Toxicological investigations on silicon carbide.

2. In vitro cell tests and long term injection tests

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Abstract

Silicon carbide (SiC) dust and other dusts for comparison were injected intratracheally at a high dose (50 mg) into rats and the response of the lungs and the lymph nodes was studied after an appropriate experimental period. The indices studied were: histological changes in the lung and lymph nodes, organ weights, the formation of collagenous fibres, and the appearance of quartz typical areas. According to several epidemiological investigations and previous experimental animal studies, SiC produces silicogenic (fibrogenic) effects. No changes in the tissues studied in terms of damaging fibrogenic effects could be found after eight months (first series) and three and 12 months (second series). In particular, the histological findings and the absence of quartz typical areas as well as the quantitative determination of collagen fibres show that SiC had no harmful effects on tissues. Based on these results, the extent to which other exposures during the production of SiC can be responsible for the established radiological alterations is discussed. Without doubt the following may be confounders: SiC fibres, crystalline SiO₂ (quartz, cristobalite, tridymite), and possibly gaslike emissions (SO₂). From the hygienic medical point of view the workplaces during SiC manufacture should be examined carefully. The substance SiC dust as such can be considered as inert from the experimental results based on qualitative and extremely sensitive procedures. A revision of the present threshold value for SiC in the German MAK list is called for.

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Epidemiological data have indicated some weak silicogenic changes in the lungs of silicon carbide (SiC) workers; it cannot be ruled out that confounders such as tridymite are present. Experimental assays for evaluating weak fibrogenic effects of mineral dusts are basically performed with the lymph node test. The dust samples are instilled into the lung in a relatively high dose; the bioassay includes the evaluation of histological changes in lung and lymph node tissue, organ weights, and quantitative evaluation of the lymph node tissue. In particular, the appearance of quartz typical areas is considered a very sensitive index for early fibrogenic events. The disadvantage of this method is the circumvention of all defensive reactions of the lung parenchyma such as elimination by alveolar macrophages. Mainly non-specific epiphenomena due to the overload situation have to be carefully evaluated when drawing conclusions as to the human exposure situation.

As well as qualitative data from inhalation experiments, the results of intratracheal instillation should also give information on minute fibrogenic effects of the samples investigated. Concerning the range of the questionable fibrogenic effects in vitro testing of the dust samples has been carried out; the capacity of the alveolar macrophages to release activated oxygen species is related to some extent to killing of bacteria and function as scavengers in the alveolar cavity. The production of tumour necrosis factor- α is related to the silicogenic capacities.

Materials and methods

DUST SAMPLES

The dust samples used were: SiC (F 1200 grün, Elektroschmelzwerk, Kempten, Germany), untreated clay, ground clay, and tempered clay, kaolinite (DMT, Essen, Germany), and Dörentruer quartz (DQ12, DMT, Essen, Germany). The dust samples were separated to give an equal respirable particle size distribution (DMT, Essen, Germany). The mean grain diameter was $<3 \mu\text{m}$.

IN VITRO METHODS

H₂O₂ Release

A test system based on the method of De la Harpe and Nathan,¹ modified to the needs of evaluation of the effects of dust, was used to measure H₂O₂ release from alveolar macrophages after exposure to dust. Briefly, alveolar macrophages from guinea pigs were introduced into wells of microtitre plates (300 000 per well); two hours after settlement and conditioning the dust samples were added at doses of 20 and 60 µg/300 000 cells respectively and incubated for 16 hours. The assay mixture for the determination of H₂O₂ was scopoletin, peroxidase and phorbo-12-myristate-13-acetate. The results were expressed as nmol H₂O₂ per well.

TNF-α

Mineral dusts—All mineral dusts were suspended at a concentration of 10 mg/ml in phosphate buffered saline and stored at -20°C. Immediately before use the suspensions were sonicated three times for five seconds and diluted with culture medium to the appropriate concentration. The culture medium was Dulbecco's modification of Eagle's medium (DMEM) (Biochrom, Berlin, Germany). It was supplemented with 10% (v/v) fetal calf serum, 1 mM sodium pyruvate (Biochrom), 50 mM L-glutamate, 50 U/ml penicillin G, and 50 mg/ml streptomycin (all from Gibco/BRL, Eggenstein, Germany).
Bone marrow macrophage differentiation culture—Briefly, bone marrow cells from CBF1-mice were seeded in teflon foil bags (3 × 10⁶ cells in 50 ml macrophage differentiation medium in each bag). The bags were sealed and incubated for 11–15 days. At the time of harvesting, the cultures represented an almost pure macrophage population (>98% purity).

Macrophage activation—Bone marrow macrophages were seeded in microtitre plates at a density of 10⁶/ml (2 × 10⁵/well). The mineral dust suspensions were added to the cultures together with 0.25 U α-antitrypsin/ml to a final volume of 200 µl/well. After a 24 hour incubation, the supernatants were tested for TNF-α activity.

TNF-α assay—A total of 10⁴ L929 cells/well were incubated with 100 µl supernatant (final volume) for 44 hours and pulsed for an additional four hours with 0.3 mCi ³H-thymidine/well. The labelled L929 cells were harvested (Automash II cell harvester; Dynatech, Denkendorf, Germany) on glass fibre filters (Skatron Inc, Sterling, VA, USA). After addition of scintillation liquid (Rotiscint 11; Roth, Karlsruhe, Germany), the incorporated ³H-thymidine was counted in a β scintillation counter. The test was performed in quadruplicate. The specificity for TNF-α was checked in a parallel experiment by adding rabbit anti-

mrTNF-α antiserum (Innogenetics, Antwerp, Belgium) at a final dilution of 1:500.

ANIMALS

One hundred and sixty four female SPS Wistar rats weighing about 200 g at the start of exposure were obtained from Lippische Versuchstierzucht (Charles River Co Germany). The rats were fed and housed under normal laboratory conditions (12 hour light/dark cycles; water and food ad libitum).

ANIMAL EXPOSURE GROUPS: INTRATRACHEAL INJECTION

First series

Under light ether anaesthesia 30 rats received a single dose of 50 mg SiC suspended in 0.5 ml physiological saline by intratracheal injection. In addition 30 rats injected with 0.5 ml physiological saline served as control animals.

Second series

The same procedure and same dusts as in the first series were used. In addition 30 rats were given an intratracheal injection of 2 mg quartz DQ12 suspended in 0.5 ml of physiological saline.

HISTOLOGY OF THE LUNGS

Three and eight months after exposure, 15 rats per group were killed with an overdose of pentobarbital and exsanguinated; the trachea was cannulated, and the lungs were degassed and inflated with 4% neutral formalin at a pressure of 20 cm water. The lungs were removed from the thorax and fixed in 4% neutral formalin in situ.

Horizontal sections (7 µm) of the left lobe (upper, middle, and lower region) of the lung were prepared by routine histology (paraffin embedding). For histological examination of the lung tissue sections of each region were stained with haematoxylin and eosin. Other sections were stained with Sirius red F3BA (Polyscience, USA) for viewing of collagen fibres in the tissue. Sirius red stain enhances the natural physical property of birefringence of the collagen fibres. Therefore collagen fibres are recognisable as bright shining structures in lung tissue under the microscope with polarised light. This staining technique displays a high affinity for collagen fibres and gives pictures of high contrast (see fig 4). The following staining procedure was used. Deparaffinised sections were stained for 30 minutes with a mixture of saturated aqueous picric acid and 0.1% Sirius red. Excessive stain was washed out with distilled water. After dehydration the sections were embedded in Eukitt (Kindler, Germany) and covered with a cover slip. The sections were viewed and photographed with a Leitz microscope (Leitz Orthoplan, Wetzlar, Germany).

HISTOLOGY OF THE LYMPH NODES

After dissection of the lung the mediastinal lymph nodes were removed and fixed in 4% formalin for further histological examination. Sections (7 μm) of the complete lymph nodes were prepared by routine histology (paraffin embedding) and stained with haematoxylin and eosin. Additional sections were stained with Sirius red F3BA according to the method described earlier (see fig 6). These were used for quantification of collagen fibres in the tissue of the lymph nodes with a Quantimet 970 automatic image analyser (Cambridge Instruments, UK). This analyser detects structures on the basis of grey level discrimination, whereby structures to be measured are selected by a preset grey level.

For quantifying collagen fibres, the complete sections of the lymph nodes were measured under polarised light with a 16x objective. The individual fields measured were $820 \times 640 \mu\text{m}$ ($800 \times 625 \text{ pixel}$) in size.

The total area of lymph node tissue and the total area of collagen fibres in relation to the tissue area as well as the distribution of the thicknesses of collagen fibres were measured. For statistical analysis the data obtained by image analysis were transposed from original measured pixels to $\text{m}\mu$.

The statistical calculations were done with the Statistical Analysis System (SAS, version 6; SAS Institute; USA). To test for group differences, a

two sided variance analysis specific for unbalanced data was used. Group mean differences were tested with a t test with Bonferroni significance levels from BMDP (BMDP, Statistical Software, 1983 USA).

Results

TOXICITY

Two procedures were chosen for *in vitro* studies of toxicity: (1) the H_2O_2 release test; (2) the release of $\text{TNF-}\alpha$ from alveolar macrophages. The various dusts were studied at doses ranging from 60 down to $20 \mu\text{g}/10^6$ cells.

Quartz at a concentration of $60 \mu\text{g}$ gave a complete inhibition of stimulation of H_2O_2 -release and the lower concentration of $20 \mu\text{g}$ quartz resulted in about 40% reduction. Corundum and SiC, both at a concentration of $60 \mu\text{g}$, showed no difference compared with untreated cells both for the temporal course of events as well as end point measurements (fig 1).

 $\text{TNF-}\alpha$

Quartz concentrations up to $10 \mu\text{g}/\text{well}$ led to a significant growth inhibition compared with the controls. When tempered and ground clay and pure clay were compared with SiC, SiC did not result in an apparent growth inhibition of L 929 cells at doses up to $50 \mu\text{g}/\text{well}$ (table).

Figure 2 summarises the results of two series of intratracheal injections of the test substance SiC (50 mg). The first series represents the lymph node response after eight months. The second series contains the results of the three and 12 month experimental period. In this case, an additional group was introduced as a positive control, namely quartz DQ12 at a dose of 2 mg. SiC led to a slight increase in average lymph node weights. This rise could be attributed to the natural response to an inert dust deposit. Essentially, no alterations in lymph node weights were found over the period from three to 12 months. By contrast, even the

Figure 1 *In vitro* testing of SiC, corundum, and quartz guinea pig alveolar macrophages; H_2O_2 release.

Release of $\text{TNF-}\alpha$ assessed by growth inhibition of L-929 cells

Mine dust	$\mu\text{g dust/well}$	L929-growth (cpm)	
		Mean	(SD)
Control	—	57.5	(7.3)
Quartz DQ12	10	36.0	(1.6)
	20	15.8	(1.0)
	50	9.4	(1.5)
	100	13.7	(2.5)
	10	58.1	(5.7)
SiC	20	51.8	(4.3)
	50	46.1	(4.6)
	100	35.3	(1.4)
Tempered clay	10	56.2	(3.4)
	20	50.3	(4.1)
	50	17.8	(0.8)
	100	0.6	(0.0)

small amount of 2 mg quartz clearly gave an increase in lymph node weight (fig 2). Histological investigations showed a completely inert deposition of SiC dust in the lung and the lymph nodes. The dust was compactly located without accompanying cellular responses so that in particular no granulocytes were found. Collagen development was not seen and could not be identified even with the aid of morphometric analysis (figs 3–6).

Discussion

The experimental animal data are based on two separate series of studies involving various methods of exposure and, in part, different assessment indices. The central issue of the toxicological analysis was the extent to which SiC possesses fibrogenic or silicogenic properties. These terms are not always completely congruent in published work: a fibrosis with nodular connective tissue-like lesions in the perivascular and peribronchial lymphatic regions of the lung similar to that of silicosis differs histopathologically and clinically from diffuse interstitial pulmonary fibrosis (DIPF), which appears as chronic inflammation of the alveolar septa of the lungs, and scarring is largely confined to the septal tissue. The causes are various—namely, asbestos, cytostatica (for example, bleomycin), radiation induced fibrosis, or idiopathic pulmonary diseases (including sarcoidosis). The pathological end point of all these noxious compounds is scarring, histologically recognisable by an increase in collagen.

The two series of studies involved an inhalative burden to the animals' lung from an intratracheal injection of a high dose of 50 mg per animal. Also, *in vitro* testing was performed. To substantiate and control the detectable changes in the alveolus *in vitro* tests were used that assess the specific interac-

Figure 2 Lymph node wet weight: first series eight months after intratracheal injection; second series three and 12 months after intratracheal injection

Figure 3 Light microscopical photograph of lung tissue (stained with haematoxylin and eosin) viewed under normal light.

Top: tissue eight months after injection of 50 mg SiC. Note the compact dust deposits (S) in the lung.
Bottom: saline injected control.

tions between dust and alveolar macrophages with particular respect to the dose. Cytopathogenicity (H_2O_2 release test) as well as stimulation of alveolar macrophages to produce $TNF-\alpha$ were used as criteria of *in vitro* effects.

The *in vitro* studies both showed maximal cytopathogenicity for the reference dust, quartz DQ12, at the basal dose of $60 \mu g/10^6$ cells.

Lower doses of quartz showed graded, weaker effects. By contrast, SiC had no effect on the indices studied at the standard dose of $60 \mu g$. We assume that these test indices in general reflect the pathogenic effects of mineral dusts and, to a lesser extent, the specific cytotoxic effect.^{2,3} For example, in these cases there is a direct correlation of cytopathogenic indices with that of dust penetrating the lymph nodes (lymphotropism) as well as the ability to evoke graded reactions in quartz typical regions of the lymph nodes.⁴

Figure 4 Light microscopical photograph of lung tissue (stained with Sirius red) viewed under polarised light (bright shining structures: silicon carbide particles and collagen fibres). Top: tissue eight months after injection of 50 mg SiC. Note the compact dust deposits (S) in the lung. Bottom: saline injected control.

Figure 5 Light microscopical photograph of lymph node tissue (stained with haematoxylin and eosin) viewed under normal light. Top: tissue eight months after injection of 50 mg SiC. Note the compact dust deposits (S) in the lymph node. Bottom: saline injected control.

Among several alveolar macrophage-derived fibrogenic factors now identified, TNF seems to play a key part in that a single instillation of silica in mice leads to a pronounced increase in lung TNF production. Also, silica induced lung fibrosis is almost completely prevented by TNF antibodies.^{5,6,7}

To better characterise the mechanism of test dust dependent alveolar macrophage activation, we have investigated the effects of silica and SiC on the secretion of TNF- α from isolated alveolar macrophages. In the case of quartz, this response was dose dependent and no further increase in TNF secretion was seen at the highest dose used, apparently as a result of direct cellular toxic effects. By contrast, incubation of alveolar macrophages with SiC did not result in enhanced TNF secretion compared with controls even at the highest dose of 50 $\mu\text{g}/10^6$ cells.

ANIMAL STUDIES

Assessment criteria are the tissue response of the lung itself as well as lymph nodes associated with the lung. These nodes accumulate all dusts drained lymphatically from the lung and concentrate these particles in lymph node tissue. This response is particularly evident in the rat after exposure to quartz, the classic silicogenic dust. The following indices are considered to be quartz typical lymph node reactions: increase in lymph node weight, collagen formation, and formation of so-called quartz typical areas.^{8,9} In our studies, the lowest dose of quartz in the intratracheal injection eliciting a distinctly positive reaction in the three indices was 0.01 mg.¹⁰ Other studies have shown, however, that doses of 0.005 mg of quartz per rat result in a still perceptible positive marginal response.¹¹ No significant reaction was seen after intratracheal application of 50 mg SiC per animal; the minimal weight increase

Figure 6 Light microscopical photograph of lymph node tissue (stained with Sirius red) viewed under polarised light (bright shining structures are SiC particles and collagen fibres)
 Top: tissue eight months after injection of 50 mg SiC. Note the compact dust deposits (S) in the lymph node.
 Bottom: saline injected control.

must be considered to be the result of the tissue response to the dust load. A completely inert deposition of dust in the tissue could be seen from histological sections, where neither collagen fibres nor quartz typical areas were found. These findings on SiC agree qualitatively with those of other studies in which high intratracheal applications of SiC did not result in pathological effects.

A hypothetical effect of more than three orders of magnitude must be defined in relation to the lowest effective quartz doses. An epidemiologically based threshold value for quartz is 0.15 mg/m^3 in Germany and 0.1 mg/m^3 in the USA. Hence a value of 100 mg/m^3 for SiC could be extrapolated as non-fibrogenic.

Further findings were obtained from the inhalation experiments. Cellular responses such as the occurrence of granulocytes are taken as signs of an inflammatory reaction, and considered to be the starting point of the fibroblastic response to fibro-

genic dusts such as quartz^{12,13} or asbestos.¹⁴ No increase in granulocytes was seen in groups exposed to pure SiC by contrast with animals subjected to quartz. The values correspond to those of the control animals as well as those found in the BAL fluid of healthy test persons.¹⁵ A weaker inflammatory reaction compared with that of quartz was also seen in animals exposed to kaolinite. This shift in cellular composition is a highly sensitive index.

A strong quartz dependent increase in granulocytes was found by Bruch¹⁶ in other studies with authentic colliery mixed dusts in which the quartz content ranged between 2% and 10%. A striking phenomenon was the decrease in the total cell count three days after completing quartz inhalation in animals.

In this case, we assumed that an inhibition of reactive cell recruitment becomes recognisable because of toxic effects of dust exposure. One important component of the lung microenvironment is the amount and composition of lung surfactant factor (LSF) in humans. High exposure to quartz dust results in an increase in LSF in the lung representative of an acute silicosis.¹⁷⁻²⁰ In animal experiments, these compounds arising in the alveolar cavity after quartz exposure were identified as LSF phospholipids with an altered composition.²¹⁻²⁴ This rise is explained by an increase in the number of the cell class responsible for LSF synthesis—namely, the pneumocyte type II.²⁵⁻²⁷ Our own investigations on the composition of LSF phospholipids indicate an extreme sensitivity on the part of this index towards toxic dusts.²⁸ Against this background, the unaltered ratio of phosphatidyl glycerol: phosphatidyl inositol in the LSF subfractions is particularly predictive. This finding is in good agreement with the data from cells. No toxic effects of SiC could be found under these conditions.

The results of dust retention after inhalation exposure to the various dusts can be interpreted in conjunction with LSF responses. Initially, the elimination of the SiC quartz admixture is greater than that of the dusts in the other groups. This can be attributed to the initially higher stimulation of cells in the alveolus.

This previously unknown finding was reported by us earlier^{29,30} and was able to be confirmed in several other inhalation experiments.³¹ The results obtained at the end of the study—that is, 90 days after inhalation—are of a decisive nature. In this case, there is a distinct retardation in the elimination of quartz whereas that of SiC progresses undiminished.

Based on the consistent data from two independent studies as well as the negative results of the Begin group³² it can be concluded that SiC as such shows no fibrogenic effect; moreover no sign of cell

stimulatory effects could be seen. No statements can be made as to the variability of the product; in particular it should be pointed out that production related admixtures or finest SiO₂ thin layers have yet to be ruled out.³³

Without doubt, other specific silicogenic substances may be facultatively present in the use of SiC—for example, during the production of SiC in certain places of work.³⁴ Individually it is not always possible to determine the extent to which the quartz doses mentioned truly reflect a previous load possibly arising 10 or more years earlier.

More recent publications on the carcinogenic effect of "hard dusts"³⁵ require classification, particularly with respect to the exact analytical nature of the substances used in those studies.

In conclusion, SiC dust produces no tissue damage in animal studies; a re-evaluation of the hygienic situation of SiC production work places seems appropriate; the various varieties of commercial SiC products should generally be tested as to their possible silicogenic or other impurities; and a reclassification of existing hygienic standards in Germany for fibrogenic and silicogenic effects should be considered.

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