$ free cells. Slate dust stimulated the number of free cells even after 24 hr of exposure and the effect was at its maximum after 4 days.

Thus, during the early response to a foreign body, there is enhanced assembly of wandering cells from circulation. By the end of 90 days, the increase in cell population is, however, only 2-fold. Decrease of cells with time may be indicative of the potential cytotoxic effect of the dust.

Acid Phosphatase Activity

Changes in the overt activity of acid phosphatase at different times of exposure are shown in Figure 2. Only negligible free activity of acid phosphatase was detected in the acellular fraction obtained from the control animals. Thus, in none of the animals was the activity more than 8% of the total activity.

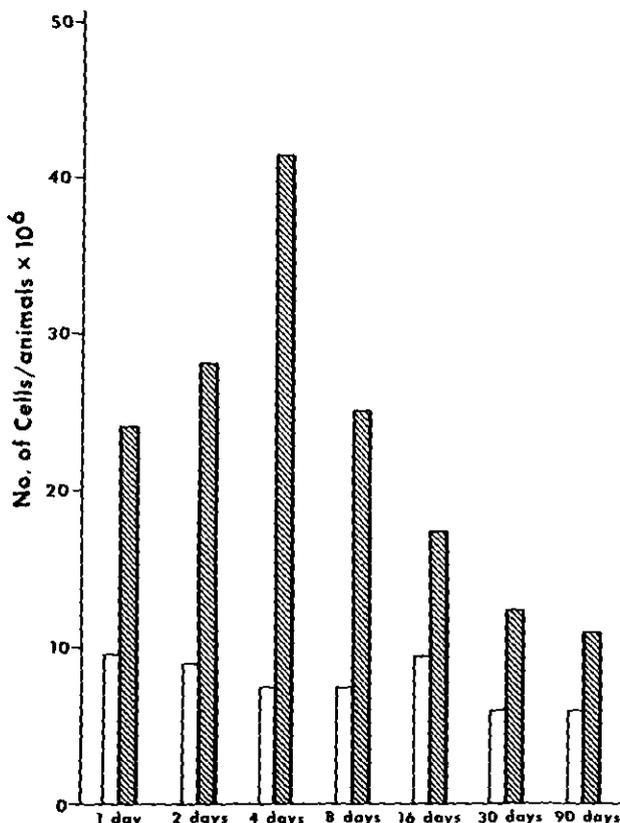


FIGURE 1. Changes in the number of free cells in the lung of normal and slate dust-exposed albino rats: (□) control; (▨) experimental.

The cells were mostly undamaged and the lysosomes retained their membrane-bound latency. The small amount of free activity detected could be attributed to some membrane damage to the cells during preparation. This activity was relatively constant throughout the experiment. It can be assumed, therefore, that the changes encountered with lavage from the experimental animals represent a true physiological adaptation to stress induced by slate dust.

Lavage from dust-treated animals showed uniformly high phosphatase activity. This trend of enhanced activity persisted during the entire period of treatment.

In the cellular fraction, the increase in acid phosphatase was steady up to 8 days, but declined thereafter. Even then, at all stages, the activity was relatively higher in the experimental group. In Figure 3, in the case of controls, the activation by 0.5% Triton-X 100 exposure was 3 ± 0.37 for the entire period. Beyond 4 days, the extent of activation tended to decrease for the experimental animals, gradually reaching 1.95 by 90 days. It is interesting to note

that the change in free activity was correlated with the gradual loss of latency of the cellular function. The latency is not totally broken even at the most advanced state since a doubling of activity occurs on detergent treatment. Apparently, slate dust *in vivo* under the conditions of the present experiment caused partial macrophage membrane damage. Thus, in spite of its high cytotoxicity and hemolytic activity, slate dust is apparently not able to break

Activation is expressed as activity with Triton X-100/activity without detergent.

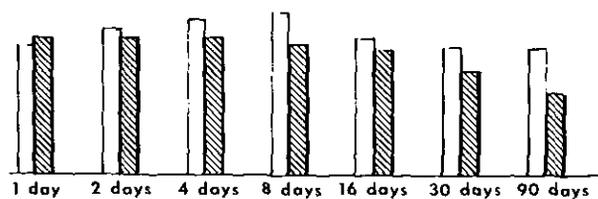


FIGURE 3. Activation of acid phosphatase activity of cellular fraction from lung lavage of normal and slate dust-exposed rats: (□) control; (▨) experimental.

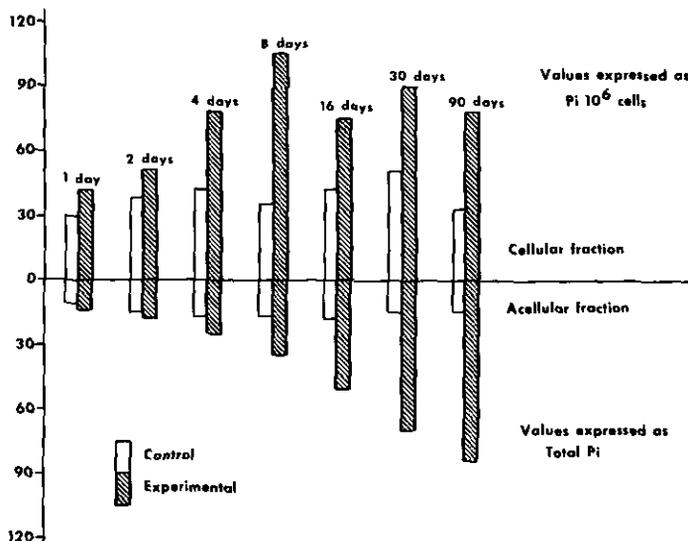


FIGURE 2. Acid phosphatase activity in cellular and acellular fractions in normal and slate dust-exposed rats.

Table 1. Total phospholipids in acellular and cellular fractions of lung lavage in normal and slate dust-treated rats.

	Pi, $\mu\text{g/l}$ total lung lavage						
	1 day	2 days	4 days	8 days	16 days	30 days	90 days
Acellular fraction							
Normal	61.48	56.256	64.13	58.176	58.86	43.464	48.67
Treated	22.08	17.913	47.112	52.296	45.834	110.35	147.84
Cellular fraction^a							
Normal	12.05	8.25	12.22	9.88	14.80	11.02	7.55
Treated	16.17	10.02	21.73	12.73	15.60	10.47	9.244

^aValues in cellular fraction are expressed as μg total Pi.

internal membranes of macrophages. Release of lactic dehydrogenase (unpublished data) also indicated plasma membrane damage.

Phospholipids in Lung Lavage

The phospholipid content of cellular fraction was almost the same in both cases, except at 4 days (Table 1). When phospholipid content was compared, it varied from 0.92 to 1.86 $\mu\text{g}/10^6$ cells for controls, the minimum and maximum being at 2 days and 30 days. For the slate-treated animals, the values were in the range of 0.51 and 0.84, the two extreme values being recorded at 2 days and 16 days. Thus, phospholipid contents of macrophages vary considerably during the cellular response to slate dust; for instance, being 75% higher on day 4, presumably due to larger number of cells.

In the acellular fraction, the treated animals had only a third of the phospholipid as compared to controls at 1 and 2 days. Some absorptive removal of nonmembrane phospholipid by the dust may account for it. Subsequently, the phospholipid in this fraction was enhanced. This may be due to availability of more cells and damage to membranes, so that by 8 days it was almost similar in controls. Subsequently, the content increased dramatically and at 90 days, it was approximately 300% of control. The differences become more marked when the phospholipid contents of acellular fraction are expressed as a percent of total content of lavage. In all the stages, 80-87% of total phospholipid was in acellular fraction in controls. For the experimental group, it was distinctly lower, i.e., 58, 64, 69 and 80% at 1,

2, 4 and 8 days, respectively. Later, it increased, reaching 91 and 94% at 30 and 90 days, respectively. Initial decrease could be due to its inactivation by macrophage phospholipase (20) and later increase a defense adaptation to protect cells against damage.

Sialic Acid Contents

After 2 days, sialic acid content per 10^6 cells of cellular fraction decreased, indicating alterations in biomembrane function and turnover. In acellular fraction of normal lung lavage, sialic acid was not detectable. In acellular fraction of treated animals, it appears after 4 days of dust exposure and increased up to 90 days (Table 2).

Since sialic acid is known to be released from membranes during cytotoxic effects of membrane damaging toxicants, this may be indicative of damage of macrophages. Removal of sialic acid could only be due to activation of sialidase (21), and this removal could make the cells even more vulnerable to damage. It will be interesting to explore the biochemical significance of this release as an index of cytotoxicity and of serum sialic acid levels in the development of slate toxicity.

Protein Content

In the cellular fraction there was an increase in protein content in experimental animals, as compared to controls, up to 4 days (Table 3). This increase of over 2-fold could only indicate additional synthesis of protein and not absorption of acellular protein on dust. The macrophages assembled for

Table 2. Total sialic acid contents in the lung lavage of normal and slate dust-exposed rats.

	Sialic acid, μg^a						
	1 day	2 days	4 days	8 days	16 days	30 days	90 days
Cellular fraction							
Normal	31.105	18.75	23.07	39.4	19.05	28.12	15.25
Treated	28.12	17.24	15.13	17.20	9.40	12.16	7.55
Acellular fraction							
Normal	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Treated	N.D.	N.D.	N.D.	3.26	2.99	1.99	4.01

^aValues are expressed as μg sialic acid/ 10^6 cells in cellular fraction whereas total sialic acid content is given in acellular fraction.

^bNot detectable.

Table 3. Protein contents in cellular and acellular fractions of lung lavage in normal and slate dust-treated rats.

	Protein, mg^a						
	1 day	2 days	4 days	8 days	16 days	30 days	90 days
Cellular fraction							
Normal	0.299	0.233	0.271	0.427	0.1895	0.152	0.261
Treated	0.625	0.608	0.658	0.408	0.278	0.392	0.2903
Acellular fraction							
Normal	0.135	0.241	0.162	0.205	0.162	0.156	0.098
Treated	0.275	0.235	0.143	0.196	0.293	0.294	0.176

^aValues in cellular fraction is expressed as mg protein/ 10^6 cells and in acellular fraction as total protein value in mg .

Table 4. Effect of slate dust on Glu-6-phosphate dehydrogenase activity, reduced glutathione content of erythrocytes and protein content in plasma.

	1 day	2 days	4 days	8 days	16 days	30 days	90 days
Glu-6-phosphate dehydrogenase activity, μ mole NADP reduced/min/mL RBC suspension.							
Normal	0.491	0.402	0.315	0.380	.383	0.446	0.419
Treated	0.393	0.380	0.277	0.280	0.234	0.275	0.369
Reduced glutathione, mg/100 mL packed cells							
Normal	53.39	45.32	48.73	39.08	46.87	47.80	46.56
Treated	52.14	45.52	39.11	40.50	41.25	38.18	34.21
Plasma Protein, mg protein/mL of plasma							
Normal	54.63	46.10	60.5	53.90	52.60	55.95	48.15
Treated	55.81	50.04	53.07	43.75	46.95	47.11	42.05

foreign body response could accomplish *de novo* synthesis of additional and new proteins to meet metabolic requirements for phagocytosis. Also, expressed in terms of whole lavage from one animal, the protein content is not merely due to increased cell populations. The maximum increase was at 4 days, 3-fold on a cell basis and 13-fold on a whole animal basis. In the acellular fraction also, there was a general increase in protein content, especially at 16 days, indicating release of cellular constituents. This is in agreement with the data on acid phosphatase and sialic acid. As with acid phosphatase, the acellular fraction contained only a small proportion of protein so that membrane damage and release is not very high.

Changes in Blood

In order to see whether the changes in lavage are reflected in circulation, some of its parameters were measured (Table 4). Erythrocyte Glu-6-P dehydrogenase tended to decrease, the maximum being 40% at 16 days. This, along with the RBC fragility *in vivo* (unpublished data), shows membrane sensitivity to the hemolytic effects of slate dust (22). The reduced glutathione content in RBC and plasma proteins showed a general tendency for decline in spite of the wide fluctuation in values from sample to sample. Thus, passage through dust-laden lungs and any impact of the lung lesion on the hemopoietic system could be reflected in blood chemistry.

Conclusion

In the case of slate dust exposure, the initial foreign body response consists mainly of the collection of scavenger cells and reaches a maximum at 4 days. Subsequently, as a result of cytotoxicity and possibly any damage to the lung tissue and the hemopoietic system, the number of free cells decreases with the progress of the toxic conditions. The increase in acid phosphatase activity in the acellular fraction and the gradual decrease of activation in the cellular fraction suggest a cytotoxic ac-

tion of slate dust *in vivo*. The release of sialic acid in the acellular fraction also indicates the alterations of biomembranes.

The above results, along with parallel studies on the hemolytic and solubility properties of slate dust (8), clearly suggest its toxicity *in vivo* for the pulmonary system in macrophages and in the blood. These facts and the similarity of many parameters to a cytotoxic, carcinogenic dust chrysotile, indicate that further in-depth *in vivo* studies are necessary to elucidate the total toxic action of slate dust and the negative impact of this agent on public and occupational health.

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REFERENCES

1. Moores, S. R., Black, A., Evans, J. C., Holmes, A., and Morgan, A. The effect of quartz, administered by intratracheal instillation, on the rat lung. *Environ. Res.* 24: 275-285 (1979).
2. Begin, R., Rola-Pleszczynski, M., Sirosis, P., Lemaire, I., Nadeau, D., Bureau, M. A., and Masse, S. Early lung events following low dose asbestos exposure. *Environ. Res.* 26: 392-401 (1981).
3. Beg, M. U., Farooq, M., Saxena, V., Rahman, Q., Viswanathan, P. N., and Zaidi, S. H. Biochemical studies on the cellular and acellular fraction of lung lavage and its relation with lung tissue in experimental asbestosis. In: *Environmental Pollution and Human Health* (S. H. Zaidi, Ed.), INSDOC, New Delhi, India, 1977, pp. 355-367.
4. Beck, E. G. Reactions of macrophages cultivated *in vitro* towards particulate and fibrous dusts. In: *Environmental Pollution and Human Health* (S. H. Zaidi, Ed.), INSDOC, New Delhi, India, 1977, pp. 233-247.
5. Admis, V., and Timar, M. Studies on the effect of quartz, bentonite and coal dust on macrophages *in vitro*. *Brit. J. Exptl. Pathol.* 59: 411-415 (1978).
6. Heppleston, A. G., Fletcher, K., and Wyatt, I. Changes in the composition of lung lipids and the "turnover" of dipalmitoyl lecithin in experimental alveolar proteinosis induced by inhaled quartz. *Brit. J. Exptl. Pathol.* 55: 384-395 (1974).
7. Wallace, W. E., Hedley, L. C., and Weber, K. C. Dipalmi-

- toyl lecithin surfactant adsorption by Koalin dusts *in vitro*. *J. Colloid Interface Sci.* 51: 535-537 (1975).
8. Singh, S. V., Viswanathan, P. N., and Rahman, Q. Interaction between erythrocyte plasma membrane and silicate dusts. *Environ. Health Perspect.* 51: 55-60 (1983).
 9. Zaidi, S. H. Experimental methods. In: *Experimental Pneumoconiosis*. Johns Hopkins Press, Baltimore, 1969, pp. 35-49.
 10. Brain, J. D. Free cells in the lungs: Some aspects of their role, quantitation and regulation. *Arch. Intern. Med.* 126: 477-487 (1970).
 11. Oser, B. L. Determination of serum phosphatase activity: In: *Hawk's Physiological Chemistry* 14th ed. (B. L. Oser, Ed.), McGraw-Hill, New York, 1965, pp. 1118-1121.
 12. Fiske, C. H., and Subbarow, Y. The colorimetric determination of phosphorus. *J. Biol. Chem.* 66: 375-400 (1925).
 13. King, E. J., Stancy, V. D., Holt, P. F., Yats, D. M., and Pickles, D. The colorimetric determination of silicon in the microanalysis in biological and mineral dusts. *Analyst.* 80: 441-453 (1955).
 14. Krantz, M. J., and Lee, Y. C. Sensitive auto-analytical method for sialic acids. *Anal. Biochem.* 63: 464-469 (1975).
 15. Folch, J., Less, M., and Stanely, G. H. S. A simple method for the isolation and purification of total lipids from animal tissue. *J. Biol. Chem.* 226: 497-509 (1957).
 16. Wagner, H., Lissau, A., Holzl, J., and Horhammer, L. The incorporation of p^{32} into the inositol phosphatides of rat brain. *J. Lipid Res.* 3: 177-180 (1962).
 17. Zaheer, N., Tewari, K. K., and Kirshnan, P. S. Exposure and solubilization of hepatic mitochondrial shunt dehydrogenases. *Arch. Biochem. Biophys.* 109: 646-648 (1965).
 18. Sedlack, V., and Ludsay, R. H. Estimation of total protein bound and non-protein-S H group in tissue with Ellmanic reagent. *Anal. Biochem.* 25: 192-205 (1968).
 19. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. Colorimetric determination of protein with Folin phenol reagent. *J. Biol. Chem.* 193: 265-275 (1951).
 20. Rao, R. H., Waite, M., and Myrvik, Q. N. Deacylation of dipalmitoyl-lecithin by phospholipases A in alveolar macrophages. *Exp. Lung Res.* 2: 9-15 (1981).
 21. Harington, J. S., Miller, K., and Macnab, G. Hemolysis by asbestos. *Environ. Res.* 4: 95-117 (1971c).
 22. Rice-Evans, C., Rush, J., Omarphas, S. C., and Flynn, D. M. Erythrocyte membrane abnormalities in glucose-6-phosphate dehydrogenase deficiency of the Mediterranean and A-types. *FEBS Letters*, 136: 145-148 (1981).