

# The effects of styrene on lung cells in female mice and rats

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## Abstract

Styrene has been shown to cause an increase in the incidence of lung tumors in CD-1 mice following chronic exposure at 40 and 160 ppm, whereas no treatment-related increase in tumors in any organ was seen in rats chronically exposed to up to 1000 ppm styrene. So far most of the mechanistic studies have been performed with male animals. The aim of the present study was to further elucidate the target cell population in mouse lungs exposed to styrene, and to investigate possible differential *in vivo* effects (e.g., glutathione depletion, increased lipid peroxidation, and oxidative DNA damage). Groups of female CD-1 mice were exposed to styrene at concentrations of 0, 172 or 688 mg/m<sup>3</sup> (0, 40 or 160 ppm) for 6 h per day on 1 day, 5 consecutive days or for 20 days during a 4 week period. Groups of female Crl:CD rats were exposed to styrene at concentrations of 0, 688 or 2150 mg/m<sup>3</sup> (0, 160 or 500 ppm) for a single 6 h period or for 6 h per day on 5 consecutive days. No signs of lung toxicity were observed in rats. The cytology of cells in lung lavage fluid provided no signs of an inflammatory response in either rats or mice. In mice, both exposure levels caused decreased CC16 protein concentrations in lung lavage fluid after 1 and 5 exposures and in mouse blood serum throughout the study, suggesting that styrene may cause destruction of Clara cells in mice. Degenerative lesions in mouse Clara cells (vacuolar cell degeneration, cell necrosis) were revealed by electronmicroscopy. After 5 and 20 exposures of mice at 160 ppm, cellular crowding, expressed as an irregular epithelial lining and indicative of a very early hyperplasia was noted. Although a depletion of glutathione was noted in mouse lung homogenates after 20 exposures, there was no evidence of oxidative stress as indicated by unchanged concentrations of 8-OH-deoxyguanosine. Malondialdehyde, an indicator of lipid peroxidation, was slightly increased in mice after 1 exposure at 160 ppm only.

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## 1. Introduction

Styrene (ethenyl-benzene, CAS No. 100-42-5) is a commercially important monomer, which is used extensively in the manufacture of polystyrene resins (plastic packaging, disposable cups and containers, insulation) and is an integral part of reinforced plastics. Human exposure occurs at levels of milligrams per day during its production and industrial use and at much higher levels in the glass fibre-reinforced plastics indus-

try. Exposure to the general population occurs at levels of micrograms per day due mainly to inhalation of ambient air and cigarette smoke and intake of food that has been in contact with styrene-containing polymers (IARC, 1994).

Styrene has been tested for its carcinogenic potential in long-term studies in rats and mice. In a 2 year whole body inhalation chronic toxicity/oncogenicity study groups of 70 CD-1 mice per sex and dose level were exposed to 0, 20, 40, 80 or 160 ppm styrene for 6 h per day, 5 days per week. Exposure of these mice to concentrations of 40 ppm and higher resulted in an increased incidence of lung tumors (Cruzan et al., 2001). In female mice exposed to 20 ppm a statistically significant increase of adenomas was observed, however, a clear dose response relationship in the low and mid dose levels was not obtained. The lung tumors only appeared

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during the last part of the study as none of these tumors were observed in the intermediate sacrifice time points (12 and 18 months exposure, 10 animals per sex, dose and time point being investigated). A total of eight chronic toxicity/oncogenicity studies have been conducted in rats (reviewed by Cruzan et al., 1998) without evidence for a carcinogenic effect in the lungs of this species. Moreover, it was also demonstrated that rats could tolerate higher levels of styrene (up to 1000 ppm), whereas in mice concentrations of 200 ppm or higher resulted in death of some mice (Cruzan et al., 1997, 1998).

In the above-mentioned inhalation studies toxicity to the lungs of mice but not of rats has been reported. CD-1 mice examined immediately at the end of a 6 h inhalation exposure to 40 or 160 ppm styrene had multifocal necrosis and cell loss in bronchiolar epithelium (Green et al., 2001). Upon repeated exposures, there was decreased cytoplasmic staining of Clara cells and cell crowding in terminal bronchioles (Cruzan et al., 2001). With long-term exposure, lung histopathological changes (decreased eosinophilia, broncheoepithelial hyperplasia) have been reported for mice exposed to levels of 20 ppm styrene (Cruzan et al., 2001). Effects in alveolar cells were not seen in these studies (Cruzan et al., 1997, 2001; Green et al., 2001). The cells comprising the area of hyperplasia stained immunohistochemically with high intensity for CC10, a protein found in Clara cells. Immunostaining for surfactant A, a typical protein for alveolar Type II cells, showed that this protein was only rarely and faintly present (Cruzan et al., 2001). The particular susceptibility of mouse lung to the toxicity and carcinogenic effects of styrene has been attributed to differences in metabolism between rat and mouse. Although styrene is metabolized by pulmonary microsomes of both mouse and rat to styrene-7,8-oxide (SO), styrene metabolizing activity was reported to be about five-fold higher in the mouse than in the rat and much greater in Clara cells than in type II pneumocytes (Hynes et al., 1999). Clara cells are much more numerous in the bronchiolo-alveolar region of the mouse lung than in rat and human lung, (Hynes et al., 1999). The more toxic form, the R-enantiomer of SO (R-SO), was preferentially formed in mice, whereas the S-enantiomer of SO (S-SO) was preferentially formed in rats (Hynes et al., 1999). Tissues that are high in the CYP2F2 P450 isoform have been reported to produce a high ratio (at least 2.4:1) of R-SO compared to S-SO (Cruzan et al., 2002). CYP2F2 is predominantly found in mouse Clara cells, whereas this P450 isozyme is less prevalent in rat lung. The importance of CYP2F2 was demonstrated by Green et al. (2001); co-administration of 5-phenyl-1-pentyne an inhibitor of CYP2F2 (and to a lesser extent of CYP 2E1) in mice treated with 160 ppm styrene for 6 h/day for 4 days showed no evidence of lung cytotoxicity. This species difference in the production of

styrene oxide enantiomers means that mouse Clara cells produce approximately 15 times more R-SO than rat Clara cells.

As styrene is first metabolized to styrene oxide (SO), and, because SO has been reported to induce adducts to proteins and DNA (IARC, 1994a, 1994b) it is possible that DNA adduct formation could contribute to the carcinogenic process. SO reacts with DNA mainly at the N-7 position in guanine, but also at other sites and with other bases. Substitution occurs at both the alpha- and beta-positions of the styrene molecule. DNA adduct profiles have been assessed in total lung and lung cell fractions enriched in Type II cells and Clara cells from rats and mice at 0 and 42 h after exposure exposed to 160 ppm [ring-U-<sup>14</sup>C] styrene by nose-only inhalation for 6 h (Boogaard et al., 2000). Styrene was shown to have only very weak adduct-forming potency. In both rat and mouse lung, the two isomeric N7-guanine adducts of SO were detected at 1 per 10<sup>8</sup> bases at 0 h, and these levels halved at 42 h. For mice, DNA adduct levels in Clara cells and non-Clara cells were similar to total lung.

SO may also alkylate several nucleophilic sites in proteins, particularly cysteine sulfhydryl, histidine imidazole, lysine amino, aspartic, and glutamic carboxylic groups, and the N-terminal position (Phillips and Farmer, 1994). Following the observation that white blood cells of styrene-exposed workers showed significantly increased levels of 8-hydroxy-deoxyguanosine (8-OHdG), it has recently been proposed that styrene exposure could create an imbalance between oxidants and anti-oxidants (Marczynski et al., 2000 and Gamer et al., 2002), i.e., oxidative stress, that would lead to depletion of glutathione, increased lipid peroxidation, and oxidative DNA damage.

The aim of the present study was to obtain further evidence, by analysis of different parameters, to confirm that the Clara cells are the target cell population in mouse lungs exposed to styrene, and to investigate possible *in vivo* effects in rat and mouse lung that may be related to the carcinogenicity of styrene in mouse lung.

Therefore, the following concentrations were selected: 40 ppm because Clara cell cytotoxicity has been reported after 6 h at that level and 160 ppm as a level where a large number of tumors occurred. These dose levels also correspond to the ones used by (Green et al., 2001) in their studies on the role of Cytochrome P450 in the tumorigenic response of mice to styrene. Females were selected because they were as susceptible in their tumors to styrene as males, and, because nearly all subsequent mechanistic investigations on the tumorigenic mode of action of styrene have been performed using male mice only (Gadberry et al., 1996; Carlson, 1997; Hynes et al., 1999; Green et al., 2001). The potential of styrene to induce glutathione depletion,

increased lipid peroxidation, and oxidative DNA damage in rats and mice was investigated. The effects of styrene on Clara cells were monitored by measuring the Clara cell protein CC16 (also referred to as CCSP or CC10) concentrations in blood serum and lung lavage fluid.

## 2. Materials and methods

### 2.1. Test substance

Styrene, CAS No.: 100-42-5, produced by BASF, purity 99.9%, a colourless, clear, homogeneous liquid, stored at room temperature.

### 2.2. Animals and maintenance conditions

Female CRL:CD-1 (ICR) BR mice and Crl:CD rats were supplied by Charles River Deutschland GmbH, Sulzfeld, Germany. The age of the animals was approximately 7 weeks at delivery. The animals were singly housed in wire cages (mice: type DK I, floor area 200 cm<sup>2</sup>; rats: type DK III, floor area 800 cm<sup>2</sup>), supplied by Becker and Co., Castrop-Rauxel, Germany. Waste trays containing paper were fixed underneath the cages. The animals were maintained in an air-conditioned room at a temperature of 20–24 °C, a relative humidity of 30–70%, and a 12 h light/12 h dark cycle. The animals were adapted to these environmental conditions for 13–14 days before the start of exposure. Before the animals' arrival, the room was completely disinfected using a disinfectant ("AUTEX", fully automatic, formalin-ammonia-based terminal disinfectant, supplied by Dr. Gruß KG, Neuss, Germany). During the study, the floor and walls were cleaned weekly with a solution of 1% Mikroquat® in water. The animals were maintained on rat/mouse/hamster laboratory diet, 10 mm pellets (Provimi Kliba SA, Kaiseraugst, Switzerland) and tap water ad libitum. Food was assayed for chemical as well as for microbiological contaminants. Drinking water was regularly assayed for chemical contaminants and the presence of microorganisms.

### 2.3. Experimental design

The study was conducted in accordance with the Good Laboratory Practice provisions of the "Chemikaliengesetz" (Chemicals Act; Bundesgesetzblatt, 1994), with the OECD Principles of Good Laboratory Practice (Paris, 1981) and with the EPA Good Laboratory Practice Standards (40 CFR part 792). At the start of the exposure period, body weights of mice ranged from 28.4 to 30.2 g; body weights of rats ranged from 201.4 to 242.4 g. The mouse study consisted of nine groups

of 35 female animals, each. Three groups of mice were exposed to styrene concentrations of 0, 172 or 688 mg/m<sup>3</sup> (0, 40 or 160 ppm) for 6 h per day on working days during a 4 week period (20 daily exposures); these animals were sacrificed on the day following the last exposure (with the exception of animals assigned to glutathione measurements, which were additionally exposed on the day of sacrifice). Three groups of mice were exposed to styrene concentrations of 0, 172 or 688 mg/m<sup>3</sup> (0, 40 or 160 ppm) for 6 h on one day; these animals were sacrificed directly after the end of the exposure. Three groups of mice were exposed to styrene concentrations of 0, 172 or 688 mg/m<sup>3</sup> (0, 40 or 160 ppm) for 6 h per day on 5 consecutive days; these animals were sacrificed directly after the end of the last exposure. Fifteen mice per group were assigned to biochemical analyses in lung homogenate. Ten mice per group were assigned to blood sampling and enzyme analyses in lung homogenate. Five mice per group were assigned to lung lavage. Five mice per group were assigned to pathological examinations (Table 1).

The rat study consisted of six groups of each 10 female animals. Three groups of rats were exposed to styrene concentrations of 0, 688 or 2150 mg/m<sup>3</sup> (0, 160 or 500 ppm) for 6 h on one day; these animals were sacrificed directly after the end of the exposure. Three groups of rats were exposed to styrene concentrations of 0, 688 or 2150 mg/m<sup>3</sup> (0, 160 or 500 ppm) for 6 h per day on five consecutive days; these animals were sacrificed directly after the end of the last exposure. Five rats per group were assigned to lung lavage. Five rats per group were assigned to blood sampling and pathological examinations.

### 2.4. Styrene exposure

The animals were maintained singly in wire cages and exposed to styrene in a glass-steel inhalation chamber, with a volume of ≈1.4 m<sup>3</sup> (BASF Aktiengesellschaft). The animals were acclimatized to the inhalation chamber for 2 days, during which time they received a normal air supply (pre-flow period). For each of the required styrene concentrations in the inhalation atmospheres, styrene was supplied at a constant rate to a two-component atomizer (Beckmann) of a thermostat vaporizer (BASF, about 30 °C) by means of piston metering pumps (DESAGA). The vapor/air mixture was generated by spraying the substance with compressed air into a counter current of conditioned supply air (about 50% ± 20% relative humidity, 22 °C ± 2 °C). Thereafter it was passed through an aerosol trap, further mixed with conditioned supply air, passed through the inhalation system and exhausted. The flows of supply and exhaust air as well as the pressure, temperature and relative humidity inside the inhalation chambers were measured

Table 1  
Experimental design

Group	Styrene concentration		Number of animals				
	mg/m <sup>3</sup>	ppm	Total	Biochemical analyses <sup>a</sup>	Blood sampling and enzyme analyses <sup>a</sup>	Lung lavage	Pathology
<i>Mice</i>							
Main groups (20 exposures)							
0	0	0	35	15 <sup>b</sup>	10	5	5
1	172	40	35	15 <sup>b</sup>	10	5	5
2	688	160	35	15 <sup>b</sup>	10	5	5
First satellite groups (1 exposure)							
0.1	0	0	35	15 <sup>b</sup>	10	5	5
1.1	172	40	35	15 <sup>b</sup>	10	5	5
2.1	688	160	35	15 <sup>b</sup>	10	5	5
Second satellite groups (5 consecutive exposures)							
0.2	0	0	35	15 <sup>b</sup>	10	5	5
1.2	172	40	35	15 <sup>b</sup>	10	5	5
2.2	688	160	35	15 <sup>b</sup>	10	5	5
<i>Rats</i>							
Main groups (5 consecutive exposures)							
0	0	0	10	–	5 <sup>c</sup>	5	5
1	688	160	10	–	5 <sup>c</sup>	5	5
2	2150	500	10	–	5 <sup>c</sup>	5	5
First satellite groups (1 exposure)							
0.1	0	0	10	–	5 <sup>c</sup>	5	5
1.1	688	160	10	–	5 <sup>c</sup>	5	5
2.1	2150	500	10	–	5 <sup>c</sup>	5	5

<sup>a</sup> In lung homogenate (enzyme analyses performed in mice only).

<sup>b</sup> The first five animals were used for determination of GSH after being additionally exposed on the day of examination.

<sup>c</sup> Animals also used for pathology.

and stored in intervals of 10 s using a PC based automated monitoring system. The air flows were regulated with this system based on the set chamber pressure. The supply air flow was 28 m<sup>3</sup>/h, corresponding to an air change of 20-fold per hour. This air change rate guaranteed the attainment of the steady state concentrations within some minutes after the start of exposure.

### 2.5. Analytical determination of styrene concentrations

The styrene concentrations in the inhalation atmospheres were determined daily, by gas chromatography, on two measured absorption samples in 2-propanol from the breathing zone of the animals per styrene treatment group. The analytical determinations were performed under the following conditions:

Column	metal
Length	3 m
Internal diameter	2 mm
Separation phase	20% Silicon UCC W982
Support	Chromosorb W AW DMCS HP 80/100 mesh

Gas chromatograph: Hewlett-Packard 5840 A  
Operating conditions:

Carrier gas	He
Carrier gas flow rate	25.8 ml/min
Hydrogen	30 ml/min
Air	240 ml/min
Furnace temp. end	120 °C
Detector	FID
Detector temperature	200 °C
Injector temperature	200 °C

During the set up of the analytical procedure a calibration curve was prepared in the solvent with the test substance to be investigated to show linearity in the suitable concentration range of the samples. The gas chromatograph was calibrated by means of calibration solutions of styrene in 2-propanol. Accurately weighed amounts of styrene in concentration ranges of 2.08–5.21 mg/50 ml in the mouse study and 6.18–16.48 mg/50 ml in the rat study were used for this purpose. Calibration curves were calculated (curve adjusted using the least squares method) showing a highly linear correlation.

For routine analysis during test period, an one-point calibration of the analytical procedure was prepared at

least for each analytical campaign. For the analysis of the samples, an external standard procedure was used. The 50 ml graduated flask was filled up to the calibration mark with 2-propanol. The injection volume was 1  $\mu$ l. Mass values of Styrene were obtained from the area integrals using the calibration point. The concentrations of the test groups were calculated from these mass values in relation to the injected volume and the sample volume of the inhalation atmosphere.

One control group sample was analyzed on one day per week during the exposure period. The constancy of styrene concentrations in the inhalation atmospheres throughout each exposure was monitored continuously by a total hydrocarbon analyzer (Testa 123), recorded using line recorders, and transferred to the automated measuring system.

## 2.6. Clinical observations

The general state of health of the animals was checked at least daily. On styrene exposure days, these clinical examinations were performed before, during and after exposure. During exposure only a groupwise examination was possible. Body weights were determined at the start of the preflow period, at the start of the styrene exposure period, and then once weekly.

## 2.7. Biochemical parameters in lung homogenates

Determination of 8-hydroxy-deoxyguanosine (8-OHdG) in lung DNA, an indicator of oxidative DNA damage, was performed as described by Dahlhaus and Appel (1993); Dahlhaus et al. (1994) and Fiala et al. (1989). Lipid peroxidation was measured with the thiobarbituric acid assay (TBA) according to the method described by Preece et al. (1988). This method determines the amount of thiobarbituric acid-reactive material derived from lipid peroxidation in tissues, e.g., malondialdehyde. GSH was measured photometrically after reaction with Ellman's reagent (Sedlak and Lindsay, 1968). Superoxide dismutase was determined colorimetrically as described by Nebot et al. (1993). Glutathione peroxidase was determined by a kinetic UV test at 340 nm as described by Paglia and Valentine (1967). Glutathione reductase was determined by a kinetic UV test at 340 nm as described by Goldberg and Spooner (1983). Catalase was determined by a kinetic UV test at 240 nm as described by Aebi (1983). The lungs were removed within 2 h after sacrifice. The lungs used for enzyme analyses were frozen and stored at  $-80^{\circ}\text{C}$  for a maximum of 8 days. Homogenates were prepared immediately after thawing and analyzed within 2 h after preparation. The lungs used for determination of the biochemical parameters were homogenized and analyzed immediately after removal.

## 2.8. Examination of lavage fluid

The animals used for lung lavage were killed by exsanguination from aorta abdominalis and vena cava under Narcoren<sup>®</sup> anesthesia and the lungs were lavaged by four instillations of physiologic saline (1 ml per instillation in mice and 5 ml per instillation in rats) immediately after sacrifice. The lavage fluid sampled during the first installation was used for the determination of humoral parameters immediately after sampling. An automatic analyzer (Cobas Fara II; Hoffmann-LaRoche, Grenzach, Germany) was used to measure protein (Fujita et al., 1983), lactate dehydrogenase (DGKC-Method, 1972), alkaline phosphatase (DGKC-Method, 1972) and  $\gamma$ -glutamyltransferase (Szasz, 1974) activities. *N*-acetyl- $\beta$ -D-glucosaminidase (Yakata et al., 1983) and catalase (Aebi, 1983) were analyzed with a spectrophotometer (DM 4; Zeiss, Oberkochen, Germany). Clara cell specific protein (CC16) and lysozyme quantification was performed at the University of Louvain, Brussels, in samples shipped frozen on dry ice, using automated latex immunoassays based on anti-rat CC16 and anti-rat lysozyme antibodies, which cross-react very well with mouse CC16 or lysozyme, respectively. They were calibrated with standards of purified proteins (Bernard and Lauwerys, 1983; Halatek et al., 1998), determined in lavage samples directly without pretreatment except for a dilution to reach the working range of the standard curves. Glutathione was measured using the Bioxytech<sup>®</sup> GSH-400 assay of Oxis International Inc., Portland, OR, USA. The lavage fluid sampled during the second to fourth installation was pooled and used for the determination of cytology parameters. Total cell counts were determined in a Neubauer cell chamber. Cyto centrifuge preparations were prepared from the fresh samples, stained according to Wright and evaluated microscopically (Warheit and Hartsky, 1993) for macrophages, polymorphonuclear neutrophils, lymphocytes, and eosinophils.

## 2.9. Examination of blood serum

Blood was sampled within 2 h after the scheduled sacrifices by decapitation under Narcoren<sup>®</sup> anesthesia from the mice used for determination of enzyme activities in lung homogenates, and by puncture of the retro-orbital plexus under Narcoren<sup>®</sup> anesthesia from the rats designated for pathology. Serum was prepared by centrifugation. CC16 quantification was performed at the University of Louvain, Brussels using automated latex immunoassays as described above. Serum samples, however, were pretreated to avoid possible interference from complement and chylomicrons. They were heated at  $56^{\circ}\text{C}$  for 30 min and treated with polyethylenglycol 600 and trichloroacetic acid. After overnight treatment

at 4 °C the samples were centrifuged and the protein were determined in the supernatant.

### 2.10. Pathology

At necropsy, the lungs, together with larynx and trachea, were removed. Lung weights were determined. The lungs were instilled with 5% cacodylate-buffered glutaraldehyde solution and fixed by further immersion for at least 48 h. Ten lung tissue samples of each animal of the control and high concentration group were embedded in epoxy resin. Of each animal, five-epoxy resin blocks were trimmed and semi-thin sections were made which were stained with Azure II—methylene blue—basic Fuchsin. Ultra thin sectioning and electron microscopy were performed on selected samples from mice of control and high dose group. Whenever possible, the light and electron microscopic findings in the lungs were separated according to their occurrence in different compartments, i.e. large bronchus, medium and terminal bronchiole.

### 2.11. Statistics

Body weights were analyzed with a parametric one-way analysis using the *F*-test (ANOVA) (two-sided). If the resulting *p*-value was equal or less than 0.05, a comparison of each group with the control group using the Dunnett's test (two-sided) was performed for the hypothesis of equal means (Winer, 1971; Dunnett, 1955, 1964). Biochemical parameters in lung homogenates,

clinical pathology parameters in lavage fluid and lung weights were analyzed by non-parametric one-way analysis using Kruskal–Wallis test (two-sided) and, if *p* < 0.05, pair-wise comparison of each dose group with the control group using the Wilcoxon test for the hypothesis of equal medians (Nijenhuis and Wilf, 1978; Hettmannsperger, 1984; Siegel, 1956).

## 3. Results

### 3.1. Exposure conditions

The measured styrene concentrations are shown in Table 2, the daily mean styrene concentrations and standard deviations are shown in Fig. 1A. The real time monitoring with total hydrocarbon analyzers proved the constant achievement of the daily concentrations, as shown by the small standard deviation. The air flows and chamber pressures were also constantly within the desired limits. The study means of temperature and relative humidity were in the range of 21.6–21.9 °C and 55.4–60.4%, respectively, for mice and 20.8–21.8 °C and 41–56%, respectively, for rats.

### 3.2. Mortality and clinical findings

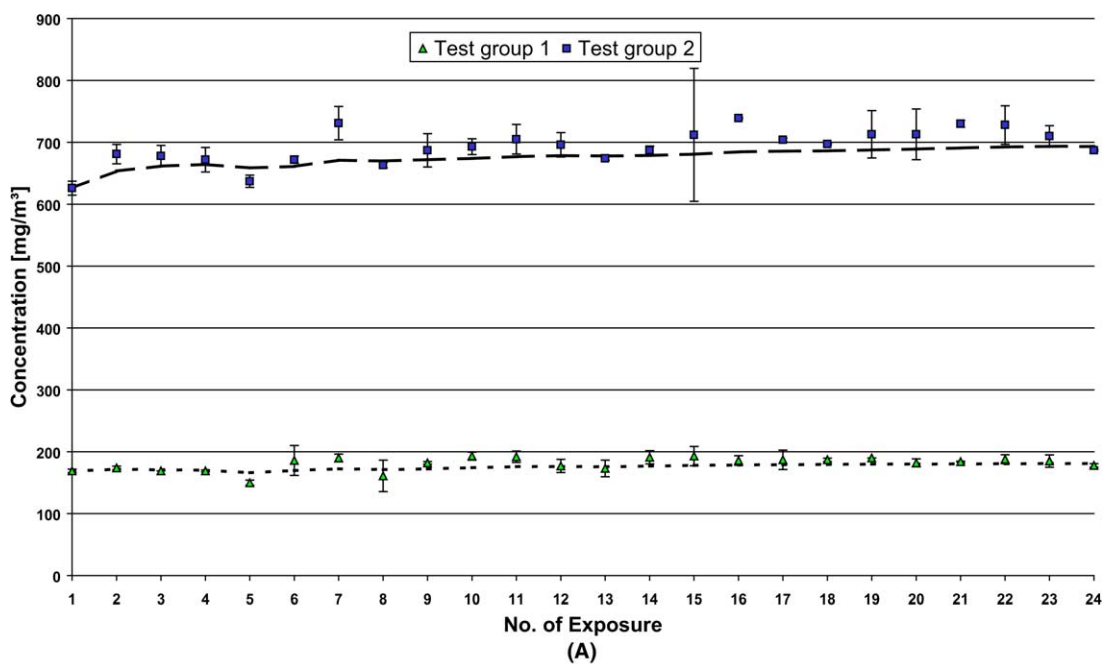
No deaths were recorded throughout the study. Squatting posture of mice at 160 ppm was observed on the fifth day of exposure. Rats at 500 ppm showed slight salivation during the first exposure and eyelid closure

Table 2  
Styrene exposure levels

Group	Target styrene concentration		Measured styrene concentration <sup>a</sup>		Total hydrocarbon measurements units <sup>b</sup>
	mg/m <sup>3</sup>	ppm	mg/m <sup>3</sup>	ppm	
<i>Mice</i>					
Main groups (20 exposures)					
1	172	40	184 ± 7.8	42.8 ± 1.81	55.0 ± 5.5
2	688	160	702 ± 20.8	163.3 ± 4.84	62.5 ± 3.7
First satellite groups (1 exposure)					
1.1	172	40	169 ± 2.8	39.3 ± 0.65	53.7
2.1	688	160	626 ± 11.3	145.6 ± 2.63	57.7
Second satellite groups (5 exposures)					
1.2	172	40	166 ± 9.3	38.6 ± 2.16	50.7 ± 6.2
2.2	688	160	659 ± 25.4	153.3 ± 5.91	59.1 ± 3.9
<i>Rats</i>					
Main groups (5 exposures)					
1	688	160	699 ± 48.9	162 ± 11.2	92.5 ± 1.7
2	2150	500	2162 ± 222	502 ± 50.9	59.2 ± 1.3
First satellite groups (1 exposure)					
1.1	688	160	693 ± 24.0	161 ± 5.6	89.1 ± 1.4
2.1	2150	500	1887 ± 83.4	439 ± 19.4	53.8 ± 1.1

<sup>a</sup> Mean ± standard deviation.

<sup>b</sup> Mean ± mean daily standard deviation (not calculated for first satellite groups in mice).



(B)

Fig. 1. (A) Time course of daily mean concentrations and standard deviations during the study in mice (the dotted lines represent the moving study mean over time) and (B) Control (1 exposure), large bronchus, mouse: no abnormalities detected. AmbF stained semithin section.

and low arousal up to the third exposure. There were no statistically significant deviations in body weights of mice. Rats at 160 ppm showed significantly lower body weights on day 4 (data not shown). Rats at 500 ppm showed significantly lower body weights on days 2 and 4 (data not shown).

### 3.3. Assays in mouse lung homogenates

Glutathione levels were significantly decreased at 160 ppm after 21 exposures. Malondialdehyde levels were significantly increased at 160 ppm after 1 exposure, but decreased at 40 and 160 ppm after 20 exposures. The levels of 8-OH-dG in lung DNA were generally lower in treatment groups with the exception of the animals

receiving 20 exposures with 160 ppm, in which they were increased without gaining statistical significance. Glutathione peroxidase was decreased at 160 ppm after five exposures. Catalase was decreased at 160 ppm after 20 exposures. Overall, no consistent concentration–time–response pattern was observed in the parameters examined in lung homogenates (Table 3).

### 3.4. Assays in rat and mouse lavage fluid and blood serum

No exposure related changes were seen in cytological examinations. Increased alkaline phosphatase activities were noted in mice at 40 ppm after 1 or 5 exposures and at 160 ppm after 1, 5 or 20 exposures. Increased  $\gamma$ -glutamyltransferase activities, though not always

Table 3  
Mouse lung homogenate assays

Group	Styrene concentration		Lung homogenate assays (means $\pm$ standard deviation and (percentage of control values))							
	mg/m <sup>3</sup>	ppm	8-OH-deoxyguanosine [ $10^5$ deoxyguanosine]	Malon-dialdehyde [nMol/g tissue]	Superoxide dismutase [U/g protein]	Catalase [mU/g protein]	Glutathione reductase [U/g protein]	Glutathione peroxidase [U/g protein]	Glutathione [ $\mu$ Mol/g tissue]	
<i>1 Exposure</i>										
0.1	0	0	4.62 $\pm$ 1.63	73.9 $\pm$ 16.7	4557 $\pm$ 390	86.5 $\pm$ 9.8	118 $\pm$ 7	521 $\pm$ 43	ND	
1.1	172	40	2.07 $\pm$ 0.82 (45)*	99.9 $\pm$ 35.5 (135)	4063 $\pm$ 501 (89)	86.5 $\pm$ 9.5 (100)	110 $\pm$ 13 (94)	507 $\pm$ 19 (97)	ND	
2.1	688	160	3.20 $\pm$ 2.60 (69)	112.4 $\pm$ 22.5 (152)*	4210 $\pm$ 665 (92)	93.8 $\pm$ 13 (109)	108 $\pm$ 16 (92)	509 $\pm$ 34 (98)	ND	
<i>5 Exposures</i>										
0.2	0	0	3.38 $\pm$ 1.61	108.5 $\pm$ 10.1	4365 $\pm$ 471	76.4 $\pm$ 8.1	118 $\pm$ 9	542 $\pm$ 37	ND	
1.2	172	40	1.63 $\pm$ 0.25 (48)	114.8 $\pm$ 9.5 (106)	4010 $\pm$ 526 (92)	74.7 $\pm$ 12.5 (98)	122 $\pm$ 8 (103)	542 $\pm$ 51 (100)	ND	
2.2	688	160	1.06 $\pm$ 0.33 (31)*	117.7 $\pm$ 18.2 (108)	4065 $\pm$ 590 (93)	66.4 $\pm$ 12.1 (87)	126 $\pm$ 13 (107)	503 $\pm$ 28 (93)*	ND	
<i>20 Exposures</i>										
0	0	0	1.3 $\pm$ 0.38	250.1 $\pm$ 28.9	5707 $\pm$ 716	113.4 $\pm$ 21.9	143 $\pm$ 18	738 $\pm$ 56	1.58 $\pm$ 0.16	
1	172	40	1.24 $\pm$ 1.34 (95)	167.6 $\pm$ 46.8 (67)*	5731 $\pm$ 386 (100)	97.1 $\pm$ 13.9 (86)	152 $\pm$ 18 (106)	793 $\pm$ 35 (108)	1.53 $\pm$ 0.18 (97)	
2	688	160	3.24 $\pm$ 2.42 (249)	173.5 $\pm$ 28.6 (69)*	5548 $\pm$ 703 (97)	87.5 $\pm$ 25.9 (77)*	148 $\pm$ 8 (104)	757 $\pm$ 56 (103)	1.11 $\pm$ 0.09 (70)*	

ND: not determined.

\* Statistically significant,  $p < 0.05$ .

statistically significant, occurred at 40 ppm after five exposures and at 160 ppm after all three exposure periods in mice. Increased lactate dehydrogenase activities were noted in mice after one exposure. Decreased total protein levels were noted in mice at 40 and 160 ppm after five exposures and in rats at 500 ppm after one exposure. A clear decrease of CC16 protein in lavage fluid was observed in mice at 40 and 160 ppm after one and five exposures. A clear decrease of serum CC16 protein was noted in mice at 40 and 160 ppm throughout the study period, the effect being most pronounced after one exposure (Table 4).

### 3.5. Pathology

Lung weights of mice were not affected by treatment. There was a significant decrease in the absolute and relative lung weights in rats at 500 ppm after one exposure.

In mice exposed to 160 ppm, the following findings were recorded: *Epithelial desquamation*: loss of epithelial cells. Granule-containing cells and other cells, which could not be properly identified, were found in lumen of airways in close proximity to epithelium. *Epithelial vacuolation* (Figs. 1B and 2): vacuolation and ballooning of airway cells, most likely secretory cells, as cilia-bearing cells were still discernible. Cells were often faintly stained. *Intracellular inclusions*: presence of small, intensely staining small inclusions in non-ciliated epithelial cells. *Blebs reduced*: the apical blebs (“domes”) of Clara cells seemed to be less prominent or even missing. *Granules reduced*: the secretory granules (most likely of Clara cells) seemed to be reduced in number and/or size. *Cellular crowding*: irregularity of airway epithelium with piling up of cells, giving the surface an undulating appearance due to increase of cell number. Composed of ciliated cells and non-ciliated large cells, sometimes having very small granules.

Microscopic examinations revealed epithelial desquamation and vacuolation in large and medium airways as well as terminal bronchioles in mice at 160 ppm after one exposure. After 5 and 20 exposures of mice at 160 ppm, cellular crowding, expressed as an irregular epithelial lining and indicative of a very early hyperplasia, and a reduction of apical blebs and secretory granules in Clara cells were detected in large and medium airways (Figs. 3 and 4). Electronmicroscopy confirmed that the Clara cells were the target cells in mice, showing either degenerative lesions (vacuolar cell degeneration, cell necrosis) or a slightly different structure (missing of electron dense, oval granules) after 5 and 20 exposures (Figs. 5–8). Histopathology revealed no styrene-related structural lesions in the bronchial and alveolar regions of the respiratory tract of rats.

Table 4  
Lung lavage fluid and blood serum assays

Group	Styrene Concentration mg/m <sup>3</sup>	Lung Lavage Fluid Assays (means ± standard deviation and (percentage of control values))										Blood Serum Assays		
		TCC [10 <sup>6</sup> /ml]	TP 8mg/l <sup>9</sup>	LDH [U/l]	ALP [U/l]	MADG [U/l]	γ-GT [U/l]	GSH μM	CC16 [mg/l]	LZ [μg/l]	CC16 [mg/l]	CC16 [mg/l]		
MICE														
1 EXPOSURE														
0	0	2.12±0.61	53±9	48.1±6.0	6.1±2.2	1.7±0.5	0.24±0.49	14.0±1.6	18.9±7.5	24.2±5.5	4.8±3.4			
1.1	172	1.65±0.89 (78)	56±15 (107)	184.3±143.3 (383)*	16.3±4.0 (267)*	2.3±0.4 (135)	0.09±0.07 (37)	18.3±3.1 (131)	8.9±5.1 (47)	26.0±7.9 (107)	0.4±0.2 (7)*			
2.1	688	2.30±0.56 (109)	43±5 (81)	153.8±28.1 (320)*	25.3±2.2 (413)*	2.3±0.5 (133)	1.28±0.76 (526)	16.7±4.0 (119)	9.1±3.0 (48)	18.9±3.8 (78)	0.6±0.5 (12)*			
5 EXPOSURES														
0	0	1.35±0.35	58±32	52.0±12.7	3.9±1.7	2.4±1.0	0.12±0.27	17.8±4.9	27.5±6.5	21.8±4.0	1.0±1.2			
1.2	172	1.37±0.20 (102)	35±39 (61)	56.3±36.8 (108)	8.9±2.5 (231)*	2.3±0.4 (99)	0.69±0.80 (572)	15.4±3.6 (87)	8.4±3.0 (31)*	20.2±4.3 (93)	0.3±0.2 (28)			
2.2	688	2.01±1.27 (149)	14±18 (24)*	57.6±27.6 (111)	26.0±6.9 (674)*	1.7±0.3 (70)	4.37±1.46 (3642)*	18.8±4.2 (106)	7.2±3.0 (26)*	21.6±5.2 (99)	0.3±0.2 (28)			
20 EXPOSURES														
0	0	4.47±1.39	41±11	49.3±7.3	9.4±1.0	1.6±0.4	0.0	31.1±4.6	4.0±1.5	14.0±2.4	0.6±0.4			
1	172	3.23±2.01 (72)	33±8 (81)	52.2±12.2 (106)	9.4±1.6 (100)	1.4±0.3 (89)	0.37±0.59	33.3±5.3 (107)	4.7±1.2 (118)	16.8±3.6 (120)	0.2±0.2 (33)*			
2	688	2.66±1.21 (59)	46±42 (113)	70.1±34.1 (142)	27.5±18.7 (293)*	1.8±0.5 (115)	1.88±0.86**	37.0±11.5 (119)	3.2±1.1 (81)	14.2±4.5 (102)	0.2±0.2 (33)*			
RATS														
1 EXPOSURE														
0	0	3.38±0.99	78±15	61.6±17.5	143.8±23.9	1.9±0.2	3.83±1.02	ND	9.2±1.2	ND	11.6±3.6			
1.1	688	3.98±1.15 (118)	66±16 (86)	48.0±15.9 (78)	145.3±33.3 (101)	2.2±0.5 (116)	5.22±1.88 (136)	ND	9.8±2.0 (107)	ND	14.6±5.3 (126)			
2.1	2150	4.05±1.75 (120)	55±11 (70)*	52.3±7.9 (85)	122.0±28.6 (85)	2.1±0.4 (107)	4.11±1.24 (107)	ND	9.2±0.7 (100)	ND	11.8±4.3 (102)			
5 EXPOSURES														
0	0	4.08±1.55	66±27	62.1±16.2	148.9±41.6	2.9±1.5	2.97±1.89	ND	11.1±2.6	ND	11.4±4.3			
1	688	2.71±0.74 (66)	53±6 (81)	48.5±6.0 (78)	118.5±19.8 (80)	2.2±0.8 (76)	4.15±1.81 (140)	ND	11.4±1.2 (103)	ND	17.0±6.7 (150)			
2	2150	3.25±0.89 (80)	45±4 (68)	55.8±6.7 (90)	128.9±23.3 (87)	2.1±0.7 (72)	5.06±1.46 (171)	ND	9.9±2.2 (89)	ND	9.4±2.6 (82)			

\* statistically significant, p < 0.05  
 ND not determined  
 TCC: total cell count  
 TP: total protein  
 LDH: lactate dehydrogenase  
 MADG: N-acetyl-β-D-glucosaminidase  
 γ-GT: γ-glutamyltransferase  
 GSH: glutathione  
 CC16: CC16 protein  
 LZ: lysozyme

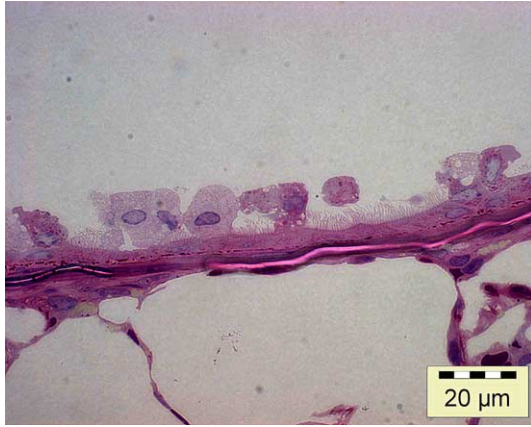


Fig. 2. Styrene 160 ppm (1 exposure), large bronchus, mouse: vacuolation, ballooning of the bronchiolar epithelium. AmbF stained semithin section.

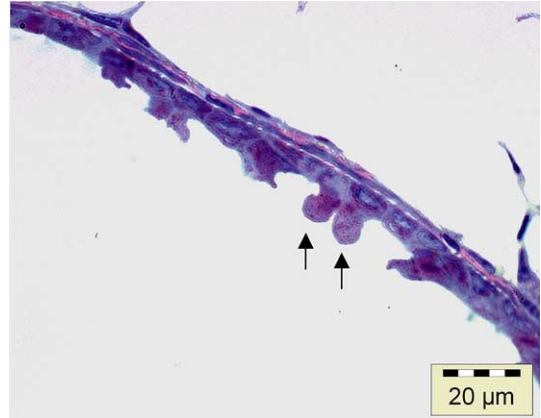


Fig. 5. Control (20 exposures), terminal bronchiole, mouse: non-ciliated (Clara) cells with apical blebs (arrows). AmbF stained semithin slides. See also Fig. 6 electron microscopic image.

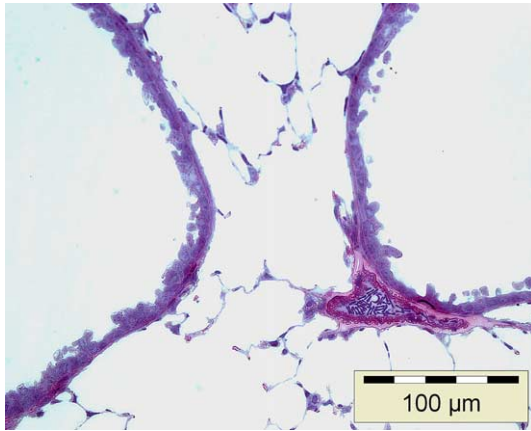


Fig. 3. Control (5 exposures), medium bronchioles, mouse: no abnormalities detected. AmbF stained semithin section.

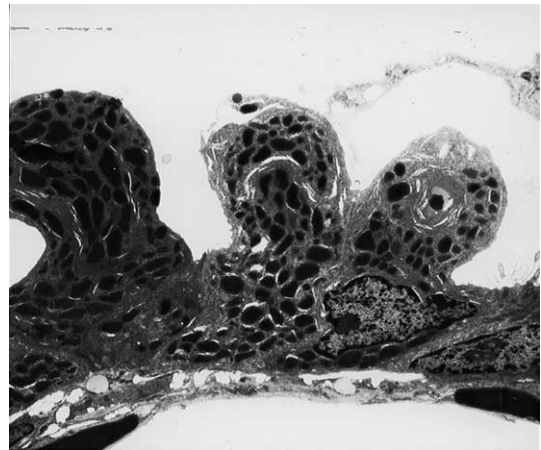


Fig. 6. Control (20 exposures), terminal bronchiole, mouse: non-ciliated (Clara) cells with apical blebs. Electron microscopic image. *Note:* electron-dense granules are abundant; original magnification: 3150x.

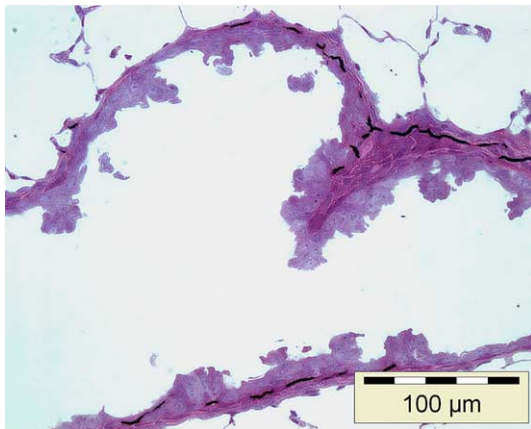


Fig. 4. Styrene 160 ppm (5 exposures), large bronchus/medium bronchiole, mouse: cellular crowding (hyperplasia/dysplasia) of bronchiolar epithelium. AmbF stained semithin section.

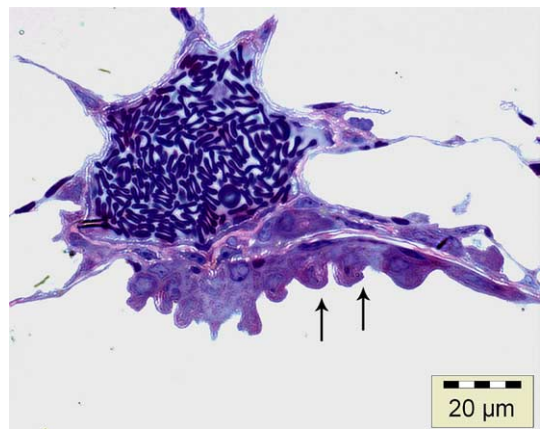


Fig. 7. Styrene 160 ppm (20 exposures), terminal bronchiole, mouse: non-ciliated cells with apical blebs (arrows). AmbF stained semithin slides. See also Fig. 8 electron microscopic image.

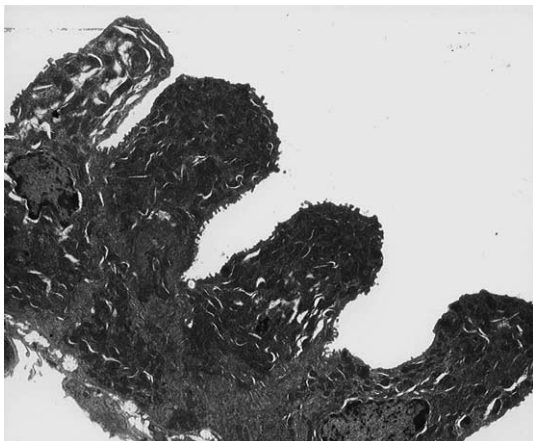


Fig. 8. Styrene 160 ppm (20 exposures), terminal bronchiole, mouse: non-ciliated cells with apical blebs. Electron microscopic image. Note: electron-dense granules are missing; original magnification: 3150 $\times$ .

#### 4. Discussion

The multiple parameters determined in lung homogenates and lavage fluid showed the expected variability. In control mice, there was a trend for decreasing 8-OH-deoxyguanosine, CC16 and lysozyme levels and a trend for increasing Malondialdehyde and GSH in lung homogenate, lavage fluid and blood serum, respectively, over time. These findings might be age related or attributed to further adaptation to the environmental conditions (Prof. Bernard, personal communication). The rather high variability of the control  $\gamma$ -GT and ALP levels over time reflects individual outlier values of these parameters in a single animal.

Although some reduction of glutathione concentration was noted in mouse lung homogenates after 4-week exposure at 160 ppm, other parameters did not indicate evidence of oxidative stress. Malondialdehyde, an indicator of lipid peroxidation, was slightly increased in mice after 1 exposure at 160 ppm only, but was decreased at both dose levels after the 4-week exposure period. No consistent concentration- and time-related changes were observed in the activities of superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase in mouse lung homogenates. Cell counts and differentials in lavage fluid provided no signs of an inflammatory response in either rats or mice.

Concentrations of 8-OH-deoxyguanosine tended to be lower in the exposed groups compared to controls, with the exception of the 160 ppm group after the 4-week exposure period. In studies reported by Boogaard et al. (2000), styrene was shown to have only very weak adduct-forming potency. In both rat and mouse lung, the two isomeric *N*7-guanine adducts of SO were detected at 1 per 10<sup>8</sup> bases at 0 h, and these levels halved at 42 h. For mice, DNA adduct levels in Clara cells and non-Clara cells were similar to total lung. The absence

of a species (rat vs. mouse) and cell (mouse Clara cells vs. mouse non-Clara cells) specific effect in DNA adduct formation is in line with the observation that the covalent binding index (CBI) was found to be the same in the lungs and liver of rats and mice (Cruzan et al., 2002; Cantoreggi and Lutz, 1993). The lack of evidence of oxidative stress and the absence of a consistent increase of DNA adducts in the present study do not support the conclusion that the carcinogenicity of styrene in mice is related to oxidative stress induced DNA adducts.

Clara cell toxicity in mice was indicated at both exposure levels by distinctly decreased CC16 protein concentrations in lavage fluid after one and five exposures and in blood serum throughout the study. The low control value in lavage after 20 exposures could on the one hand be related to high variability of that parameter; on the other hand it could reflect a physiological age-related decrease that might be partly due to a higher lung volume and thus to a higher dilution of proteins recovered in the lavage fluid. In this context, the relatively "normal" values measured at 40 and 160 ppm fit to the altered histomorphological appearance of Clara cells, i.e. the lack of typical blebs and decrease in secretory granules. This cellular morphology suggests an adapted cell population, which is less susceptible to styrene exposure. The lower decrease of CC16 in serum after 20 exposures compared to one or five exposures also fits into this picture.

CC16 is a 15.8-kDa protein secreted all along the tracheobronchial tree and especially in the terminal bronchioles where Clara cells are localized, and serves as a marker for assessing the cellular integrity or the permeability of the lung epithelium (Broeckaert et al., 2000). The serum concentrations of CC16 are decreased in subjects with chronic lung damage caused by tobacco smoke and other air pollutants as a consequence of the destruction of Clara cells. By contrast, serum CC16 increases in acute or chronic lung disorders characterized by an increased airway permeability (Broeckaert et al., 2000). Thus, the decreased CC16 serum concentrations observed in mice suggest that styrene causes destruction of Clara cells in this species. Electronmicroscopy indeed showed degenerative lesions (vacuolar cell degeneration, cell necrosis) in mouse Clara cells. The observed cellular crowding after 5 and 20 exposures, expressed as an irregular epithelial lining, indicates regenerative hyperplasia. The increased  $\gamma$ -glutamyltransferase (GGT) and alkaline phosphatase activities noted in mice at 40 ppm after 5 or 20 exposures and at 160 ppm after 1, 5 or 20 exposures presumably reflect Clara cell toxicity and (regenerative) cell proliferation, respectively (Anderson et al., 1997; Henderson, 1989). This assumption is based on the findings of Day et al. (1990), who reported increases in GGT levels in rat lung lavage fluid after systemic injection of 4-ipomeanol, which is a selective pneumotoxicant that has been shown to produce

epithelial lesions primarily in non-ciliated bronchiolar Clara cells (Boyd, 1997). Gadberry et al. (1996) and Carlson (1997) also reported increased levels of GGT and additionally of lactate dehydrogenase (LDH) in bronchoalveolar lavage fluid obtained at necropsy after intraperitoneal (Ip) administration of 600 mg/kg bw. The Ip injection of styrene oxide (SO) caused increases in GGT and LDH at lower doses (300 mg/kg bw) suggesting that SO is more toxic than styrene (Gadberry et al., 1996). Lysozyme concentration, a marker for type II pneumocyte integrity (Plopper, 1996), was not changed in mouse lung lavage fluid.

In contrast to the above changes in mice, one or five 6 h exposures of female rats to concentrations of 160 and 500 ppm styrene did not produce lung toxicity. More importantly, there was no indication of specific Clara cell damage observed in pathology or by CC16 values in blood serum as well as in lung lavage fluid which were unchanged at both concentrations and both time points.

The finding obtained in this study indicate that the cells in the lungs of female mice respond in a similar way to styrene exposure as in the male mouse, which is in line with their similar susceptibility to the carcinogenic effect of styrene. Also, the far lesser response of female rats is not in variation with the lack of a carcinogenic response of the female rat lungs to styrene exposure.

The results of this study thus suggest that the carcinogenic mode of action of styrene, or one of its metabolites, in mice may proceed along the following pathway: initial induction of a specific targeted toxicity to the Clara cells, with functional as well as morphological changes. These changes may very well trigger a cycle of regenerative cell proliferation as was previously discussed by Cruzan et al., 2002, 1997 and Green et al., 2001. Sustained cell damage and regenerative cell proliferation is a potentially non-genotoxic carcinogenic mode of action that is considered to be associated with the existence of a threshold. The findings of this study obtained at concentrations of 40 and 160 ppm in mice are consistent with the observed carcinogenicity of these exposure levels in mice. The biochemical changes observed at 40 ppm in mice suggest that at this dose level some functional adverse effects are induced in the Clara cells. These early biochemical changes in the Clara cells may very well be the initial event resulting in further changes with prolonged exposure. Cruzan et al., 2001 describe a progressive increase of lung histopathological changes in mice exposed to 20 ppm over time e.g. bronchiolar epithelial hyperplasia extending into alveolar ducts (22%, 37% and 58% in males and 0%, 37%, 36% after 12, 18 and 24 months, respectively). The absence of specific Clara cell toxicity in any of the dose levels tested in rats is also in line with the fact that styrene is not a lung carcinogen in rats. Based on these observations it can be hypothesized that the carcinogenic effect of styrene is related to the induction of

continuous Clara cell toxicity and regenerative cell proliferation. The mode of action for the development of mouse lung tumors by styrene was previously reviewed (Cruzan et al., 2002). In mice, but not rats, repeated inhalation exposures resulted in toxicity to the Clara cells of the terminal bronchioles of the lung, seen as increased cell proliferation and decreased staining of the cytoplasm, followed in order by cellular crowding, hyperplasia in terminal bronchioles, and hyperplasia in terminal bronchioles extending into alveolar ducts. The importance of cellular toxicity and subsequent cell proliferation has previously also been discussed by Cruzan et al., 1997, 2002 and Green et al., 2001. This hypothesis would explain the onset of a carcinogenic effect in mice as well as the absence of such a response in rats.

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