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Distinct Spectrum of Mutations Induced by Crocidolite Asbestos: Clue for 8-Hydroxydeoxyguanosine-dependent Mutagenesis *in Vivo*

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ABSTRACT

DNA damage due to reactive oxygen or nitrogen species is proposed to be involved in the molecular mechanism of asbestos-induced carcinogenicity. However, indications for this hypothesis came mainly from *in vitro* assays using cultured cells or cell-free systems. In the present study, the mutagenicity of crocidolite fibers and the underlying molecular mechanisms were investigated *in vivo*. Mutation frequencies were determined in DNA of omenta, a relevant target tissue for mesothelioma carcinogenesis, using *lacI* transgenic rats. The mutagenic effect of 2 and 5 mg of crocidolite asbestos was demonstrated, with a maximal relative increase in mutation frequency of 3.4 compared with the control group. The molecular analysis of the mutations revealed striking differences according to mutation types between asbestos-induced mutations and spontaneous mutations. Therefore, a specific molecular mechanism induced by crocidolite that differs from that induced by the generation of spontaneous mutations can be proposed. G to T transversions, which are known to be induced by the premutagenic DNA adduct 8-hydroxydeoxyguanosine (8-OHdG), were the most prominent mutation type (29%) within crocidolite-induced mutations. In additional experiments, 8-OHdG in DNA of omenta from rats treated with 1 or 2 mg of crocidolite asbestos was determined. Levels of 8-OHdG in animals treated with crocidolite were significantly increased compared with negative controls. These data give strong evidence for the involvement of reactive oxygen or nitrogen species in crocidolite-induced mutagenesis *in vivo*.

INTRODUCTION

Occupational exposure to fibrous dusts in humans is a cause of disorders like asbestosis and different types of cancer (1). Due to exposition pathways, fibers induce tumors mainly in lung and pleura and less frequently in pericardium and peritoneum. After penetration of the lung tissue, fibers accumulate in the pleura (2). In the mesothelial cell layer lining this cavity, in addition to other pathological effects, mesothelioma is induced. In this target tissue, as compared with the lung, clearance mechanisms are of minor importance. Therefore, fibers persist here for a long time. In human, mesothelioma arises after a long period of latency (1).

Besides proliferative and probably tumor-promoting effects (3, 4), some fibrous dusts may have genotoxic potential. The mutagenicity of fibers has been investigated using various *in vitro* assays with cultured cells (for review, see Ref. 5). The mutagenicity of chrysotile has been demonstrated by hypoxanthine-phosphoribosyltransferase assay and Southern blot analyses in a human hamster hybrid cell line (6). Together with results from S-1 mutation assays performed for several natural and man-made fibers, these results indicate that fibers induce genome or chromosome mutations rather than point mutations (7). However, these and other approaches used mainly *in vitro* systems, which only monitor direct effects of fibers on target cells. Indirect effects mediated by the interaction of two or more cell types (*e.g.,* inflammatory cells and mesothelial cells) have not been monitored with these systems. Thus far, the mutagenicity of fibrous dusts in mesothelial cells *in vivo* has not been demonstrated.

The molecular mechanisms of fiber-induced mutagenesis are not understood. Mutation assays or DNA damage monitoring assays with fibers in the presence of antioxidants indicate the involvement of ROS[2] in fiber-induced mutagenesis (8, 9). Several fibers have been demonstrated in cell-free systems to catalyze the formation of hydroxyl radicals (10, 11). The production of hydroxyl radicals in cells treated with asbestos may result in the formation of premutagenic DNA bases such as 8-OHdG. The increase of 8-OHdG in genomic DNA has been documented *in vitro* when rat pleural mesothelial cells were treated with crocidolite asbestos (12). *In vivo*, these effects may be augmented by hydrogen peroxide (H_2O_2) released from inflammatory cells that are recruited after fiber deposition in the tissue (13). Using intrinsic cellular or fiber-bound transition metals (mainly Fe) as catalyzer, hydroxyl radicals may be generated via Fenton reaction from H_2O_2, which is able to diffuse into target cells (14). In addition to ROS, reactive nitrogen species may be involved in 8-OHdG generation. In rat alveolar macrophages, asbestos fibers were able to induce nitric oxide ($NO^{\cdot}$; Ref. 15). *In vivo,* $NO^{\cdot}$ may react with superoxide anion to produce peroxynitrite, which is able to oxidize guanosine to produce 8-OHdG. Studies with human lung epithelial cells (A549) and Chinese hamster cells (V79 *hgprt*$^+$; V79 *hgprt*$^-$, *gpt*$^+$) revealed synergistic effects of NO and crocidolite asbestos with regard to induction of 8-OHdG and mutation frequencies (16, 17).

However, a causal association between fiber- or particle-induced oxygen or nitrogen species probably resulting in the formation of 8-OHdG and the manifestation of mutations *in vivo* has not yet been demonstrated. An appropriate experimental approach for *in vivo* studies is mutagenesis assays with *lacI* transgenic animals (18). In this system, bacterial reporter genes can be analyzed for mutations in every cell of the target tissue relevant for the respective exposure pathway. Using shuttle vectors, mutation frequencies are estimated in a bacterial detection system. Subsequent molecular analysis of mutation types (transition or transversion) can indicate the chemical mechanism of DNA damage in response to a certain carcinogen.

In this study, the mutagenicity of crocidolite asbestos was investigated *in vivo* in *lacI* transgenic rats. Test substances were applied by i.p. injection, which allows fibers to come directly into contact with the mesothelial cell layer of the abdomen. In earlier studies, we have demonstrated on the level of mRNA expression that mesothelioma carcinogenesis in omentum is mainly comparable with this process in pleural mesothelial cells of rats and humans (19). Furthermore, in this tissue, in preceding tumorigenicity studies, mesotheliomas have been observed most frequently (20). Mutations induced in this system were investigated on the molecular level to analyze the spectrum of mutations induced by crocidolite asbestos *in vivo*. The role of the premutagenic DNA adduct 8-OHdG in this mutagenic process was investigated by measurement of this substance in the genomic DNA of Wistar rats treated with crocidolite, using HPLC and electrochemical detection.

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[2] The abbreviations use are: ROS, reactive oxygen species; 8-OhdG, 8-hydroxydeoxyguanosine; HPLC, high-performance liquid chromatography; UICC, Union Internationale Contre le Cancer; BaP, benzo(*a*)pyrene; PFU, plaque-forming unit(s); dG, deoxyguanosine.

MATERIALS AND METHODS

Fibers and Chemicals. For i.p. treatment of animals, UICC crocidolite asbestos with the following physical parameters was used: 8×10^7 fibers/mg, considering fibers of a length:diameter ratio of >5/1, length > 5 μm, and diameter < 2 μm (21). Samples were suspended in 0.9% buffered NaCl solution in 50-ml aliquots by sonification (4 min, 100 W). BaP (Sigma Chemical Co.-Aldrich, Deisenhofen, Germany) was solubilized in a carrier substance (beeswax:tricaprylin, 20:80; Sigma Chemical Co.-Aldrich) at 50°C. The dosages of 2 mg of crocidolite and 5 mg of BaP were chosen because of their carcinogenic potency in the i.p. assay. In earlier experiments, these dosages induced tumors in about 50% of animals (20). To detect rather low mutagenic effects of crocidolite fibers, a relatively high dosage of 5 mg was applied in an additional group.

Animals and Treatments. For mutagenesis assay, age-matched, female, 8-week-old transgenic F344 rats bearing multiple copies of λ*lacI* shuttle vectors (Big Blue) were purchased from Stratagene (La Jolla, CA; animals were bred by Taconic Farms, Germantown, NY). Animals were randomly housed in individual filter-topped cages in a room maintained with 12-h light/dark cycles. After 2 weeks of adaptation, rats were injected i.p. with either 2 or 5 mg of UICC crocidolite asbestos suspended in 2 ml of NaCl solution. The negative control group was injected with 2 ml of NaCl solution. The positive control group was anesthetized by i.p. injection of 37.5 mg/kg body weight pentobarbital sodium. BaP was injected solubilized in carrier substance tempered at 50°C to form long-term depots at body temperature. For determination of 8-OHdG, an additional animal experiment was performed as described above using Wistar rats (Rj:WI Han) purchased from Elevage Janvier (Le Genest St Isle, France). Rats were i.p. injected with either 1 or 2 mg of UICC crocidolite.

Rats were sacrificed by CO_2 inhalation after the respective incubation times (see Table 1 and Fig. 1). The greater omentum (omentum majus) was carefully prepared to prevent contamination with pancreatic tissue, snap-frozen in liquid nitrogen, and stored at −80°C until DNA preparation. In addition, diaphragms were prepared, formalin-fixed, and paraffin-embedded for subsequent pathological examination. The mesothelial cell layer of the diaphragm was used as an indicator for possible malignant transformation. Because this effect was not observed, no animals had to be excluded from the analyses.

Table 1 *Mutation frequencies in omenta of crocidolite asbestos-treated animals*

Treatment group	Time after exposure (weeks)	No. of animals	No. of PFU counted	No. of mutants	Mutation frequency (group mean ± SD; $\times 10^{-5}$)	Fold increase over controls
NaCl (control)	4	5	86864	6		
			105084	3		
			61020	0		
			80208	1		
			100419	6	3.4 ± 3	
Crocidolite (2 mg)	4	5	73791	2		
			37644	1		
			77778	3		
			83998	3		
			33516	2	3.8 ± 1.3	1.1
Crocidolite (5 mg)	4	5	25700	1		
			99441	4		
			98010	3		
			68517	4		
			24606	0	3.3 ± 2.1	0.97
BaP (5 mg)	4	3	110250	40		
			56466	17		
			88389	39	36 ± 7[a]	10.6
NaCl (control)	12	5	52326	1		
			62329	0		
			128466	4		
			100748	1		
			54424	1	1.6 ± 1.2	
Crocidolite (2 mg)	12	5	106496	5		
			107388	0		
			121662	2		
			128196	4		
			133650	3	2.3 ± 1.7	1.48
Crocidolite (5 mg)	12	5	55998	4		
			168534	5		
			124563	6		
			86562	7		
			81548	4	5.5 ± 2[a]	3.44
BaP (5 mg)	12	4	61586	17		
			52668	17		
			93582	14		
			120402	20	24 ± 9.5[a]	14.8
NaCl (control)	24	4	145764	2		
			126489	1		
			112050	1		
			109782	0	0.8 ± 0.6	
Crocidolite (2 mg)	24	5	151974	4		
			152000	0		
			133542	4		
			109134	3		
			104940	5	2.6 ± 1.7	3.25
Crocidolite (5 mg)	24	5	170766	5		
			149670	4		
			105390	3		
			81072	2		
			137844	3	2.6 ± 0.3[a]	3.25
BaP	24	3	84042	40		
			93348	35		
			66330	17	37 ± 11[a]	46

[a] Significantly different from age-matched control. $P < 0.01$ (Student's *t* test).

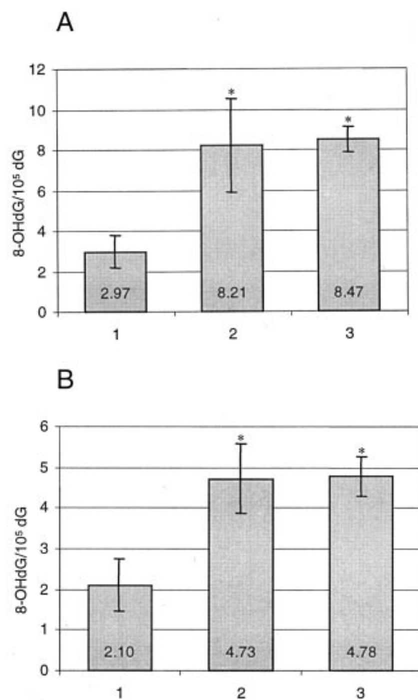

Fig. 1. Mean number of 8-OHdG molecules per 10^5 dG in DNA from rat omenta. *A,* 10 weeks after treatment; *B,* 20 weeks after treatment. *1,* NaCl-treated control animals (10 weeks, $n = 6$; 20 weeks, $n = 7$); *2,* animals treated with 1 mg of UICC crocidolite (10 weeks, $n = 6$; 20 weeks, $n = 10$); *3,* animals treated with 2 mg of UICC crocidolite (10 weeks, $n = 5$; 20 weeks, $n = 8$). *, $P < 0.01$ (Student's *t* test).

DNA Preparation and Phage Packaging. High molecular weight DNA was prepared from the complete omentum (30–80 mg) using the RecoverEase DNA isolation kit (Stratagene) according to the manufacturer's recommendations. The whole amount of DNA was subjected to packaging reactions using λ phage packaging extracts (Transpack; Stratagene) as recommended. Rescue efficiency was estimated by plating serial dilutions of the packaging reactions.

Plating Assay. The plating procedures followed the Big Blue instruction manual (22). The calculated aliquot amounting to 10,000–15,000 PFU was incubated with plating bacteria *Escherichia coli* SCS-8 and plated with NYZ top agarose including 1.5 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Stratagene) onto assay trays containing bottom agar. Trays were incubated at 37°C overnight and subsequently sectored into 36 squares of equal size. The number of viable phages was determined arithmetically after counting of at least eight representative sectors of each tray. Blue plaques indicating the mutant ($lacI^-$) phenotype were scored visually using a light box and a red transparency to enhance the color contrast of the mutant plaques. All mutant plaques were scored independently by at least two trained individuals. Intensity of the blue plaque staining as well as plaque morphology was recorded for each plaque individually. For validation of the blue plaque identification, mutant plaques were verified by replating in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Sectored plaques and pinpoint staining were not counted as mutant. Mutation frequencies were calculated by comparing the numbers of PFU and mutant plaques.

DNA Sequence Analysis. Mutant plaques from treated animals were randomly selected for sequencing from groups that exhibited mutation frequencies significantly different from those of control groups. Due to the low number of mutant plaques in the control groups, spontaneous mutations were determined from plaques of all time points (12 plaques, 4 weeks; 6 plaques, 12 weeks; 3 plaques, 24 weeks). Blue plaques were picked into individual tubes containing 50 μl of TE buffer [10 mM Tris (pH 8)-1 mM EDTA]. After incubation of samples for 5 min at 98°C and cool down to room temperature, samples were centrifuged for 5 min at 16,000 × *g*. Five μl of the supernatant were subjected to PCR. The *lacI* gene was amplified with Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany) as recommended by the manufacturer using the following primers: (*a*) forward primer, 5′-GTA-TTA-CCG-CCA-TGC-ATA-CTA-G-3′; and (*b*) reverse primer, 5′-CGT-AAT-CAT-GGT-CAT-AGC-TGT-T-3′. PCR conditions using a hot start technique were 30 cycles of 45 s at 94°C, 45 s at 50°C, and 1 min at 72°C. PCR products were diluted 1:100 and subjected to direct sequencing with nested primers. Sequencing reactions were performed using the Thermo Sequenase Kit (Amersham-Pharmacia, Freiburg, Germany) with Cy5-labeled oligonucleotides according to the kit manual. Sequencing primers were as follows: (*a*) primer 3, 5′-CG-GCG-ATG-GCG-GAG-CTG-3′; (*b*) primer 4, 5′-TGT-AAA-GCG-GCG-GTG-CA-3′; (*c*) primer 5, 5′-T-CTG-GTC-GCA-TTG-GGT-C-3′; (*d*) primer 6, 5′-CT-GCG-ATG-CTG-GTT-GCC-3′; (*e*) primer 9A, 5′-T-CCG-CTC-ACA-ATT-CCA-CA-3′; (*f*) primer 10, 5′-CAC-CAG-TGA-GAC-GGG-CA-3′; (*g*) primer 11A, 5′-AG-CCC-GGA-CTC-GGT-AAT-3′; and (*h*) primer 13A, 5′-C-CAG-CGG-ATA-GTT-AAT-GAT-CA-3′. Sequence analysis was performed using the AlfExpress automatic DNA sequencing system (Amersham-Pharmacia). Mutations were identified using Mutation Analyzer software (Amersham-Pharmacia). The sequencing strategy ensured bidirectional overlapping sequencing of about 90% of the coding sequence. Only mutations that occurred in two sequencing reactions with separate primers were accepted. Mutational spectra were established by sorting the mutations identified in the experimental groups according to position and type of mutation. Mutation spectra were compared with the Big Blue *lacI* database accessible at the Center for Environmental Health of the University of Victoria (British Columbia, Canada).

Nucleoside Preparation. Genomic DNA was isolated immediately after preparation of the tissue using the Qiagen Blood & Cell Culture DNA Maxi Kit according to the recommendations of the manufacturer (Qiagen, Hilden, Germany). DNA was then dissolved in H_2O overnight at 4°C. Fifty μg of DNA diluted in 135 μl of 20 mM $C_2H_3NaO_2$ (pH 5.0) were incubated at 95°C for 10 min. Heat-denatured DNA was then hydrolyzed to nucleotides by 20 units of nuclease P1 incubated at 65°C for 10 min in 20 mM $C_2H_3NaO_2$ containing 0.1 mM $ZnCl_2$, followed by incubation with 4 units of alkaline phosphatase for 1 h at 37°C. The hydrolysate was filtered through Microcon 30 (Amicon, Beverly, MA) before HPLC analysis to remove enzymes and other macromolecules.

Determination of 8-OHdG. A HPLC-System equipped with a diode array detector 168 set at 252 nm (Beckman, Munich, Germany), an electrochemical detector 41,000 with a glassy carbon working electrode set at a potential of 0.6 V (Chromsystems, Munich, Germany), and a Beckman Integrator (Nouveau Gold System) was used. Separation was achieved on a 250 × 4.6-mm inner diameter 7-μm C_{18} column (Adsorbosphere Nucleotide-Nucleoside; Alltech, Unterhaching, Germany) by isocratic elution (1.0 ml/min) with 12% methanol in 50 mM NaH_2PO_4 buffer containing 3 mM KCl (pH 4.5). The quantification of the nucleosides dG and 8-OHdG was performed by comparison with the corresponding standards. Levels of 8-OHdG in DNA were expressed as values per 10^5 dG.

RESULTS

Mutagenicity Assay. Mutation frequencies in the *lacI* gene were determined in DNA from omenta of *lacI* transgenic rats 4, 12, and 24 weeks after application of 2 or 5 mg of crocidolite asbestos (Table 1). Despite the relatively low amounts of tissue extracted from each animal, about 100,000 PFU, on average, were rescued. With five animals in each group, sufficient data for reliable statistical analysis were obtained. In some tissues from animals of the BaP group, DNA preparation was impaired by the organic carrier substance; however, because of the strong effects of this chemical carcinogen, significant effects could be observed.

In tissues from control animals, the spontaneous mutation rates were 3.4×10^{-5} (4 weeks), 1.6×10^{-5} (12 weeks), and 0.8×10^{-5} (24 weeks), respectively. In animals treated with the chemical mutagene BaP, a mutation frequency 10.6-fold higher than that seen in controls was observed 4 weeks after treatment. After 12 weeks, this value increased up to 14.8-fold, and it peaked (46-fold) after 24 weeks. In crocidolite-treated animals, a significant increase in mutation frequency occurred 12 weeks after treatment in the higher dosage group (5 mg; 3.44-fold). The single treatment with 2 or 5 mg crocidolite asbestos revealed a 3.25-fold increase 24 weeks after treatment. This effect proved to be significant for the animals treated with 5 mg

Table 2 *Number of mutations classified in mutation types observed in DNA from omenta of i.p. treated rats*

	G to A transition[a]	G to C transversion	G to T transversion	A to G transition	A to C transversion	A to T transversion	Insertion	Deletion[b]	Σ
Crocidolite[c]									
No.	7	4	10	1		2	1	9	34
%	20	12	29	3		6	3	26	99
BaP									
No.	11	2	8	2		2	4	2	31
%	35	6	26	6		6	13	6	98
NaCl									
No.	12	3	4				1	1	21
%	57	14	19				5	5	100

[a] Number of G to A transitions in CpG positions: crocidolite, 4; BaP, 8; NaCl, 7.
[b] Length and position of deletions are: crocidolite: 1 bp, codons 22, 58, 73, 2 × 215, 267, and 328; 2 bp, codon 297; 3 bp, codon 215; BaP: 2 bp, codon 56; 3 bp, codon 180; and NaCl: 1 bp, codon 111.
[c] $P = 0.05$ (Fisher's exact test), crocidolite compared with NaCl.

of crocidolite asbestos. Although the result for the lower dosage group did not prove to be statistically significant ($P = 0.08$), we rank this result as reliable to demonstrate the time course of mutagenicity because only one animal was seen without detectable mutations, resulting in a relatively high SD.

Mutation Spectra. To obtain indications for the molecular mechanisms of mutagenesis induced by crocidolite asbestos, mutational spectra considering the chemical nature of the mutations were determined using DNA sequence data from mutant plaques (Table 2). Comparing the frequencies of mutation types occurring in the experimental groups, certain differences can be observed. G to A transitions, which occur in the spontaneous mutations with the highest frequency (57%), are less prominent in the animal groups treated with the carcinogens. The most prominent type of crocidolite-induced mutations was G to T transversions (29%). Another important mutation type observed was deletions (1–3 bases; 26%) that were not prominent in BaP-induced and spontaneous mutations. Comparing the distribution of mutations over all possible mutation types, the statistical analysis revealed that the spectrum of mutations induced by crocidolite differs significantly from the spectrum of the spontaneous mutations ($P = 0.05$; Fisher's exact test). Comparison of reported data from a *lacI* database with our mutation spectra revealed obvious similarities regarding the spectra of spontaneous mutations. In different tissues, the most prominent mutations were G to A transition (40–60%) and G to T transversion (16–27%). The observed 57% of G to A transitions and 19% of G to T transversions for rat omentum are within this range (Table 2).

The analysis of mutational spectra with regard to the position of the mutations revealed no predominant features for crocidolite-induced mutations (data not shown). However, mutational hot spots observed in the spontaneous mutations (three mutations in position 28, codon 10) and in the BaP group (four mutations in position 65, codon 22) were not present in these positions in the crocidolite group.

8-OHdG. The possible involvement of 8-OHdG in fiber-induced mutagenesis was tested by measurement of this premutagenic DNA adduct in genomic DNA of omenta from Wistar rats 10 and 20 weeks after treatment with 1 or 2 mg of crocidolite asbestos (Fig. 1). The background level of 8-OHdG in control animals was 2.97 8-OhdG/10^5 dG (10 weeks) and 2.10 8-OhdG/10^5 dG (20 weeks), respectively. At both time points in both dosage groups, a significant increase of 8-OHdG in the genomic DNA was observed compared with the control group. The adduct levels in the 1- and 2-mg-treated groups were 8.21 and 8.47 8-OhdG/10^5 dG after 10 weeks and 4.73 and 4.78 8-OhdG/10^5 dG after 20 weeks, respectively.

DISCUSSION

The system of transgenic animals bearing the bacterial reporter gene *lacI* has been designed for *in vivo* mutagenicity testing of substances in their relevant target organs (18). With this system, point mutations and smaller insertions and deletions can be detected. In our experiments, we tried to find these kinds of mutations in rats treated with asbestos fibers in the omentum, which is the main target organ for mesothelioma carcinogenesis in the peritoneal cavity. In earlier experiments, after the chosen time points of 4, 12, and 24 weeks after treatment, no tumors in treated animals were observed (20).

The present study describes a significant increase in mutation frequencies in response to i.p. treatment with crocidolite fibers in *lacI* transgenic rats. As expected, the strong chemical mutagene BaP induced elevated mutation levels 4 weeks after treatment. This high level was increased after 12 and 24 weeks. Although the chosen dosages of 5 mg of BaP and 2 mg of crocidolite asbestos exhibited similar carcinogenic activity in earlier experiments (20), in the mutagenicity assays, we found differences in mutation frequencies between these groups. This discrepancy could be an indication of the differences in the underlying molecular mechanisms of mutagenesis of these two carcinogens. Whereas BaP is known to induce mainly bulky adducts resulting in point mutation, the primary mode of genotoxic action of crocidolite may be the induction of ROS or reactive nitrogen species that result in DNA double-strand breaks and large genomic mutations (23). However, most genome mutations such as large deletions and insertions, translocations, and aneuploidia are not effectively recovered by the *lacI* shuttle vector. Therefore, the overall mutagenesis induced by crocidolite asbestos may be underestimated. The differences in mutation frequencies may come from the high number of undetected large mutations induced by crocidolite-derived ROS. An additional indication for this hypothesis is the increase of small deletions (which can be monitored with the *lacI* assay) in the crocidolite groups observed in the molecular analysis of the mutant *lacI* genes (Table 2).

Compared to an inhalation study with crocidolite asbestos and *lacI* transgenic mice (24), a relatively high increase in mutations was observed in rat omentum. Although the fiber numbers in both studies were comparable (6×10^7 fibers/DNA preparation from mouse lung *versus* 1.6×10^8 fibers applied to the abdomen of rats), in mouse lung, an increase of only factor 1.9 has been observed 4 weeks after treatment. Twelve weeks after treatment, this effect could not be observed. These differences between the two studies may be due to differences between lung tumor and mesothelioma carcinogenesis or to species-specific reactions on fiber- and particle-induced pathogenesis. On the other hand, compared with the mouse inhalation study and with other data from rat tissues, the spontaneous mutation rate observed in omenta is relatively low (24, 25). Therefore, with this background level, it was possible to monitor low effects on mutation frequency. A trend in decreasing mutation frequencies over time was observed (Table 1). However, this effect proved not to be statistically significant, and therefore it cannot be ruled out that it is due to

experimental variability. In other studies with liver and bladder of *lacI* transgenic mice, an accumulation of mutations during aging was observed (26). Therefore, this possible phenomenon of decreasing mutations would be difficult to explain. On the other hand, when mutation frequencies of exponentially proliferating and quiescent *lacI* mouse cells were compared with regard to their response to low dosages of ethylnitrosurea, a 5-fold increase was observed in the growing cells (27). This effect partially refers to mutation manifestation from premutagenic lesions, which in quiescent cells occurs in the bacterial assay system and results in one mutant plaque for one DNA adduct. In proliferating cells after DNA replication, two mutant plaques will be derived from one adduct. Moreover, the authors speculate that unknown factors may protect quiescent cells from premutagenic DNA lesions. To our knowledge, no comparable data from other groups performing mutation analyses with omenta are available. Therefore, this phenomenon may be considered tissue specific and has to be investigated in additional studies. Considering this possible trend, the mutagenicity of crocidolite is demonstrated in the increase of the absolute mutation frequency in the 5-mg-treated group after 12 weeks and in the slight increase in the 2-mg-treated group from 12 to 24 weeks (Table 1).

The spectra of mutations induced by crocidolite asbestos and the mutations induced spontaneously exhibited significant differences when compared with each other. This indicates that mechanisms different from those leading to the induction of spontaneous mutations are induced by crocidolite asbestos. In the mouse inhalation study, however, no such differences have been observed (24). An increase of mutations of the same type as the spontaneous mutations may possibly be an effect of the enhanced proliferation rate in the target tissue after fiber application. This may be an indication for tumor promotion rather than for the mutagenic effect of asbestos (28). The difference in results between reported data and data of this study could indicate different pathways of tumorigenicity induced by crocidolite in lung and in mesothelium.

The most prominent mutation type induced by asbestos was G to T transversion. This type of mutation is often discussed in the literature as the main descendant of the premutagenic DNA adduct 8-OHdG. The most convincing data come from a study using *lacI* transgenic mice that are defective in the removal of oxidative base damage (29). In the animals lacking a DNA glycosylase specific for 8-OhdG, which is encoded by the *OGG1* gene, a dramatic increase in G to T transversions has been observed in the spontaneous mutations. 8-OHdG has been described to be induced in cell-free systems when pure DNA is incubated with crocidolite or man-made mineral fibers (30, 31). This effect has been increased by the addition of H_2O_2, which, *in vivo,* may be released by inflammatory cells. In the i.p. assay in the recent study, crocidolite asbestos treatment induced a significant increase in the 8-OHdG level (Fig. 1). Although the chosen dosages in earlier experiments induced tumor rates of 50% (2 mg) and 25% (1 mg), interestingly, no dose dependency in 8-OHdG induction was observed. 8-OHdG production in animals of the lower dosage group seems to be at the maximum, which cannot be exceeded in the higher dosage group. This result may be an indication of the dominance of a cellular reaction, such as inflammation, providing H_2O_2 and NO. A dose dependence observed in some cell culture experiments (12) that may refer to reactions mediated directly by the interaction of fibers with the target cells could not be reproduced in the *in vivo* system. The plateau effect, however, was also observed in the *in vitro* study using A549 cells (16). Mutagenic effects mediated by inflammatory cells cannot be the cause for this behavior of 8-OHdG induction in these experiments. One could speculate that inducible nitric oxide synthase (responsible for production of NO in these experiments), which was demonstrated to be induced by crocidolite, may be up-regulated to a maximum and that NO may be the limiting factor for 8-OHdG induction. An additional feature of 8-OHdG induction is the decrease of fiber-induced 8-OHdG over time. Compared with the control groups, the amount of 8-OHdG 20 weeks after treatment was lower than that after 10 weeks. A possible long-term induction of repair systems responsible for this effect should be visible in additional experiments examining the expression status of specific repair enzymes.

In conclusion, the results of this study show that crocidolite asbestos *in vivo* in the relevant target tissue for mesothelioma carcinogenesis is able to induce a significant increase in mutation frequency. The mutational spectrum gives a clear indication of a molecular mechanism that is different from the generation of spontaneous mutations. The dominance of G-T transversions and the increase of the premutagenic DNA adduct 8-OHdG after fiber treatment indicate the involvement of hydroxyl radicals in crocidolite-induced mutagenesis *in vivo*. Additional experiments investigating the cellular composition of the target tissue after crocidolite treatment should examine the hypothesis of saturation of the cellular reaction.

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REFERENCES

1. Mossman, B. T., Bignon, J., Corn, M., Seabon, A., and Gee, J. H. L. Asbestos: scientific development and implications for public policy. Science (Wash. DC), *247:* 294–301, 1990.
2. Adamson, I. Y. R., Bakowska, J., and Bowden, D. H. Mesothelial cell proliferation after instillation into mouse lung. Am. J. Pathol., *142:* 1209–1216, 1993.
3. Walker, C., Everitt, J., and Barrett, J. C. Possible cellular and molecular mechanisms for asbestos carcinogenicity. Am. J. Ind. Med., *21:* 253–273, 1992.
4. Wylie, A. G., Skinner, H. C. W., Marsh, J., Snyder, H., Garzione, C., Hodkinson, D., Winters, R., and Mossman, B. T. Mineralogic features associated with cytotoxic and proliferative effects of fibrous talc and asbestos on rodent tracheal epithelial and pleural mesothelial cells. Toxicol. Appl. Pharmacol., *147:* 143–150, 1997.
5. Jaurand, M-C. Mechanisms of fiber-induced genotoxicity. Environ. Health Perspect., *105S:* 1073–1084, 1997.
6. Hei, T. K., Piao, C. Q., He, Z. Y., Vannais, D., and Walsren, C. A. Chrysotile fiber is a strong mutagen in mammalian cells. Cancer Res., *52:* 6305–6309, 1992.
7. Okayasu, R., Wu, L., and Hei, T. K. Biological effects of naturally occurring and man-made fibres: *in vitro* cytotoxicity and mutagenesis in mammalian cells. Br. J. Cancer, *79:* 1319–1324, 1999.
8. Dong, H. Y., Buard, A., Renier, A., Levy, F., Saint-Etienne, L., and Jaurand, M-C. Role of oxygen derivatives in the cytotoxicity and DNA damage produced by asbestos on rat mesothelial cells *in vitro*. Carcinogenesis (Lond.), *15:* 1251–1255, 1994.
9. Hei, T. K., He, Z. Y., and Suzuki, K. Effects of antioxidants on fiber mutagenesis. Carcinogenesis (Lond.), *16:* 1573–1578, 1995.
10. Weitzman, S. A., and Graceffa, P. Asbestos catalyzes hydroxyl and superoxide radical generation from hydrogen peroxide. Arch. Biochem. Biophys., *228:* 373–376, 1984.
11. Maples, K. R., and Johnson, N. F. Fiber-induced hydroxyl radical formation: correlation with mesothelioma induction in rats and humans. Carcinogenesis (Lond.), *13:* 2035–2039, 1992.
12. Fung, H., Kow, Y. W., van Houten, B., and Mossman, B. T. Patterns of 8-hydroxydeoxyguanosine formation in DNA and indications of oxidative stress in rat and human pleural mesothelial cells after exposure to crocidolite asbestos. Carcinogenesis (Lond.), *18:* 825–832, 1997.
13. Goodglick, L. A., and Kane, A. B. Cytotoxicity of long and short crocidolite fibers *in vitro* and *in vivo*. Cancer Res., *50:* 5153–5163, 1990.
14. Jackson, J. H., Vollenweider, M., Hill, J., Rodriguez, H., Schwabacher, A. W., Mitra, G., and Kuo, C-Y. Stimulated human leukocytes cause activating mutations in the K-ras proto-oncogene. Oncogene, *14:* 2803–2808, 1997.
15. Thomas, G., Ando, T., Verma, K., and Kagan, E. Asbestos fibers and interferon-γ up-regulate nitric oxide production in rat alveolar macrophages. Am. J. Respir. Cell Mol. Biol., *11:* 107–715, 1996.
16. Chao, C-C., Park, S-H., and Aust, A. E. Participation of nitric oxide and iron in the oxidation of DNA in asbestos-treated human lung epithelial cells. Arch. Biochem. Biophys., *326:* 152–157, 1996.
17. Park, S-H., and Aust, A. E. Participation of iron and nitric oxide in the mutagicity of asbestos in $hgprt^-$, gpt^+ Chinese hamster V79 cells. Cancer Res., *58:* 1144–1148, 1998.
18. Provost, G. S., Kretz, P. L., Hamner, R. T., Matthews, C. D., Rogers, B. J., Lundberg, K. S., Dycaico, M. J., and Short, J. M. Transgenic systems for *in vivo* mutation analysis. Mutat. Res., *288:* 133–149, 1993.
19. Sandhu, H., Dehnen, W., Roller, M., Abel, J., and Unfried, K. mRNA expression patterns in different stages of asbestos-induced carcinogenesis in rats. Carcinogenesis (Lond.), *21:* 1023–1029, 2000.

20. Unfried, K., Roller, M., Pott, F., Friemann, J., and Dehnen, W. Fiber-specific molecular features of tumors induced in rat peritoneum. Environ. Health Perspect., *105S:* 1103–1108, 1997.
21. Timbrell, V. Characteristics of the International Union Against Cancer standard reference samples of asbestos. *In:* H. A. Shapiro (ed.), Pneumoconiosis, Proceedings of the International Conference Johannesburg 1969, pp. 28–36. Cape Town, South Africa: Oxford University Press, 1970.
22. Stratagene. Big Blue® Transgenic Rodent Mutagenesis Assay System. La Jolla, CA: Stratagene, 1997.
23. Jaurand, M. C., Kheuang, L., Magne, L., and Bignon, J. Chromosomal changes induced by chrysotile fibres or benzo-3,4-pyrene in rat pleural mesothelial cells. Mutat. Res., *169:* 141–148, 1986.
24. Rhin, B., Coulais, C., Kauffer, E., Bottin, M-C., Martin, P., Yvon, F., Vigneron, J. C., Binet, S., Monhoven, N., Steiblen, G., and Keith, G. Inhaled crocidolite mutagenicity in lung DNA. Environ. Health Perspect., *108:* 341–346, 2000.
25. de Boer, J. G., Erfle, H., Holcroft, J., Walsh, D., Dycaico, M., Provost, S., Short, J., and Glickman, B. W. Spontaneous mutants recovered from liver and germ cell tissue of low copy number lacI transgenic rats. Mutat. Res., *352:* 73–78, 1996.
26. Stuart, G. R., Oda, Y., de Boer, J. G., and Glickmann, B. W. Mutation frequency and specificity with age in liver, bladder and brain of *lacI* transgenic mice. Genetics, *154:* 1291–1300, 2000.
27. Bielas, J. H., and Heddle, J. A. Proliferation is necessary for both repair and mutation in transgenic mouse cells. Proc. Natl. Acad. Sci. USA, *97:* 11391–11396, 2000.
28. Ames, B. N., and Gold, L. S. Too many rodent carcinogens: mitogenesis increases mutagenesis. Science (Wash. DC), *249:* 970–971, 1990.
29. Klungland, A., Rosewell, I., Hollenbach, S., Larsen, E., Daly, G., Epe, B., Seeberg, E., Lindahl, T., and Barnes, D. Accumulation of premutagenic DNA lesions in mice defective in removal of oxidative base damage. Proc. Natl. Acad. Sci. USA, *96:* 13300–13305, 1999.
30. Leanderson, P., Söderkvist, P., Tagesson, C., and Axelson, O. Formation of 8-hydroxydeoxyguanosine by asbestos and man made mineral fibers. Br. J. Ind. Med., *45:* 309–311, 1988.
31. Adachi, S., Yoshida, S., Kawamura, K., Takahashi, M. Uchida, H., Odagiri, Y., and Takemoto, K. Induction of oxidative DNA damage and mesothelioma by crocidolite, with reference to the presence of iron inside and outside of asbestos fiber. Carcinogenesis (Lond.), *15:* 753–758, 1994.