EFFECT OF QUARTZ DUST DQ 12 ON HUMAN MONOCYTES/MACROPHAGES *IN VITRO* —AN ELECTRON MICROSCOPICAL STUDY

HEIDRUN BEHRENDT • N.H. Seemayer

Medical Institute of Environmental Hygiene at the University
Auf'm Hennekamp 50, D-4000 Düsseldorf, FRG

INTRODUCTION

Quartz particles are highly cytotoxic to rodent macrophages in vitro.[4,9,10] This cytotoxic effect is thought to be mediated by the lysis of phagolysosomal membranes through ingested quartz particles.[2] In comparison to cultured rodent macrophages human monocytes/macrophages are more resistant to quartz-induced cytotoxicity in vitro.[6] Exposure of human macrophages to quartz mainly leads to the formation of fibroblast-proliferating factors and to the release of mediators which activate neutrophils to generation of oxygen species.[3,8] This stimulatory action of quartz dust DQ 12 on human macrophages in vitro is morphologically paralleled by the development of a labyrinth of dilated vacuolar spaces within the cells.[1] Sequential analysis of quartz-exposed human macrophages by means of transmission electron microscopy further indicates the manifestation of specific autophagolysosomal processes which result in a vacuolar network filled with degradation products intrinsic to the cell in addition to quartz particles. From this study,[1] it has been concluded that human macrophages in vitro develop a protection mechanism against toxic quartz particles which is initiated by phagocytosis and which results in consecutive cell stimulation.

In the present study we report about surface alterations in human monocytes/macrophages in vitro which are induced by quartz DQ 12 and which further explain the considerably higher resistance of human macrophages to quartz particles in vitro.

MATERIALS AND METHODS

Cell Cultures

Isolation of human monocytes from peripheral blood in Ficoll-Hypaque gradient and cultivation of monocytes to maturation of cells with characteristics of macrophages has already been described in detail.[6,7]

For transmission electron microscopical studies 25×10^6 mononuclear cells were seeded in tissue culture flasks (Falcon 3013), for scanning electron microscopy 2.5×10^6 cells were distributed on tissue culture plate with 24 wells (Falcon 3047, Multiwell tissue culture plate) containing round glass coverslips with a diameter of 12 mm. After removing of non-adherent cells monocytes were cultivated for 7–14 days to differentiate into mature macrophages as already reported.[6,7] The cells were then incubated with 100 µg quartz DQ 12 (particle size $<5\mu m$) per ml medium for 2, 24 and 48 hours. Cell viability has been tested by dye exclusion test.

Transmission Electron Microscopy

For transmission electron microscopy the cells were briefly fixed *in situ* by the addition of 2% buffered glutaraldehyde. After gently shaking to remove the cells from their substrate the cells were spinned down, pelleted and postfixed with osmium tetroxide. They were then dehydrated in a graded series of ethanol, and embedded in Araldite. Ultrathin sections were investigated in a Philips 400T electron microscope.

Scanning Electron Microscopy

For scanning electron microscopical investigations the cells were fixed *in situ* with buffered glutaraldehyde, post-fixed with osmium tetroxide and dehydrated in a graded series of ethanol. After short immersion in hexamethyldisilazane the cells were air dried according to the method of Nation,[5] coated with gold, mounted and analyzed in a Philips SEM 515 electron microscope.

RESULTS

At concentrations which are highly cytotoxic to guinea pig macrophages, human monocytes/macrophages react with a considerable higher survival rate (Figure 1). Macrophages which had been exposed to 100 µg/ml quartz DQ 12 for 48 hours display a vacuolar network filled with flocculent material and quartz particles (Figure 2) but no signs of cytotoxicity. No disruptions of lysosomal membranes were ever detected. The morphological picture rather results from processes of cell activation induced by quartz particles in vitro. The dilated vacuolar network is open to the extracellular space (Figure 2, Figure 3), so that the intravacuolar degradation products as well as the quartz particles are exposed to the extracellular micro-environment.

Connections between quartz-induced labyrinth formation and extracellular space—supposed to occur for the analysis of transmission electron micrographs[1]—are also evident in cell samples which have been exposed to 100 µg/ml quartz DQ 12 for 24 hours and subsequently investigated by scanning electron microscopy. As is indicated in Figure 6, deep in-

dentations or 'holes' can be seen on the surfaces of nearly all macrophages. One single foramen is usually characteristic for these cells. Since the formation of foramina can never be observed in cultured control macrophages (Figure 4) nor in cells being exposed to shorter times (2 hours, Figure 5) of the same quartz dust concentration, 'holes' are characteristically late phase alterations in otherwise vital cells. A comparison between Figure 3 and Figure 6 clearly indicates the similarity between sections through parts of dilated vacuoles containing fingerprint-like structures[1] and the formation of a foramen on their surface.

DISCUSSION AND CONCLUSIONS

Results obtained by scanning electron microscopy (presented in this paper) and by transmission electron microscopy[1] indicate that cultured human monocytes/macrophages display unique features upon contact with toxic quartz particles. The higher resistance of the cells to quartz concentrations which are toxic for animal cells is underlined by a special mechanism of phagocytosis in combination with autophagolysosomal processes[1] and cell secretion.[3,8] Similar observations have never been reported to occur in silica-exposed rodent macrophages. Therefore, the effects demonstrated by us are species specific characteristics of human cultured monocytes/macrophages which have been exposed to quartz particles.

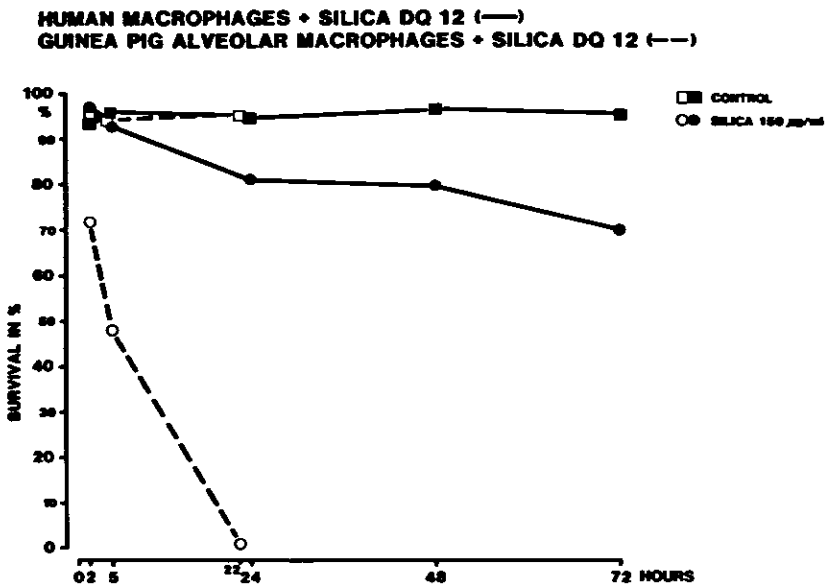

Figure 1. Effect of quartz DQ 12 on the survival rate of guinea pig alveolar macrophages and of human macrophages *in vitro*.

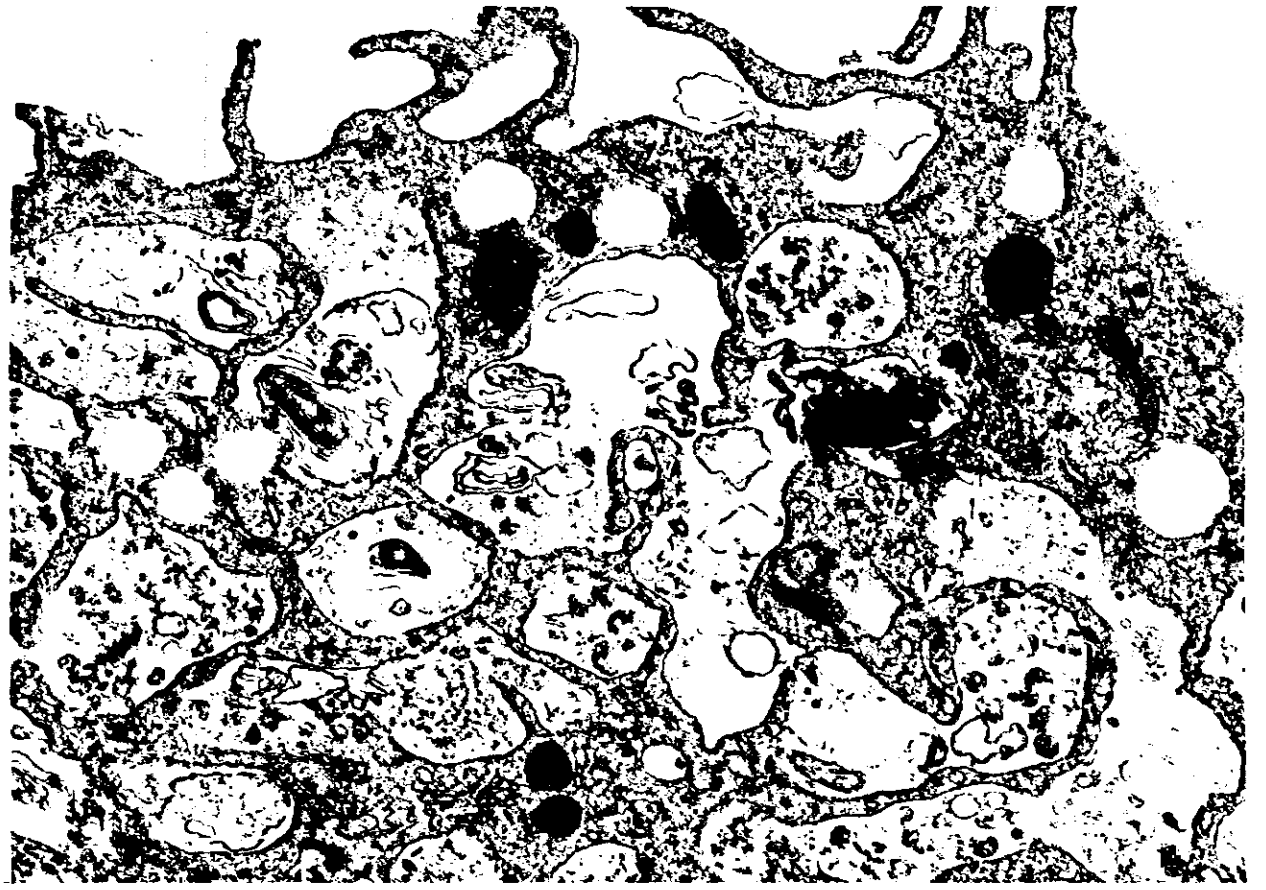

Figure 2. Part of a 12 days old human macrophage after incubation with 100 µg/ml quartz DQ 12 in vitro. Development of a vacuolar network containing quartz particules and flocculent material. Transmission electron micrograph. Magn. x25000.

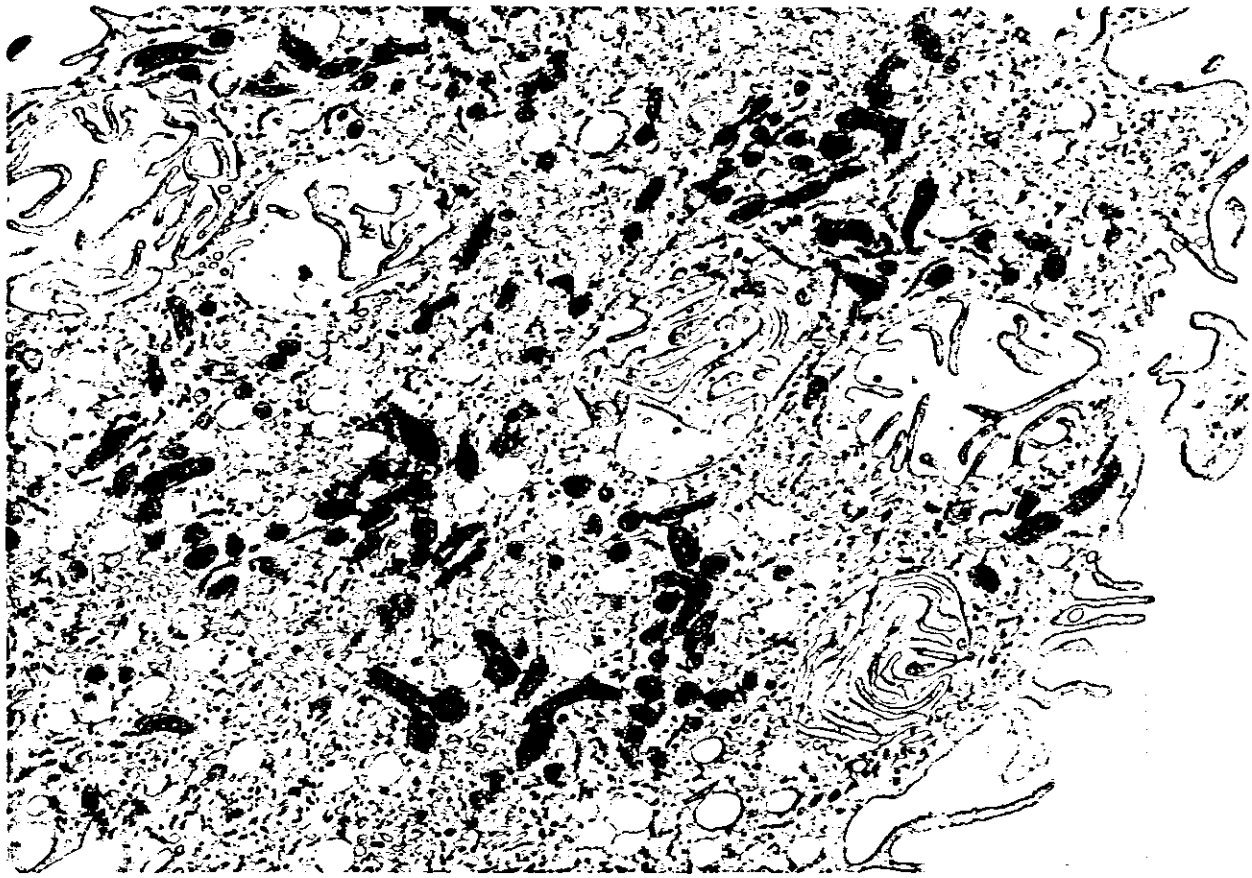

Figure 3. 12 days old human macrophage after incubation with 100 µg/ml quartz DQ 12 in vitro. Section through a 'hole.' Transmission electron micrograph. Magn. x9200.

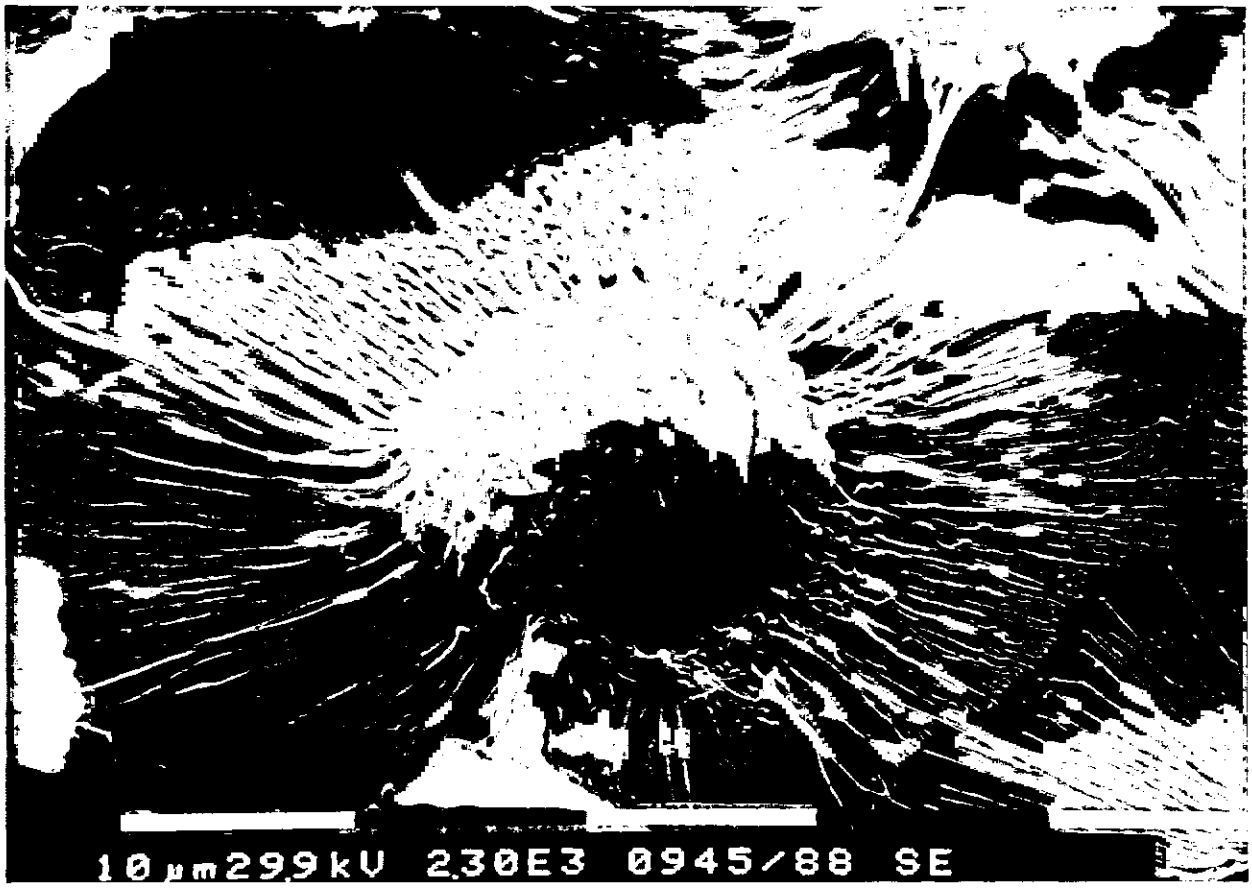

Figure 4. Human macrophages from a 12 days old culture. Scanning electron micrograph.

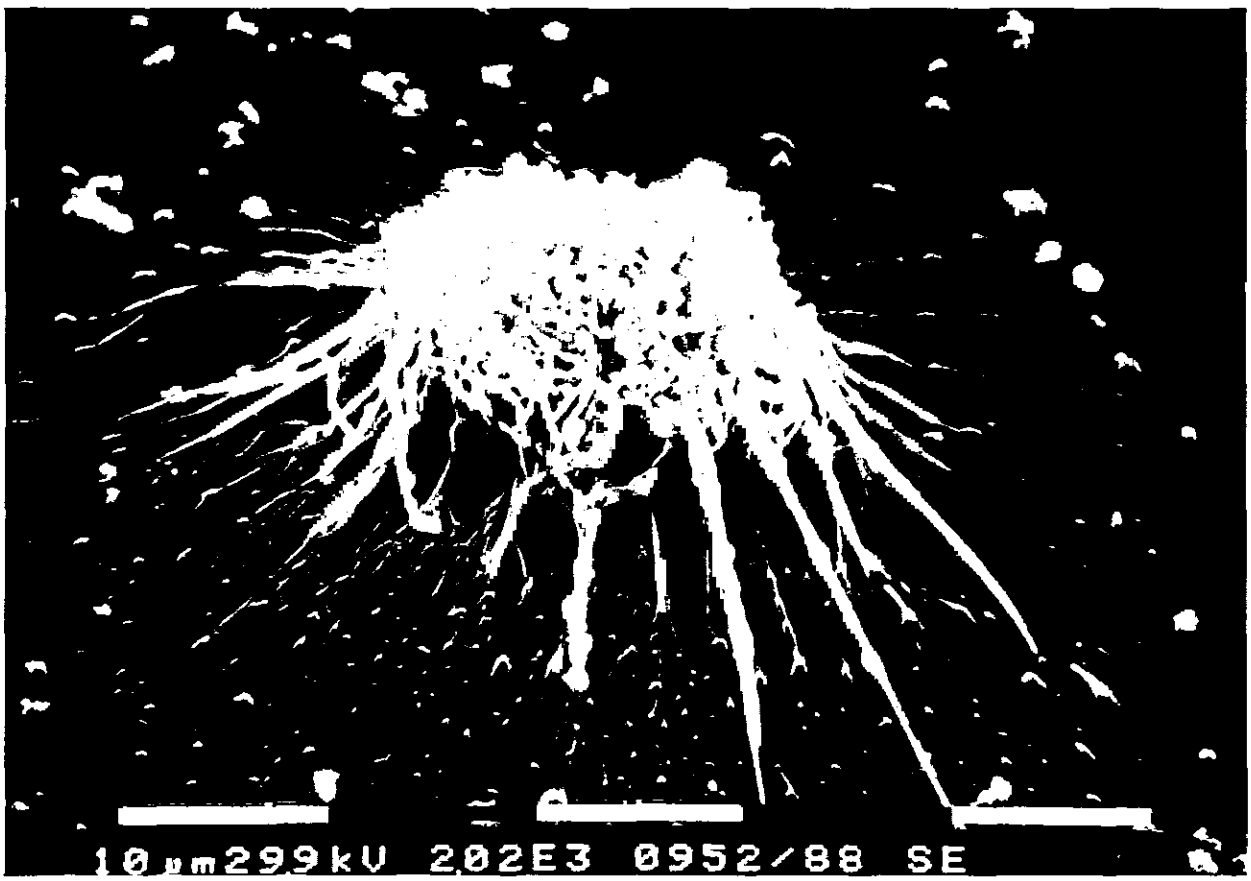

Figure 5. Scanning electron micrograph of a human macrophage after exposure to 100 µg/ml quartz DQ 12 for 2 hours in vitro.

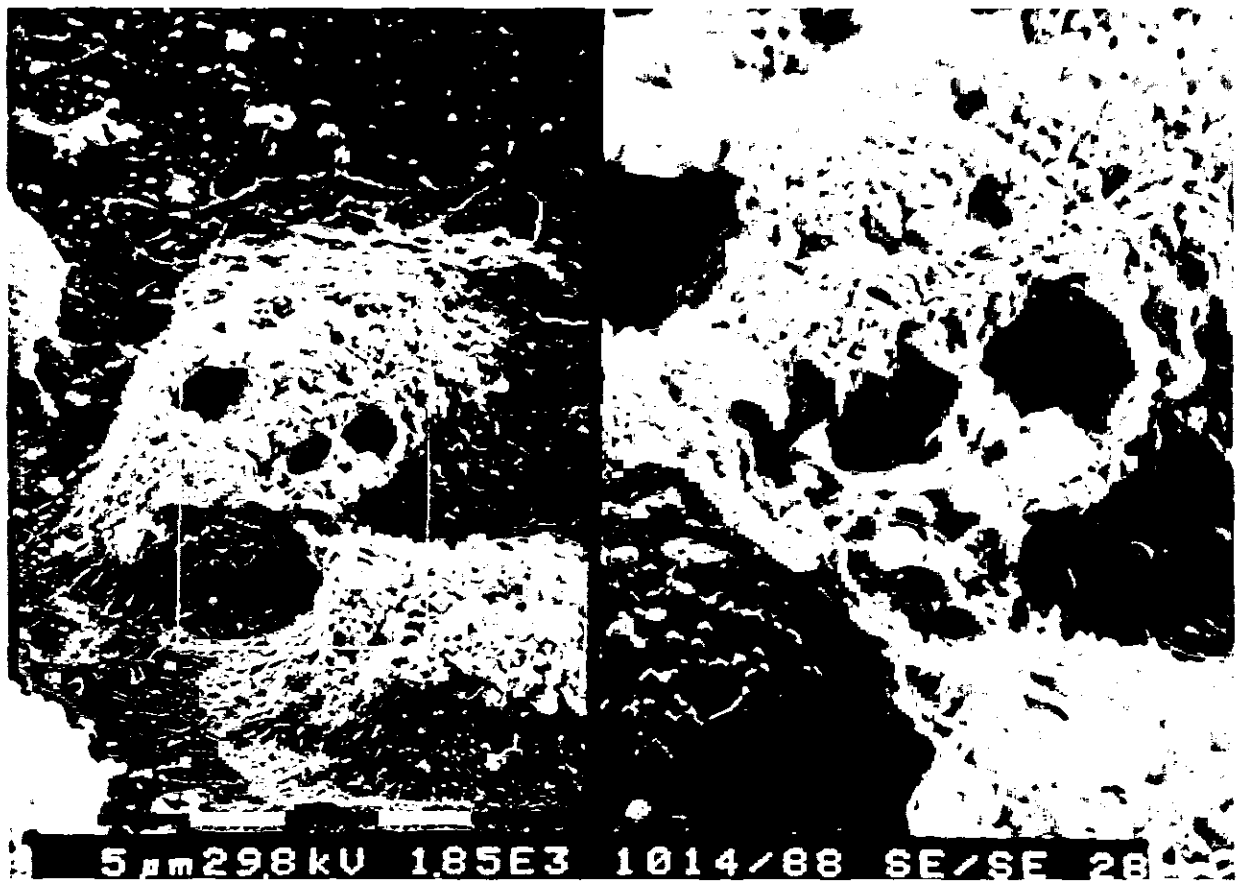

Figure 6. Scanning electron micrograph of a human macrophage after exposure to 100 µg/ml quartz DQ 12 for 24 hours, displaying characteristic 'holes.'

REFERENCES

1. Behrendt, H., Seemayer, N.H., Braumann, A., Nissen, M.: Electron microscopy investigation of the effect of quartz dust DQ 12 on human monocytes/macrophages in vitro. *Silicosis Report North-Rhine Westfalia*. Vol. 16, 171-183 (1987).
2. Comolli, R.: Cytotoxicity of silica and liberation of lysosomal enzymes. *J. Pathol. Bacteriol.* 93:241-253 (1967).
3. Maly, E., Seemayer, N.H., Braumann, A., Behrendt, H., Manojlovic, N., Maly, F.E.: The significance of immunomodulative cytokines for the development of silicosis. I. Biological characterization of the granulocyte-activating mediator from human monocytes/macrophages treated with quartz dust DQ 12. *Silicosis Report North-Rhine Westfalia*. Vol. 16, 157-170 (1987).
4. Morgan, D.M.L., Allison, A.C.: Effects of silica and asbestos on alveolar and peritoneal macrophages: a comparative study. In: *The in vitro effects of mineral dusts*, pp. 75-80. R.C. Brown, I.P. Gormley, M. Chamberlain, R. Davies, Eds. Academic Press, London (1980).
5. Nation, J.L.: A new method using hexamethyldisilazane for preparation of soft insect tissues for scanning electron microscopy. *Stain Technol.* 58:347-351 (1983).
6. Seemayer, N.H., Braumann, A.: Investigation on the cytotoxic effect of quartz dust DQ 12 and coal mine dusts on human macrophages. *Silicosis Report North-Rhine Westfalia*. Vol. 15, 301-320 (1985).
7. Seemayer, N.H., Braumann, A.: Effects of particle size of coal mine dusts in experimental anthrocosilicosis: In vitro studies on human macrophages. *Ann. Occup. Hyg.*, in press.
8. Seemayer, N.H., Braumann, A., Maly, E.: Development of an 'in vitro' test system with human macrophages and fibroblasts for analysis of the effect of quartz dusts and coal mine dusts. I. Formation of a fibroblast proliferation factor. *Silicosis Report North-Rhine Westfalia*. Vol. 16, 143-155 (1987).
9. Seemayer, N.H., Manojlovic, N.: Biological effects of coal mine dusts on macrophages. The in vitro effects of mineral dusts, pp. 5-12. R.C. Brown, I.P. Gormley, M. Chamberlain, R. Davies, Eds. Academic Press, London (1980).
10. Ziesche, R., Wenners, J., Behrendt, H.: Morphological investigations on the initial effects of quartz DQ 12 on membranes in vitro. *Silicosis Report North-Rhine Westfalia*, Vol. 15, 331-344 (1985).

MEDIATORS(S) FROM HUMAN MONOCYTES/MACROPHAGES INDUCED BY QUARTZ DUST DQ12 OR COAL MINE DUST TF-1 ARE LEADING TO RELEASE OF OXYGEN RADICALS FROM HUMAN GRANULOCYTES

E. MALY • N.H. Seemayer • Heidrun Behrendt • N. Manojlovic • F.-E. Maly*

Medical Institute of Enviromental Hygiene at the University, Gurlittstr. 53, D-4000 Düsseldorf, F.R.G.
*Institute of Clinical Immunology, Inselspital Bern, CH-3010 Switzerland

INTRODUCTION

Pneumoconiosis is a chronic inflammatory and fibrotic lung disease caused by long-term inhalation of quartz dust or quartz containing dust. Inhaled dust particles with a mass median aerodynamic diameter less than 5 µm may overcome the mechanisms of lung clearance and gain access to regional lymphatic tissue, hilar lymph nodes and subpleural aggregates. Here we find the typical fibrotic nodules, which consist of quartz dust laden alveolar macrophages, lymphocytes, polymorphonuclear granulocytes and fibroblasts. Alveolar macrophages seem to play a central role in the development of fibrotic tissue changes in the lung. Several authors reported that alveolar macrophages can influence the activity of inflammatory processes by production and secretion of soluble cytokines.[4,5,6,8,11] Particular attention must be paid to the activation of polymorphonuclear granulocytes (PMN), since an increased immigration of PMN in the lung tissue, especially in the early phases of pneumoconiosis, has been reported by several investigators.[3,7,8] Human PMN are potent inflammatory cells and their importance in the development of pneumoconiosis may be due to the effects of secretory products of activated PMN as f.e. collagenases, elastases, proteolytic enzymes, and especially reactive oxygen radicals (ROS).[20] Besides, activation of prolylhydroxylase, a key enzyme in collagen synthesis, by superoxide anion has been reported.[2] Therefore, we investigated whether cultivation of human monocytes/macrophages under exposure to quartz dust DQ12 or a typical coal mine dust from the Ruhr-area could lead to the liberation of mediators, which in turn could activate PMN. Since toxicity of PMN is largely dependent on their generation of free oxygen radicals, we examined the activation of the oxidative burst of PMN by supernatants of quartz or coal mine dust exposed cultures of human mononuclear cells. Furthermore, we studied the morphological changes of PMN under influence of quartz dust induced mononuclear cell supernatants with a transmission electron microscope.

MATERIALS AND METHODS

Cell Cultures

Isolation of human monocytes and PMN from the peripheral blood of healthy donors by Ficoll-Hypaque density centrifugation as well as the maturation of monocytes to cells with characteristics of macrophages have been described in detail.[17]

Dust Samples

Quartz dust DQ12 was used as fibrogenic stimulus for cultures of human mononuclear cells. This is Dörentruper crystall quartz flour (grinding No.12) with a particle size 5 µm. Furthermore a typical coal mine dust (TF-1, fraction BAT-II) from a colliery of the Ruhr-area was tested. It is characterized by a high mineral content (95 weight%), a quartz content of 10.6 weight% and a grain size distribution from 0.5 to 2.5 µm.[18] Electrocorund (BAR 3 S, particle size < 5 µm) was used as non cytotoxic control.

Detection of Free Oxygen Radicals

Measurement of formation of reactive oxygen species, in particular of superoxide anion, from activated PMN was performed using lucigenin dependent chemiluminescence (CL).[1] For this purpose we either used a 6-channel luminometer (LB9505, Berthold, Wildbad, FRG) or a microtiterplate image luminometer (C-1966, Hamamatsu Photonics Europe, Herrsching, FRG) as reported earlier.[12,14,15] Furthermore, we studied the activation of the oxidative burst of PMN applying the cytological nitroblue tetrazolium reduction capacity test (NBT test): the percentage of detectable "formazan-cells"—associated with the uptake of nitro-blue tetrazolium and its reduction to formazan—reflects the activation of PMN by oxidative processes.[10]

Biological and Biochemical Characterization of Mediators

In order to approach the nature of the mediator(s) in the supernatants of dust treated cultures of mononuclear cells we analyzed the dose effect relationship, the thermostability, and the effect of treatment of the mediators with various enzymes.[13,14] Preliminary estimation of the molecular weight of the mediator(s) involved were performed using HPLC gelfiltration techniques (TSK 2000) as reported previously.[14] In order to evaluate the cellular origin of the mediator(s) responsible for the activation of PMN we isolated monocyte depleted and monocyte enriched cell suspension and incubated them with quartz dust DQ12 (50 µg/ml, 24h). Cellular composition of isolated cell suspensions was deter-

mined by cell surface marker analysis with an Ortho cytofluorograph (model 50 H) using FITC-labelled mouse monoclonal antibodies as described earlier.[14]

RESULTS

Supernatants of quartz dust DQ12 treated mononuclear cells are capable of stimulation of human PMN to a highly significant and long lasting chemiluminescence, which reflects the release of superoxide anion from activated PMN.[1] The mediator(s) in the supernatants responsible for this effect were called "Granulocyte Activating Mediator(s)." "GRAM". However, this activation of PMN was strictly dependent on the sort and dose of dust used for production of supernatants. Highest values of CL of PMN were obtained with supernatants from mononuclear cells exposed to 50–100 µg/ml quartz dust DQ12 for 24 hours. Even the tested coal mine dust TF-1 was able to release GRAM's from mononuclear cells,[14] but in contrast to quartz dust DQ12 peak values of CL of PMN were obtained using 200 µg/ml TF-1 for production of supernatants. Interestingly, electrocorund, known for its non-fibrogenic behaviour, was unable to release GRAM's from mononuclear cells.[14] Furthermore, we analysed the influence of the time of exposure of quartz dust DQ12 to mononuclear cells on the release of GRAM's. After an incubation period of only 4 hours low amounts of GRAM's were detected. Highest values were measured after an incubation period of 24 hours.[14] After 48, 72, and 96 hours of incubation release of GRAM's was reduced to approximately 50% of the peak value obtained after 24 hours. Therefore, supernatants harvested from cultures of mononuclear cells exposed to 50 µg/ml quartz dust DQ12 for 24 hours were used as "standard-GRAM" for further characterisation of GRAM. Exposure of PMN to "standard-GRAM" in the NBT-test led to a threefold increase in the formation of formazan-cells.[12,14] This result again underlines the activation of the oxidative burst of PMN by GRAM's. We also analyzed the morphological changes of PMN exposed to GRAM with a transmission electron microscope. We found, that in contrast to control cells PMN which were exposed to GRAM for 15 min. present marked signs of chemotactic activity as can be seen by changes of the cell shape and development of a leading lamella. After an incubation period of 45 min. PMN seem to enlarge to some extent. After 60 min. of incubation we found a reduction of chemotactic activity. Additionally, we detected a loss of intracytoplasmatic granules, indicating the release of lysosomal products.[12,14] In further studies we investigated some biological and biochemical properties of GRAM. The chemiluminescence inducing activity of standard-GRAM was detectable up to a dilution 1:16.[14] Activity of GRAM was progressively diminished by heat treatment and was abolished after boiling of supernatants.[14] After treatment of GRAM with hydrolytic enzymes and subsequent testing of the remaining CL induction on PMN, it was demonstrated that GRAM is relatively stable towards ribonuclease, neuraminidase and trypsin, whilst chymotrypsin and protease significantly reduced the activity.[13,14] Preliminary estimations of the molecular weight of GRAM using HPLC gelfiltration techniques indicated that the chemiluminescence inducing activity of GRAM is probably caused by two substances with a m.w. of about 10 kDa and 20 kDa resp.[14] In further studies we investigated the cellular origin of GRAM. Therefore, we incubated monocyte enriched and monocyte depleted cell suspensions with 50 µg/ml quartz dust DQ12 for 24 hours. Supernatants were harvested and tested on their ability to induce chemiluminescence of PMN. Results demonstrated that monocyte depleted cell cultures (95% lymphocytes, 5% monocytes/macrophages) were unable to release sufficient amounts of GRAM. Cultures of mononuclear cells consisting of 17% or 50% of monocytes/macrophages were strong inducers of release of GRAM.[14] Data suggest that monocytes/macrophages and not lymphocytes are the main producers of GRAM.

DISCUSSION AND CONCLUSION

Results presented demonstrate that human monocytes/macrophages in culture release a soluble mediator(s) following incubation with quartz dust DQ12 or coal mine dust TF-1. The mediator(s) stimulates human PMN to the release of reactive oxygen species, especially of superoxide anion. Therefore, we named this mediator(s) "Granulocyte Activating Mediator(s)," "GRAM". Some important characteristics of GRAM are summarized in Table I: Human monocytes/macrophages are able to release GRAM's if stimulated with low amounts of fibrogenic dust particles (quartz dust DQ12 or coal mine dust TF-1 were tested). The relative thermoresistance of GRAM and the sensitivity of GRAM against treatment with protease or chymotrypsin suggest a protein nature of GRAM. Preliminary determination of the molecular weight of GRAM indicates that two molecules (or two parts of one molecule) with a m.w. of approximately 10 kDa and 20 kDa resp. are responsible for the observed chemiluminescence. Besides the enhancement of the oxidative burst of PMN by GRAM we observed the induction of stong chemotactic changes as well as the release of lysosomal products from GRAM treated PMN in a time dependent manner by ultrastructural analysis. Taken together our results present an *in vitro* example of possible non-direct mechanisms of quartz- and coal mine dust pathogenicity. Monocytes/macrophages, when treated with low amounts of quartz dust DQ12 or coal mine dust TF-1 release a soluble cytokine like mediator(s), which then in turn activates human PMN to production of reactive oxygen species. Oxygen species could either directly be toxic and thus lead to alveolar damage[9,16] or could enhance activity of prolylhydroxylase, a key enzyme in collagene synthesis.[2] Furthermore, the chemotactic effects of GRAM on PMN may explain the immigration of PMN in the early phases of silicosis.

In previous studies we reported that human monocytes /macro-phages under influence of quartz- or coal mine dust release a "Fibroblast Proliferation Factor," "FPF".[17,19] The question whether FPF and GRAM are identical has not yet been investigated. Therefore, further research concerning biological and biochemical properties of the mediators described is needed. Our investigations as done so far point to new non-direct, cytokine mediated mechanisms of pneumoconiosis. Measurement of the release of such

Table I

Important Characteristics of Quartz and Coal Mine Dust Induced Granulocyte Activating Mediator(s)

GRANULOCYTE ACTIVATING MEDIATOR (GRAM)

Source:	Human monocytes/macrophages
Inducing Agent:	Quartz dust DQ12, Coal mine dust TF-1
Molecular Weight:	- one (part of a) molecule with m.w. about 20 kda - another (part of a) molecule with m.w. just <10 kda (preliminary estimation, reduced by data from HPLC gel-filtration)
Stability:	56°C, 60 min - 37% loss of activity 80°C, 60 min - 58% loss of activity 100°C, 60 min - 81% loss of activity
Sort of molecule:	Protein nature
Target cells:	human polymorphonuclear granuloctes (PMN)
Effects:	Induction of chemiluminescence of PMN Formation of Formazan cells morphological changes of PMN
Results:	- Metabolic activation with release of oxygen radicals (superoxide anion) from PMN - chemotactic activation of PMN

mediators may be helpful to estimate the noxious effects of respirable particles.

REFERENCES

1. Allen, R.C.: Lucigenin chemiluminescence: A new approach to the study of polymorphonuclear leukocyte redox activity. *Bioluminescence and Chemiluminescence*. Ed. by M. A. DeLuca and W. D. McElroy. Academic Press New York, 63-73 (1981).
2. Bathnagar, R.S.: The role of superoxide in oxidant-induced pulmonary fibrosis. *Biochemical effects of Environmental Pollutants*. Ed. by S. D. Lee. Ann Arbor Science Publ.,Ann Arbor, Mich. 47-58 (1977).
3. Behrendt, H., Friedrichs, K.H.: Effect of low dose and short-time inhalation of silica dust on the morphology of rat lungs. *Aerosols: Formation and Reactivity*. Pergamon Journals Ltd., GB, 279-282 (1986).
4. Berardi, M., Begin, R., Rola-Plescynski, M.: Stimulation of neutrophil chemiluminescence by alveolar macrophages from patients exposed to asbestos. *In vitro effects of mineral dusts*. Ed. by E.G. Beck and J. Bignon., Springer Verlag Berlin-Heidelberg, FRG, 83-91 (1985).
5. Bittermann, P.B., Rennard, S.I., Huninghake, G.W., Rystal, R.G.: Human alveolar macrophage growth factor for fibroblasts: regulation and partial characterisation. *J. Clin. Invest.* 70:806-822 (1982).
6. Bittermann, P.B., Adelberg, S., Crystal, R.G.: Mechanisms of pulmonary fibrosis: spontaneous release of the alveolar macrophage-derived growth factor in the interstitial lung disorders. *J. Clin. Invest.* 72:1801-1813 (1983).
7. Dauber, J.H., Rossman, M.D., Daniele, R.P.: Pulmonary fibrosis: Bronchoalveolar cell types and impaired function of alveolar macrophages in experimental silicosis. *Environ Res.* 27:226-236 (1982).

8. Davis, G.S.: Pathogenesis of Silicosis: Current concepts and hypotheses. *Lung* 164 No. 3:139-154 (1986).
9. Fox, H.B., de Togni, P., Babior, B.M.: Phagocytes and carcinogenesis. Immunology Today Vol. 6 No. 11:327-328 (1985).
10. Gifford, R.H., Malawista, S.E.: The nitroblue tetrazolium reaktion in human granulocytes adherent to a surface. *Yale J. of Biology and Medicine* Vol. 45:119-132 (1972).
11. Lugano, E.M., Dauber, J.H., Daniele, R.P.: Silica stimulation of chemotactic factor release by guinea pig alveolar macrophages. *J. of the Reticuloendothelial Society* Vol. 30:381-390 (1981).
12. Maly, E., Seemayer, N.H., Braumann, A., Behrendt, H., Manojlovic, N., Maly, F.-E.: Die Bedeutung von immunmodula- tiven Zytokinen fuμr die Silikoseentstehung. I. Biologische Charakterisierung eines Granulozyten-Aktivierenden Mediators aus Quarzstaub DQ12 behandelten menschlichen Mono- zyten/Makrophagen. *Ergebnisse von Untersuchungen auf dem Gebiet der Staub- und Silikosebekampfung im Steinkohlenbergbau* Bd.16 201-211 (1986).
13. Maly, E., Braumann, A., Seemayer, N. H., Maly, F.-E.: Induction of chemiluminescence of human granulocytes by supernatants of human mononuclear cultures after treatment with quartz dust DQ12. *Bioluminescence and Chemiluminescence*. New Perspectives. Ed. by J. Schlmerich, R. Andreesen, A. Kapp, M. Ernst and W. G. Woods. J. Wiley & Sons, Chichester-New York 121-124 (1987).
14. Maly, E.: Generation of free oxygen radicals from human polymorphonuclear granulocytes by cytokines from human mononuclear cells, treated with quartz dust DQ12 or coal mine dust TF-1.- New aspects in pathogenesis of pneumoconiosis. *Zbl. Bakt. Hyg. B* (in press).
15. Maly, F.-E., Urwyler, A., Rolli, H. P., Dahinden, C.A. De Weck, A.L.: A single-photon-imagimg system for simultaneous quantitation of luminescent emissions from multiple samples. *J. of Analytical Biochemistry* 168:462-469 (1988).
16. McGoowan, S.E., Murray, J.J.: Direct effects of neutrophil oxidants on elastase-induced extracellular matrix proteoysis. *Am. Rev. Respir. Dis. Vol.* 135:1286-1293 (1987).
17. Seemayer, N.H., Braumann, A.: Untersuchung uber die zyto- toxische Wirkung von Quarz- und Grubenstauben auf menschliche Makrophagen in vitro. In: *Ergeb. Unters. Geb. Staub- und Silikosebekämpfung im Steinkohlenbergbau. Silicosis Report North- Rhine Westphalia*, Vol 15 ,Essen : Steinkohlenbergbauverein, p. 301-320 (1985).
18. Seemayer, N.H.: Importance of grain size and mineral content of coal mine dusts for cytotoxicity on macrophages in vitro. *In vitro effects of mineral dusts*. Ed. by E. G. Beck and J. Bignon, Springer-Verlag Berlin-Heidelberg 497-504 (1985).
19. Seemayer, N.H., Braumann, A., Maly, E.: Entwicklung eines "in vitro" Testsystems mit menschlichen Makrophagen und Fibro- blasten zur Analyse der Wirkungen von Quarz- und Grubenstauben. I. Bildung eines Fibroblasten-Proliferationsfactors. *Ergebnisse von Untersuchungen auf dem Gebiet der Staub- und Silikosebekämpfung im Steinkohlenbergbau* Bd. 16 191-199 (1987).
20. Weiss, S.J., Lobuglio, A.F.: Biology of disease: Phagocyte generated oxygen metabolites and cellular injury. *Laboratory Investigation* Vol. 47 No. 1:5-19 (1982).

CYTOTOXICITY AND SPECTROSCOPIC INVESTIGATIONS OF ORGANIC FREE RADICALS IN FRESH AND STALE COAL DUST

N.S. DALAL* • B. Jafari* • V. Vallyathan† • F.Y.H. Green†

*Chemistry Department, West Virginia University, Morgantown, WV 26506, USA
†Department of Pathology, West Virginia University, Morgantown, WV 26506, USA

INTRODUCTION

The mechanistic details of the biological events leading to coal workers pneumoconiosis (CWP) are not yet fully understood, despite several decades of extensive epidemiologic[1,2] and laboratory studies.[2-6] Epidemiologic studies[1,2] have shown, for example, that the incidence and severity of CWP differ markedly in the different regions and mines at comparable exposures, but laboratory investigations[2-6] have demonstrated only partial correlation of the epidemiologic data with the differences in the mineral composition and the rank of coal mined. In particular, while epidemiologic data indicate[1,2] direct correlation of the prevalence and severity of CWP with the rank (i.e., % carbon content) of coal, this correlation has not been established by laboratory[4-6] and animal exposure[3] studies. In order to explain these results, in 1980 Artemov and Reznik[7] suggested that perhaps they arise from the fundamental differences in the surface properties of the coal dusts inhaled by the coal miners and those used in the laboratory studies: while the miners inhale freshly fractured coal particles, henceforth called the 'fresh' coal dust, the laboratory studies generally utilize 'aged' coal dusts (i.e., dusts that have been stored for many days or even longer). Reznik and Artemov[7] used electron spin resonance (ESR) spectroscopy to show that the mechanical crushing of some Soviet Union coals generated organic free radicals and that the concentration and the decay times (hence the reactivity) of these radicals were higher for the coals of higher ranks. Since some of the radicals decayed within a few minutes in air, the authors surmised that while these radical species could lead to certain specific pathogenic reactions at the sites of mining operations, this might not be the case in the laboratory studies, due to the conventional use of 'stored' dusts which would be expected to contain significantly smaller free radical concentrations. This is consistent with the recent findings that, standardized, aged coal dusts exhibit minimal cytotoxicity.[8]

While the above mentioned work of Artemov and Reznik[7] did suggest a possible new clue to the pathogenesis of CWP (i.e., the role of the coal based free radical species), no direct biological/cytotoxicity data were provided to show that 'fresh' coal dusts were, indeed, more pathogenic than the 'stale' ones. Because of the rather significant implications of the Artemov-Reznik hypothesis to the understanding of the biochemical mechanism and, hence, the strategies for the eventual containment of CWP, we have initiated a comparative study of the free radical formation and cytotoxicity properties of freshly crushed coal particles. As done by Artemov and Reznik,[7] we have used the ESR technique as the direct method of measuring the concentration and decay kinetics of the coal based free radicals. Our preliminary ESR studies on two Pennsylvania coals, a bituminous (carbon content 72%) and an anthracite (95% carbon) coal, have confirmed the Artemov-Reznik finding that the crushing-induced free radical sites are higher on the coals with higher carbon content (i.e., higher rank).[9-11] In the present work we describe our more recent results of a parallel study of the time dependence of the decrease in the free radical content of a freshly made anthracite dust (as measured via ESR) and that of the dust's cytotoxicity potential as measured by the extent of hemolysis of (sheep) erythrocytes. We have also investigated the effects of free radical scavengers on the cytotoxicity potential of the dust and deduce that indeed the free radicals could play a significant role in the initial events in the mechanism of the cytotoxic effects due to the inhalation of coal dust inhalation.

MATERIALS AND METHODS

Reagents

The anthracite coal (#PSOC-867 Carbon content = 95%) was obtained from the Generic Respirable Dust Technology Center, Pennsylvania State University, University Park, Pennsylvania, USA. The samples were received as particles of about 5 mm (longest) dimension. These particles were hand crushed using an agate mortar-pestle arrangement to sizes smaller than 20 microns. A mixed particle size, rather than a specific size fraction, was used in our studies as an effort to simulate the rather random, respirable size coal dust particles in the mining operations. Superoxide Dismutase (SOD) and catalase were purchased from Sigma and were used as received.

ESR Measurements

ESR spectroscopy was used for identifying the crushing-induced coal radicals, and to follow their concentration. The ESR measurements were made with a Bruker ER-200D spectrometer operating at X-band ($\approx$9.5 GHz) frequencies, with

100 kHz field modulation. The magnetic field is controlled via a linearized Hall probe (Bruker, model ER031M) and calibrated with a self-tracking NMR gaussmeter (Bruker, model ER035M). The microwave frequency was measured with a Hewlett-Packard 5340A digital frequency counter. All ESR measurements were carried out at room temperature.

Hemolysis Measurements

Hemolytic activity of the coal dust was measured, following an established method,[12] as the amount of hemoglobin released from a 4% suspension of sheep erythrocytes after incubation with 5 mg and 10 mg of coal dust for one hour at 37°C. The hemoglobin release was estimated via the absorbance at 540 nm using a Gioford spectrophotometer. The procedure was phosphate buffer solution as a negative control (background) and 0.5% Triton-X-100 as a positive control (100% hemolysis). The percentage of hemolysis was calculated as follows:

$$\% \text{ Hemolysis} = (I_{coal} - I_{neg}) / (I_{pos} - I_{neg})$$

where I_{coal} is the absorbance after incubation with the silica dust, while I_{neg} and I_{pos} are those with buffer only and 0.5% Triton-X-100, respectively.

RESULTS AND DISCUSSION

Radical Concentration vs. Crushing Under Nitrogen

Figure 1 shows three typical, first derivative, ESR spectra of the Pennsylvania anthracite coal (PSOC-867). Figure 1 (a) corresponds to the radicals from the stale, uncrushed particles while (b) and (c) are the spectra from 200 x 200 mesh (smaller than 40 micron) and the 400 x 400 mesh (smaller than 20 microns) particles. All of the signals are assigned to the highly delocalized carbon-centered organic free radicals, based on the measured g-value of 2.0029, Lorentzian lineshapes with peak-to-peak widths of about 1 Gauss.[13] Since the lineshapes and widths of all three spectra are essentially the same, the peak-to-peak heights of the first derivative spectra are proportional to the radical concentration in the respective preparations. It is evident that the smaller the particle size the larger the free radical concentration. The measured radical concentrations for all three samples are present in Table I.

Radical Decay in Air

As noted earlier,[9,13] the free radical signals decreased upon exposure of the samples to air, or oxygen. In order to investigate the effect of the crushing in air, as would be the case in the mining environment, some particles (the more shiny ones) were crushed in air to sizes of smaller than 25 microns and kept in air contact during ESR measurements every five minutes over 170 hours, without disturbing the sample or the spectrometer settings. Figure 2 shows three typical ESR spectra taken dependence of the free radical concentration. We note here that the radical concentration was measured from the areas under the ESR signal via double integration of the derivative peaks. It is seen from Figure 2 that the radical decay pattern seems to exhibit an oscillatory behavior up to about 24 hours after which the decay is monotonous. While much more detailed experimentation is necessary to establish the origin of this complex decay kinetics, it was found reproducible in two independent sets of measurements. Table II lists some of the selected data points. While the mechanism for the complex decay kinetics is not clear, a somewhat similar fluctuational behavior was noted in the hemolysis measurements as discussed next.

Hemolysis By Fresh vs. Stale Coal Dust

In order to determine if the freshly-crushed coal particles, containing higher amounts of free radicals, are more cytotoxic than the same particles on storage, we carried out hemolysis measurements on dust particles from the same stock as used in the above discussed radical kinetics. The hemolysis measurements were made for two dust concentrations at specific times (0–1/2, 4, 24, and 96 hours) after crushing. The average hemolytic activity was determined as 24.5% for the 5 mg/ml and 45.3% for the 10 mg/ml coal dust samples for the 0–1/2 hour period. The other measured values are included in Table III and represented graphically in Figure 3. Both Table III and Figure 3 reveal that the hemolytic activity decreased significantly as a function of the dusts' storage in both air and in a phosphate buffered saline (PBS) solution. It is also seen that the air stored dust samples exhibited a faster decrease in the hemolytic activity as compared to the PBS-stored samples.

Effect of Radical Scavengers

The above ESR measurements on 'fresh' coal dust demonstrated that the free radical sites on the coal particles react with oxygen in the air, and that this reaction increases the cytotoxicity of the dust particles. In order to find further clues to the biochemical mechanism of the oxygen-radical involvement in the cytotoxicity, hemolysis measurements were made in the presence of several oxygen-radical scavengers as discussed below.

Superoxide Dismutase (SOD)

The superoxide dismutase (SOD) was the first radical quencher enzyme tested for its effect on the hemolysis because SOD is known to provide an enzymatic defense mechanism against oxygen toxicity.[14] Thus if oxygenated radicals contribute to the toxicity of the fresh coal dust as measured via hemolysis, the addition of SOD should cause a decrease in the hemolysis. Table IV shows the results of addition of 0.5 mg/ml of SOD to the fresh coal dust samples prior to incubation with the sheep erythrocytes. As before, the hemolysis measurements were made at the dust concentrations of 5 mg/ml and 10 mg/ml. Indeed the addition of SOD causes a significant drop in the hemolytic potential of the fresh coal dust. The results indicate a significant role of the superoxide-based radicals in the hemolysis by coal dust.

Catalase

As an aid in understanding the above results with SOD, we investigated the effect of catalase, an enzyme known to offer protection against the hydrogen peroxide (H_2O_2) toxicity, by breaking down H_2O_2 into H_2O and O_2. Table IV also includes the hemolysis results in the presence of 0.5 mg/ml of added catalase. It is clearly seen that the addition of catalase decreases the hemolytic activity even more than done by SOD.

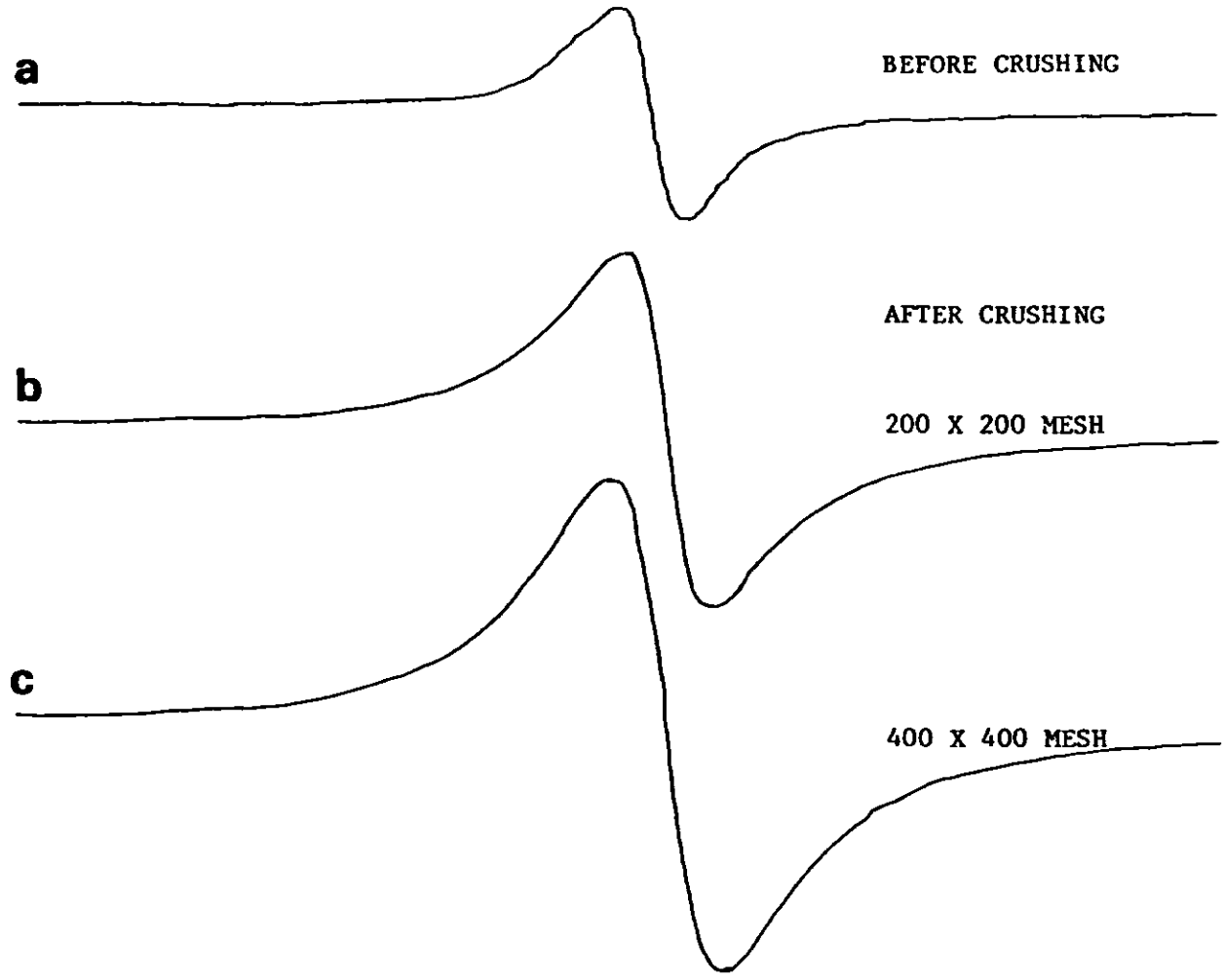

Figure 1. Time dependence of the decay of radicals in air.

Table I

Effect of Crushing on the Anthracite (PSOC-867) Coal		
	Size	Spin/Gram
Before Crushing		4.8×10^{16}
After Crushing	200 x 200 mesh	1.5×10^{17}
Before Crushing		7.9×10^{16}
After Crushing	400 x 400 mesh	4.3×10^{17}

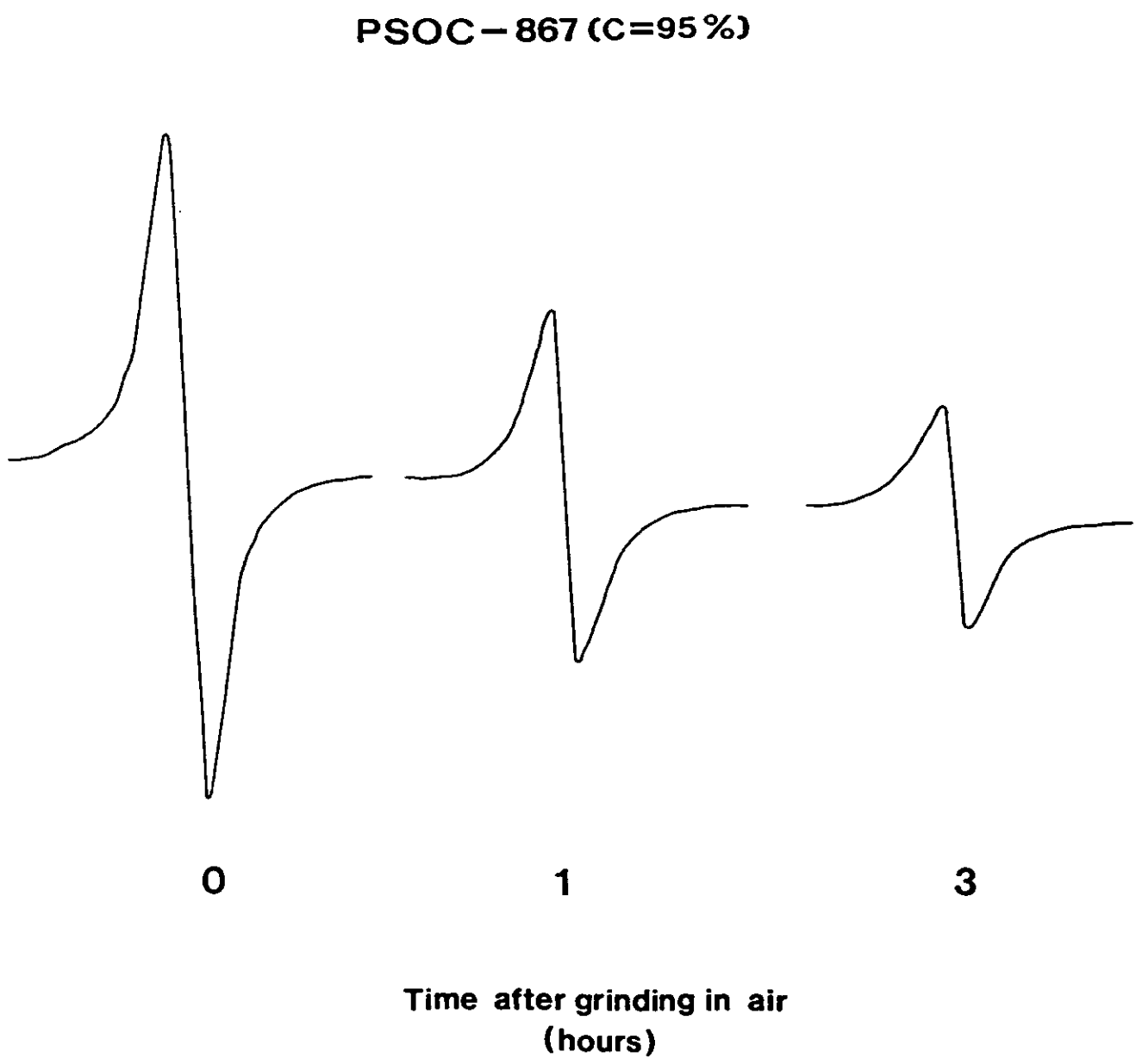

Figure 2. ESR spectra showing the formation of radicals on crushing.

Sodium Benzoate

As a third test, we investigated the hemolytic activity of the fresh dust in the presence of sodium benzoate, a compound often used tp specifically quench ·OH radicals in biological systems.[14] Sodium benzoate was added at two different concentrations, 0.1 mg/4 ml and 0.01 mg/4 ml. The results are presented in Table IV wherefrom it is clear that sodium benzoate decreases the hemolytic activity in a dose-response manner but with much less efficiency than catalase or SOD. These results suggest that the ·OH radicals are not the main species in the mechanism of the membrane cytotoxicity of the fresh anthracite dusts.

Table II

Decay of Radicals in an Anthracite Coal Crushed and Kept in Air	
Time (hours)	Spins/Gram
0	4.28×10^{17}
10	4.33×10^{17}
20	4.44×10^{17}
50	4.31×10^{17}
100	4.07×10^{17}
170	3.35×10^{17}

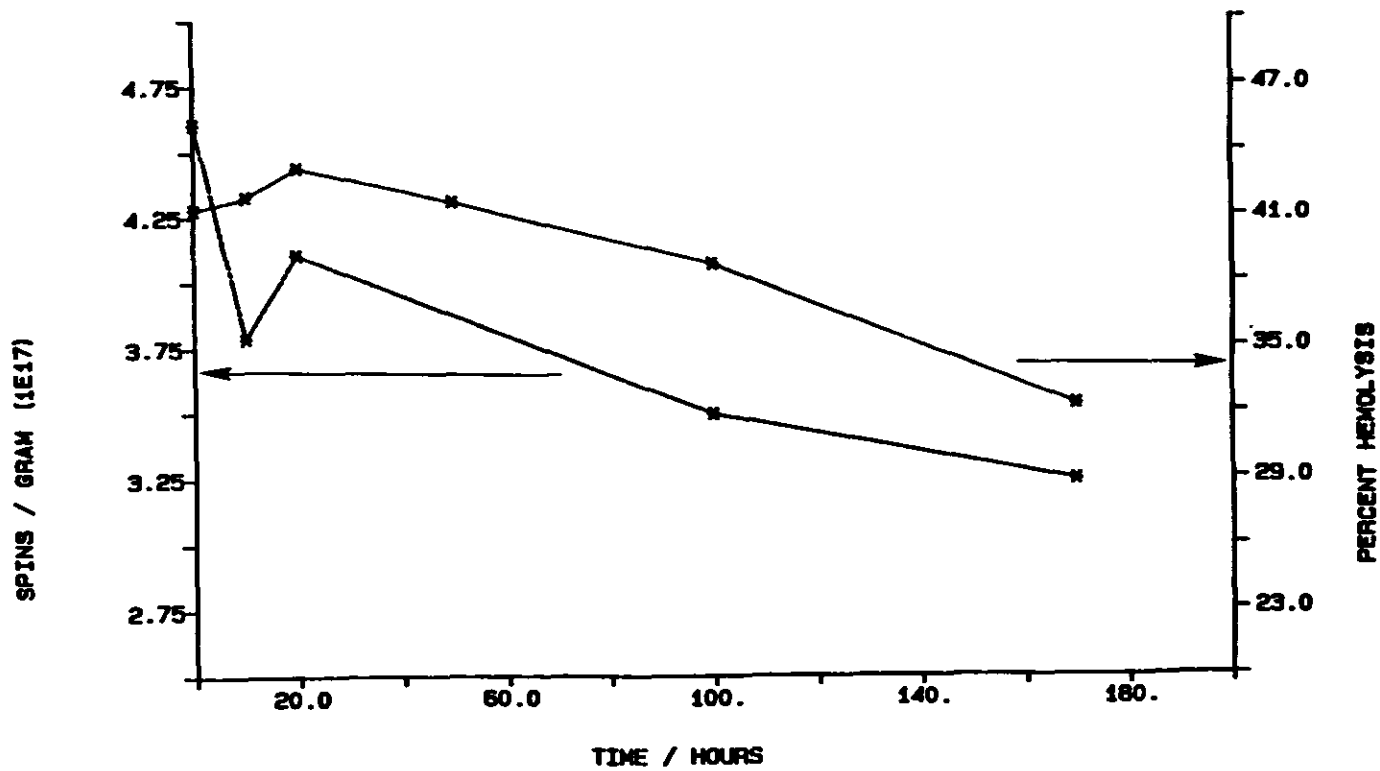

Figure 3. Correlation of the radical concentration measured by ESR, and the toxicity deduced from hemolysis studies of fresh anthracite coal as a function of time.

Table III

Percent Hemolysis Data for an Anthracite Coal (PSOC-867) Crushed in Air

Time/Hours	Percent Hemolysis			
	5 mg/ml AIR	5 mg/ml BUFFER	10 mg/ml AIR	10 mg/ml BUFFER
0	24.5	24.5	45.3	45.3
4	18.5	20.0	35.5	37.5
24	20	21.7	39.3	41.0
96	17.3	20.0	32.0	40.0
176	17.0	20.5	29.0	40.0

Table IV

Effect of SOD, Catalase, and Sodium Benzoate on Hemolysis of Coal Dust

Compound	Concentration	Percent Hemolysis
SOD	5 mg/ml	31.1
	5 mg/ml + SOD	8.7
	10 mg/ml	46.3
	10 mg/ml + SOD	24.3
Catalase	5 mg/ml	29.7
	5 mg/ml + Catalase	10.6
	10 mg/ml	47.2
	10 mg/ml + Catalase	23.3
Sodium Benzoate	5 mg/ml	32.5
	5 mg/ml + 0.1 mg Sodium Benzoate	22.9
	5 mg/ml + 0.1 mg	26.5
	10 mg/ml	45.3
	10 mg/ml + 0.1 mg Sodium Benzoate	31.9
	10 mg/ml + 0.01 mg Sodium Benzoate	38.4

Oxygen Atmosphere

In order to further ascertain if oxygen plays a direct role in the cytotoxicity, not involving the mechanism of any oxygenated species, we carried out comparative hemolysis studies of coal dusts particles under flowing nitrogen gas (to exclude oxygen) and, separately, in air. Moreover the measurements were made for two different particle sizes, 200 x 200 mesh (<40 microns) and 400 by 400 (<25 microns). As shown in Figure 4. The results show that the participation of oxygen is as important to the mechanism of the fresh dust's cytotoxicity as measured by hemolysis, in conformity with the conclusions from the above discussed measurements employing the oxygen radical quenchers.

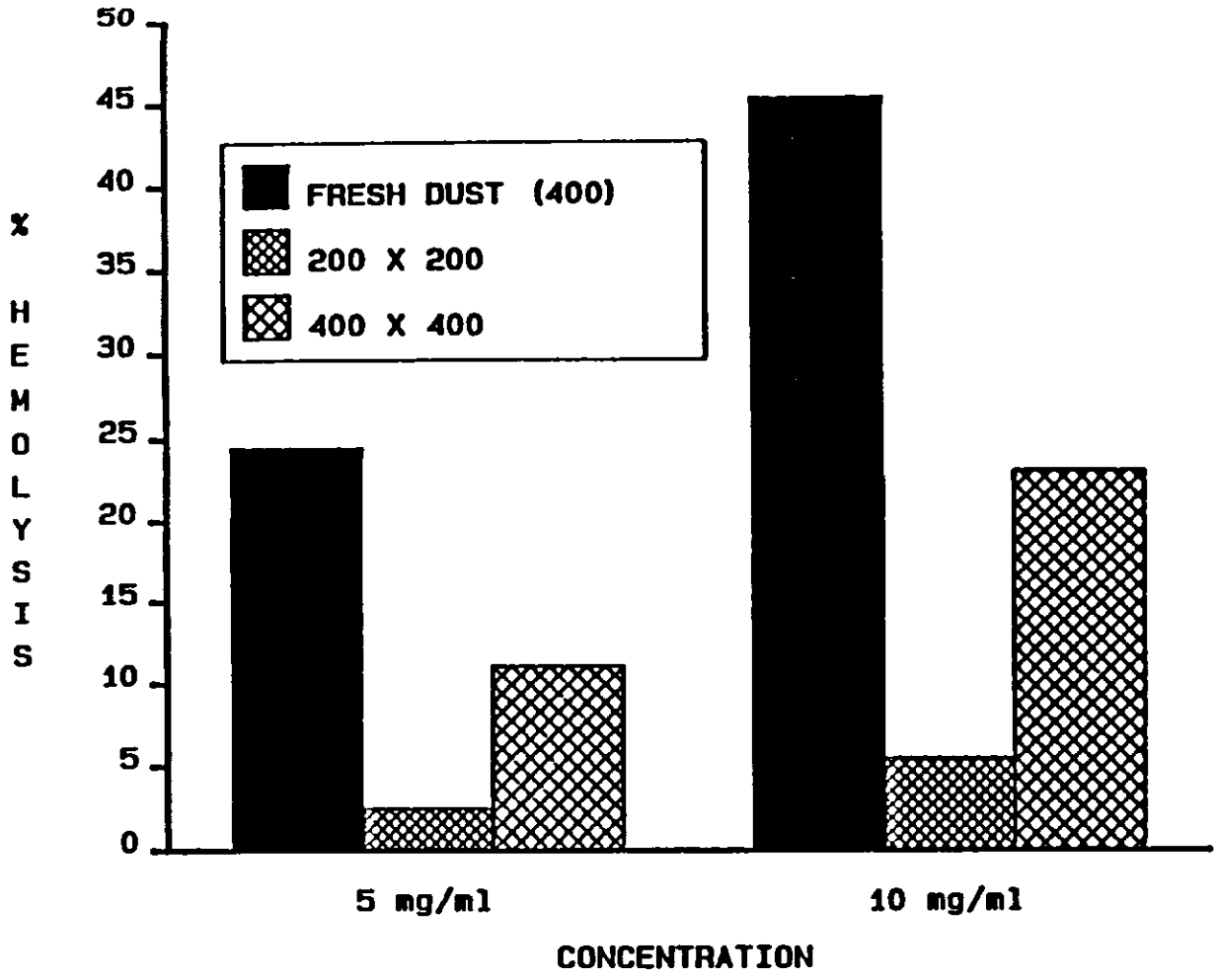

Figure 4. Effect of particle size and nitrogen atmosphere on the hemolysis by the fresh anthracite dust. The data indicates mean of 7 experiments with freshly prepared dust.

Conclusions

The above results suggest that freshly made anthracite coal dusts are more cytotoxic than the 'stale' dusts from the same stock, and that surface oxidation reactions involving free radical sites on the coal particles play a significant role in the dusts cytotoxicity.

REFERENCES

1. Hurley, J. F.: Simple Pneumoconiosis and Exposure to Respirable Dust: Relationships from twenty-five years' Research at Ten British Coal Mines. Institute of Occupational Medicine Report, No. TM/79/13 (1979).
2. Reisner, M.T.R., Robock, K.: Results in Epidemiological, Mineralogical and Cytotoxicological Pathogenicity of Coal-Mine Dusts. *Inhaled Particles,* Vol. 4, p. 703 (1977).
3. Heppleston, A.G.: Changes in the Lungs of Rabbits and Ponies Inhaling Coal Dust Underground. *J. Path. Bact.* 67:349-359 (1958).
4. Hatch, G.E., Gardner, D.E., Menzel, D.B.: Stimulation of Oxidant Production in Alveolar Macrophages by Pollutant and Latex Particles. *Environmental Research* 23:121-136 (1980).
5. Christian, R.T., Nelson, J.B.: Coal: Response of Cultured Mammalian Cells Corresponds to the Incidence of Coal Workers' Pneumoconiosis. *Environmental Research,* 15:232-241 (1978).
6. Christian, R.T., Nelson, J.B., Cady, T.E., Larson, E., Bingham, E.: Coal Workers' Pneumoconiosis: In Vitro Study of the Chemical Composition and Particle Size as Causes of the Toxic Effects of Coal. *Environmental Research,* 20:358-365 (1979).
7. Artemov, A.V., Reznik, L.A.: Questions on the Methodology of Biological Investigations of the Fibrogenicity of Coal Dust. *Gib. Tr. Professor Zabol.,* 2:51-55 (1980).
8. Vallyathan, V., Schwegler, D., Reasor, M., Stettler, L., Green, F.Y.H.: Comparative In Vitro Cytotoxicity and Relative Pathogenicity of Mineral Dusts: paper presented at the International Symposium on Inhaled Particles, Cambridge, England (1985) (in press).
9. Dalal, N.S., Suryan, M.M., Vallyathan, V., Green, F.Y.H., Jafari, B., Wheeler, R.: Detection of Reactive Free Radicals in Fresh Coal Mine Dust and Its Implications to Pneumoconiosis. Paper presented at the International Symposium on Inhaled Particles, Cambridge, England (1985).
10. Dalal, N.S., Suryan, M.M., Jafari, B., Shi, X., Vallyathan, V., Green, F.Y.H.: Electron Spin Resonance Detection of Reactive Free Radicals in Fresh Coal Dust and Quartz Dust and Its Implications to Pneumoconiosis and Silicosis. Proc. Int. Symp. on Respirable Dusts in the Mineral Industries, Pennsylvania State University, University Park, PA (USA). pp. 24-29, (1986).
11. Jafari, B.: EPR-Biochemical Studies of Free Radicals in Pneumoconiosis. MS Thesis, Chemistry Department, West Virginia University Morgantown, WV (USA), (1986).
12. Harringt, J.S., Miller, K., McNab, G.: Hemolysis by Asbestos. *Environ. Res.* Vol. 4, 95-117 (1971).
13. Retcofsky, H.L.: Magnetic Resonance Studies of Coal. *Coal Science,* 1:43-82 (1982) and references therein.
14. Bohinski, R.C.: Modern Concepts in Biochemistry. McGraw Hill, Chapter V (1980).

ACKNOWLEDGEMENTS: This research has been supported by the Department of the Interior's Mineral Institute program administered by the Bureau of Mines through the Generic Mineral Technology Center for Respirable Dust under the grant number G1135142.

EVIDENCE FOR FREE RADICAL INVOLVEMENT IN THE TOXICITY AND CARCINOGENICITY OF CHROMATE DUSTS

XIANGLIN SHI • Nar S. Dalal

Department of Chemistry, West Virginia University, Morgantown, WV 26506, USA

INTRODUCTION

Epidemiologic studies of workers in chromate-ore related industries,[1-3] and stainless steel welding and related occupations[4] have shown that they have about 20–40 times higher risk of throat or respiratory track cancer than controls. Although the actual carcinogenic substances were not identified in these statistical studies, Cr(VI) compounds (for example, calcium chromate, zinc chromate, and lead chromate) were implicated as the causative agents, whereas Cr(III) compounds were not suspected as carcinogens.[5] These suggestions were supported by laboratory studies wherein many Cr(VI) compounds produced sarcomas at the implant or injection sites.[1,5] Squamous cell carcinomas and adenocarcinomas closely resembling human lung cancer were induced by intrabronchial implants of calcium chromate in rats,[6,7] whereas Cr(III) oxide and Cr(III) sulfate did not induce any tumor formation.[6,7]

While the mechanism of the chromate-induced carcinogenicity is not fully understood, it is generally thought[8] that it involves some damage to DNA. Specifically, it has been reported that: a) the chromate ion, henceforth referred to as chromate, can pass through the cell membrane and enter the cell while CR(III) does not,[9] b) chromate does not interact with either native or denatured DNA[10] while Cr(III) does,[11] amd c) the final Cr-DNA complex isolated from cellular reactions of chromate is Cr(III)-DNA, with Cr(III) binding to the phosphate groups.[12,13] Thus the important question is: since Cr(III) cannot pass through the membranes, how does Cr(III)-DNA complex form? For this to happen, Cr(VI) must be reduced to its lower oxidation states,[14] ultimately to Cr(III), by some reductants in the cellular environment. Unless this reduction occurs, the DNA would not be damaged and therefore no chromate carcinogenicity would ensue. Thus the reduction of chromate to its lower oxidation states seems to be a key step in the chromate carcinogenicity.[15] One of the major reductants in cellular environments is thought to be glutathione (GSH), both outside and inside the cells.[16-18] Some evidence for the role of GSH in the chromate toxicity was provided by recent studies showing that exposure of hamster cells to non-toxic levels of added selemite increases the levels of GSH as well as the Cr(VI)-induced DNA strand breaks,[19] and that such DNA strand breaks in hepatocytes also change in direct proportion to the GSH content.[20,21] These observations were interpreted as implying that the reduction of chromate by GSH to some reactive intermediate is an important step in the chromate carcinogenicity.[20,21] In the present undertaking[22] we have used electron spin resonance (ESR) and spin-trap methodology to investigate the reduction of chromate by GSH and find evidence for the involvement of the glutathionyl radical (GS•) as well as Cr(V)-intermediates.

MATERIALS AND METHODS

ESR spectra were obtained at X-band (~9.7 GHz) using a Bruker ER200D ESR spectrometer. The magnetic field was calibrated with a self-tracking NMR Gaussmeter (Bruker, Model ER035M) and the microwave frequency was measured with a Hewlett-Packard (Model 5340A) frequency counter. The spin probes, α-(4-pyridyl-1-oxide)-N-tert-butylnitrone (4-POBN) and 5,5-dimethyl-1-pyrroline-1-oxide (DMPO), were purchased from Aldrich, and used without further purification since very weak or no spin-adduct signals were obtained from the purchased sample when used alone. $K_2Cr_2O_7$, purchased from Fisher, was used as a source for the chromate ion. All measurements were made at room temperature.

RESULTS AND DISCUSSION

Figure 1 shows some typical ESR spectra obtained. While an aqueous solution of 0.1 M spin trap, 4-POBN, containing either chromate or GSH alone, did not give any ESR signal, mixtures of chromate, GSH and 4-POBN together gave a spectrum which was composite of the spin adduct signal (sharp doublets of triplets) and those of Cr(V) (the broad peaks at g = 1.995 and g = 1.985)[23-26] (Figure 1a). About ten minutes later, when the signal from Cr(V) had decayed, a clear spectrum at g = 2.0061, consisting of only doublets of triplets, was obtained, which is assigned to the 4-POBN-GS adduct because of its strong similarity to the spectrum reported earlier[25] for the same adduct, the GS• radical being produced via reaction of GSH with α-chromanoxyl radical. The analysis of the spectrum (doublets of triplets) in Figure 1a gave the nitrogen hyperfine coupling a_N = 15.0 G and proton hyperfine coupling a_H = 2.3 G, which compare well with those (a_N = 15.13 G and a_H = 2.32 G) reported earlier.[25]

Additional support for this identification was obtained from spin-trap studies with DMPO. The ESR spectrum obtained using DMPO was composite of that of the spin adduct, a 1:2:2:1 quartet, and that of Cr(V), the broad peaks at g = 1.995 and g = 1.985, Figure 1b. The analysis of the spin-adduct spectrum gave a_N = 15.2 G and a_H = 15.9 G. These values are fairly close to those (a_N = 15.4 G and a_H = 16.2 G) reported earlier[26,27] for the DMPO-GS spin adduct.

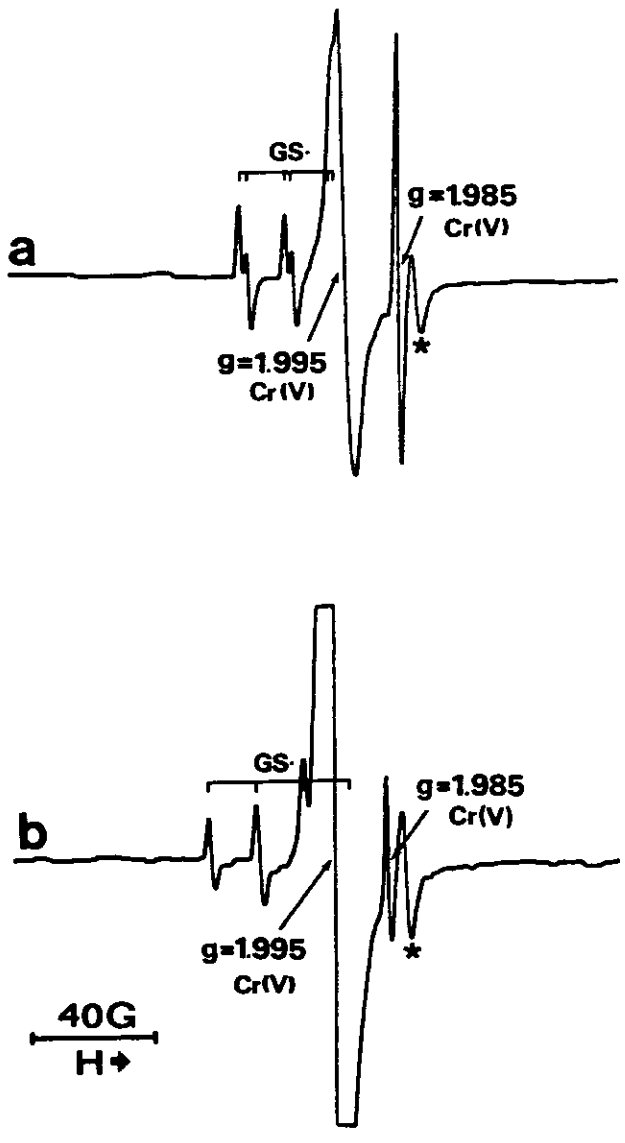

Figure 1. ESR spectra recorded 2 minutes after mixing a solution of [$K_2Cr_2O_7$] = 0.015 M, [glutathione] = 0.15 M; (a) [4-POBN] = 0.1 M; pH = 4.0; (b) [DMPO] = 0.1 M; pH = 7.2. The asterisks indicate minor Cr(V) species.

Moreover the spin-adduct spectrum showed a rapid decrease with time essentially as described previously.[26,27]

The spin-trap studies showed that an increase in the amount of GSH causes to an increase in the spin adduct ESR signals until the intensity leveled off at a molar ratio of about fifteen to one of GSH to $K_2Cr_2O_7$. No spin-adduct ESR signal was detected for the molar ratio of less than one. We also find direct evidence for the formation of a fairly long-lived Cr(V) intermediate, but at molar ratios of higher than one of GSH to $K_2Cr_2O_7$, in contradictiom to an earlier report.[16] In agreement with other studies,[23,24] however, several different Cr(V) complexes were observed depending on the reaction conditions, as indicated by asterisks in Figure 1. We were able to isolate the dominant, g = 1.995, species with a yield of about 50 percent. The measured g-values for the powder spectrum are $g_{\parallel}$ = 2.007 and $g_{\perp}$ = 1.989, with little variation with temperature from 115 to 310 K. These values are typical of Cr(V) solids.[28] ESR measurements on samples redissolved in water gave spectra identical with those from the reaction mixtures (before isolation), showing the stability of this isolated product, and reaffirming its Cr(V) identification.

The above results also help understand two recent reports[20,21] showing that increased levels of GSH in the cells result in increased DNA damage by Cr(VI). Our detection of the formation of GS · and Cr(V) at high GSH levels, as true for the *in vivo* conditions,[10] suggests that the synergistic reactions of the Cr(V) intermediate and GS · are perhaps responsible for the increased Cr(VI)-induced DNA strand breaks at high GSH levels. It is thus felt that these results open up new avenues for understanding the mechanism of chromate-related carcinogenesis.

REFERENCES

1. Enterline, P.E.: Respiratory Cancer among Chromate Workers. *J. Occup. Med.* 16:523-526 (1974).
2. Langard, S., Norseth, T.: A Cohort Study of Bronchial Carcinomas in Worker Producing Chromate Pigments. *Br. J. Ind. Med.* 32:62-65 (1975).
3. Hedenstedt, A., Jenssen, D., Lidesten, B.M., Ramel, C., Rannug, U., Stern, R.M.: Mutagenicity of Fume Particles from Stainless Steel Welding. *Scand. J. Work, Environ. Health* 3:203-211 (1977).
4. Baetjer, A.M.: Pulmonary Carcinoma in Chromate Workers. *Arch. Ind. Hyg. Occup. Med.* 2:487-504 (1950).
5. Furst, A., Schlauder, A.M., Sasmore, D.P.: Tumorigenic Activity of Lead Chromate. *Cancer Res.* 36:1779-1783 (1976).
6. Lane, B.P., Mass, M.J.: Carcinogenicity and Cocarcinogenicity of Chromium Carbonyl in Heterotopic Tracheal Crafts. *Cancer Res. 37:.* 1476-1479 (1977).
7. Stoner, G.D., Shimikn, M.B., Troxell, M.C., Thompson, T.C., Terry, L. S.: Test for Carcinogenicity of Metallic Compounds by the Pulmonary Tumor Response in Strain A Mice. *Cancer Res.* 36:1744-1747 (1976).
8. Biabchi, V., Celotti, L., Lanfranchi, G., Majone, F., Montaldi, A., Sponaza, G., Tamino, G., Venier, P., Zaevtedeschi, A., Levis, A.G.: Genetic Effects of Chromium Compounds. *Mutat. Res.* 117:279-300 (1983).
9. Jennette, K.W.: Chromate Metabolism in Liver Microsomes. *Biol. Trace Element Res.* 1:55-62 (1979).
10. Tsapakos, M.J., Wetterhahn, K.E.: The Interaction of Chromium with Nucleic Acids. *Chem.-Biol. Interactions.* 46:265-277 (1983).
11. Frenderson, M.B., Zeff, L.A.: The Effect of Cr(III) upon the Thermal Denaturation of DNA. *Bioorganic Chem.* 13:24-33 (1985).
12. Huff, J.W., Sastry, K.S., Gordon, M.P., Wacker, W.E.C.: The Action of Metal Ions on Tobacco Mosaic Virus Ribonucleic Acid. *Biochemistry* 3:501-506 (1964).
13. DePhamphiles, M.L., Cleland, W.W.: Preparation and Properties of Chromium(III)-Nucleotide Complexes for Use in the Study of Enzeyme Mechanisn. *Biochemistry* 12:3714-3724 (1973).
14. Tasapakos, M.J., Hampton, T.H., Wetterhahn, K.E.: Chromium(VI)Induced DNA Lesions and Chromium Distribution in Rat Kidney, Liver, and Lung. *Cancer Res.* 43:5662-5667 (1983).
15. Connett, P.H., Wetterhahn, K.E.: Metabolism of the Carcinogen Chromate by Cellular Constituents. *Structure and Bonding;* 54:93-124 (1983).
16. Connett, P.H., Wetterhahn, K.E.: In Vitro Reaction of Carcinogen Chromate with Cellular Thiols and Carboxylic Acids. *J. Am. Chem. Soc.* 107:4282-4288 (1985).
17. Connett, P.H., Wetterhahn, K.E.: Reaction of Chromium(VI) with Thiols: pH Dependence of Chromium(VI) Thiol Ester Formation. *J. Am. Chem. Soc.* 108:1842-1847 (1986).
18. Weigand, H.J., Ottenwalder, H., Bolt, H.M.: The Reduction of

Chromium to Chromium(III) by Glutathione: An Intracellular Redox Pathway in the Metabolism of the Carcinogen Chromate. *Toxicology* 33:341-348 (1984).
19. Sugiyama, M., Ando, A., Furuno, A., Furlong, N.B., Hidaka, T., Ogura, R.: Effects of Vitamin E, Vitamin B_2 and Selenite on DNA Single Strand Breaks Induced by Sodium Chromate(VI). *Cancer Lett.* 38:1-7 (1987).
20. Wetterhahn, K.E., Connett, P.H.: Metabolic Action and DNA Damage Were Important to the Carcinogenicity of Cr(VI). *Trace Subst. Environ. Health* 18:154-162 (1984).
21. Cupo, D.Y., Wetterhahn, K.E.: Modification of Chromium(VI)-Induced DNA Damage by Glutathione and Cytochromes P-450 in Chicken Embryo Hepatocytes. *Proc. Natl. Acad. Sci. USA* 82: 6755-6759 (1985).
22. Pre/iminary results have been presented at the International Conference on Medical, Biochemical and Chemical Aspects of Free Radicals. April 9-13, 1988, Kyoto, Japan.
23. O'Brein, P. Chromium(V) Can Be Generated in the Reduction of Chromium(VI) by Glutathione. *Inorg. Chim. Acta* 108:L19-20 (1985).
24. Goodgame, D.M.L., Joy, A.M.: Relatively Long-Lived Chromium(V) Species Are Produced by the Action of Glutathione on Carcinogenic Chromium(VI). *J. Inorg. Biochem.* 26:219-224 (1986).
25. Niki, E., Tuschiya, J., Tanimura, R., Kamiya, Y.: Regeneration of Vitamin E from Alpha-Chromanoxyl Radical by Glutathione and Vitamin C. *Chemistry Lett.* 789-792 (1982).
26. Harman, L.S., Carver, D.K., Schreiber, J., Mason, R.P.: One- and Two-Electron Oxidation of Reduced Glutathione by Peroxidase. *J. Biol. Chem.* 261:1642-1648 (1986).
27. Stock, B.H., Schreiber, J., Guenat, C., Mason, R.P., Bend, J.R., Eling, T.E.: Evidence for a Free Radical Mechanism of Styrene-Glutathione Conjugate Formation Catalyzed by Prostaglandin H Synthase and Horseradish Peroxidase. *J. Biol. Chem.* 261:15915-15922 (1986).
28. Dalal, N.S., Millar, J.M., Jagadeesh, M.S., Seehra, M.S.: Paramagentic resonance, Magnetic Susceptibility, and Antiferromagentic Exchange in a Cr^{5+} Paramagnet: Potassium Perchromate. *J. Chem. Phys.* 74:1916-23 (1981).

Abbreviations used here: GSH, glutathione; GS·, glutathionyl radical; 4-POBN, α-(4-pyridyl-1-oxide)-N-tert-butylnitrone; DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; ESR, electron spin resonance.