RELEASE OF A "FIBROBLAST PROLIFERATION FACTOR" FROM HUMAN MACROPHAGES IN VITRO TREATED WITH QUARTZ DUST DQ 12 OR COAL MINE DUSTS

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INTRODUCTION

Alveolar macrophages are the primary target for the noxious effect of quartz—and coal mine dusts in human and animal lung. Macrophages produce a very large number of more than 50 "biofactors" or "mediators" which participate in various inflammation and immunological regulation processes.[16,26] Heppleston and Styles[9] reported in 1967 that after incubation with quartz dust, peritoneal macrophages of the rat produce a factor which stimulates chicken fibroblasts to collagen synthesis. After initially negative reports[8] Heppleston's findings have been confirmed by various investigators using diverse in vivo and in vitro cell systems.[1,5,11,12,27] Recently, evidence is accumulating about the formation of mediators in human monocyte and macrophage cultures following stimulation with soluble and particulate agents which stimulate fibroblasts to cell replication.[3,6,7,13,19] Various reports suggest, that human monocytes and macrophages have the ability to generate multiple "fibroblast activating factors," depending on culture conditions, agents used for stimulation, cell type and assay employed for activity evaluation.[3,6,7,13,19,26] As an extension of our report of 1986,[23] this paper presents results on the formation of a "Fibroblast proliferation factor" in human monocyte/macrophage cultures following incubation with quartz dust DQ 12 and coal mine dusts TF-1 from the Ruhr-area (FRG).

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 elsewhere.[22] The cell line FH-3 (human embryonal skin fibroblasts) and the cell line (FH-27) (human embryonal lung fibroblasts) were obtained from Biochrom, Berlin (FRG). The cell line WI-38 (human embryonal lung fibroblasts) were purchased from Flow Laboratories, Meckenheim, FRG and the cell line MRHF (human foreskin dermal fibroblasts) from Api-BioMerieux Nürtingen, FRG. The cell lines of human lung and dermal fibroblasts were cultured in Dulbecco's modified Minimum essential medium with 10% foetal calf serum and antibiotics (Pencillin 100 I.U/ml, Streptomycin 100 μg/ml).

Mine Dust and Control Dust

The TF-1 dust, fraction BAT-II from the Ruhr region was kindly supplied by the Hauptstelle for Staubbekämpfung und Pneumokonioseverhütung des Steinkohlenbergbauvereins in Essen. This is a mine dust with a high mineral content of 95 wt % and a quartz content of 10.6 wt %, with a particle size distribution of 0.5–2.5 μm.[20]

Quartz dust DQ 12 was used as the toxic dust (positive control). This is Dorentruper crystall quartz flour (grinding no. 12) with a particle size 5 μm.

Preparation of Supernatants from Cultures of Human Monocytes/Macrophages

Dust samples were suspended in Iscove medium or RPMI-1640 medium without an addition or with 1% newborn calf serum. The samples were subjected to ultrasonic treatment (Sonifier B-12 from Branson Sonic Power Company, USA) in order to achieve a uniform distribution of the particles and to destroy germs. Suspended dust samples in concentrations as described under "Results" were added to cultures of human monocytes/macrophages. After an incubation period of 24 hours at 37°C, the culture supernatants were centrifuged for 15 min at 3,000 rpm and then filtered through Millipore filters (pore size 0.45 μm). The supernatants were then deep frozen at -20°C until used.

Chemicals and Equipment

Fibroblast growth factor (FGF) was purchased from Sigma, Munich (FRG) and Boehringer, Mannheim (FRG), Platelet derived growth factor (PDGF), porcine, Speywood Laboratories was obtained from Sebak Company, Aidenbach, FRG. Ultrafree PF Filter Units of 10.000 and 30.000 NMWL and Centrifugal Ultrafree Filter Units of 10.000 and 30.000 NMWL were commercially available from Millipore, Eschborn, FRG.

Determination of Cell Growth of Human Dermal and Lung Fibroblasts

Human fibroblast cell lines (FH-3, MRHF, FH-27, WI-38) were detached with a trypsin-Versene mixture and adjusted to a cell count of 4–6 $\times$ 10^4 cells/ml in Dulbecco's MEM with 1% foetal calf serum or with 10% serum (2% foetal and 8% newborn calf serum), corresponding to dermal and lung fibroblasts, respectively. 1 ml of this cell suspension per well was transferred to a tissue culture plate with 24 wells (Falcon 3047 MultiWell tissue culture plate) or to LAB-Tissue Culture Chamber (4 chamber, LT-4804). 24 hours later cell cultures were re-fed with Dulbecco's MEM with 0.5% foetal calf

serum or with 0.15% bovine serum albumin (Boehringer Mannheim, FRG) and kept for 2–4 days to obtain "quiescent" cultures. The macrophage supernatants were added in an amount of 0.2 ml per well or chamber. In each case 4 cultures were used per measurement point. The protein determination according to Lowry in the modification of Oyama and Eagle[18] was carried out as already described earlier.[21] A protein calibration curve was plotted with "pure" bovine serum albumin from Serva, Heidelberg (FRG). For morphological evaluation of cell cultures by light microscopy we used a standard procedure of fixation and staining with Bouin's solution and hematoxylin-eosin or with methanol and Giemsa.

Statistical Analysis

For statistical analysis data were computerized and mean values and limits of confidence were determined. Futhermore, Bartlett test for equal variances, one-way analysis of variance and Student's t-test were performed.

RESULTS

Human fibroblast cultures, "quiescent" or showing only slight cell replication were used to quantify the "fibroblast proliferation activity" of supernatants from treated and untreated human macrophage cultures. The results of such an experimental set-up are shown on Figure 1. Human macrophages were cultivated for 7 days. Thereafter cells were treated with quartz dust DQ 12 for 24 hours in Iscove medium without serum at a concentration of 30 μg/ml per approximately 1×10^6 cells. Supernatants were collected as outlined in Materials and Methods. To cell cultures of human dermal fibroblasts (FH-3) supernatants of untreated and quartz dust DQ 12 treated macrophages were added. After a culture period of 6–8 days on an average, the protein content of the cultures was determined by the method of Lowry in the modification of Oyama and Eagle.[18] Column 1 (Figure 1) shows an untreated FH-3 fibroblast culture. The amount of protein in μg/ml is given as a criterion of cell growth. Column 2 illustrates the cell growth of the fibroblast culture to which the supernatants of the untreated macrophage culture had been added. No increase in cell growth is seen in comparison with the control. Column 3–6 (Figure 1) represent the growth of human dermal fibroblast culture FH-3 to which the supernatant of a macrophage culture treated with 30 μg/ml DQ 12 had been added in various dilutions. The potent "proliferation-stimulating activity" of this supernatant led nearly to doubling of the protein content of these cultures at a dilution of 1:5 in the course of 7 days in comparison with the control (Column 3). But also at higher dilutions of 1:10 up to 1:40 the "proliferation-stimulating activity" of this supernatant can be seen (Column 4–6).

The "proliferation-stimulating activity" of supernatants of human macrophages (age 7 days) to which the coal mine dust TF-1, fraction BAT-II had been added for 24 hours was tested in further experiments. For this purpose, supernatants of untreated human macrophage cultures and of those treated with coal mine dust TF-1 (30 μg/ml, 24 hours) were added to human fibroblast cultures (line FH-3) and cultured at 37°C for 7 days. The results are shown on Figure 2. Column 1 represents the cell control, column 2 shows the cell growth of the fibroblasts after addition of the supernatant of untreated macrophages. Column 3–6 (Figure 2) represent the cell growth following addition of supernatant of macrophages treated with coal mine dust TF-1. In comparison with the cell and macrophage control, there is a significant increase in cell growth of the human dermal fibroblasts FH-3 which had been incubated with the supernatant of macrophages treated with coal mine dust TF-1. A similar "proliferation-stimulating effect" was observed with human diploid lung fibroblasts WI-38 treated with supernatants from another batch of human macrophages incubated with coal mine dust TF-1 (30 μg/ml, 24 hours). We made further attempts to characterize the factor produced by quartz and coal mine dust exposed human macrophages. We found that the factor is still active after

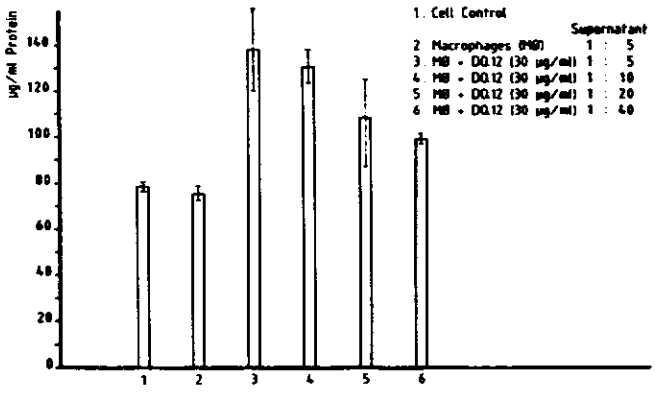

Figure 1. Cell growth of human fibroplasts of cell line FH-3 following incubation with the supernatant of an untreated (column 2) human macrophage culture and one incubated with quartz dust DQ 12 (column 3–6) in various dilutions. Mean values and confidence limits are shown.

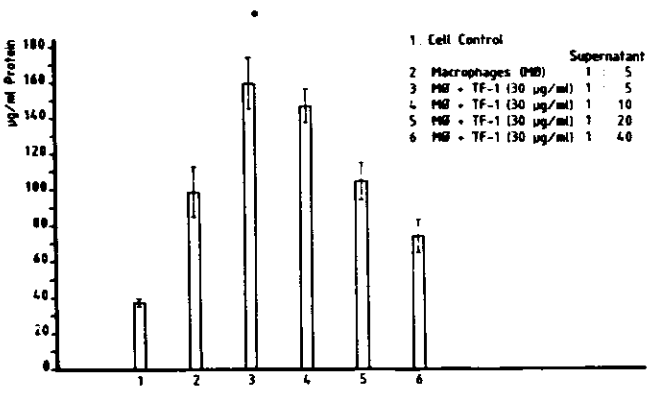

Figure 2. Cell growth of human fibroplasts of cell line FH-3 following incubation with the supernatant of an untreated (column 2) and one treated with coal mine dust TF-1, BAT-II (column 3–6) in various dilutions. Mean values and limits of confidence are presented.

incubating at 56°C for 60 min. For estimation of the approximate molecular weight of the factor we utilized Ultrafree PF Filter Units (Millipore) of 10.000 and 30.000 NMWL (nominal molecular weight limits) and corresponding Centrifugal Ultrafree Filter Units (Millipore) of 10.000 and 30.000 NMWL. Results based on induced cell multiplication of non-replicating MRHF and FH-3 fibroblasts and on stimulation of DNA synthesis of WI-38 cells by the supernatant of quartz dust DQ 12 exposed human macrophages larger and smaller than 10.000 and 30.000 NMWL indicate a molecular weight of the factor of more than 30 KDa. In further experiments we incubated non-replicating WI-38 cells for various lengths of time (2, 4, 16 and 24 h, 7 days) with supernatants of quartz dust DQ 12 exposed macrophages. Results revealed that a continuous presence of the factor is necessary for cell replication. This assumption is also supported by measuring the number of DNA synthesizing cells of WI-38 cells exposed for various lengths of time to supernatant. Only continuous presence of the factor led to a high number of DNA synthesizing cells.[10] After removal of the factor the DNA synthesis ceased very rapidly. To elucidate the nature of the "fibroblast proliferation factor" we performed "complementation tests" according to Stiles et al.[25] and Bitterman et al.[3] Addition of fibroblast growth factor or of platelet derived growth factor (PDGF) to no replicating WI-38 or MRHF-cells enhanced the cell growth significantly in presence of supernatants of quartz dust DQ 12-treated human macrophages. Similar results were obtained with WI-38 cells in presence of supernatant of coal mine dust TF-1 treated human macrophages. Addition of FGF or PDGF led to a remarkable enhancement of cell multiplication.

DISCUSSION AND CONCLUSION

Results presented demonstrate that human macrophages in culture obtained by cultivation and differentiation of blood monocytes, form a soluble factor(s) following incubation with quartz dust DQ 12 or coal mine dust TF-1. This factor stimulates "quiescent" or only moderately replicating human lung and dermal fibroblasts to a considerable cell multiplication. Therefore, we designated the "factor" as "fibroblast proliferation factor" (FPF).

As we earlier reported,[23,24] the process of fibroblast multiplication stimulated by FPF could also be visualized by morphological criteria, such as increased rate of DNA synthesis[10] and of mitosis and by manifestation of high cell density of cultures.

The "dual control model of growth regulation" suggests[25] that growth factors can be classified either as "competence factors" or as "progression factors." While cells require only transient exposure to "competence factors," i.e. PDGF, FGF, "progression factors" are required continuously for DNA synthesis and cell replication. Results suggest a classification of FPF as a "progression factor" because a continuous exposure was required for cell multiplication of fibroblasts and in view of an enhanced growth after addition of FGF or PDGF in a serum-free medium "complementation test."

Bitterman et al.[3] reported that human alveolar macrophages obtained by lung lavage and incubated in vitro with soluble and particulate agents, release an alveolar macrophage derived growth factor (AMDGF), exhibiting activity as a "progression factor" for human lung fibroblasts. The molecular weight of AMDGF of 18 kDa differs from the MW of FPF, preliminary estimated as more than 30 kDa. Thermal stability of FPF (56°C, 60 min) is resembling stability of an alveolar macrophage factor from silica instilled rats, isolated by Benson et al.,[2] inducing elevated DNA synthesis of rat lung fibroblasts. Beside FPF in supernatants of quartz DQ 12 or coal mine dust TF-1 treated human monocytes/macrophages a "Granulocyte activating Mediator" (GRAM) was detected[14,15] causing a long lasting Lucigenin-dependent chemiluminescence of human granulocytes.

In several studies "fibroblast growth factors" have been described, which were induced in cultures of human monocytes and macrophages by various soluble and particulate agents, i.e. by zymosan, phytohemagglutinine, concanavalin A, endotoxin, immune complexes, staphylococci and quartz dust (Bitterman et al.,[3] Dohlman et al.,[6] Glenn and Ross,[7] Leslie et al.,[13] Schmidt et al.,[19] Seemayer et al.[23,24]

The growth stimulation of fibroblasts by activated or damaged macrophages is of great importance for fibrotic lung processes especially silicosis and appears to be a generally applicable, pathobiological principle.[4]

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USE OF A SENSITIVE ELECTRO-OPTICAL METHOD TO QUANTIFY SUPEROXIDE PRODUCTION FROM SINGLE PULMONARY ALVEOLAR MACROPHAGES EXPOSED TO DUSTS *IN VITRO* OR *IN VIVO:* SOME CURRENT EXPERIMENTAL AND MODEL RESULTS

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ABSTRACT

This laboratory has developed a sensitive electro-optical method to quantify the initial rate (R) and total amount (MAX) of superoxide (O_2^-) produced by single pulmonary alveolar macrophages (PAM). The method uses a microscope-based TV system to visualize PAM in culture, and to video record the images during the time the cells produce O_2^-. MAX and R are calculated from measurement of temporal changes in optical density in the images due to precipitated diformazan formed by the reaction of O_2^-, produced by each PAM, with nitroblue tetrazolium present in the culture medium. To date, values of R and MAX, measured due to adherence of PAM to the dish, have been compared to values obtained when quartz, coal mine dust (CMD), and kaolin were added to the medium (*in vitro*). R and MAX have also been calculated for PAM lavaged from animals exposed to quartz and CMD in the WVU Inhalation Facility. Presently, experiments are being done using serum which will permit PAM to be restimulated by different dusts to help establish a dose response relationship and a means to study the role of lung surfactant on modifying the acute toxicity of inhaled dusts. Ultimately, this methodology should provide useful insight into establishing the role of O_2^- in PAM dysfunction due to inhalation of dusts, and in lung diseases such as pneumoconioses.

INTRODUCTION

PAM are free cells found in the lungs which protect the lungs by removing foreign debris and bacteria. This is accomplished, in part, by the process of phagocytosis, whereby foreign matter is internalized into vesicles known as primary phagosomes. Phagocytosis also involves the chemical breakdown of inhaled dusts and the killing of microbes. Detoxification is aided by the respiratory burst, which is a metabolic response of the cell to foreign substances, that results in the production of highly reactive oxidizing agents from the partial reduction of extracellular oxygen to superoxide (O_2^-) at the surface of the plasma membrane. Superoxide undergoes either spontaneous or enzyme catalyzed dismutation reactions to form hydrogen peroxide (H_2O_2) and subsequently hydroxyl radical ($OH^\bullet$) and singlet oxygen ($O_2{}^1$). However, while these oxygen metabolites aid in the killing of microbes they also may destroy endogenous tissue. For example, O_2^- has been linked to the aging process and to many diseases including emphysema, diabetes, and cancer.[4] Therefore, a better understanding of the production of O_2^- is extremely important since an abnormally low production could result in damage by inhaled dusts and bacteria while an abnormally high production could result in direct damage to the lung tissue by the phagocytes.

Inhalation of respirable sized mineral dusts, such as quartz (silica), coal mine dusts (CMD), and asbestos, results in various pulmonary disorders. PAM are thought to play an important role since evidence suggests that the first step in fibrogenesis is an interaction of dust particles with PAM. Generally, *in vivo* exposure of animals to mineral dusts results in increased respiratory burst activity, migratory patterns, phagocytic behavior, and secretory potential of PAM, and for this reason these have been implicated to be pivotal events in the pathogenesis of pulmonary diseases.[1] In addition to stimulation of resident PAM, an influx of monocytes into the lung and/or production of new macrophages generally occurs in response to fibrogenic dust inhalation. Further, PAM may attract and stimulate fibroblasts, which normally synthesize proteins and collagen during repair of tissue, by secreting chemoattractants and enzymes. Thus, PAM may be involved in pulmonary disorders through failure or partial loss of their defensive capability, or indirectly, through release of other mediators.

The major objective of this project is to develop a better understanding for the role O_2^- production by PAM plays in the development of pulmonary disease following exposure to inhaled dusts. Specifically, it is not understood whether certain dusts (or constituents of dusts), inhaled over a period of time, can alter the ability of this cell-type to remove foreign material from the lung. Also, it is not known whether dysfunction of PAM occurs which may result in an underproduction or an overproduction of O_2^-; either of which may be harmful to normal lung tissue.

A novel technique has been developed to quantify O_2^- production by *single* PAM which has permitted study of the

effects of different dusts, concentrations of dust, and time of exposure on O_2^- release by PAM. A multifaceted approach has been used which encompasses the following: (1) study of O_2^- production by single PAM isolated in culture, when contacted directly with different concentrations of dusts suspended in the medium (*in vitro*); (2) study of O_2^- production by PAM after *in vivo* exposure to dusts in inhalation chambers for known periods of time; and (3) development of a mathematical model to describe the kinetics of production by single cells. These studies ultimately will provide a rapid, quantitative assay to determine the effect of toxic dusts on O_2^- production by PAM.

METHODOLOGY

The methodology for measuring O_2^- production from single PAM using NBT reduction was developed previously.[2,3] Briefly, 3 ml of NBT solution (37 C) was placed in culture dishes and then 0.4 ml of a cell suspension ($2–5 \times 10^5$ PAM) added. A layer of paraffin oil was placed on top of the aqueous layer. The dish was placed on a temperature controlled stage of an inverted microscope and trans-illuminated at 550 nm. In the presence of a strong reducing agent, such as O_2^- produced due to PAM adherence to the culture dish, soluble NBT is reduced to a diformazan precipitate which can be measured spectrophotometrically. PAM were visualized (20x) and images, containing at least 6 well-separated PAM, were televised and video recorded for 40 min. Recorded images were played back through electronic instrumentation which permitted determination of optical density (OD) changes for individual cells due to precipitated diformazan. The OD was converted to the mass of diformazan produced versus time, from which MAX was determined directly, and then the data fit to a phenomenological equation from which R was calculated.

RESULTS

In Vitro Experiments

The effects of acute *in vitro* exposure to respirable quartz and kaolin on O_2^- production during adherence of PAM to culture dishes were tested using low (0.025 mg/ml) and high (0.05 mg/ml) dust concentrations. The low dose of quartz decreased the maximum amount of O_2^- produced (MAX) 38% compared to control while the high dose did not. However, the maximum rate of diformazan production, R, decreased 31% and 24% for the low and high dose, respectively. *In vitro* exposure of PAM to the high dose of sonicated CMD suspensions resulted in increased O_2^- production. In contrast, kaolin, a non-fibrogenic dust did not significantly change either MAX or R. These results suggested that O_2^- production may be a better indicator of pathogenicity and PAM dysfunction than cell death, which gives comparable results for quartz and kaolin. Interestingly, no changes in O_2^- production were observed following *in vitro* exposure of PAM to quartz, kaolin, or CMD in the presence of a surfactant (tween 80), suggesting that lung surfactant may alter the acute toxicity of dusts.

Previously, PAM could not be restimulated after adherence. However, it has been recently shown that O_2^- production from adherent PAM is possible when cells are incubated with serum.[3] Serum alone did not stimulate adherent PAM indicating serum is necessary but not sufficient for stimulation. This finding supports the idea that *in vivo* serum may condition PAM to produce O_2^-.

In Vivo Experiments

The effects of *in vivo* exposure of animals to respirable quartz (20 mg/m^3, 16 hr/d, 5 d/wk of MIN-U-SIL 10, 95% <5 μm) was tested by housing animals (2-4 weeks) in the WVU inhalation facilities. Control (no quartz) animals also were kept in identical inhalation chambers for 2-4 weeks. Following exposure, animals were removed from the inhalation chambers and housed in animal-care facilities for 3, 10, or 31 days post-exposure. This approach permitted analysis of the effects of length of *in vivo* exposure and post-exposure time on PAM analyzed for O_2^- production. Overall, respirable quartz increased MAX 36% and R 29% compared to control animals. Importantly, PAM from exposed animals showed an increased O_2^- production for up to 10 days after 2-4 weeks of exposure followed by a return to control levels by 31 days. Interestingly, the 3 day group suggested that there was activation and/or recruitment of PAM. *In vivo* exposures performed using CMD (20 mg/m^3, 16 hr/d, 7 d/wk of Pittsburgh BOM Dust 2020, 100% 4-6 um) also showed increased production at 10 days post-exposure, with large amounts of CMD phagocytosed by 31 days. Similarly, the data showed an activation and/or recruitment of PAM.

Theoretical Model Development

A kinetic model was developed to describe the production of O_2^- by *single* PAM. The kinetic model considered three reactions: (1) the production of extracellular O_2^- from the reduction of oxygen by NADPH oxidase using intracellular NADPH as the substrate, (2) the subsequent dismutation of O_2^- to form H_2O_2, and (3) the reaction of O_2^- and NBT. NBT specificity of O_2^- was analyzed by comparing experimental results, in the presence and absence of superoxide dismutase (SOD) which catalyzes the dismutation of O_2^- to H_2O_2. Measured PAM heterogeneity (without SOD) was accounted for in the model by varying the concentration of intracellular NADPH, its rate of depletion, and the concentration of NADPH oxidase. Model predictions compared well with experimental results except when SOD was present. Experiments showed only a 50-60% decrease in diformazan production using SOD. This discrepancy may be due to diffusional limitations which occur since SOD is a much larger molecule (34 kD) compared to NBT (818 D). In addition, the cell surface is both ruffled and negatively charged, which may introduce steric hindrances and/or electrostatic effects since SOD is also negatively charged.

CONCLUSIONS

In vitro assays on large numbers of cells in culture using hemolysis of red blood cells or release of enzymes from PAM following dust exposure have been used to analyze cytotoxicity. In such systems, kaolin has been found to have an activity comparable to quartz on a mass basis. However, *in vivo*,

quartz is highly fibrogenic resulting in silicosis while kaolin is not. Therefore, the assay results do not correlate with the *in vivo* effects of quartz and kaolin. However, in this study, *in vitro* exposure to kaolin did not significantly alter O_2^- production compared to control PAM. The results obtained correlate more closely with *in vivo* exposure effects and help support the usefulness of this quantitative superoxide assay in evaluating the effects of respirable dusts on PAM. This suggests O_2^- production may play an important role in silicosis and other respiratory diseases. In addition, results from experiments with surfactant support the concept that lung surfactant may alter the toxicity of dusts inhaled into the lung.

Interestingly, *in vivo* exposure to quartz resulted in increased O_2^- production rather than the decrease observed *in vitro*. While this difference may be due to a change in cellular function from the *in vivo* to the *in vitro* environment, it is also possible the *in vitro* response may be due to an initial (acute) response produced immediately after contacting the cells with dusts. In contrast, the *in vivo* responses may have resulted from the longer contact time since animals were exposed 2-4 weeks followed by a 3-31 day post-exposure period before O_2^- analysis. Thus, it may be that quartz causes an initial injury to PAM resulting in decreased O_2^- production followed by recruitment or activation of PAM having increased production capabilities. This is supported by the fact that PAM analyzed 3 days post-exposure exhibited a wider range in O_2^- production than the control or 10 and 31 day groups. Specifically, perhaps two populations of PAM are present: (1) cells injured by initial or long-term dust contact, resulting in decreased O_2^- production release; and (2) recruited or activated PAM with an increased O_2^- production.

In summary, a sensitive, quantitative assay to study *individual* PAM function related to O_2^- production has been developed which shows that respirable dusts do affect O_2^- release by PAM. The *in vitro* results provide a basis for quantifying the *acute* effects of dust-cell contact (or constituents of dusts) on O_2^- release by PAM. Importantly, the addition of serum to the culture medium permits restimulation of the *same* cell by different dusts after adherence of the cell to the culture dish. The *in vivo* results provide a basis for critically examining the effects of long-term exposure to airborne dusts on O_2^- production by PAM, and continued refinement of the methodology will provide a means to assess and improve present understanding of the phagocytosis process in health and disease.

Based on the results and conclusions obtained to date, continuing work is focused on several specific objectives. Experimental work is designed to critically examine the effects of repeated exposure to different dusts (and concentrations) on the ability of the same cell to produce and release O_2^-. Concomitantly, the ability to maintain animals for long periods of time in the inhalation chambers will permit evaluation of the effects of chronic exposure to dusts. This approach has the potential to provide information not possible using population measurements and to critically assess and ultimately lead to improved clinical therapies for treatment of pulmonary disorders.

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AN ATTEMPT FOR EVALUATION OF THE ALVEOLAR DUST DEPOSITION ON THE BASE OF THE PARTICLE SIZE DISTRIBUTIONS OF LUNG DUSTS

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INTRODUCTION

The experimental and theoretical studies of the way human lungs behave at different size distributions of the offered dusts and form the alveolar depot—the major determinant of the development of lung diseases, did not offer completely defined results. Regardless of the recent advances in this field, such as the derived curves of extrathoracic, tracheobronchial and alveolar deposition of inhaled particles and the lot of unified parameters, the criteria for respirable dust sampling and assessment of dust hazards etc.[9] many problems did not find satisfactory answers.

The progress of technics and electronics eliminates the mass of difficulties in the particle size analysis and in the complicated calculations of the selective deposition of particles in the respiratory tract.

Presently, the most important problems derive from the impossibility to characterize precisely the entire dust inhaled by workers during their lifetime. The experimental studies in vivo on humans and animals, on models of the respiratory tract, as well as mathematical models providing a precise but valid information for the moment,[6] are unable to assess yet the long-term retention of dust in the lungs as a response of the inhaled particles.

A very specious solution of these problems seems to be the study of dusts recovered post mortem from the lung tissue of dust-exposed workers and to compare them with the airborne dusts offered to the respiratory tract.Of course, a method of approach like this also has its disadvantages mainly in the assessment of the inhaled, resp. airborne dust. But it is possible after analyzing a significant number of airborne dust samples, collected in the work environment during a long period of time and including all technological processes used on the areas where employees had worked, to obtain a more or less rough approximation to reality. So, the respiratory tract with its special features and intersubject variability depending on individuals and experimental design, can be considered as a "black box" with known "inlet" and "outlet." Many authors have used lung dusts in their investigations and a lot of them support the reliability of the information received about the alveolar deposition of airborne particles.[2,5,8]

Another important limitation of this experimental design can be the impossibility for assessment of particle aggregations and the kinetic behaviour of aggregates. Possible errors can be eliminated to some extent by analyzing fully dispersed samples of airborne and lung dusts using the same method for particle analysis.[2]

When the results from different studies of dust deposition in the respiratory tract are to be compared, it is particularly important to bear in mind that the main curves used nowadays as criteria for dust hazard evaluation[9] are derived with monodispersed aerosols, while the determination of alveolar dust deposition with lung dust studies (representing a long-term retention of dust in lungs) is based on the inhalation of polydisperse aerosols.

Diu and Yu[3] proposed a new mathematical model of polydisperse aerosol deposition in human respiratory tract, proving more or less considerable differences varying with the polydispersity. The model needs a validation on experimental studies. Investigation of lung dusts should be convenient for this purpose.

The mass particle size distributions of 47 samples of total airborne dust in the work environment of three mines—24 from a polymetal ore mine (PMM), 18 from copper mine A (CMA) and 5 from copper mine B (CMB) were determined. Full-shift sampling was performed at normal passing technological processes according to the actual standard in the country.[1] Samples were collected in different intervals of time during 10 years.

Fifty-nine dusts, recovered post mortem by the formamid method of Thomas and Stegemann[12] from the lungs of miners with entire length of service in the same mine, were analyzed for determination of the particle size distributions. Forty three of them were from the PMM group, 11—from CMA and 5—from CMB groups.

The particle size analyses were performed by two methods based on different principles: centrifugal sedimentation in Joyce-Loebl disc centrifuge[4] analyzing particles in class intervals of Stokes diameters 0.01–20 μm and automatic counting of particles in liquid media with Coulter Counter (CC) using tube aperture 50 μm, analyzing particles in class intervals of UDS diameter 0.7–25 μm. Dust samples were suspended in filtrated 0.1% solution of sodium hexametaphosphate in distillate water. A lot of the analyses were performed with the same suspension. The Stokes particle size distributions were calculated in aerodynamic diameters using the density data for every dust. Particle size distributions were plotted on log-probability graph paper.

Mean values of the groups of airborne and lung dusts from the three mines were calculated, as well as the standard deviations and the confidence limits intervals. The standard deviations of the groups of airborne and lung dust from every mine

were compared statistically by means of the Fischer criterion.

The alveolar deposition was determined by the method described by Leiteritz, Einbrodt and Klosterkötter.[5] The enrichment of fine sizes of each fraction of the lung dust particle size distribution was calculated as a ratio of the corresponding airborne dust fraction. This is the so-called enrichment factor. The relative alveolar deposition of each fraction is the quotient for the enrichment factors and the maximum enrichment factor which is taken to be = 1.

RESULTS AND DISCUSSION

Particle Size Distributions of Airborne and Lung Dusts

It was found that the mean values of the mass median aerodynamic diameters of the particle size distributions (MMAD) of the three groups of mine airborne dusts are in the class intervals 3–5 μm, analyzed by both methods. The mean geometric standard deviations (σg) of the groups were varying from 4.4 to 5.3 for Joyce analysis and from 2.2 to 2.3 for Coulter Counter analysis. The airborne dust samples analyzed with Joyce were reduced to 5 combined samples for each mine, because the analysis needs about 50 mg of dust.

The mean value of MMAD of the particle size distributions of lung dusts of the three groups of miners was varying from 1.9 to 2.6 μm by both analyses and σg—from 3.3 to 3.6 and from 1.6 to 1.8 by Joyce and CC analyses, respectively.

The maximum frequency percentage of the particle size distributions, as well as the other data about them are presented in Table I.

Relative Alveolar Deposition

It was found that the maximum enrichment factors for the 3 groups of airborne and lung dusts are in the class interval 1.2–2.4μm. The mean values for each size range of the enrichment factor were calculated and a curve of the relative alveolar deposition was derived (Figure 1). The theoretical

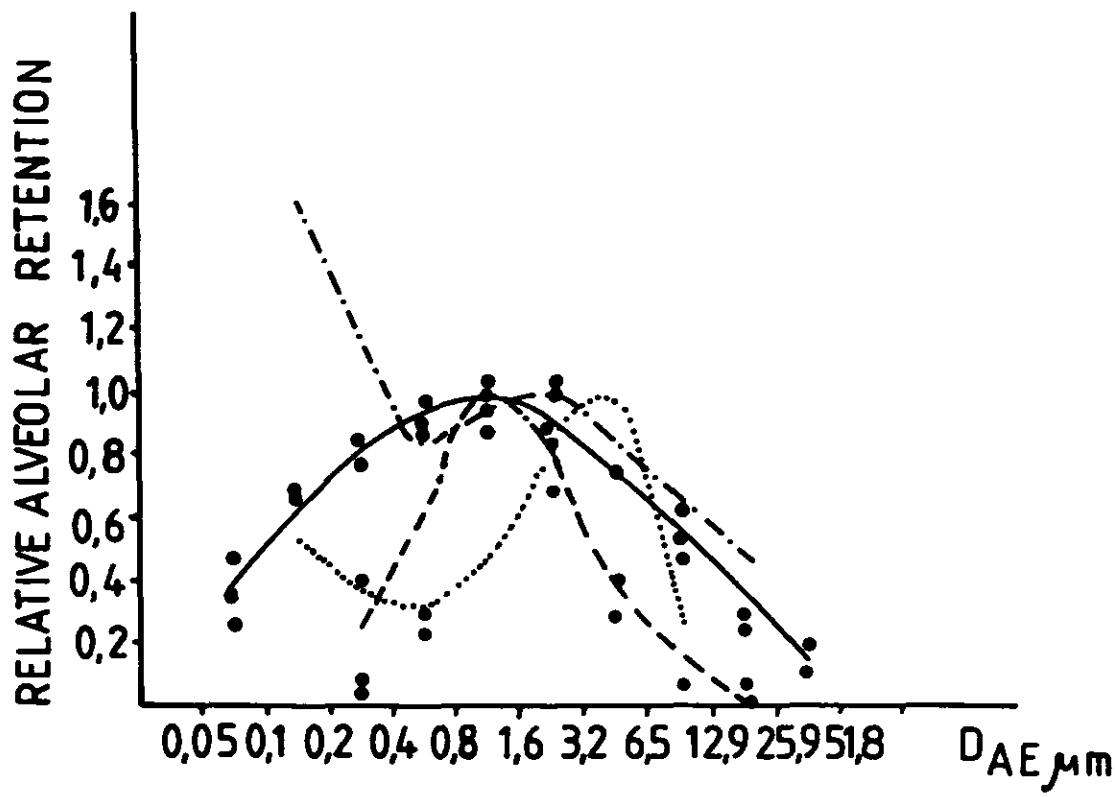

Figure 1. Curves of relative regional alveolar retention of airborne dusts from three ore mines derived on the basis of particle size distribution of lung dusts.

LEGEND

———— Mean retention curve derived by Joyce analysis
– – – – – – – – Mean retention curve derived by CC analysis
— · — · — · — Deposition curve of ICRP[9]
. . . . . Predictive deposition curve of Yu[13]

Table I

Characteristics of the Mass Particle Size Distributions of Airborne Dusts and Lung Dusts of Miners from Three Ore Mines Analyzed with Joyce-Loebl Disc Centrifuge and Coulter Counter

Mine	Parameters	Airborne dusts		Lung dusts	
		Joyce	Coulter	Joyce	Coulter
PMM	Mean MMAD, μm	3.30	3,95	2.13	2.49
	Confid. lim. μm	± 3.01	± 2.45	± 1.60	± 1.50
	σ_g	5.30	2.08	3.60	1.66
	Confid lim.	± 2.68	± 0.66	± 2.26	± 0.41
	Anal.No	5	24	43	43
	Max.fr.%	32	37	23	53
	Int.,μm	1.6-6.5	3.2-6.5	1.6-3.2	1.6-3.2
CMA	Mean MMAD, μm	2.90	3.74	1.86	2.31
	Confid. lim. μm	± 2.49	± 2.48	± 1.70	± 1.08
	σ_g	5.00	2.00	2.35	1.83
	Confid. lim	± 2.66	± 0.71	± 1.52	± 0.51
	Anal.No	5	18	11	11
	Max.fr %	34	36	42	44
	Int;μm	1.6-6.5	3.2-6.5	0.8-3.2	1.6-3.2
CMB	Mean MMAD	3.92	4.82	2.53	2.57
	Confid. lim. μm	± 3.61	± 3.45	± 3.48	± 2.34
	σ_g	4.40	2.10	3.60	1.63
	Conf.lim.	± 2.61	± 1.01	± 2.57	± 0.66
	Anal.No	5	5	5	5
	Max.fr.%	36	37	21	50
	Int; μm	1.6-6.5	3.2-6.5	1.6-3.2	1.6-3.2

curve of Yu[13] and the ICRP curve of Task Group[9] were also plotted on Figure 1 for comparison, recalculated in the same way.

The relative alveolar depositions with the minimum and maximum values of airborne and lung dusts of the three mines were calculated separately. The values' dispersion is shown on Figure 1 and the data are presented in Table II.

DISCUSSION

The data obtained showed some differences in the particle size distributions of the same dusts, analyzed by both methods. The MMAD determined by Joyce are finer than those determined by CC, while for σg the contrary was proved. In principle this was expected because the instrumental limitations of CC in sizing of fine particles (smaller than 0.7 μm) as well as Joyce unreliability in sizing of coarse particles (larger than 15–20 μm) are known. But it is necessary to emphasize the fact for two reasons: on one hand to demonstrate the considerable influence on the calculated relative alveolar deposition of the different particle size distributions used, and, on the other hand—to draw the attention of specialists on the need of good knowledge about the limitations of every method or instrument used for particle size analysis, when interpretation of data has to be performed. Thereby useless contradictions due to underestimation of the last reason could be avoided.

Another fact of interest is the fine dispersity of airborne dusts (mean MMAD vary from 3 to 5.0 μm, with real maximal value of the groups examined—5.2 μm by CC analysis). In most of the studies known these values are much higher but they concern coal mine dusts.[2,5,8] Ore mine dusts are expected to be finer and the authors of this paper have many studies in this field.[4,10] They have found a considerable presence of submicron fraction of mineral origin in ore mine airborne dusts, as proved with X-ray analysis,[11] the same being evidenced for lung dusts, also.

The determination of the alveolar dust deposition by using lung dusts and work environment airborne dusts is connected with some preliminary assumptions: the particle sizes are the leading factor for the dust deposition in the respiratory tract; the "inlet" and the "outlet" dusts, e.g. the airborne and lung dusts are representative and reliable; alveolar deposition, the retention, respectively, of dust is more real when lung dusts are used because the long-term clearance as a total and the changes of dust parameters after continued staying in lung are included.

The data obtained for alveolar deposition (more correctly it should be called alveolar long-term retention) differ to some extent from the referred in the main studies performed—experimental, calculated and predicted. The maxima of the relative alveolar retentions calculated as mentioned above, are

Table II

Size Range Intervals of the Calculated Maximal Enrichment Factors with the Mean and Real Values of MMAD and σg of the Particle Size Distributions of Airborne and Lung Dusts from Three Ore Mines by Joyce and CC Analyses

Mine	Values of parameters used	Calculated maximal enrichment factors	
		Joyce analysis size intervals	CC analysis size intervals
PMM	Mean	1.6-3.2 μm	1.6-3.2 μm
	Min	0.4-0.8 μm	0.4-0.8 μm
	Max	1.6-3.2 μm	3.2-6.5 μm
CMA	Mean	0.8-1.6 μm	0.8-1.6 μm
	Min	0.1-0.2 μm	0.2-0.4 μm
	Max	1.6-3.2 μm	0.4-0.8 μm
CMB	Mean	0.8-1.6 μm	1.6-3.2 μm
	Min	0.1-0.2 μm	0.8-1.6 μm
	Max	0.8-1.6 μm	1.6-3.3 μm

found to be in the same size range intervals as the maxima of the particle size distributions of lung dusts or are removed toward the fine size ranges—Figure 1 and Table II.

These results are in correspondence with the mathematical model of Diu and Yu.[3] To airborne dust with σg significantly higher than 2 results a different alveolar dust retention in comparison with the deposition curve of Yu,[13] predicted for monodispersed aerosols—retention curve calculated with Joyce data on Figure 1. This curve is quite similar in the size range interval 0.5–1.6 μm to the ICRP curve[9] derived from a lot of different studies. On the contrary, the CC retention curve does not differ in some of the size range intervals considerably from the curve of Yu. The values of σg of airborne dusts analyzed by CC are about 2. Morrow[7] affirms that inhaled dust with $\sigma g < 2$ have similar deposition in respiratory tract to this of monodispersed aerosols.

CONCLUSIONS

The experimental curve of long-term alveolar retention of inhaled dust from the working environment derived on the basis of the particle size distributions of miners' lung dusts from three ore mines proved that the real alveolar deposition and retention is different from the predicted deposition of monodispersed aerosols.

The study was performed with a comparatively great number of airborne and lung dust samples and can be regarded as reliable.

It is obvious that the polydispersity of dust samples has more considerable influence on the alveolar deposition and retention than it was estimated till now—a problem disregarded to some extent in the experimental studies.

Modern technologies and the mechanizing and automation of working processes lead to increasing of the polydispersity of airborne dusts and namely of the fine particles share. This fact inevitably will lead to some changes in the understandings about the evolution of the hazard and imposes more attention to be paid to the problems of particle size analyses and the interpretation data.

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THE BIOLOGICAL EFFECT OF PARTICLE SIZE DISTRIBUTION OF QUARTZ COMPONENT IN POLYMINERAL DUSTS

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Experimental and epidemiological studies have shown that not in all of the industrial dusts a correlation between the content of respirable quartz and fibrogenicity existed.[1,9,14,21]

This means that the mass of respirable quartz, which is the basis of contemporary norms for quartz containing dusts in a majority of countries, is not the solely sufficient criterion for their fibrogenicity. During the last 10-15 years the efforts of a number of authors were directed toward the study of the properties of the surface of quartz dusts and finding quantifiable indicators for their fibrogenic activity. A relationship was defined between pathogenic activity of quartz dusts and: concentration of hydroxyle groups,[17] activating energy of the surface,[12,13] free quartz surface,[2,7,8] particles size distribution.[22,23] etc. [6,16]

Most of the experiments studying the influence of the particle size of quartz dust have been performed with pure quartz dusts. Results of experiments with coal quartz containing dust of different particle size are reported, but they do not present data on particles size distribution of the quartz content.[2,15]

The results of investigations performed by different authors are consistent and could be summarized as follows: A maximum cytotoxic and fibrogenic effect is manifested by quartz particles of a size from 2-3 to 0.5-1 μm; at larger or smaller size a decrease in the activity is observed.

Investigations on the size of respirable industrial dusts, as well as of lung dusts from deceased exposed workers show the presence in them of mineral particles of a size within comparatively wide range (from more than 10 μm to 0.01 μm diameter).[5,10,19]

On the other hand, it is well known that the sizes of quartz particles usually differ more or less from those of the other components of the mixed dust.

In the literature available to us we did not find research data on particle size distribution of the quartz component in mixed dusts. Gade and Luft,[4] working out a IR-spectrophotometric method for quantitative determination of quartz in industrial dusts by use of double absorption peak, found a relationship between particle size and the ratios of the absorbances at the double absorptions at 798^{-1} and 775^{-1} cm to the minimum absorbance between the two absorbance peaks. However, they proposed this method with a view at correction of the influence of the particle size upon the results at quantitative determination of the quartz. The above relationship was later proved by Dodgson and Whittaker[3] for pure quartz. At mixed dusts the absorbance could be influenced also by interfering minerals,[13,11] which would require the use of correction coefficients, making the method complicated and unreliable.

The aim of the present work was to characterize the particle size distribution of the quartz component of mixed dusts and to assess its biological role by an analysis of: lung dust from decreased exposed miners and some respirable industrial dusts.

MATERIALS AND METHODS

Dusts extracted from the lungs of 26 workers from lead-zinc, copper and uranium mines and tunnels were investigated, as well as samples of respirable dust from the work environment in lead-zinc mines, dina plant and foundry.

X-ray and morphological findings of silicotic changes of different degrees were proved in almost all of the deceased miners with exception of 8 of them, whose lungs contained less than 235 mg quartz.

The lung dust was extracted by formamide digestion after Thomas and Stegemann. Then they were ashed at 600°C and the residue subjected to an analysis of its particle size distribution and mineral composition. For determination of the particle size distribution of the quartz in the mixed dusts the following procedure was applied: the dust sample, suspended in 0.1% water solution of sodium hexametaphosphate was fractionated within the range of 8.9–0.24 μm stockes diameters, calculated on the base of the specific weight of the quartz by sedimentation in a disc centrifuge. The suspensions of the fractions were run through Sartorius membrane filters of 0.05 μm pores size. After that, the membrane filters with the fractions were ashed at 600°C, the residues weighed and the quartz quantity for each fraction determined by IR-spectroscopy. The absorption peak at 695 cm^{-1} was used, because of its being less particle size dependent in the range of 9 to less than 1 μm. To enhance the peak height the ordinate expansion of 5 x was used. So a sensibility of 0.03 mg quartz in the specially prepared pellet was attained. The quartz quantities in it varied from 0.03 to 0.4 mg, the confidence limits at $p = 95\%$ being less than 0.011 mg.

On the basis of the quartz quantities determined in the fractions, the mass particle size distribution of the quartz component was plotted on log-probability paper.

RESULTS

The data of the total lung dust, as well as the parameters of its particle size distribution and that of the quartz components, in particular, are shown in Table I.

It may be seen in the Table that the quantity of the extracted and ashed dust was varying from about 1 to 24 g, in 20 of the cases being between 1 and 10g. The mass median unit density sphere (UDS) diameter of the majority of the dusts analyzed (17 from 26) is between 3 and 5 μm, in 5 of them it is more than 5 up to a maximum of 6.8 μm and in 4—less than 3 μm with a minimum down to 2.4 μm. These data are consistent with the results obtained by Tcherneva[18] in measuring equivalent volume diameter of lung dusts from deceased miners of similar exposure by using Coulter-counter.

The quartz percentage of the dusts mentioned in Table I varies from about 6 to 40% and most frequently between 10 and 30%, its quantity in 22 of the lungs being less than 3 g, in 12—less than 1 g and in 4—between 3 and 5 g.

The mass particle size distribution of the quartz component plotted on the log-probability paper shows a log normal distribution. On Figure 1 the quartz component distribution in three of the dusts examined is illustrated.

The particle size distribution in 19 of the samples investigated is characterized by a larger mass median UDS diameter as compared with that of corresponding total lung dust (See Table I). In 7 of the samples the mass median UDS diameter of the quartz component and that of the total dust, respectively, could be accepted as almost equal, since they differ only by 0.1 to 0.4 μm.

The mass median UDS diameter of the quartz component in all 26 samples examined was over 3 μm; in 10 of the samples it was between 3 and 5 μm and in 16-larger than 5 μm.

With very few exceptions, the median geometric standard deviations of the distribution of the quartz component were smaller than those of the corresponding total lung dust. Although that the quartz particles in general are larger than those of the other mineral components in the dust and are characterized with a more limited polydispersity, significant quantities of quartz particles with a UDS under 1 μm were found. The mass of the submicron fraction was varying from 3.2 to 15% from the whole mass of the quartz. In 16 of the samples it was between 5 and 10%; in six—more than 10% and only in 4 it was less than 5%. These data are evidencing significant individual differences in the particle size distribution of quartz component in lung dusts.

Table I

Characteristics of Lung Dusts and Their Quartz Component

Case No	Whole extracted lung dust				Quartz component			
	Mass mg	Mass median UDS diameter μm	σ_g	Quartz content %	Mass, mg	Mass median UDS diameter μm	σ_g	Submicron fraction %
43	1632	5.5	5.9	7.2	118	10.5	3.8	3.2
74	1148	3.6	3.4	12.3	141	4.8	3.0	7.2
75	1635	4.4	4.8	5.7	92	4.8	3.0	7.2
76	355	3.7	3.9	14.5	51	5.8	3.1	5.7
77	14897	3.6	3.8	13.5	2011	4.8	3.0	7.2
107	24024	3.6	3.8	17.8	4276	3.2	3.2	15.0
109	2950	2.6	5.6	17.8	525	3.3	3.5	16.0
110	8165	5.5	4.7	29.9	2441	7.0	4.7	10.0
111	8450	2.8	4.1	28.1	2374	4.0	3.5	13.0
112	2294	3.9	4.6	16.0	367	5.6	3.5	8.0
113	4482	4.4	4.8	35.8	1605	5.3	3.3	8.0
114	5782	4.2	3.9	27.6	1596	7.4	3.7	6.4
115	5796	3.6	4.3	43.8	2539	7.5	3.3	4.5
117	1222	3.6	4.3	17.4	213	7.5	3.1	4.0
119	17882	3.9	4.2	28.0	5007	3.5	3.2	13.0
120	15029	3.6	4.3	27.5	4133	5.2	3.3	6.0
125	1083	5.2	6.3	13.2	143	5.5	3.4	8.0
126	1396	8.1	6.8	16.8	235	8.2	3.9	6.0
129	1240	3.7	5.3	13.6	169	6.0	3.5	7.5
140	11470	2.4	3.8	26.5	3040	4.0	3.1	10.5
141	7209	4.7	5.9	15.3	1107	4.6	3.3	9.0
142	3522	4.2	7.8	17.4	613	5.5	3.4	8.0
143	7068	3.4	4.6	21.9	1548	5.2	3.3	7.5
144	2062	6.8	6.0	13.3	274	9.0	3.8	4.6
147	8626	4.1	4.4	25.9	2234	5.4	3.4	8.0
148	7736	2.9	4.8	30.7	2375	3.7	13.0	13.5

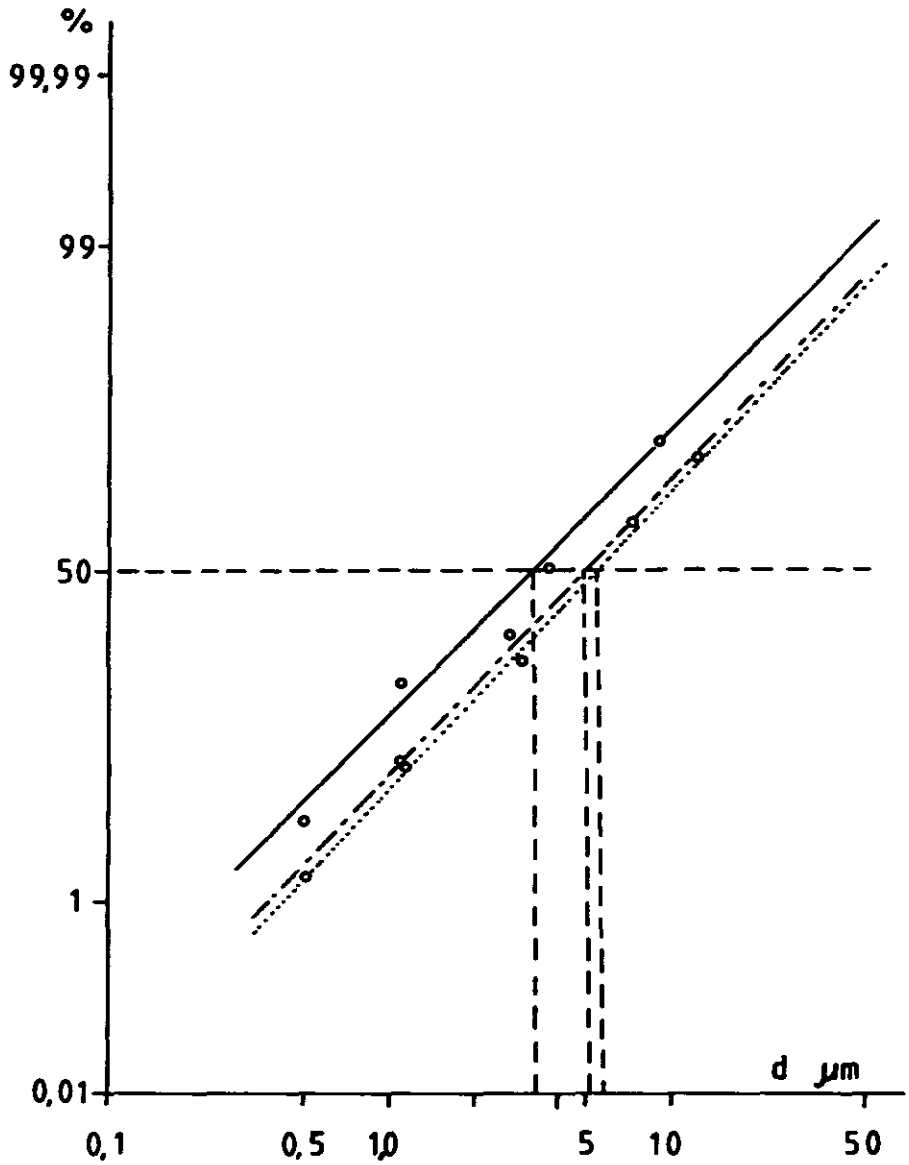

Figure 1. 1. Mass particle size distribution curves of the quartz component of three lung dusts.

Case No. 107 — — — — —
74 —·—·—·—·
120 ·····

d — Mass median UDS diameter
% — Mass percentage of quartz fraction

Research data for respirable industrial dusts are shown in Table II. In these samples significant differences were not obtained between the mass median UDS diameters of the whole dust and of the quartz component. Comparatively near were proved to be the values for these parameters measured for the three sorts of dust (between 5,3 and 6.8 μm), in spite of the differences in their composition. The percentage of quartz particles with UDS diameter less than 1 μm was between 6.8 and 13%.

For assessment of the influence of the particle size of the quartz component on fibrogenicity of mixed dusts, their characteristic data and resulting fibrosis changes in the lung tissue were processed by the program product "Statgraf." The following dust parameters were included in the analysis: total quantity of the extracted dust, its residence time in the lungs, % of the free crystalline silica content, total free crystalline silica content and quantity of the fraction with sizes between 3 and 0.5 μm. Slightly higher correlation coefficient—0.84, was obtained for the quartz fraction within 3—0.5 μm, as compared with 0.83 for the total quartz quantity.

DISCUSSION

The results obtained illustrate the possibility of determining the particle size of quartz component in mixed dusts as a help for the better characterization of both lung and industrial or experimental dusts.

Evidently, this method has the all well-know shortcomings, related with the procedure of lung tissue digestion and dust extraction, ashing at 600°C and preparing liquid suspension for centrifuging etc. In spite of these limitations, we are of the opinion that this method could be a help in elucidating the relationship between the properties of quartz-containing dusts and their pathogenicity.

In our attempt to assess the biological significance of this parameter on the basis of the 26 lung dusts investigated we did not obtain a significant increase of the coefficient of correlation. This fact is perhaps due to the high coefficient of multiple correlation for a comparatively small number of subjects on the background of the number of the variables investigated. The studies continue with a larger group of lung dust cases.

Table II

Characteristics of Respirable Industrial Dusts and Their Quartz Component

Sort of dust	Silica content	Parameters of particle size distribution				
		Respirable dust		Respirable quartz		
	%	UDS mass median diameter	σ_g	UDS mass median diameter	σ_g	submicron fraction
		μm		μm		%
Ore mine	9.6	5.9	4.5	6.0	3.3	6.8
Dinas	65.4	5.3	4.1	5.5	3.7	9.0
Foundry	5.3	6.2	12.4	6.6	5.4	13.0

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CHEMILUMINESCENCE AND BIOLOGIC REACTIVITY OF FRESHLY FRACTURED SILICA

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INTRODUCTION

Silicosis is the chronic fibrosing disease of the lungs caused by the inhalation of crystalline silica. However, inhalation of crystalline silica, may induce three distinctly different disease patterns; i.e., chronic silicosis, accelerated silicosis, and acute silicosis. These three disease patterns differ in their pathologic characteristics, clinical symptoms, onset of disease, and mortality. Many studies in the past have focused on the elucidation of mechanisms involved in the development of chronic silicosis. We recently reported that freshly fractured silica has surface properties that could make it more reactive with lung tissue than aged silica, and that this unique reactivity of freshly fractured silica may be involved in the pathogenesis of acute silicosis (Dalal et al., 1986; Vallyathan et al., 1988; Shi et al., 1988). Because freshly fractured silica could contain some moieties in an "excited state," due to the breakage of silicon-oxygen bonds, we postulated that the de-excitation of these moieties could produce light which could be monitored by a luminescence measurement technique. In addition, if this "excited state" of silica could trigger a greater biologic response by phagocytic cells, this activation could be monitored by a chemiluminescence technique in the presence of appropriate enhancers.

This investigation describes for the first time the use of luminescence in the study of freshly fractured silica and its properties. Enhanced biologic reactivity of the freshly fractured silica with alveolar macrophages was monitored by the chemiluminescence technique.

MATERIALS AND METHODS

Native silica was obtained from the dust bank of the Generic Respirable Dust Technology Center, Pennsylvania State University, State College, PA, and ground for 30 min in an agate ball mill. It was then sieved through a 20 micron mesh filter and used within 10 min as fresh silica or stored in glass bottles for aging. X-ray energy spectrometry and X-ray powder diffraction studies on representative samples were made to confirm the mineralogic purity. All the silica samples were found to be 99% pure with minimal detectable contamination by metal ions.

Luminescence of freshly ground silica was measured from 5 g samples in plastic scintillation vials using a Packard Liquid Scintillation Counter operated in the out-of-coincidence mode. After grinding, the samples were stored in dark for 10 min, and luminescence was monitored over a period of several hours and days. The effect of aqueous solutions on the generation of luminescence was studied in HEPES-buffered medium (145 mM NaCl, 5 mM KCl, and 10 mM HEPES, pH 7.4). Freshly ground silica (5 g) was added to 5 ml of HEPES-buffered medium, the vials stored in dark for 10 min, and samples counted at various time intervals. The effect of scavengers on luminescence was investigated by the addition of 5 g freshly ground silica to HEPES-buffered medium containing 125 μg/ml superoxide dismutase (SOD), 125 μg/ml catalase, or 100 mM 5,5-dimethyl-1-pyrroline-1-oxide (DMPO).

Biologic reactivity of freshly ground silica was monitored as lucigenin-enhanced chemiluminescence at 37°C using a Berthold Luminometer, Model 9500. Alveolar macrophages were obtained by the broncho-pulmonary lavage of Sprague-Dawley male rats with calcium and magnesium-free Hank's balanced salt solution. Repetitive lavages were pooled (total volume of 80 ml) and was sedimented by centrifugation at 500 g for 5 min. Cells were washed and resuspended in HEPES-buffered medium containing 1 mM calcium and 5 mM glucose. With the aid of trypan-blue and hemocytometry, cell viability and counts were determined miscroscopically. Results of these studies indicate that approximately 90% of the lavaged cells were viable alveolar macrophages. Samples of alveolar macrophages (1×10^6) were then incubated with 20 μg/ml silica in 0.5 ml HEPES-buffered medium at 37°C. The buffer contained 2.5×10^{-8} M lucigenin as an enhancer of chemiluminescence. Silica-induced reactivity of alveolar macrophages was then monitored over time for 40 min.

RESULTS

Figure 1 shows the results of luminescence studies on freshly ground silica in comparison with that of aged dust. Aged dust, on the other hand, showed a steady minimal baseline intensity of light emission, most likely due to "fluorescence." This intensity of light emission was greater when samples were not dark equilibrated for 10 min. Therefore, we consider that, this basal emission to be due to fluorescence excited by the ambient light. The data clearly indicate that freshly ground silica emitted substantially more light than aged silica. The intensity of this light emission declined with time after grinding and exhibited half-life of approximately 40 min.

In order to find whether contact with a biologic medium would quench the luminescence instantaneously, luminescence

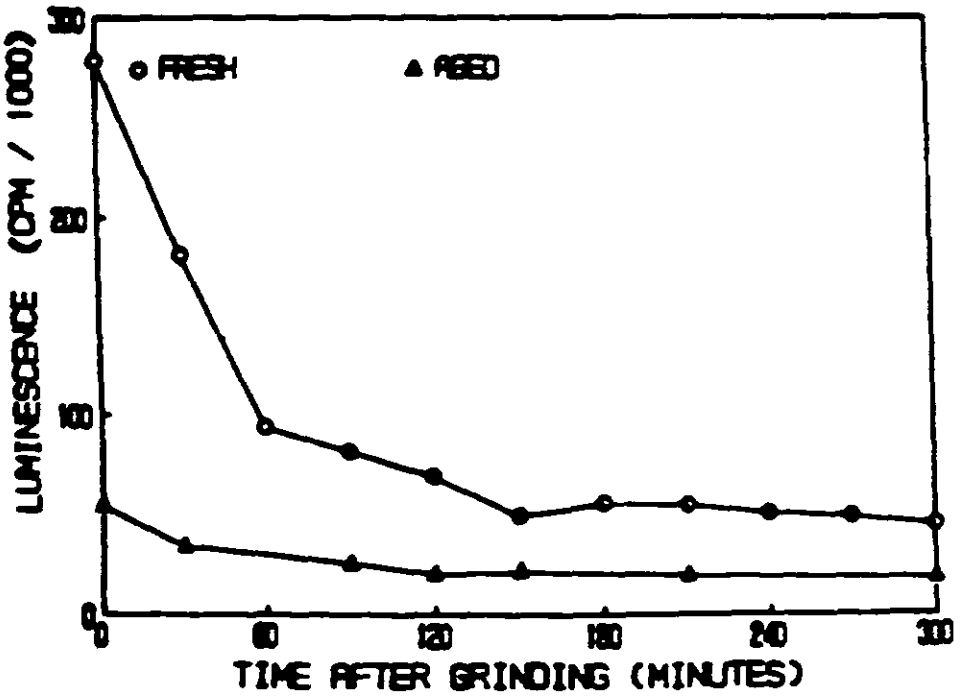

Figure 1. Luminescence of freshly ground and aged silica compared to show the greater intensity of time dependent luminescence associated with freshness.

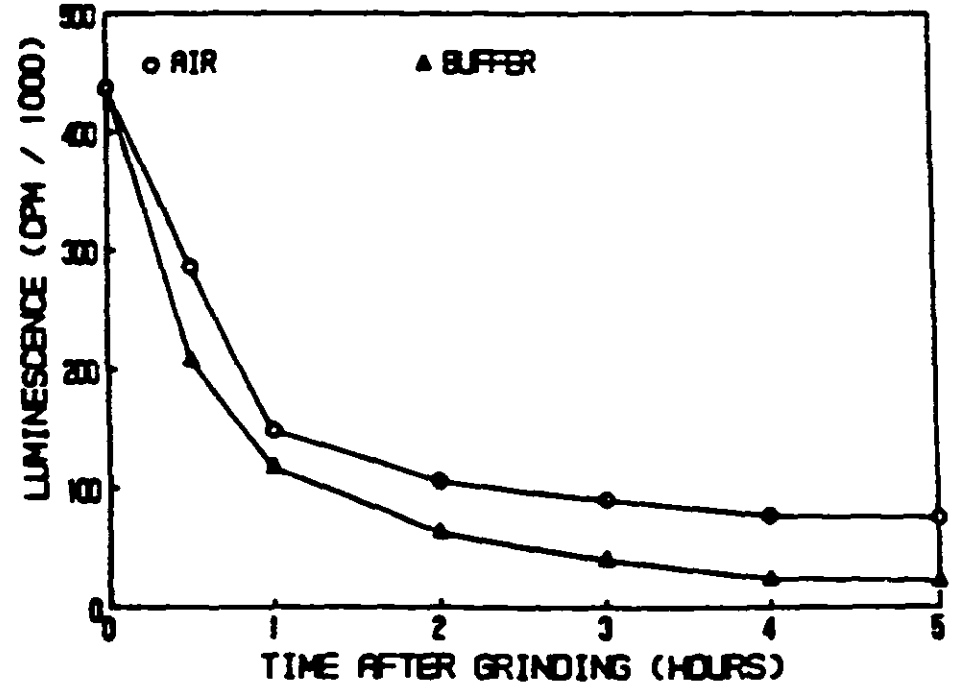

Figure 2. Luminescence of freshly ground silica in air and in HEPES-buffered medium.

measurements were made on freshly ground silica dispersed in HEPES-buffered medium. It is evident from the data presented in Figure 2 that fresh silica suspended in the biologic medium emits substantial light suggesting that the reactive surface sites on freshly ground silica are not immediately quenched after contact with biologic media.

Effect of free radical scavengers on the light emission intensity is presented in Figure 3. It is seen that luminescence of freshly ground silica was inhibited by approximately 71% with SOD, while catalase and DMPO inhibited the light emission by 88% and 97%, respectively.

Figure 4 shows the effect of freshly ground silica on alveolar macrophages stimulation and resulting release of reactive species of oxygen, monitored as chemiluminescence in the presence of an enhancer, lucigenin. Alveolar macrophages incubated with freshly ground silica (20 μg/ml) generated chemiluminescence which peaked approximately 8 min after exposure to silica. This silica-induced activation of alveolar macrophages was substantially greater with freshly ground silica as compared to silica aged for 24 and 48 hours (Figure 4).

DISCUSSION

Data from the present study indicate that freshly fractured silica emits light which can be monitored by luminometry or a liquid scintillation technique. These studies also indicate that the luminescence generated by silica is not quenched instantly in biologic medium but can be inhibited to a substantial degree by SOD, catalase, and DMPO. These results suggest that excited surface sites result from the cleavage of silica and that these surface sites can react with aqueous media to form reactive oxygen species as a source of emission of light detected by the luminescence technique. We have previously shown that silicon-oxygen radicals and possibly O_2 radicals are formed during grinding of silica (Dalal et al., 1986; Shi et al., 1988). We have also shown that these radicals undergo a time dependent decay in ambient air (Dalal et al., 1986; Shi et al., 1988). We tentatively assign the light emission from the freshly fractured silica to the de-excitation of these radicals and/or the silicon-oxygen radicals.

Our studies also indicate a correlation between light emission by freshly fractured silica and potential for biologic reactivity. The increased activation of alveolar macrophages induced by freshly ground silica as monitored by chemiluminescence indicates excessive secretion of reactive oxygen species during phagocytosis. We have shown previously that generation of reactive species of oxygen on silica during grinding can cause the generation of OH radicals. The presence of these increased cytotoxicity reactive species has been related to lipid peroxidation (Vallyathan et al., 1988). Results of the present study support our hypothesis that the reactive species associated with fresh silica together with those generated by alveolar macrophages in response to fresh silica may induce an oxidant stress and overwhelm the protective anti-oxidant systems of lung in occupational exposures, such as sandblasting, tunnelling, drilling, or silica flour mills where freshly fractured silica dust is generated. We, therefore, conclude from these studies that oxidant stress may play a role in the etiology of acute silicosis.

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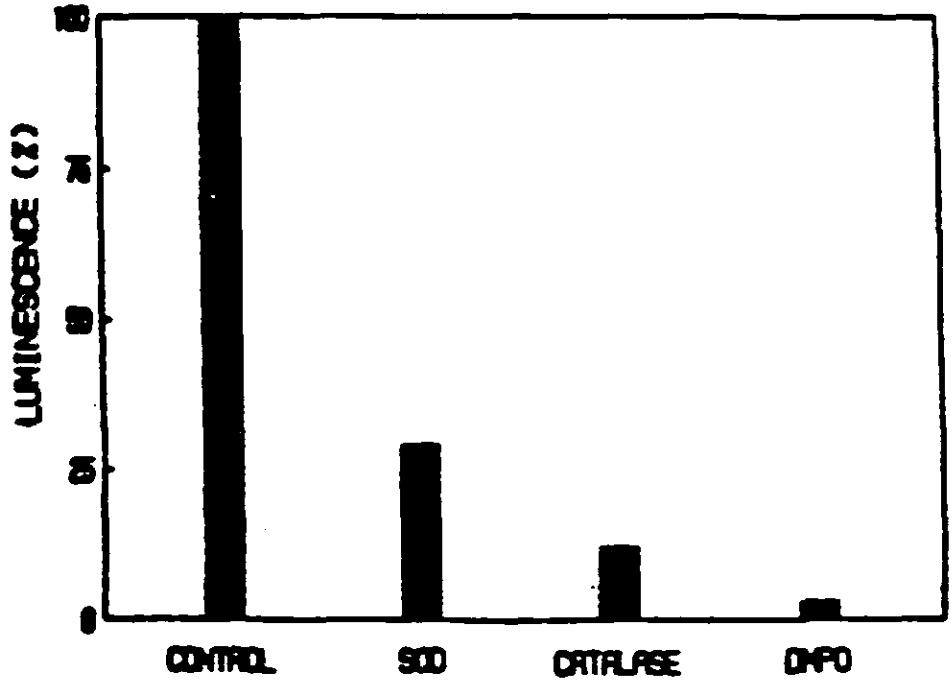

Figure 3. Effect of SOD, catalase, and DMPO on luminescence by freshly ground silica.

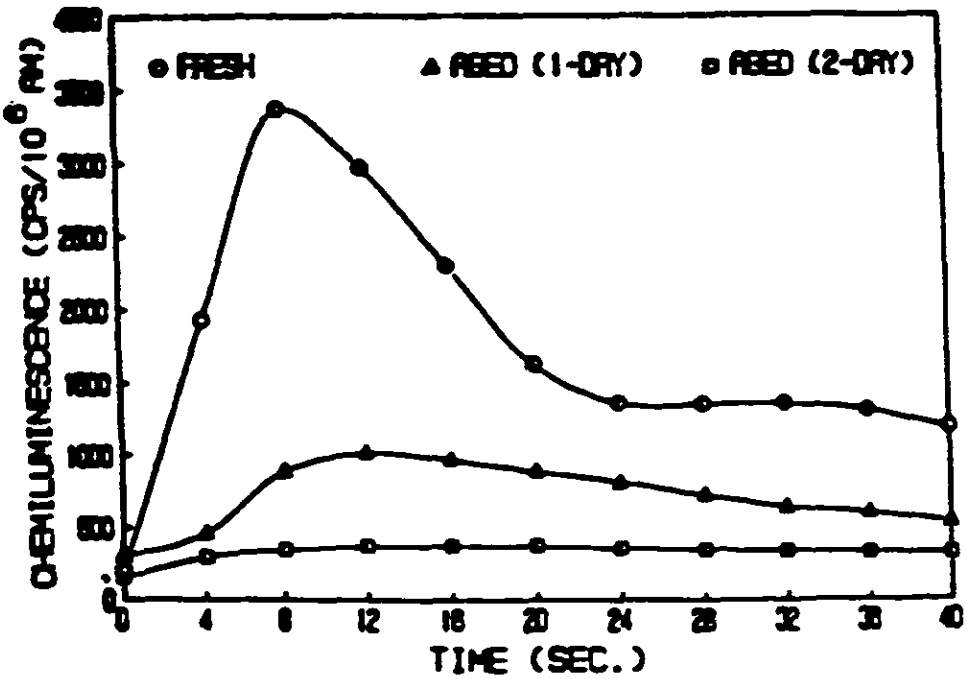

Figure 4. Effect of freshly ground and aged silica on chemiluminescence by alveolar macrophages.

THE INJURIOUS EFFECT OF QUARTZ ON CELL MEMBRANES AND THE PREVENTIVE EFFECT OF ALUMINIUM CITRATE AGAINST QUARTZ

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ABSTRACT

The injurious effect of quartz on the membranes of macrophages as well as erythrocytes and the anti-injurious effect of aluminium citrate (Al citrate) were examined. The comparative study with titanium dioxide was carried out simutaneously. The results from the present study show that quartz can cause the increases of fluidity and permeability of macrophage membranes and reduce membrane-bound water of erythrocytes, resulting in the membrane dehydration. Furthermore, quartz can change electrophoretic behavour of macrophages by increasing the negative charge density and electrokinetic potential on these cells surface. The effect of titanium dioxide on cell membranes however is very different from quartz in intensity and kinetics, and is not affected by Al citrate. The relationship between these effects was discussed and a possible mechanism was proposed for the interaction of quartz with membrane lipids resulting in membrane damage.

The preventive effect of Al citrate against membrane damage by quartz was also demonstrated. In general, the addition of Al citrate can recover all alternations caused by quartz, so that the stability and order structure of cell membranes were maintained. A hypothesis about the action of Al citrate on the surface of quartz particles to exert its anti-injurious effect was postulated in this paper.

INTRODUCTION

It is generally accepted that the cytotoxic effect of quartz on alveolar macrophages is a key step in the pathogenesis of silicosis.[1-2] The cytotoxic mechanism postulated by Allison depends on mainly the release of hydrolytic enzymes from lysosomes after the phagocytosis of quartz by macrophages followed by cell damage.[3] However, a question that remains unanswered is whether the toxic particles directly damage the plasmic membranes of macrophages. It is well known that the contact of quartz with the cell membranes is the first event during the process of phagocytosis, no matter how the particles are uptaken into the interior of these cells. For this reason, it is desirable to elucidate the molecular interactions between quartz and cell membranes from the viewpoint of membrane toxicology.

The therapeutic effects of Al citrate on the experimental animal and patients with silicosis have been demonstrated in our previous experimental studies and clinical observations. It was also found that Al citrate is able to prevent effectively macrophages from the cytotoxicity of quartz instead of the inhibition of fibrosis.[4-6] It is, therefore, necessary to clarify its pharmacology with the goal being to provide the experimental and theoretical evidence for screening the preventive measurments and therapeutic drugs for silicosis.

On the other hand, titanium dioxide, a less toxic and usually classified as "inert dust,"[7] was also studied in this work for comparsion.

MATERIALS AND METHODS

Macrophages were harvested from lung of guinea pig through lavage. The erythrocyte membranes of rabbit were prepared as described elsewhere.[8]

Quartz (99% pure) was supplied by Hygiene Institute of Chinese Prophylatic Medical Center. Particles diameter is less than 5 μm, among which 89.3% is less than 2 μm. Titanium dioxide with the same purity and size was selected as a control. Al citrate with Al of 9.26% was supplied by Pharmaceutical Factory of Beijing Medical University. Fluorescence probe, 1,6-diphenyl-1,3,5-hextriene (DPH) was purchased from Sigma. Adenosine 5'-triphosphate disodium salt (ATP) was produced by Shanghai Biochemical Institute of Academia Sinica.

Fluorescence polarization was determined by spectrophotofluometer Model MPF-4. Potassium (K+) content of cells was detected by Fire Atomic Absorption Spectrophotometer Model Y-3.[9] Na^+-K^+-ATPase activity was determined using the method described by Pan H.Z.[10] Viscosity of medium and surface charge of cells were measured by viscosimeter Model E and Cell Electrophoresis Autotimer Model SX-2, respectively.[11] Membrane-bound water was determined employing the method of sorption isotherms and Nicolet Fourier Transform Infrared Spectrometer Model 5DX.[8-12]

There were on the average five samples in each group. Data

were presented as mean + standard error and significance was estimated by analysis of variance. Pairing data about fluidity and permeability were treated by linear correlation and regression.

RESULTS

Cell Membrane Lipid Fluidity

We began with the examination of membrane fluidity of macrophages by measuring fluorescence polarization P and microviscosity of membrane-bound DPH. As shown in Figure 2, P values of quartz I and II groups dropped down continuously with the cultural time, resulting in more fluid membranes. It is important that the effect of quartz on membrane fluidity is not only time-dependent, but also dose-dependent (Figure 1). However, the change of membrane fluidity by titanium dioxide is much lower than that of quartz group and tends to be recovered rapidly (Figure 1).

Compared with quartz control, fluidity was decreased (e.g. P value raised) when quartz plus Al citrate was added into the cells simutaneously, although Al citrate did not affect membrane fluidity alone. Similarly, the effect of Al citrate against quartz is dose-dependent (Figure 1).

Permeability of Cell Membrane to K^+

Table I presents the differences between the groups treated by several ways in membrane fluidity and permeability to K^+. It is interesting that the increased pemeability of macrophage membranes to K^+, that is, K^+ content of the cells was reduced, by quartz was accompanied with increasing membrane fluidity. Statistic analysis indicates the effects of quartz on both these properties of macrophage membranes exhibit very siginificant correlation (for instance, using η and K^+ as X and Y, respectively, $r=0.917$, $P< 0.001$, $Y=9.059X-0.011$) (Figure 3).

Like that on fluidity, Al citrate did not influence permeability of macrophage membranes to K^+ by itself, but it prevented acting efficiently against the effect of quartz, except that macrophages were pretreated with Al citrate (Table I).

It is seen from Table I that membrane permeability of titanium dioxide group was lowered only slightly and it seems that no exact relationship exists between the changes of fluidity and permeability. Another important difference from quartz is that the effects of titanium dioxide are unable to be affected by Al citrate.

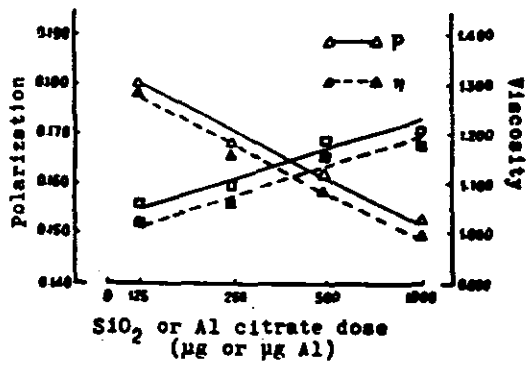

Figure 1. Dose-effect relationships of the effect of SiO_2 on fluorescence polarization (P) of macrophage membrane-bound DPH and lipid viscosity (η) and the antagonistic effect of Al citrate against SiO_2.

Δ——Δ SiO_2: □——□ SiO_2 (1 mg) +Al citrate
▲——▲ ■——■

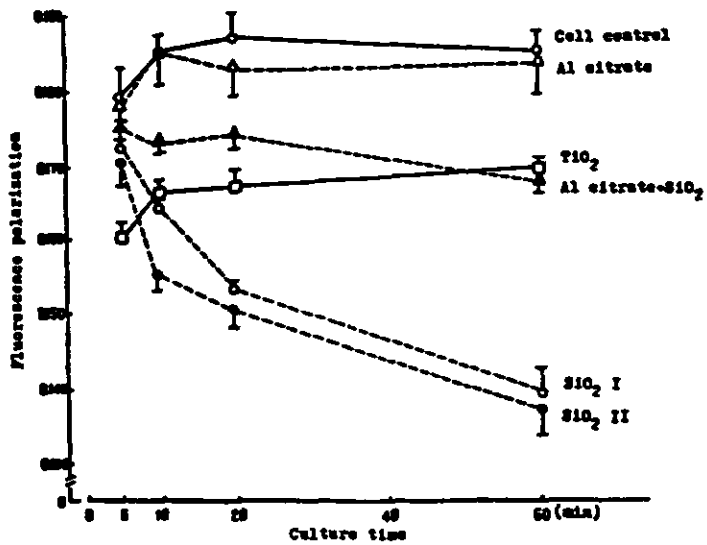

Figure 2. Kinetic curve of DPH fluorescence polarization labelled in macrophage membrane

SiO_2I: the simultaneous addition of DPH and SiO_2 to cell medium

SiO_2II: the addition of SiO_2 to cell medium followed by the addition of DPH

The dose of SiO_2 or TiO_2 was 1 mg; the dose of Al citrate was 0.5 mg Al.

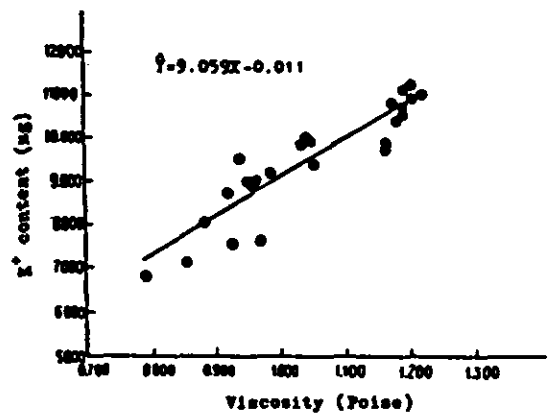

Figure 3. Scatter diagram of membrane lipid viscosity with K^+ concentration of macrophages administrated with SiO_2.

It may be involved in K^+ permeability, however, no change in the activity of Na^+-K^+-ATPase could be found after the treatment of three cell preparations with quartz (Table II), indicating that the increased permeability is related closely to the change in lipid fluidity.

Membrane-bound Water

Membrane hydration of quartz group at the defferent ralative humidities (RH), particularly at higher RH, was reduced markedly from sorption isotherms curve (Figure 4) and data listed in Table III. In IR spectra, VOH shifts largely to lower frequency (Figure 5) and the results represented in Figures 5 and 6 are identical, for instance, at 76% RH, the hydration and VOH peak position in both of control and quartz groups are 18.8% and 3535 cm^{-1}, and 10.1% and 3447 cm^{-1}, respectively. It is clear that the dehydration of cell membranes was caused by quartz and has a significant does effect relationship (Table IV).

Whereas membrane hydration in either quartz plus Al citrate group or the pretreated quartz group with Al citrate is higher than quartz control (Table III) and their VOH peak position shifts towards the higher frequency (Figures 5, 6). The effect of Al citrate against quartz exists also a dose-effect relationship (Table IV). Membrane-bound water under the treatment by the different ways is presented in Figure 7. The similar results are found from two quartz groups pretreated with Al citrate and $AlCl_3$. However, the effect of titanium dioxide on membrane-bound water is not only lower than quartz, but also was not recovered by the pretreatment of Al citrate (Figure 7).

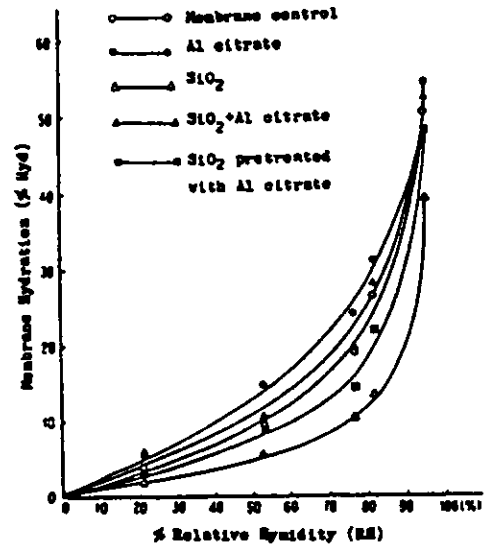

Figure 4. Sorption isotherm of water on red blood cell membranes treated by different ways at 20°C.

20mg SiO_2: 8.333 mg Al(Al citrate)

Table I

Polarization (P) of Membrane-bound DPH and Its Lipid Microviscosity (η) with K^+ Content of Macrophages

Groups	time (min)	P $\bar{X}\pm SE$	η $\bar{X}\pm SE$	K^+ $\bar{X}\pm SE$
Control	20	0.185±0.003	1.352±0.042	11.557±0.149
	60	0.184±0.002	1.329±0.018	11.533±0.099
SiO_2	20	0.152±0.002	0.984±0.019	9.239±0.192
	60	0.141±0.003	0.885±0.031	7.431±0.205
SiO_2+Al citrate	20	0.171±0.001	1.183±0.007	10.612±0.227
SiO_2 pretreated with Al citrate	20	0.172±0.001	1.195±0.010	10.694±0.254
Cell pretreated with Al citrate	20	0.185±0.004	1.350±0.043	11.608±0.181
Cell pretreated with Al citrate+SiO_2	20	0.154±0.003	1.000±0.029	9.498±0.205
TiO_2	20	0.164±0.002	1.104±0.019	10.616±0.264
	60	0.163±0.002	1.096±0.025	9.384±0.231
TiO_2+Al citrate	20	0.164±0.002	1.111±0.022	10.549±0.258
TiO_2 pretreated with Al citrate	20	0.163±0.001	1.100±0.015	10.647±0.279

K^+($\mu g/2x10^6$ cell); 1mg SiO_2 or TiO_2; 0.5mg Al

Table II

Na^+-K^+-ATPase Activities (μM Pi/mg protein) of Three Cell Preparations

Groups	adhesion cell	suspension cell	cell homogenate
	$\bar{X}\pm SE$	$\bar{X}\pm SE$	$\bar{X}\pm SE$
Control	0.672±0.099	0.806±0.144	1.156±0.190
SiO_2	0.646±0.113	0.794±0.142	1.099±0.232
Al citrate	0.616±0.099	0.799±0.137	1.136±0.057
SiO_2+Al citrate	0.666±0.119	0.794±0.128	1.162±0.168
TiO_2	0.627±0.095	0.862±0.192	1.098±0.187

The doses of SiO_2 and Al citrate were 300μg and 125μg Al respectively; The enzymatic activities were determined at 1 hr. of culture.

Table III

Hydration of Erythrocyte Membranes of Several Groups at the Different Relative Humidity (RH)

Groups	Hydration(%)				
	95%RH	81%RH	76%RH	52%RH	20%RH
Control	50.2	26.1	18.8	9.2	4.0
SiO_2	39.1	13.3	10.1	5.8	2.3
Al citrate	54.1	30.9	23.8	14.7	5.7
SiO_2+Al citrate	52.0	28.1	19.4	10.6	5.8
SiO_2 pretreated with Al citrate	47.5	21.6	14.3	9.1	3.2

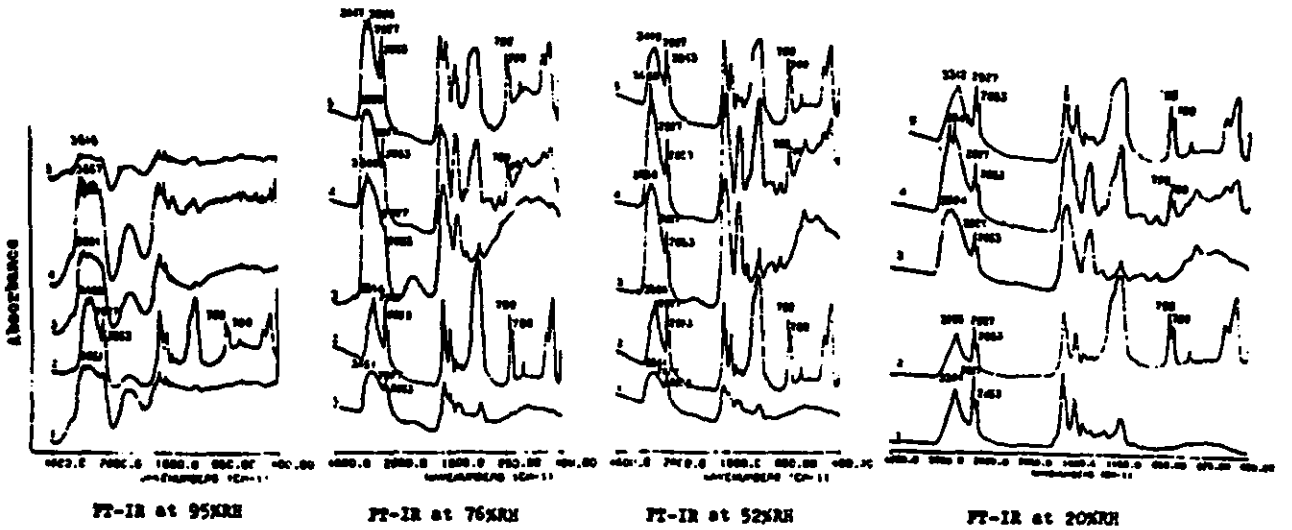

Figure 5.

1. Membrane control
2. SiO_2
3. Al citrate
4. Al citrate + SiO_2
5. SiO_2 pretreated with Al citrate
2. Omg SiO_2: 0.833 mg Al

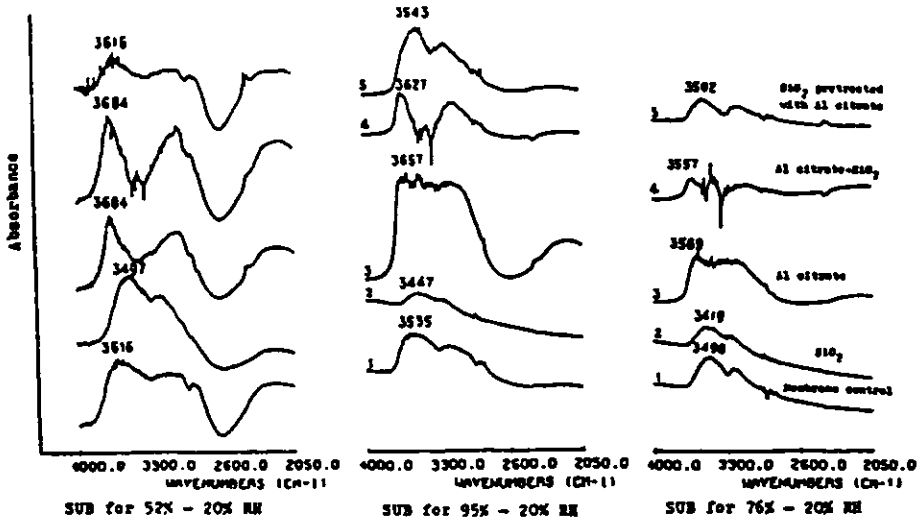

Figure 6.

Table IV

The Effects of Different Doses of SiO_2 and Al Citrate on Membrane-bound Water

SiO_2(mg)	$\nu_{OH}(cm^{-1})$	Al(mg)	$\nu_{OH}(cm^{-1})$	
0.5	3395	0.417	3419	
1.0	3364	0.833	3558	3460
2.0	3314	1.668	3607	3493
3.0	3288			

* SiO_2(2.0mg)+Al citrate

Cell Membrane Charge

As shown in Figure 8, electrophoretic mobility of macrophages sped up rapidly following the addition of quartz. The result indicates that the interaction of quartz with macrophage surface causes increasing negative electrophokinetic potential (ξ-potential) and charge density on the membrane surface. Similar to that on membrane fluidity and permeability, the effect of quartz on membrane charge of macrophages has also significant time-dependent and dose-dependent relationships (Figures 8, 9).

Al citrate can decrease electrophoretic mobility of macrophages by itself like its effect on membrane-bound water. The effect of quartz is almost abolished by the addition of a high dose of Al citrate (Figure 9). Of particular interest, the effect of quartz on membrane charge can be decreased by the pretreatment with Al citrate (Figure 8).

As illustrated in Figure 8, the increment by titanium dioxide is lower and its kinetics are very different from that of quartz, although it increased also electrophoretic mobility of macrophages.

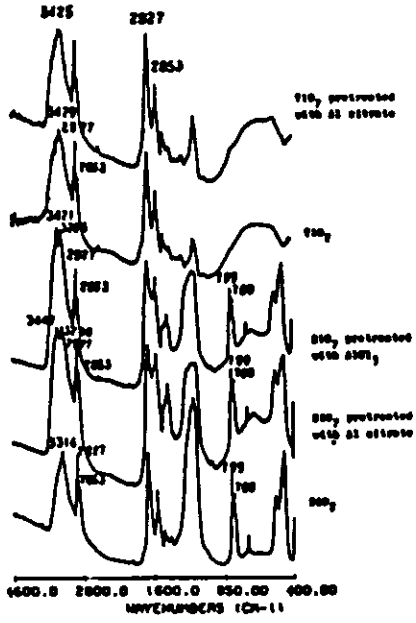

Figure 7. PT-IR or erythrocyte membranes treated by the different ways at 76% RH.

2.0 mg SiO_2 or TiO_2

DISCUSSION

In previous studies, the cytotoxicity of quartz on macrophages was evaluated usually by measuring the activities of LDH and ACP and cell death rate.[3,6,13-15] The enzymatic activities may reflect indirectly the palsmic and lysosomal membranes damage caused by quartz, but their changes did not occur until after one hour of the incubation of cells with quartz. It is obvious that the indirect interaction between quartz and macrophage membranes, particularly its early effect need to be observed in order to establish the injurious effect of quartz on the membranes. It is for this purpose that the present studies was carried out.

Membrane fluidity plays an important role in membrane function.[16,17] Fluorescence Probe DPH used in this experiment can be inserted into the hydrocarbon region of lipid bilayers and fluorescence polarization depends on microviscosity of that region. The decrease of P under the action of quartz elucidates that the motion of lipidic molecules was increased due to the lowered membrane lipid microviscosity, resulting in disruption of membrane structure. Moreover, the fact that quartz can increase fluidity of liposomes prepared from lecithin and cholesterol also suggests that quartz interacts mainly with membrane lipids.[18]

In regard to the study of permeability, we have demonstrated that the reduction of K^+ content in macrophages can anticipate the enhanced activities of LDH and ACP in culture medium following the addition of quartz to these cells and is responsible for the cytotoxicity.[6] The present paper establishes further the correlation between both changes of permeability of macrophage membranes to K^+ and their mambrane fluidity by quartz. Likewise, the mechanism of the increased permeability is considered to be associated with the effect of quartz on membrane lipids, but not on Na^+-K^+-ATPase.

Bound water is a major component of biological membranes and is required for the structural stability of lipid bilayers and the normal function. A novel information about the effect of quartz on membrane "water structure" was obtained from the experiment of membrane-bound water of erythrocytes. The membrane IR spectra show hydration-dependent changes in the stretching vibration band of bound water, namely VOH shifted to the lower frequency with decreasing hydration. The result from subtract spectra (SUB), which can exclude absorbance of several groups besides water at 3000-3800 cm^{-1} region, is consistent with the effect. A turning point of membranes hydration from sorption isotherms curve is at about 76% RH, at which hydration of normal erythrocytes membranes is 18.8% and its VOH peak position is 3535 cm^{-1}, whereas hydration of quartz group is only 10.1%, and its VOH peak position exhibits red shift to 3447 cm^{-1}. The decrease of membrane-bound water does not provide lipidic molecules with a necessary condition required for hydrophilic and hydrophobic interactions, so that the order degree of biomolecular layers was not maintained. Indeed, Clifford et al have found the changes of structure, such as phase separation of cholesterol from lipid, in membrane dehydration.[19] Thus dehydration by quartz is associated with increasing fluidity or permeability. On the other hand, charges on the membrane surface will alter relatively because the dehydration has made water molecules separate from some groups on membranes which are bound to them. This is further supported by the results from cell electrophoretic experiments.

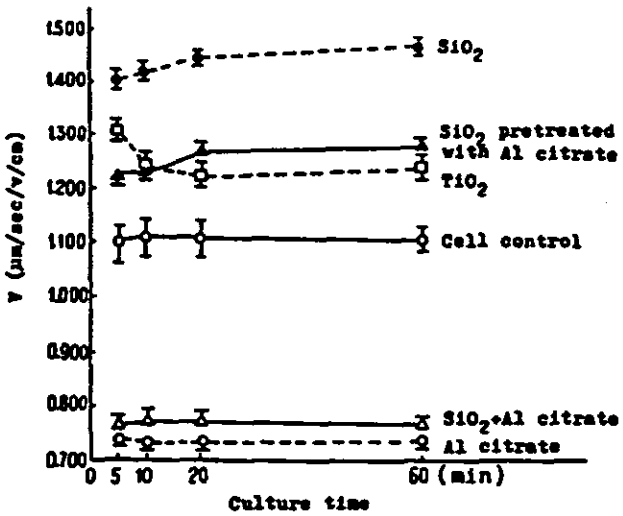

Figure 8. Kinetic curve of electrophoretic mobility (V) of macrophages treated by different ways.

500μg SiO_2 or TiO_2; 250 μg Al

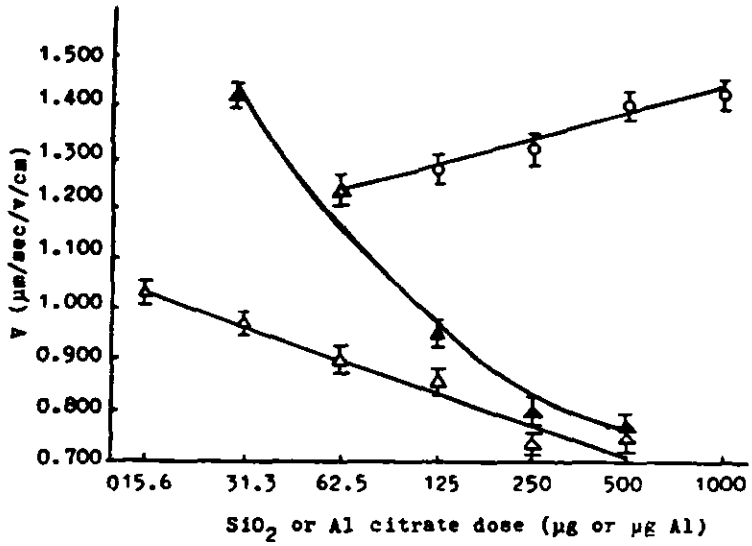

Figure 9. Dose-effect relationships of SiO_2, Al citrate and SiO_2 (500 μg) + Al citrate on electrophoretic mobility (V) of macrophages.

O—O SiO_2; Δ—Δ Al citrate; ▲—▲ SiO_2 + Al citrate

It seems more possible that quartz interacts with the positively charged groups, such as $-N^+(CH3)3$ riched in membrane phospholipids, so positive charge on the cell surface is neutralized partly and negative charge density increases relatively. Nash et al and Depasse et al presented the indirect evidence that quartz is easy to attract amide phosphate and quatenary ammonium groups and suggested that the attraction is responsible for haemolysis of quartz toward erythrocytes.[20-21]

Compared to quartz, the effects of titanium dioxide on cell membranes are not only much lower in intensity, but also very different in kinetics, for instance, the changes of fluidity, permeability and cell electrophoresis can not enhance permanently with the culture time, whereas tended to recover rapidly and remained constant. Of interest, the results from morphology is quite in accordance with biophysical and biochemical determinations.[22] Whether the membrane damage is caused will depend on physical and chemical properties of different particles. The fact that the effects of titanium dioxide on cell membranes were not affected by Al citrate may give some insight to the difference between quartz and titanium dioxide in their surface structure and affinity for ions, such as $-N^+(CH3)3$ and $A13^+$.

Another part of this paper focuses on the anti-injurious effect of Al citrate and its mechanism. In general, the increased membrane fluidity, permeability and negative charge density were declined, but the decreased membrane hydration were enhanced following the addition of Al citrate, so that the function, stability and order structure of cell membranes can be recovered and maintained. The observation by scanning electron microscope convinced us of the antagonistic effect of Al citrate once more.[22]

The mechanism is discussed through the compared antagonistic effects of several ways of the administration. It seems that Al citrate will affect membrane-bound water and charge by it self if the addition of it into cell medium without washing, but the its effect of disappeared after the cells were washed.[11] These findings suggest that Al citrate combines with certain membranes, even though the combination is not firm and matters little to its effect against quartz. No preventive effect was found in fluidity and permeability experiments of macrophages pretreated with Al citrate. Moreover, Al citrate alone did not influence these properties of macrophage membranes. From these it is considered at least that the preventive effect of Al citrate is not produced by its direct action on cell membranes.

The preventive effects of Al citrate and $AlCl_3$ were examined through the pretreatment of particles. The results show that this pretreatment way can effectively resist membrane damage by quartz. On the other hand, the fact that AlC13 exhibits a similar action indicates that the pharmalogical effective component of Al citrate is mainly Al itself, which explains why many kinds of soluble Al agents processes a similar effect of treatment for silicosis. Attention should be paid to the potential significance of the special action of Al on quartz in preventive and therapeutic silicosis.

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