OXYGEN RADICAL GENERATION BY ASBESTOS AND ITS CORRELATION TO CYTOTOXICITY

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INTRODUCTION

Etiologic identification of occupational minerals as pathogenic in humans usually requires epidemiologic, pathologic, toxicologic and animal experimental studies. Epidemiologic studies are often initiated by potential health concerns prompted by cytotoxicity, animal experimentation and/or case reports of overt human health effects. Because we need to know in advance the biosafety of the materials we work with, bioassays and animal experiments to predict the health effects in humans would be of great value. Animal experimental studies are time consuming, relatively expensive and the results are often controversial and difficult to extrapolate to humans due to differences in the responsiveness between species. Disease prediction by cytotoxicity tests would be beneficial because in vitro bioassays are relatively inexpensive, rapidly concluded for the valuable depictions of disease mechanisms and biologic interactions.

In this context during the last few years, molecular toxicology has gained substantial support as a valuable tool in risk evaluation and prediction of adverse health effects of toxic substances in humans. The recognition of mechanisms involved in the initiation of disease through molecular reactions has become relevant to the evaluation of pathogenic risk . Therefore in risk evaluations of chemicals, minerals and possibly in future studies of man-made mineral fibers and untested materials, it is important to pursue some advances in molecular toxicology to enhance our understanding of disease initiation. To test the validity of a possible correlation of cytotoxicity we have attempted, in

NATO ASI Series, Vol. H 85
Cellular and Molecular Effects
of Mineral and Synthetic Dusts and Fibres
Edited by J. M. G. Davis and M.-C. Jaurand

this study, to evaluate asbestos minerals with known fibrogenic potential and fiberglass, using a battery of in vitro bioassays.

There is considerable debate concerning the mechanism of iron-stimulated biologic generation of oxygen radicals and the associated lipid peroxidation in the pathogenicity and carcinogenicity of asbestos (Craighead et al.1982; Mossman et al.1989; 1990). All types of asbestos have been shown to cause fibrotic and malignant pulmonary diseases in humans and animals (Craighead et al.1982). Several investigators have proposed that amphiboles are more fibrogenic and carcinogenic than serpentines because of their higher iron content and potential to produce more oxygen radicals (Craighead et al.1982; Mossman et al.1988; 1989; Kamp et al.1992). On the other hand several studies have suggested that dissimilar physical and chemical characteristics of amphiboles and serpentines are important determinants of cytotoxicity, pathogenicity and carcinogenicity (Mossman et al.1990). In conventional cytotoxicity assays these dissimilar physical and chemical characteristics of minerals play an important role giving false positive and false negative results. We have shown in a recent study that in addition to the physical characteristics, the ability of asbestos to generate oxygen radicals through surface based iron and by frustrated phagocytosis may play an important role in cell injury (Vallyathan et al.1992).

The objective of the present study was to assess the potential role of several asbestos fiber types in the generation of hydroxyl radicals (·OH) from hydrogen peroxide (H_2O_2) and to correlate, if possible, in vitro cytotoxicity, lipid peroxidation potential and in vitro generation of oxygen radicals during phagocytosis. Known mass concentrations of asbestos were mixed with H_2O_2 in the presence of a spin trap to capture the ·OH generated. The stable radical adduct produced was measured in an electron spin resonance (ESR) spectrometer. The signal peak heights of the radical adduct provided a quantitative measure of the ·OH generated. For correlation, cytotoxicity was measured as red blood cell hemolysis and release of alveolar macrophage (AM) enzymes after exposure to minerals. Lipid

peroxidation, as a measure of radical-induced initial cellular membrane injury, was determined using a non-cellular in vitro assay.

MATERIALS AND METHODS

Asbestos used in this study originated from different geographic areas. They were obtained from: chrysotile (Quebec, Canada); amosite (Transvaal, South Africa); crocidolite (Kalahari Desert, South Africa). In addition, Code 100 fiberglass was obtained from Johns Manville, Denver, CO. All types of asbestos were originally obtained by the National Institute of Environmental Health Sciences (Research Triangle Park, NC) and was chemically and physically characterized in detail (Campbell et al.1980). Scanning electron microscopic analyses with the aid of x-ray spectrometric analyses on Nucleopore filters were made to confirm the size characteristics of fibers. Surface area measurements were made by the nitrogen adsorption technique.

Electron Spin Resonance (ESR) Measurements:

Spin trapping of radicals generated from the minerals was made in a total volume of 1 ml containing 1 mg dust, 100 μl 1 M 5,5,-dimethyl-1-pyrroline-1-oxide (DMPO) and 100 μl 0.1M H_2O_2. The reaction was initiated in a plastic syringe by the addition of H_2O_2, mixed well for 10 sec and filtered through a 0.45 μm nylon Acrodisc filter attached to the syringe. ESR measurements were made on 250 μl samples using a flat quartz cell. The measurements were completed within 7 min after mixing H_2O_2, using a Varian E 109 ESR operating at x-band ($\sim$9.4 GHz) frequency. A microwave power of 50 mW and modulation amplitude of 2 G were found to be adequate for the optimal development of peak heights without compromising on spectral resolution.

Cytotoxicity Measurements:

Hemolysis of red blood cells was measured as an index of cellular membrane damage. The

test system consisted of 2 ml PBS, pH 7.4, containing 1 mg mineral and 2 ml of a 4% suspension of sheep erythrocytes. The percent hemolysis was measured according to the method described earlier (Vallyathan et al.1988).

Alveolar Macrophage enzyme Studies:

For enzyme studies, AM were harvested from pathogen free male Fischer 344 rats by repetitive pulmonary lavages using a calcium and magnesium free Hank's balanced salt solution. AM were sedimented by centrifugation and suspended in HEPES buffered solution containing 10 mM glucose. Cell viability counts were made by the trypan blue dye exclusion method. Approximately 95% of the cells obtained by this technique were AM.

For enzyme studies, AM (2×10^6) were incubated with 1 mg dust at 37° C for 2 hr in a water bath with gentle mixing. Percent of LDH, β-NAG and β-GLUC released from the AM into the medium were determined according to the method described earlier (Vallyathan et al.1988).

Lipid Peroxidation:

Mineral-induced lipid peroxidation, of the polyunsaturated lipid linoleic acid, was measured by monitoring the production of malondialdehyde generated in one hour according to the method of Hunter et al (1963). The reaction mixture, in a total volume of 1 ml, contained 1 mg mineral and 10 μl emulsified linoleic acid in phosphate buffer at pH 7.4. The reaction was terminated at the end of one hour by the addition of 0.3 ml 5 N HCl and 0.625 ml 40% trichloroacetic acid. The tubes were mixed and 0.625 ml of 2% thiobarbuturic acid was added and heated in a water bath at 95° C for 20 min. The thiobarbuturic acid reactive substances developed a color which was measured at 535 nm after cooling and centrifugation for 10 min at 600 x g. Malondialdehyde produced was calculated from a standard curve. Control experiments were carried out without minerals and in the presence of an antioxidant, butyl hydroxy toluene, to inhibit lipid peroxidation.

ESR Measurements of Oxygen Radicals During Phagocytosis:

Asbestos stimulated generation of oxygen radicals by rat AM was studied in the presence of a spin trap PBN. AM (1 X 10^6) were incubated with 250 ug dust and 0.1M PBN for 30 min. Reaction was terminated by the addition of 5 ml chloroform:methanol (3:1) and the total lipids extracted. The chloroform was evaporated and the lipid reconstituted in 0.5 ml chloroform and read in a ESR spectrometer as described earlier (Vallyathan et al.1992).

RESULTS

Physical and Chemical Characteristics of Dusts:

The physical and chemical characteristics of the minerals used in this study are presented in Table 1.

Mineral	Length (um)	Diameter (um)	Surface Area (m^2/G)	Iron %	MnO %
Fiberglass	>10	>0.25	4.3	>0.1	ND
Amosite	>41	0.53	4.0	34.61	2.66
Crocidolite	>10	0.27	9.8	18.32	0.08
Chrysotile	>21	0.17	24.9	2.93	0.06

ND-Not detected

The particle size and the size distributions differed considerably among the various types of minerals. Surface area measurements, by nitrogen adsorption, also showed wide differences in surface area reflecting the variability in size (Table 1).

·OH Radical Generation:

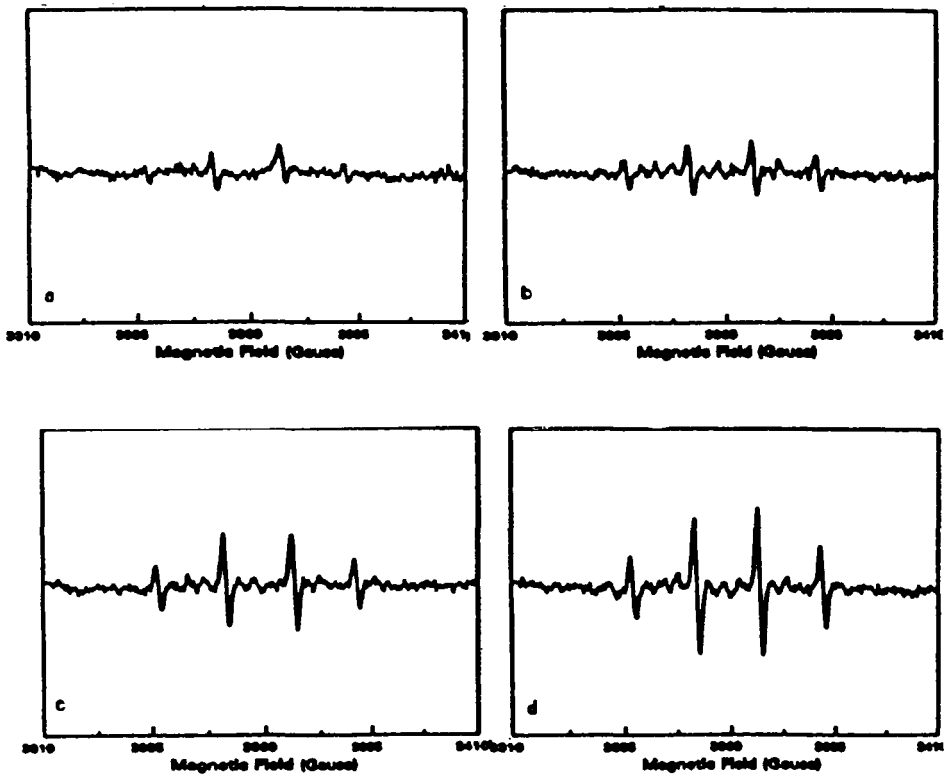
Minerals reacted with H_2O_2 in the presence of an ·OH radical trap DMPO to produce DMPO-

OH radical adducts showing a typical 1:2:2:1 quartet signal. All the ESR signals were similar except for differences in peak intensities (Fig.1a-1d). The ESR spectra obtained for amosite was 375% bigger than that of chrysotile. Crocidolite asbestos produced a signal 166% bigger than that of chrysotile. Fiberglass generated signals barely detectable above the background level produced by DMPO and H_2O_2. The signals produced by minerals in the presence of DMPO was centered around g= 2.0059 + 0.0003 with a splitting constant of 14.9 G, very characteristic of the DMPO-OH adduct (Buettner,1987). To further confirm the generation of ·OH radicals, a competitive ·OH radical scavenger, ethanol, was added in increasing concentrations to the reaction mixture. Addition of ethanol to the dust-DMPO-H_2O_2 mixtures resulted in the generation of a six-line spectrum characteristic of DMPO ethanolyl radical adducts. Metal chelator deferoxamine and ·OH radical scavengers such as catalase and sodium benzoate inhibited ·OH radical generation significantly (80-90%).

The relative potential of all minerals in equal mass concentration (1 mg/ml) to generate ·OH radicals from H_2O_2 is illustrated in Figure 2. It is clearly evident that amosite and crocidolite asbestos, which contained higher concentrations of iron, generated greater amounts of ·OH radical. However, iron concentration of minerals showed no direct correlation to the ·OH radical generation. This non-correlation was more evident when the ·OH radical generation by the minerals was expressed per unit surface area. On a unit surface area basis ($0.1 m^2$), amosite generated the strongest signal, whereas crocidolite and chrysotile produced much weaker signals. The ESR signal generation thereby showed no direct correlation with total iron content.

Cytotoxicity Studies:

The hemolysis potential of all the minerals in equal mass concentration (1 mg/ml) is presented in Figure 3. Chrysotile was the most hemolytic followed by amosite and crocidolite. Hemolytic potential on an equal surface area basis ($0.1 m^2$) showed that amosite was the most hemolytic followed by crocidolite, chrysotile and fiberglass. Release of the cytosolic enzyme LDH and lysosomal enzymes β-GLUC and β-NAG in equal mass concentrations (1 mg/ml) are shown in Figures 4-6. Amosite, crocidolite and chrysotile induced the release of the cytosolic enzyme LDH in



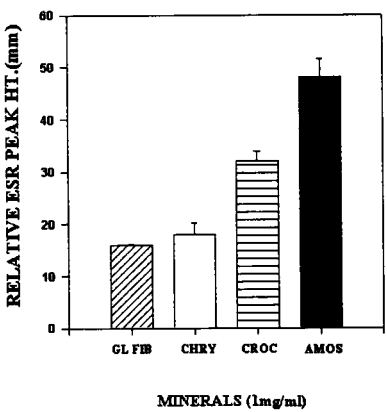


Fig.1 Electron spin resonance spectra obtained from mixtures of minerals, H_2O_2, and spin trap DMPO. Fig.1a. Spectrum obtained from 1mg fiberglass. Fig 1b. Spectrum obtained from 1 mg chrysotile. Fig.1c. Spectrum obtained from 1mg crocidolite. Fig.1d. Spectrum obtained from 1mg amosite.

Fig.2-Graph showing the relative intensities of DMPO OH adducts formed from 10 mM H_2O_2 and 1mg dust samples in the presence of 100mM DMPO.

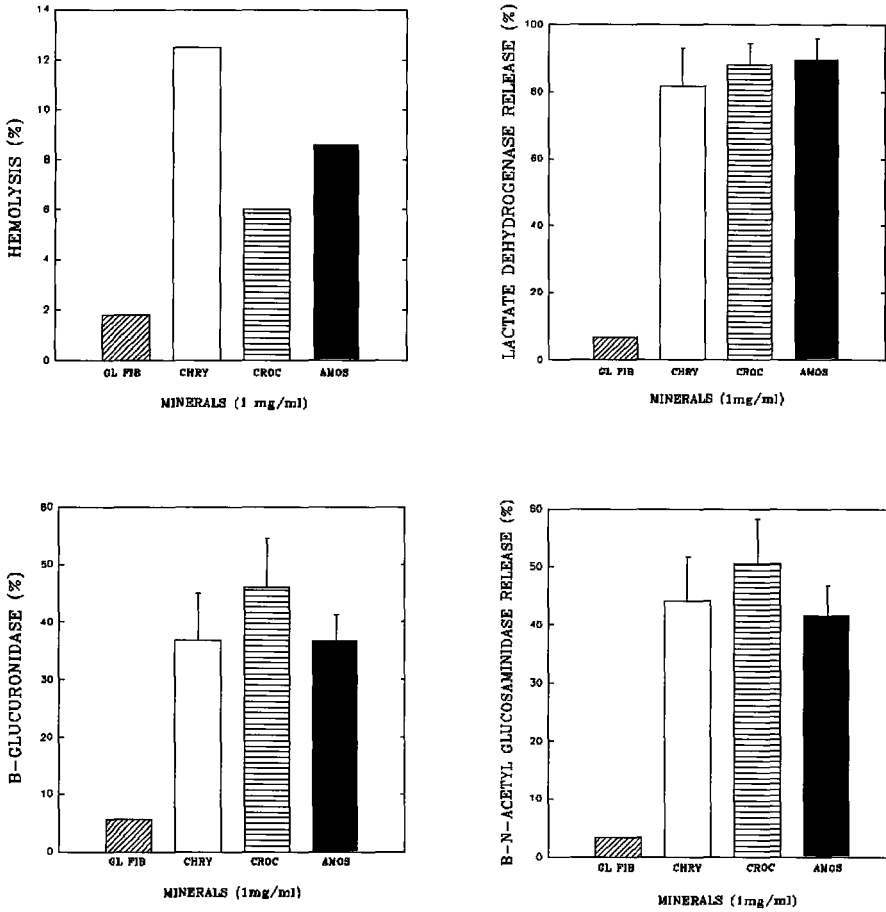
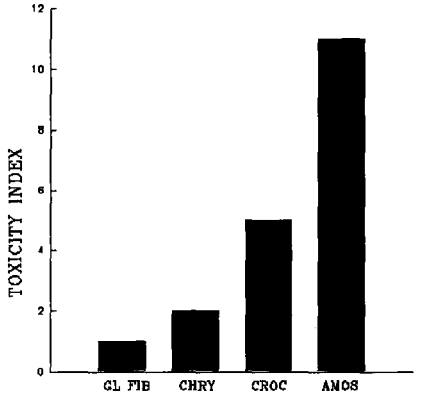
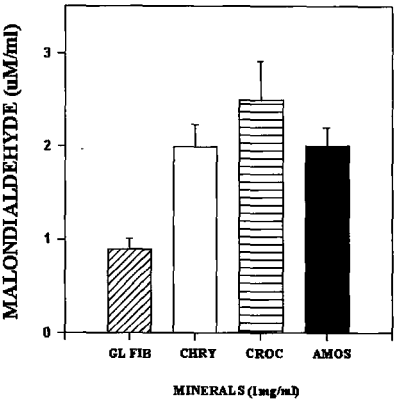
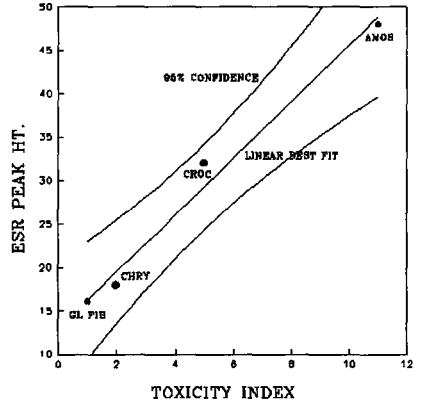


Fig.3 Hemolytic potential of minerals in equal mass concentration. Results presented are the means of (N=5).

Figs.4-6 Release of lactate dehydrogenase (Fig.4) β-glucuronidase (Fig.5) and β-N-acetyl glucosaminidase (Fig.6) from alveolar macrophages ($2x10^6$)/2hr. Results presented are means (N = 5) and SD.







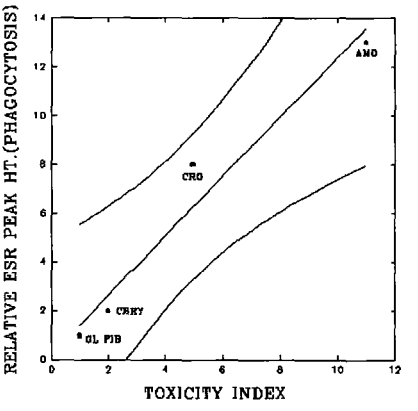


Fig.7 Combined cytotoxicity index of minerals on an equal surface area basis ($0.1m^2$). Cytotoxicity index was derived by adding the data on hemolysis and enzymes and normalizing to 1 for inert dust fiberglass.

Fig.8 Lipid peroxidation potential of minerals in equal mass (1mg) concentrations.

Fig.9 Cytotoxicity index of minerals based on equal surface areas ($0.1m^2$) vs ESR peak heights based on 1 mg mass. Toxicity index showed a good correlation with $^{\bullet}OH$ radical generation.

Fig.10 Relative ESR peak heights vs toxicity index. ESR peak and toxicity index normalized to 1 for fiberglass on an equal surface area basis. The data indicates good correlation.

comparable levels. However, the selective releases of lysosomal enzymes β-GLUC and β-NAG were more pronounced by crocidolite compared to amosite and chrysotile. On an equal surface area basis amosite was the most cytotoxic followed by crocidolite, chrysotile and fiberglass.

Combined Cytotoxicity Index:

Combined cytotoxicity index of hemolysis and three enzymes on an equal surface area basis ($0.1m^2$) are illustrated in Figure 7. The data on an equal surface area basis clearly indicates that amosite and crocidolite are the most cytotoxic and chrysotile and fiberglass are least cytotoxic.

Lipid peroxidation by minerals in equal mass concentration showed crocidolite as a strong inducer of lipid peroxidation followed by amosite and chrysotile (Figure 8). However, when compared on an equal surface area basis, amosite was found to be a stronger inducer of lipid peroxidation followed by crocidolite and chrysotile.

Cytotoxicity VS ·OH Radical Generation by Minerals:

Figure 9 illustrates a direct relationship between the combined cytotoxicity index calculated on an equal surface area basis ($0.1\ m^2$) and ESR peak heights generated from minerals using equal masses ($r^2 = 0.99$, $p<0.01$). There was also no correlation between cytotoxicity index and ESR data calculated on an equal mass basis.

Cytotoxicity VS Oxygen Radical Generation during Phagocytosis:

The oxygen radicals generated during the phagocytosis of minerals is presented in Figure 10. The cytotoxicity index and the ESR peak heights are based on equal surface area ($0.1\ m^2$) basis. The data was normalized to 1 for fiberglass. The ESR peak generated as a result of the combined contributions of mineral and cells correlated well with cytotoxicity ($r^2=0.98$, $p<0.02$).

DISCUSSION

The goal of this study was to investigate the potential use of existing in vitro bioassays to evaluate their applicability to predict fibrogenicity of mineral dusts. This is particularly important because of the proposed ban on the use of asbestos by the United States Environmental Protection

Agency and the manufacturing and introduction of several asbestos substitutes for commercial use. Therefore an attempt was made to identify bioassays that would provide an alarm and reasonable justification for initiating further investigations. A group of well characterized asbestos particles was tested using a battery of cytotoxicity assays, including an assay for the ability of minerals to generate oxygen radicals by themselves and during phagocytosis.

The results suggest that several mechanisms are likely to be involved in the generation of oxygen radicals during asbestos cell interactions. Results with the iron chelator deferoxamine indicate that bioavailability of iron on the surface of asbestos is a major factor in the production of oxygen radicals. These data are also supported by our previous studies showing the augmentation of oxygen radical production when asbestos was treated with chelators such as DETAPAC thereby making iron available for the Fenton reaction (Vallyathan et al.1992).

Asbestos was shown to generate ·OH radicals from H_2O_2 by Weitzman and Gracefa (1985). Potential of asbestos to induce cytotoxicity and lipid peroxidation was shown by several investigators (Weitzman and Weitberg,1985; Rahman and Casciano,1985; Mossman et al.1988). From these studies, it is evident that asbestos particles cause the generation of oxygen free radicals and that these radicals are known to cause cell injury and disease. It is not clear what mechanisms are involved in the generation of oxygen free radicals during the interactions of asbestos and phagocytic cells. First it is evident that iron (Fe^{3+}) present in the asbestos is a major contributing factor in the generation of oxygen radicals (Mossman et al.1988). Another major source of oxygen radical generation is the "respiratory burst" resulting from the phagocytosis of asbestos fibers. The ability of asbestos to perpetuate the generation of oxygen free radicals as a result of cell death and recycling of fibers from repetitive phagocytosis may be a major factor. Finally, frustrated phagocytosis by several phagocyte commonly observed on long asbestos fibers may be a major source of oxygen free radicals.

From the results of these studies made on reference asbestos, we consider it is more valuable to study lipid peroxidation potential and generation of oxygen radicals during phagocytosis as

predictive bioassays for fibrogenicity screening. The value of these predictive bioassays depends on their ability to estimate the potential fibrogenicity of untested materials. In order to establish that goal these bioassays must be tested and validated with several known fibrogenic reference materials.

REFERENCES

Buettner GR. Spin trapping: ESR parameters of spin adducts. Free Rad Biol Med 3: 259-303, 1987.

Cambell WJ, Huggins CW and Wylie AG.Chemical and physical characterization of amosite, chrysotile, crocidolite and non-fiborous tremolite for oral ingestion studies by the National Institute of Environmental Health Sciences. U.S.Department of Interior,R18452,pp1-63,-1980.

Craighead JE, Abraham JL, Churg A, Green FHY, Kleinerman J, Pratt PC, Seemayer TA, Vallyathan V and Weill H. Asbestos-Associated Diseases. The pathology of asbestos asoociated diseases of the lungs and pleural cavities: Diagnostic criteria and proposed grading schema. Arch Path Lab Med 106: 544-596,1982.

Hunter FE Jr, Gebicki JM, Hoffsten PE, Weinstein J and Scott A. Swelling and lysis of rat liver mitochondria induced by ferrous ions. J Biol Chem 238: 828-832, 1963.

Kamp DW,Graceffa P, Pryor W and Weitzman S. The role of free radicals in asbestos induced diseases. Free Rad Biol Med 12:293-315,1992.

Mossman BT, Marsh JP, Shatos MA, Doherty J, Gilbert R, and Hill S. Implication of active oxygen species as second messengers of asbestos toxicity. In: Asbestoc Toxicity, Editors Fisher GL and Gallo MA, Dekker, New York, NY, 1988, pp 157-180.

Mossman BT, Gee J. Asbestos related diseases. NEJM 320:1721-1730, 1989.

Mossman BT, Bignon J, Corn M, Eaton A and Gee J. Asbestos. Scientific developments and implications for public policy. Science 247:294-301, 1990.

Rahman Q and Casciano DA. Involvement of superoxide radical in the toxicity of mineral fibers. In: In Vitro Effects of Mineral Dusts. Springer-Verlag, Berlin, Germany, pp483-488, 1985.

Vallyathan V, Mega JF, Shi X and Dalal NS. Enhanced generation of free radicals from phagocytes induced by mineral dusts. Am J Respir Cell Mol Biol 6:404-413, 1992.

Vallyathan V, Schwegler D, Reasor M, Stettler L, Clere J and Green FHY. Comparative in vitro cytotoxicity and relative pathogenicity of mineral dusts. Ann occup Hyg 32:279-289, 1988.

Weitzman SA and Graceffa P. Asbestos catalyzes hydroxyl and superoxide radical generation from hydrogen peroxide. Arch Biochem Biophys 228:373-376, 1984.

Weitzman SA and Weitberg AB. Asbestos-catalyzed lipid peroxidation and its inhibition by deferoxamine.Biochem J 225:259-262, 1985.

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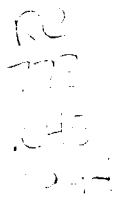
Springer-Verlag
Berlin Heidelberg New York London Paris Tokyo
Hong Kong Barcelona Budapest
Published in cooperation with NATO Scientific Affairs Division

Proceedings of the NATO Advanced Study Institute on Cellular and Molecular Effects of Mineral and Synthetic Dusts and Fibres, held in Paris, France, October 11–13, 1993



ISBN 3-540-58063-8 Springer-Verlag Berlin Heidelberg New York
ISBN 0-387-58063-8 Springer-Verlag New York Berlin Heidelberg

CIP data applied for

Typesetting: Camera ready by authors
SPIN 10122375 31/3130 - 5 4 3 2 1 0 - Printed on acid-free paper

PREFACE

The Fifth International Workshop on "Cellular and Molecular Effects of Mineral and Synthetic Dusts and Fibres" was held on October 11-13, 1993 in Paris, France. The emphasis of the NATO Advanced Research Workshop was the use of cell and organ culture and lavage cell populations obtained from man and laboratory animals to elucidate cellular and molecular events occurring after their interaction with fibrous and non-fibrous particulates including metal compounds. In four sessions, an international representation of scientists from 18 countries (Australia, Belgium, Canada, Finland, France, Germany, Hungary, India, Israel, Italy, Japan, Netherlands, Norway, South Africa, Sweden, Switzerland, UK, USA) presented research findings in the following areas:

- Cellular and metabolic changes caused by mineral dusts.
- Molecular changes and DNA alterations produced by mineral dusts.
- In vivo dust-related pathological processes. Correlations between in vitro and in vivo data.
- Physico-chemical properties of minerals in relation to their biologic effects.

The organising committee for this workshop was:
Co-Chairs: M.C. Jaurand (France) and J.M.G. Davis (United Kingdom)

G. Barlovatz-Meimon (France)
J. Bignon (France)
B. Fubini (Italy)
Q. Rahman (India)
R. Bégin (Canada)
R.C. Brown (UK)
B.T. Mossmann (USA)

The workshop was sponsored by the Advanced Study ARW Program of NATO. Additional financial sponsorship was also obtained from the following:

- Agence de l'Environnement et de la Maîtrise de l'Energie
- Communauté Economique Européenne
- Ministère du Travail, de l'Emploi et de la Formation Professionnelle
- Université Paris Val de Marne
- Association Française de l'Amiante
- Council for Tobacco Research
- Du Pont de Nemours
- Joint European Medical Research Board
- European Ceramic Fibres Industries Association
- North American Insulation Manufacturers Association
- Procter & Gamble Company

The editors wish to thank Springer-Verlag for their interest in publishing this state of the art volume on cellular and molecular interaction between mineral and organic dusts and cells.

The Sixth International Workshop on "Cellular and Molecular Effects of Mineral and Synthetic Dusts and Fibres" will be held in the USA in 1997 and co-chaired by K.E. Driscoll and G. Oberdorster.

John MG Davis
Marie-Claude Jaurand

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