Environmental Health Perspectives
Vol. 51, pp. 141-146, 1983

Evidence for a Dose-Dependent Inflammatory Response to Quartz in the Rat Lung and Its Significance in Early Changes in Collagen Metabolism

by S. E. Sykes,* A. Morgan,* S. R. Moores,* S. T. Jones,* A. Holmes* and W. Davison*

The response of the rat lung to a range of doses of quartz at 50 and 100 days after its administration by intratracheal instillation has been assessed by bronchopulmonary lavage. The effects on the number of polymorphonuclear leukocytes (PMN), lymphocytes and macrophages are described. In addition the concentrations of soluble protein and hydroxyproline and the activities of lactate dehydrogenase, PZ peptidase and collagenase in lavage fluid supernatants were measured and an assessment of the hydroxyproline content of recovered cells was made. Finally PZ peptidase and collagenase were assayed in PMN-enriched cell fractions and in samples obtained from short-term culture of recovered macrophages. There was a dose-dependent increase in the recovery of all three cell types, and in the amounts of lactate dehydrogenase, protein and hydroxyproline in lavage fluids, which showed no signs of resolution over the 100-day period studied. Measurements of PZ peptidase and collagenase suggested that the PMN, not the macrophages, are the major source of these degradative enzymes. The relevance of these findings with regard to the importance of PMN in quartz-induced fibrosis is discussed.

Introduction

The prolonged inflammatory response which occurs both in experimentally induced fibrosis in animal models and in patients with idiopathic pulmonary fibrosis has now been well documented (*1, 2*). In previous studies, we have shown that following the instillation of a number of mineral dusts there was a marked influx of polymorphonuclear leukocytes (PMN) into the alveolar spaces, which reached a maximum at approximately 24 hr after dust administration. For nonfibrogenic materials, this acute inflammatory response was short-lived, and the free cell population of the lung returned to normal within a few weeks. For quartz, however, the influx of PMN was followed by a subacute inflammatory response which persisted over a period of at least three months (*3*).

The mechanism by which this prolonged inflammation can result in the accumulation of fibrotic tissue remains unknown. Gadek et al. (*4*) have suggested that disturbances of the normal balance between collagen synthesis and degradation may play an important role, and it is now well established that both PMN and macrophages do possess collagenase and neutral protease activities (*5, 6*). If the influx of PMN in the presence of quartz is able to bring about alterations in collagen metabolism, then we would expect to see some correlations between PMN numbers in the lung and evidence of tissue damage.

In this study we have looked at the dose dependence of the subacute cellular response of the rat lung to increasing amounts of quartz and at the associated biochemical changes. In addition, both PMN-rich cell preparations and samples obtained from short-term culture of lavaged macrophages have been assessed for collagenase and PZ peptidase activities. The significance of these results with regard to the role of the PMN in quartz-induced fibrosis is discussed.

*Environmental and Medical Services Division, Atomic Energy Research Establishment, Harwell, Oxfordshire, UK.

Materials and Methods

Instillation and Lavage

The techniques of instillation and bronchopulmonary lavage have been described previously (*7*). In this study, Dowson and Dobson superfine quartz was administered to rats weighing about 350 g to give doses of 0.5, 1.5, 3.5 and 5.0 mg. These quantities were fractionated so that one-third of the dose was given in each of three instillations over a period of one week. Control animals received sterile saline. At each dose level, animals were killed at 50 and 100 days after dust administration. Excised lungs were lavaged, initially with balanced salt solution (BSS, Gibco-Biocult) and then with physiological saline. Recovered cells were identified and counted as described by Morgan et al. (*7*).

Measurements on Lavage Fluid Supernatants

The concentrations of protein and hydroxyproline and the activity of lactate dehydrogenase (LDH) were measured in all the supernatants of the BSS washes. PZ peptidase and collagenase were measured in supernatants from animals given saline or 5 mg quartz only.

LDH was assayed by the method of Wroblewski and LaDue (*8*) as modified by Moores et al. (*9*). Protein was assayed by the method of Lowry et al. (*10*). Hydroxyproline was determined by a modification of the method of Stegemann and Stalder (*11*) as described by Moores et al. (*12*).

PZ peptidase activity was assessed by the lysis of the PZ substrate, 4-phenylazo-benzoxy carbonyl-L-prolyl-L-leucyl-L-glycl-L-prolyn-D-arginine dihydrate (Sigma Chemical Co., London, U.K.), as described by Wunsch and Heidrich (*13*) and modified by Hurych et al. (*14*). 1 mU of activity is defined as the rate of hydrolysis of the substrate of 1 nmole/min at 37°C.

Collagenase activity was assayed by using ^{14}C-acetylated collagen. Since no attempt to analyze the products of digestion of the ^{14}C-collagen was made, the term "collagenase" in this report has been used to include all collagenolytic activities in the samples. The methods of preparation of the ^{14}C-labeled collagen and the assay for collagenase were modifications of those of Cawston and Barrett (*15*) as described by Sykes et al. (*16*). Results are given as percentage lysis of the ^{14}C-collagen and as μg collagen degraded per hr/10^6 cells, as appropriate.

Measurements on Samples from Cell Cultures

Cells recovered from animals given 5 mg of quartz and from saline controls were also taken for short-term culture from animals killed at 30, 50 and 100 days after instillation (*16*). In brief, cells were suspended in medium containing 15% serum and transferred to medical flats. After 2 hr, nonadherent cells (more than 85% PMN) were removed and fresh medium added. The PMN-enriched cell fraction was stored and assayed for PZ peptidase and collagenase activity. After 24 hr the cell culture media were changed to serum-free Dulbecco's medium and samples collected for assay after a further 48 hr. At the 100 day time point, at the termination of culture, the macrophages were lysed in 0.1% Triton and the cell extracts frozen at −20°C.

Results

Lung Weights

At both 50 and 100 days after instillation, there was a dose-dependent increase in the fresh weights of the lungs. As shown in Figure 1, there was an excellent correlation between the increases in lung fresh weights (expressed as a percentage of body weight) and the amounts of quartz instilled at both 50 and 100 days. The correlation coefficients were 0.99 and 0.98, respectively.

Recoveries of Free Cells

As shown in Figure 2, very few PMN or lymphocytes were recovered at either time point from saline instilled animals. For experimental animals the recoveries of PMN and lymphocytes were dose dependent and increased with time after instillation.

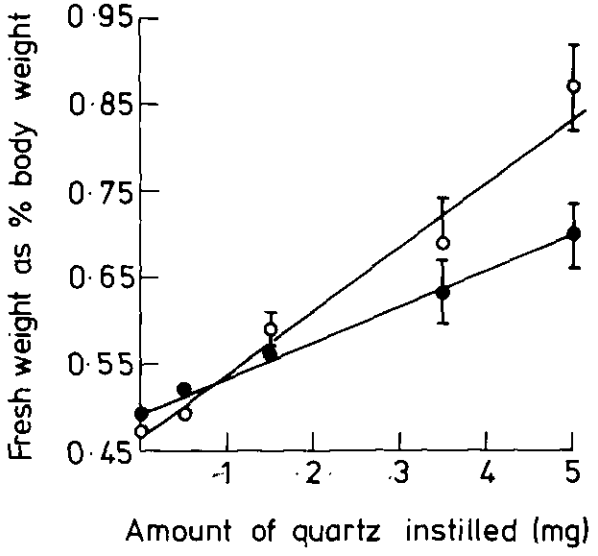

FIGURE 1. Correlation between lung weights, expressed as % body weights and the amounts of quartz instilled at (●) 50 and (○) 100 days after dust administration.

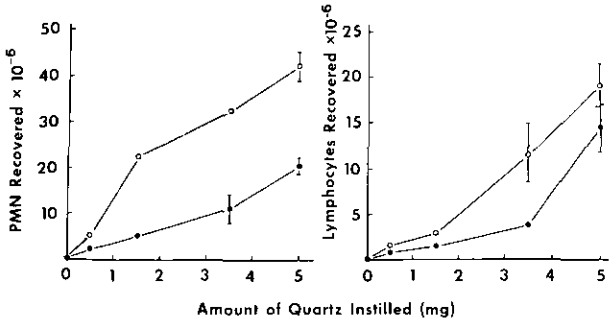

FIGURE 2. Numbers of PMN and lymphocytes recovered from the lungs of rats at (●) 50 and (○) 100 days after the instillation of increasing amounts of quartz. Values are means ± SEM.

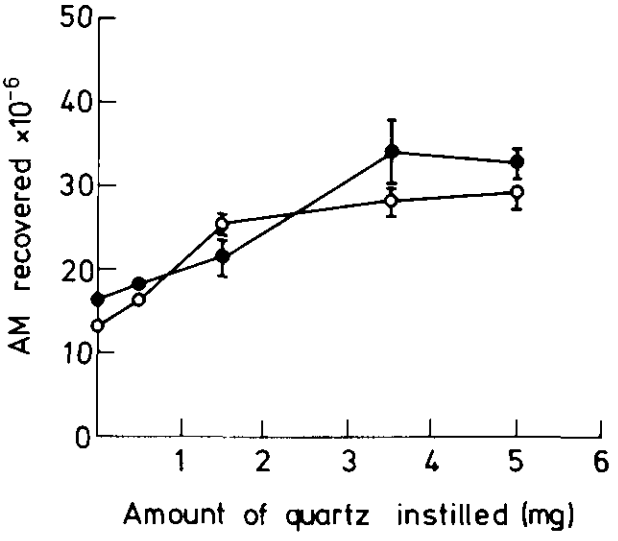

FIGURE 3. Numbers of macrophages recovered from the lungs of rats at (●) 50 and (○) 100 days after the instillation of increasing amounts of quartz. Values are means ± SEM.

In all cases the numbers of PMN were much greater than the numbers of lymphocytes.

The recoveries of alveolar macrophages are given in Figure 3. Up to 3.5 mg, there was a dose-dependent increase in the numbers of AM which did not differ significantly between the two time points. There was no further change in cell recovery above the 3.5 mg dose level. The numbers of multinucleate cells represented less than 2% of the total in all cases and there was no significant change in their incidence with increasing amounts of quartz instilled.

Measurements in Cell-Free Lavage Fluid

The activity of LDH in lavage fluid supernatants from control animals was relatively constant with a mean value of about 10 mU/mL. For experimental animals, LDH rose rapidly with increasing amounts of quartz at both 50 and 100 days after instillation. There was a close correlation (r = 0.93) between the activity of LDH in the supernatants and the recoveries of PMN and lymphocytes (see Fig. 4), suggesting that a major part of the free LDH originated from the PMN or PMN and lymphocytes.

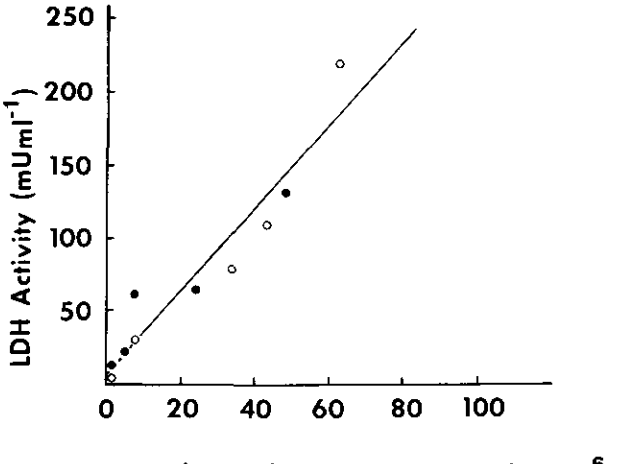

FIGURE 4. Correlation between the LDH activity of lavage supernatants and the numbers of PMN and lymphocytes recovered at (●) 50 and (○) 100 days after the instillation of increasing amounts of quartz.

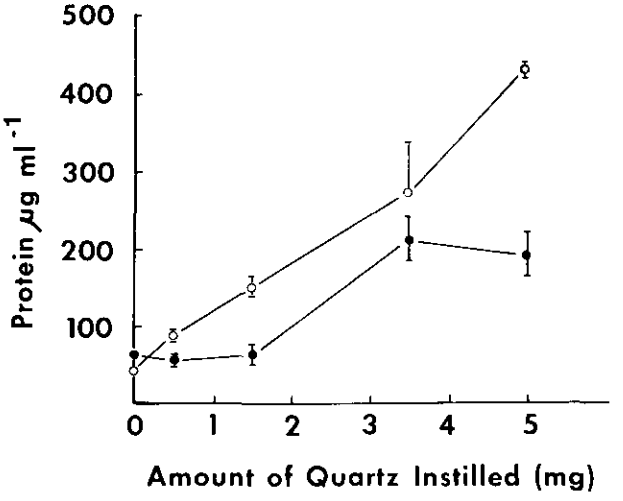

FIGURE 5. Concentrations of soluble protein in lavage fluid supernatants at (●) 50 and (○) 100 days after the instillation of increasing amounts of quartz. Values are means ± SEM.

The effects of increasing amounts of quartz on the concentration of soluble protein in lavage fluids are shown in Figure 5. At 50 days there was no increase in the amount of protein with doses of 0.5 and 1.5 mg quartz but at 100 days, the dose-response relationship was nearly linear.

The concentrations of hydroxyproline in lavage fluids are given in Figure 6. At 50 days there was a linear relationship between hydroxyproline concentration and the amount of quartz administered, with a correlation coefficient of 0.97. At 100 days, values were significantly ($p < 0.01$) greater than at 50 days for doses exceeding 0.5 mg, and there was some leveling off in the response above a dose level of 1.5 mg.

The activity of a peptidase able to cleave the PZ substrate was measured at 30, 50 and 100 days following the instillation of 5 mg of quartz. The results are given in Table 1. For control animals, the mean PZ peptidase activity was always less than 10 mU/mL. For experimental animals, activity increased with time from 27 mU/mL at 30 days to 160 mU/mL at 100 days.

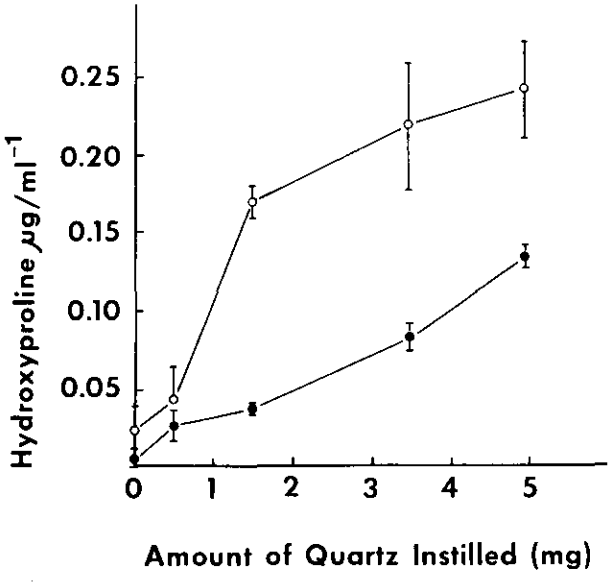

FIGURE 6. Concentrations of hydroxyproline in lavage fluid supernatants at (●) 50 and (○) 100 days after the instillation of increasing amounts of quartz. Values are means ± SEM.

Table 1. PZ peptidase activity in lavage fluid supernatants following the instillation of saline and in lavage fluid supernatants and PMN following the instillation of 5 mg of quartz.[a]

Time after instillation, days	Lavage fluid supernatants: Saline, mU/mL	Lavage fluid supernatants: Quartz, mU/mL	Nonadherent cells, mU/10⁶ PMN
30	2.7 ± 1.0 (2)	27 ± 0.5† (3)	54 ± 8 (2)
50	2.6 ± 1.4 (2)	37 ± 6* (3)	64 ± 13 (3)
100	9.6 ± 3.6 (2)	161 ± 3* (2)	116 ± 14 (2)

*$p < 0.05$ compared to the saline control.
†$p < 0.001$ compared to the saline control.
[a]Values are means ± SEM of the number of observations given in parentheses.

Lavage fluid supernatants were also assayed for collagenase, but no significant levels of activity were detected.

PZ Peptidase and Collagenase Activity in PMN and Alveolar Macrophages

When cells recovered by lavage from animals given 5 mg of quartz were cultured, the alveolar macrophages adhered readily to the surface of the culture plates, and at least 85% of the cells left in suspension were PMN. The nonadherent cells were collected and assayed for PZ peptidase and collagenase activities. For both enzymes, results have been calculated as the mean activity per 10^6 PMN on the basis of (a) the large excess of these cells over other cell types present and (b) from the much higher activity found in PMN than in AM.

The PZ peptidase activities of PMN recovered at various times after the instillation of 5 mg of quartz are given in Table 1. At 30 and 50 days, values were approximately constant at around 60 mU/10^6 cells but by 100 days they had doubled. Measurements of PZ peptidase on AM at the termination of culture gave values of <1 mU/10^6 cells.

The collagenolytic activities of the PMN are given in Table 2. The mean activity represented the lysis of 31% of the labeled collagen under the assay condition described which is equivalent to the lysis of 1.7 μg collagen/hr/10^6 PMN. Unlike PZ peptidase there was no change in activity with time after instillation. Measurements of collagenase activity in AM at the termination of culture gave rates of collagen lysis of 0.08 ± 0.01 and 0.05 ± 0.01 μg/hr/10^6 cells for cells from quartz-treated animals and saline controls, respectively. These differences were not significant.

PZ Peptidase and Collagenase in Culture Media

PZ peptidase was not detected in any of the samples of media taken from macrophage culture throughout the period of study.

Measurements of collagen lysis by culture media are given in Table 2. Higher levels of collagenolytic activity were present in media collected from experimental culture than from saline controls but there was no change in activity with time after instillation.

Histology

Lungs from saline-instilled animals appeared normal. In experimental animals, deviations from this pattern increased in severity with increasing

Table 2. Collagenase activity in PMN and in macrophage culture media following the instillation of saline or 5 mg of quartz.[a]

Time after instillation, days	Collagen lysis, %/10⁶ PMN	Collagen lysis, % mL culture medium	
		Saline	Quartz
30	28.5 ± 3.3 (2)	1.4 ± 1.4	10.3 ± 0.3*
50	36.9 ± 6.1 (2)	5.1 ± 1.8	9.4 ± 0.9
100	26.1 ± 5.5 (3)	1.6 ± 0.8	9.2 ± 3.1

*$p < 0.05$ compared to the saline control.
[a]Values are means ± SEM of the number of observations given in parentheses.

amounts of quartz administered. In all cases there was cellular infiltration into alveolar spaces and alveolar walls (mainly mononuclear cells and PMN) and at the two high doses of 3.5 and 5 mg, there were focal lesions, cellular nodules containing collagen fibers and general disruption of the lung architecture.

Discussion

In this study, we have shown that the exposure of rats to quartz by intratracheal instillation gives rise to an inflammatory response which is not resolved and indeed increases in size and complexity at least up to 100 days after dust administration. In the dose range chosen, there was no threshold level below which the long-term cellular response was not apparent, and at both the 50 and 100 day time points there was a dose-dependent increase in the recovery of cells from quartz-treated animals compared to controls.

Analyses of fluids obtained by bronchopulmonary lavage have shown that the main cell types contributing to the inflammatory response were mononuclear cells (macrophages and lymphocytes) and large numbers of PMN. Dauber et al. (*17*) have looked at the effects of quartz in the guinea pig lung and have also reported large increases in the numbers of PMN recovered from experimental animals compared to controls, although in their study there was little or no change in the number of macrophages and lymphocytes recovered.

The use of levels of free LDH in the airways as an indicator of cell damage has been described previously (*18*). In addition, in a number of experimental models it appears that there is a good correlation between the amounts of LDH present and the numbers of PMN in the lung (*9, 19*). In the present study, there was a good correlation between the LDH activity in the lavage fluids and the numbers of PMN recovered, suggesting that even at these later times, much of the free LDH is derived principally from these cells.

The influx of PMN in pulmonary inflammatory reactions has now been well documented (*20, 21*), but the precise mechanisms involved in this response are not clear. Hunninghake et al. (*22*) and Kazmierowski et al. (*20*) have described the production of a chemotactic factor which is specific for PMN and which is stimulated by phagocytosis of particulate material (*23*). In addition, Miller et al. (*24*) have described the production of a quartz-induced factor from guinea pig AM which is chemotactic for other resident macrophages, and Dauber and Daniele (*25*) have identified chemotaxins for PMN, macrophages and lymphocytes from cultures of guinea pig macrophages.

The alveolar macrophage may therefore play a critical role in developing the inflammatory response. Its ability to produce specific chemotaxins means that it is well equipped to recruit new cells into the lung in an attempt to deal with the insult. At later times the effects of the macrophages may be modified by serum-derived chemotactic agents and by factors from other cell types such as the PMN themselves or the fibroblasts.

The results described in the present study also suggest that the inflammatory response may be central in initiating early fibrotic changes in the lung. High levels of hydroxyproline in lavage fluids were clearly dependent on the amounts of quartz administered, suggesting that increased numbers of inflammatory cells were associated with increased amounts of hydroxyproline in the alveolar spaces.

The main source of this hydroxyproline is likely to arise from the degradation of lung collagen and elastin and, in support of this, high levels of PZ peptidase activity were found both in lavage fluid supernatants and in PMN-rich cell preparations. The significance of PZ peptidase in collagen degradation has not been well defined. It is considered to be a gelatinase, unable to cleave native collagen but active in digesting degraded fragments which are not readily attacked by other proteases. In a number of pathological conditions, increased PZ peptidase activity has been found concurrently with increased collagen degradation (*26*).

Finally, although no significant collagenase activity was found in lavage fluids, quite high rates of collagen lysis were obtained with the PMN cell preparations. It is likely that these measurements are the products of activity of more than one enzyme, since the presence of polymorph elastase able to attack collagen has been well documented (*27*). In addition, significant amounts of collagenolytic activity were detected in culture media collected from macrophages isolated from experimental animals, whereas little or no activity was detected from corresponding control cultures. Thus, activated macro-

phages can produce collagen-degrading enzymes at rates comparable to the activity found in PMN.

In previous studies, the importance of the inflammatory response in initiating the development of pulmonary fibrosis has not been well defined. In some experimental models, a key role has been ascribed to alveolar macrophages by their ability to produce specific fibrogenic factors which can stimulate fibroblasts directly. The results of the present study indicate an important role for the PMN, since these cells possess high levels of at least two enzymes concerned directly with the degradation of connective tissue. Since their numbers are dependent on the amounts of quartz administered and they increase up to at least 100 days after instillation, they could bring about destruction of lung connective tissue which was also dose dependent. This is supported by the results of hydroxyproline measurements. Assuming that a fine balance between collagen degradation and synthesis does indeed exist (4), then we can expect a similar dose dependence in the deposition of new collagen leading to the dominance of synthesis over degradation and the development of fibrotic tissue.

The authors acknowledge the support of the Institute of Occupational and Environmental Health, Montreal (Project No. 28). They would also like to thank Dr. J. C. Wagner, MRC Pneumoconiosis Unit, who provided the quartz, Dr. B. Sykes, John Radcliffe Hospital, Oxford, for the gift of rat type I collagen and to acknowledge the technical assistance of Ms. S. Carver and Mrs. L. Halstead.

REFERENCES

1. McCullough, B., Collins, J. F., Johansson, W. G., and Grover, F. L. Bleomycin-induced diffuse interstitial pulmonary fibrosis in baboons. J. Clin. Invest. 61: 79-88 (1978).
2. Hunninghake, G. W., Kawanami, O., Ferrans, V. J., Young, R. C., Roberts, W. C., and Crystal, R. G. Characterization of the inflammatory and immune effector cells in the lung parenchyma of patients with interstitial lung disease. Am. Rev. Resp. Dis. 123: 401-412 (1981).
3. Sykes, S. W., Morgan, A., Evans, J. C., Evans, N., Holmes, A., and Moores, S. R. Use of an *in vivo* test system to investigate the acute and sub-acute responses of the rat lung to mineral dusts. In: Ann. Occup. Hyg. 26: 593-605 (1982).
4. Gadek, J. E., Kelman, J. A., Fells, G., Weinberger, S. E., Horwitz, A. L., Reynolds, H. Y., Fulmer, J. D., and Crystal, R. G. Collagenase in the lower respiratory tract of patients with idiopathic pulmonary fibrosis. New Engl. J. Med. 301: 737-742 (1979).
5. Marom, Z., Weinberg, K. S., and Fanburg, B. L. Effect of bleomycin on collagenolytic activity of the rat alveolar macrophage. Am. Rev. Resp. Dis. 121: 859-867 (1980).
6. Murphy, G., Reynolds, J. J., Bretz, V., and Boggiolini, M. Collagenase is a component of the specific granules of human neutrophil leucocytes. Biochem. J. 162: 195-197 (1977).
7. Morgan, A., Moores, S. R., Holmes, A., Evans, J. C., Evans, N., and Black, A. The effect of quartz administered by intratracheal instillation on the rat lung. I. The cellular response. Environ. Res. 22: 1-22 (1980).
8. Wróblewski, F., and LaDue, J. S. Lactate dehydrogenase activity in blood. Proc. Soc. Exp. Biol. Med. 90: 210-213 (1955).
9. Moores, S. R., Black, A., Evans, J. C., Evans, N., Holmes, A., and Morgan, A. The effect of quartz administered by intratrachael instillation on the rat lung II. The biochemical response. Environ. Res. 24: 275-285 (1981).
10. Lowry, O. H., Rosebrough, N. L. J., Farr, A. L., and Randall, R. J. Protein measurement with the Folin-phenol reagent. J. Biol. Chem. 193: 265-275 (1951).
11. Stegemann, H., and Stalder, K. Determination of hydroxyproline. Clin. Chim. Acta 18: 267-273 (1967).
12. Moores, S. R., McFarland, C. D., and Wood, J. C. An improved technique for the determination of collagen in lung tissue. AERE Report 9351 (1979).
13. Wünsch, E., and Heidrich, H. G. Zur quantitaven bestimmung der kollagenase. Z. Physiol. Chem. 333: 149-151 (1963).
14. Hurych, J., Mirejovska, E., Kobrle, V., and Rencara, J. Enzyme changes during experimental silicotic fibrosis. Environ. Res. 25: 424-433 (1981).
15. Cawston, T. E., and Barrett, A. J. A rapid and reproducible assay for collagenase using 1-^{14}C acetylated collagen. Anal. Biochem. 99: 340-345 (1979).
16. Sykes, S. E., Moores, S. R., and Jones, S. T. Dose-dependent effects in the sub-acute response of the rat lung to quartz. II. Evidence for early changes in collagen metabolism. Exptl. Lung Res., in press.
17. Dauber, J. H., Rossman, M. D., and Daniele, R. P. Pulmonary fibrosis: bronchoalveolar lavage cell types and impaired function of alveolar macrophages in experimental silicosis. Environ. Res. 27: 226-236 (1982).
18. Henderson, R. F., Damon, E. G., and Henderson, T. R. Early damage indicators in the lung. I. Lactate dehydrogenase activity in the airways. Toxicol. Appl. Pharmacol. 44: 291-297 (1978).
19. De Nicola, D. B., Rebar, A. H., and Henderson, R. F. Early damage indicators in the lung. V. Biochemical and cytological response to NO_2 inhalation. Toxicol. Appl. Pharmacol. 60: 301-312 (1981).
20. Kazmierowski, J. A., Gallin, J. I., and Reynolds, H. Y. Mechanism for the inflammatory response in primate lungs. J. Clin. Invest. 59: 273-281 (1977).
21. Shaw, J. O. Leucocytes in chemotactic fragment-induced lung inflammation. Am. J. Pathol. 101: 283-302 (1980).
22. Hunninghake, G. W., Gallin, J. I., and Fauci, A. S. The *in vivo* and *in vitro* generation of a neutrophil chemotactic factor by alveolar macrophages. Am. Rev. Resp. Dis. 117: 15-23 (1978).
23. Gadek, J. E., Hunninghake, G. W., Fells, G. A., Zimmerman, R. L., Keogh, B. A., and Crystal, R. G. Evaluation of the protease-antiprotease theory of human destructive lung disease. Bull. Eur. Physiopath. Resp. 16 (Suppl.): 27-40 (1980).
24. Miller, K., Calverley, A., and Kagan, E. Evidence of a quartz-induced chemotactic factor for guinea-pig alveolar macrophages. Environ. Res. 22: 31-39 (1980).
25. Dauber, J. H., and Daniele, R. P. Secretion of chemotaxins by guinea pig lung macrophages. Exptl. Lung Res. 1: 23-32 (1980).
26. Ryan, J. N., and Woessner, J. F. Oestradiol inhibits collagen breakdown in the involuting rat uterus. Biochem. J. 127: 705-713 (1972).
27. Starkey, P. M., and Barrett, A. J. Human lysosomal elastase. Catalytic and immunological properties. Biochem. J. 155: 265-271 (1976).