Determination of low level exposure to volatile aromatic hydrocarbons and genotoxic effects in workers at a styrene plant

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Abstract

Objectives—Low exposures to volatile aromatic hydrocarbons and cytogenetic effects in peripheral white blood cells were determined in 25 healthy workers employed in different areas of a styrene production plant in the former German Democratic Republic. The results were compared with 25 healthy unexposed controls (matched for age and sex) employed in the same company.

Methods—The concentrations of aromatic hydrocarbons determined from active air sampling in all areas of the factory (styrene: 73–3540 $\mu\text{g}/\text{m}^3$ (<0.01–0.83 ppm); ethylbenzene 365–2340 $\mu\text{g}/\text{m}^3$ (0.08–0.53 ppm); benzene 73–3540 $\mu\text{g}/\text{m}^3$ (<0.02–1.11 ppm); toluene 54–2960 $\mu\text{g}/\text{m}^3$ (0.01–0.78 ppm); xylenes 12–94 $\mu\text{g}/\text{m}^3$ (<0.01–0.02 ppm)) were considerably lower than in the pump house (>4000 $\mu\text{g}/\text{m}^3$ styrene, ethylbenzene, benzene, and toluene; >500 $\mu\text{g}/\text{m}^3$ xylenes), which was only intermittently occupied for short periods. Passive personal monitoring, bio-monitoring of exhaled air and metabolites (mandelic, phenylglyoxylic, trans, trans-muconic, hippuric, *o*-, *m*- and *p*-methylhippuric acids, and phenol) in urine samples collected before and after an eight hour working shift was used to assess individual exposure. Questionnaires and examination of company records showed that the historical exposure was far higher than that measured. Genotoxic monitoring was performed by nuclease P1-enhanced ^{32}P -postlabelling of DNA adducts in peripheral blood monocytes, and DNA single strand breaks, sister chromatid exchange, and micronuclei in lymphocytes. The content of kinetochores in the micronuclei was determined by immunofluorescence with specific antibodies from the serum of CREST patients.

Results—No genotoxic effects related to exposure were detected by DNA adducts or DNA single strand breaks and sister chromatid exchange. The only effect related to exposure was an increase in kinetochore positive micronuclei in peripheral lymphocytes; the frequency of total micronuclei in peripheral lymphocytes did not change. Smoking was confirmed by measurement of plasma cotinine, and no confounding effect was found on any of the cytogenetic variables. **Conclusions**—Low occupational exposure

to styrene, benzene, and ethylbenzene did not induce alterations of genotoxicological variables except kinetochore positive micronuclei. This is the first reported use of the CREST technique for an in vivo study in occupational toxicology, which thus could serve as a valuable and sensitive technique for toxicogenic monitoring.

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Occupational exposure to benzene is associated with cytogenetic abnormalities and an increased risk of leukaemia in humans.¹ For alkylbenzenes such as toluene and xylenes, inadequate evidence exists for their carcinogenicity in experimental animals and humans.²

Styrene has mutagenic properties that are principally attributed to its main active metabolite, styrene-7,8-oxide.³ Styrene acts directly in vitro inducing sister chromatid exchange⁴ and chromosomal aberrations⁵ in human whole blood cultures. In workers exposed to styrene, styrene-7,8-oxide adducts have been detected in DNA isolated from lymphocytes^{6–8} and in haemoglobin.⁹ Cross sectional occupational studies have shown increased sister chromatid exchange in lymphocytes of workers exposed to concentrations of styrene of at least 170–213 mg/m^3 ,^{11,12} but at lower exposures no increase is found.^{9,13} Similarly, increases in chromosomal aberrations in lymphocytes are only observed with exposure to relatively high concentrations (112–435 mg/m^3) of styrene.¹⁴ In stimulated lymphocytes without cytokinesis block, significant increases in the percentage of micronucleated lymphocytes have been detected in workers exposed to styrene at 47–55 mg/m^3 .^{9,15} At concentrations as low as 11 ppm (0.6–44 ppm), significant increases in DNA single strand breaks in lymphocytes occur.⁹ A summary of genotoxicological studies on styrene exposure has recently been published.¹⁰ Despite the range of cytogenetic effects induced by exposure to styrene, no epidemiological data are available to support an increased risk of cancer in workers exposed to styrene.

Ethylbenzene is devoid of biological activity in the Ames test and genotoxicity in both experimental in vitro systems¹⁶ and animal

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models.¹⁷ Neither the United States Environmental Protection Agency nor the International Agency for Research on Cancer has classified ethylbenzene as having carcinogenic potential, because of lack of data.

Toluene on its own is devoid of biological activity and does not induce mutations in the Ames test, or sister chromatid exchange, or chromosomal aberrations in experimental *in vitro* systems.¹⁸ Cytogenetic effects found in workers exposed to both toluene and benzene are most probably due to benzene, and it is doubtful whether toluene induces somatic cell damage in humans.¹⁸

Xylenes are also devoid of biological activity and do not induce mutations in the Ames test²⁰ or micronucleation in bone marrow polychromatic erythrocytes after being given intraperitoneally to mice.¹⁷ Xylenes do not induce sister chromatid exchange or chromosomal aberrations in human lymphocytes *in vitro*²¹ or cytogenetic effects in lymphocytes of human volunteers after experimental exposure to 174 mg/m³ of xylene (15% *o*-, 25% *p*-, and 60% *m*-xylene) for seven hours a day over three consecutive days.²²

In our study, the effect of low occupational exposure to a mixture of styrene, ethylbenzene, benzene, toluene, and xylenes in 25 styrene production workers and 25 non-exposed controls employed at a styrene production plant in the former German Democratic Republic is reported. Exposure was determined by passive and personal air sampling for each subject, biomonitoring of aromatic hydrocarbons in exhaled air, and excretion of urinary metabolites. Cytogenetic studies are reported in which both the short and long term effects of exposure to low concentrations of these aromatic hydrocarbons are determined with nuclease P1-enhanced ³²P-postlabelling of DNA adducts in peripheral blood monocytes as well as DNA single strand breaks, sister chromatid exchange, and micronucleation in lymphocytes. The influence of smoking as a possible confounding factor of genotoxic effects is also reported.

Materials and methods

SUBJECTS

The study was conducted over one working week in July 1991 in a styrene production plant in the former German Democratic Republic. Twenty five exposed workers (mean age 38.8, range 20–58) were compared with 25 non-exposed control employees (mean age 39.3, range 19–59) working at the same company. All subjects were matched for age and sex (17 men and eight women). Information was obtained by questionnaire concerning diet, cigarette and alcohol consumption, use of medication, and possible exposure to aromatic hydrocarbons outside the workplace. Occupational histories and historical exposure were evaluated by examination of company records. The exposed workers were employed in five different areas of the styrene production plant (oven house, production control, storage facility, distillation area, pump house)

and the non-exposed controls in the company medical service, fire brigade, and the security service that manned the factory gates.

The questionnaire showed that the average time of employment of the 25 exposed workers in the plant was 18 years with a historic exposure of one to 34 years. The smoking habits were similar: exposed group, 13 smokers (mean 15.2 cigarettes/day, range 4–40); control group, 17 smokers (mean 17.1 cigarettes/day, range 10–25). Determination of plasma cotinine, a nicotine metabolite, confirmed the similarity of both study groups (exposed smokers *v* control smokers: 280 (202) *v* 243 (132) ng cotinine/ml plasma). Apart from the self reported tendency for higher consumption of alcohol in the non-exposed control group, no further noteworthy differences were apparent from the questionnaires.

EXPOSURE MEASUREMENT

Air samples were collected with graphitised charcoal tubes (type 1-010R2, Analyt GmbH, Mühlheim, Germany) and a model PCXR7 SKC aircheck sampler (DEHA Haan and Wittmer GmbH, Friolzheim, Germany). Intermittent sampling (one minute sample collection every 10 minutes) at a flow rate of 100 ml/min over a period of one hour was performed at each of the five site areas (net sampling time six minutes a tube) and for three hours at each of the workplaces of the controls (18 minutes a tube). Analysis of aromatic hydrocarbons was performed by microwave desorption (Rektorik, Geneva, Switzerland) directly into a gas chromatograph-mass spectrometer (GC-MS; HP 5890/-MSD 5870, Hewlett-Packard GmbH, Bad Homburg, Germany). The limit of detection was 1 µg/m³.

Personal exposure to aromatic hydrocarbons was determined by passive sampling with ORSA 5 personal samplers (Dräger AG, Lübeck, Germany). Personal samplers were worn by each subject over the entire eight hour working shift and styrene, ethylbenzene, benzene, and toluene were measured by the standard NIOSH method.²⁴

COLLECTION AND ANALYSIS OF EXHALED SAMPLES

Samples exhaled after the shift were collected on graphitised charcoal tubes (type 1-010R2, Analyt GmbH) for two minutes at a flow rate of 0.5 ml/min with a modified sampling device.²⁵ Analysis of coded samples under blind conditions was performed by microwave desorption GC-MS.

COLLECTION AND ANALYSIS OF URINE SAMPLES

Urine samples were collected from each subject at the beginning and end of the working shift. Metabolites of styrene and ethylbenzene (mandelic and phenylglyoxylic acids, benzene (phenol), toluene (hippuric acid), and xylenes (*o*-, *m*-, and *p*-methylhippuric acids), were measured by methods published by the Analytical Chemistry Working Group of the Deutsche Forschungsgemeinschaft Commission

for the investigation of health hazards of chemical compounds in the work area.²⁶ Trans,trans-muconic acid, another benzene metabolite, was measured by the method of Ducos.²⁷ Urinary concentrations were expressed after correction for creatinine, which was determined by the Jaffé method with a commercial kit (Merck, Darmstadt, Germany). All samples were coded and analysed under blind conditions.

COLLECTION OF BLOOD SAMPLES FOR GENOTOXIC STUDIES

Blood samples (50 ml) were drawn from the cubital vein into coded heparinised syringes at the end of the working shift. Samples were transported in ice to the laboratory with as little vibration as possible. White blood cells for genotoxic studies were isolated within four to five hours of sample collection. Blood samples were also used to confirm smoking state by measurement of cotinine in plasma with a modified radioimmunoassay.²⁸

NUCLEASE P1-ENHANCED ³²P-POSTLABELLING OF DNA ADDUCTS IN MONOCYTES

White blood cells were isolated from 5 ml of whole blood by a modified Ficoll-Paque density centrifugation.²⁹ Isolated leucocytes were cultured and monocytes purified from residual lymphocytes for ³²P-postlabelling.³⁰ The DNA was isolated by proteinase K digestion³¹ and was measured by fluorescence spectrometry with calf thymus DNA (Boehringer Mannheim GmbH, Germany) as a standard.³² The postlabelling method was adjusted to the detection of benzene related DNA adducts.³³ The DNA samples (8 µg DNA) were evaporated to dryness and digested overnight at 37°C in a total volume of 7 µl containing 0.21 U micrococcal nuclease (Sigma, Deisenhofen, Germany), 0.58 mU spleen phosphodiesterase (Boehringer Mannheim GmbH), 17 mM disodium succinate, and 8 mM CaCl₂. Nucleotides were measured by the absorbance at 260 nm of a 2 µl aliquot of the digest.³⁴ The digest was treated with 5 µl of an aqueous solution containing nuclease P1 (1 U; Sigma), sodium acetate (0.12 M), and ZnCl₂ (0.09 mM). The samples were incubated at 37°C for one hour and the reaction terminated by adding 2 µl of 0.5 M tris base. The DNA samples (5 µl) were labelled with ³²P by adding 25 µl of labelling buffer (200 mM bicine, 100 mM MgCl₂, 100 mM dithiothreitol, 10 mM spermidine), 25 µl of carrier free [³²P]ATP (20 µCi; Amersham Buchler, Braunschweig, Germany), 7.5 µl T4 polynucleotide kinase (6.7 U; Boehringer Mannheim GmbH) and 13.4 µl H₂O and labelled nucleotides resolved by four dimensional chromatography on PEI cellulose thin layer chromatography (TLC) sheets (Macherey-Nagel, Düren, Germany).³³ The adducts were detected by screen intensified autoradiography for 72 hours at 80°C (Xomat 5, Kodak, Rochester, NY, USA). The radioactive areas were excised and the radioactivity measured by scintillation spectrometry. Adduct concentrations, expressed

as relative adduct labelling were calculated from adduct count rates and the specific activity of [³²P]ATP.³⁵ The specific activity of [³²P]ATP was determined by measuring incorporation of ³²P into 1 pmol of deoxyadenosine 3'-monophosphate (dAp). The dAp was labelled in exactly the same way as the samples and diluted in 1.5 ml bicine (200 mM) before it was put on the TLC sheet. The [³²P]ATP was excised after one-dimensional chromatography (pH 6.8; 0.28 M ammonium sulphate, 0.05 M disodium hydrogen phosphate). The amount of deoxyribonucleoside 5'-monophosphates (dNp) on the chromatograms was calculated with the estimate that 1 µg DNA = 3240 pmol DNA-P. A calibration curve was constructed with known amounts of digested DNA to determine the exact amounts of sample digest. The adduct nucleotides in three areas of the chromatogram (A, B, C), and the remaining area (D), except for radioactive areas to the top and side of the origin (fig), were excised and counted. Two aliquots of one sample of DNA were labelled and the average amount of radioactivity of the two autoradiograms was calculated.

LYMPHOCYTE MICRONUCLEUS ASSAY

Lymphocytes were isolated from 5 ml of whole blood by a Ficoll-Paque density centrifugation²⁹ and washed twice with pH 7.2 phosphate buffered saline (PBS). Micronucleated lymphocytes were measured with a modified cytokinesis-block method.³⁶ Cells were cultured at 37°C for 72 hours in 5 ml of chromosome medium 1A (Gibco, Karlsruhe, Germany) that contained 10 ml/l phytohaemagglutinin. Cytochalasin B (3 µg/ml final concentration; Sigma) was added after 44 hours of incubation. After 72 hours cells were washed in hypotonic saline for eight minutes, fixed in methanol/glacial acetic acid (4:1), and harvested on to slides. Slides were dried and stained for 15 minutes in 4',6-diamidino-2-phenylindole (DAPI, 5 mg/l; Serva Feinbiochemica, Heidelberg, Germany). Two thousand binucleated cells per sample were scored blindly by the same reader. Micronuclei had to meet the following criteria: (a) DAPI positive, (b) less than one third the diameter of the main nucleus, (c) in the same plane of focus, (d) have the same texture as the main nucleus, and (e) have a smooth oval or round shape.

IMMUNOFLUORESCENT STAINING OF KINETOCHORE POSITIVE MICRONUCLEI

Kinetochores positive micronuclei were determined by CREST antibody labelling.³⁷ Cultures for determination of kinetochores positive micronuclei were established as already described. After culture, cells were fixed with ethanol (95%), washed with PBS (pH 7.2), and collected by centrifugation (10 minutes, 140 g). Cells were transferred to a 1.5 ml vial, resuspended in 1 ml calcosin-Raynaud's phenomenon-oesophageal dysmobility-sclerodactyly-telangiectasia syndrome of scleroderma (CREST; Chemicon

Representative ^{32}P -postlabelling autoradiograms of peripheral monocyte DNA adducts in exposed people (top) and controls (bottom). The areas A, B, and C represent areas with adduct nucleotides that were separately evaluated (table 4).

International, Temecula, CA, USA) antibody solution (CREST serum 1:500 diluted with pH 7.2 PBS containing 1% albumin) and incubated at 37°C for one hour. Cells were centrifuged (three minutes, 550 g), washed twice with PBS containing 1% albumin and incubated at 37°C for 45 minutes in 1 ml of rhodamine conjugated rabbit-antihuman IgG (1:500 diluted with pH 7.2 PBS containing 1% albumin). Cells were harvested on to slides and stained with DAPI, then immersed for 20 seconds in dansylchloride (0.3 mg/ml, 2:1 NaHCO_3 saturated ethanol/0.9% NaCl)

to make the cytoplasm visible. Two thousand binucleated cells per sample were scored blindly by the same reader for kinetochore positive micronuclei.

SISTER CHROMATID EXCHANGE

Whole blood (1 ml) cultures were established in 5 ml of chromosome medium 1A (Gibco) that contained 15 $\mu\text{g/ml}$ 5-bromo-2-deoxyuridine (BrdUrd). Cultures were incubated for 72 hours at 37°C under conditions of dim light. Colcemid (0.1 $\mu\text{g/ml}$) was added to each culture two hours before the end of

Table 1 Active air sampling of aromatic hydrocarbons at different working sites

Exposure group and location	Time period	Concentrations of aromatic hydrocarbon at different workplaces ($\mu\text{g}/\text{m}^3$)					
		Styrene	Ethylbenzene	Benzene	Toluene	o-Xylene	m- or p-Xylene
Exposed workers:							
Oven house	0921-1021	200	940	325	150	9.5	12.5
	1021-1121	220	1090	210	175	14.7	17.1
	1121-1221	355	1120	725	265	18.8	15.7
Production control	0927-1027	810	835	2320	1010	8.5	42.1
	1030-1130	2570	1360	950	655	8.1	11.9
	1130-1230	505	420	970	710	6.0	64.0
Storage facility	0921-1021	73	670	340	25.5	6.5	5.6
	1027-1127	265	1180	1570	250	13.4	5.5
	1127-1227	235	1230	2280	54.1	17.6	23.2
Distillation area	1244-1344	3040	365	2230	2850	44.1	50.0
	1344-1444	3540	2340	3260	2960	30.6	6.2
	1444-1544	1840	1440	2120	2200	15.3	40.9
Pump house*	1231-1331	>4000	>4000	>4000	>4000	>300	>500
	1331-1431	>4000	>4000	>4000	>4000	>300	>500
	1431-1531	>4000	>4000	>4000	>4000	>300	>500
Control workers:							
Medical centre	1605-1820	49.2	290	180	85	15.6	21.2
Fire station	1332-1635	11.7	145	58.7	125	16.6	18.3
Security office	1021-1321	38.8	250	67.1	47.1	15.5	16.8

*All measurements performed within the pump house exceeded the sampling capacity of the sampling device.

incubation. Lymphocytes were washed with hypotonic saline, harvested on to slides, and air dried chromosome preparations were made. The slides were stained for five minutes in acridine orange ($10 \mu\text{g}/\text{ml}$) and washed twice in phosphate buffer (pH 6.0). All slides were scored blindly by the same reader. Only 15 well differentiated diploid second division metaphases with at least 40 chromosomes were scored.

DNA SINGLE STRAND BREAKS

Lymphocytes were isolated by the Ficoll-Paque method and DNA single strand breaks determined immediately afterwards with a modified version of the nick translation assay.³⁸ Each determination was performed seven times and averaged. The coefficient of variation was below 15% for multiple determinations. A second lymphocyte sample was cultured for 12 hours at 37°C in sterile filtered Dulbecco's modified Eagle's medium (DMEM, Gibco) buffered to pH 7.5 with 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES), 1.0 g/l NaHCO_3 and supplemented with 10% fetal calf serum (FCS; Gibco), 0.1 g/l streptomycin, 0.1 g/l neomycin, and 7.78×10^4 U/l penicillin. After culture, the nick translation assay was performed to determine the capacity of DNA for repair.

STATISTICAL ANALYSIS

As the individual groupings were sometimes small and the distributions not always normal, the Mann-Whitney rank sum test was used to compare exposure, alveolar, and urinary metabolite concentrations in the exposed and control groups. The *t* test was used to compare genotoxic data. A test was considered significant with $P < 0.05$.

Results

EXPOSURE ASSESSMENT

Table 1 shows the concentrations of aromatic hydrocarbons in ambient air of the different production plant areas (oven house, production control, storage facility, distillation area, pump house) and control working areas (medical centre, fire station, security office). Active air sampling showed the maximum concentrations within the pump house, where only a few workers were present for short periods. The concentrations of aromatic hydrocarbons were significantly higher than in the control areas and in the case of the pump house exceeded the upper limit of the detection method.

Personal monitoring with ORSA 5 passive samples confirmed that the workers were exposed to far higher concentrations of aromatic hydrocarbons (styrene 0.31 (0.88) ppm;

Table 2 Aromatic hydrocarbon concentrations in exhaled air after the shift

Exposure group	Subjects n	Concentrations of aromatic hydrocarbon in exhaled air after the shift (mean (SD) $\mu\text{g}/\text{m}^3$)					
		Styrene	Ethylbenzene	Benzene	Toluene	o-Xylene	m- or p-Xylene
Exposed workers:							
Total group	25	83.9 (167.6)**	95.5 (78.2)***	30.9 (17.0)***	25.5 (9.8)***	3.4 (1.3)	3.7 (2.0)
Smokers	17	79.9 (188.1)	104.0 (91.2)	27.2 (14.5)**	21.8 (9.0)*	3.5 (1.3)	3.5 (1.8)
Non-smokers	8	92.4 (112.1)	77.5 (31.1)	38.8 (19.1)**	23.9 (11.1)*	3.3 (1.3)	4.0 (2.4)
Non-exposed controls:							
Total group	25	30.2 (52.0)	20.7 (10.8)	5.5 (3.8)	14.3 (11.2)	3.5 (2.1)	4.0 (2.5)
Smokers	13	25.3 (44.8)	20.3 (6.5)	7.6 (3.8)	17.8 (14.5)	3.6 (1.9)	3.8 (2.2)
Non-smokers	12	35.5 (58.4)	21.2 (14.1)	3.2 (2.0)	10.6 (2.7)	3.4 (2.4)	4.2 (2.8)

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; paired differences between exposed and unexposed groups for individual aromatic hydrocarbons by Mann-Whitney rank sum test.

Table 3 Aromatic hydrocarbon metabolites in urine

Exposure group	Subjects n	Aromatic hydrocarbon metabolites in urine (mean (SD) mg/g creatinine)							
		Mandelic acid	Phenylglyoxylic acid	Benzoic acid	Hippuric acid	<i>o</i> -Methyl-hippuric acid	<i>m</i> - or <i>p</i> -Methyl-hippuric acid	Trans, trans-muconic acid	Phenol
Exposed workers:									
Total group:	25								
Before shift		13.3 (20.4)***	10.7 (21.5)	12.4 (13.0)	1269 (958)	3.3 (6.5)	3.4 (4.3)	0.85 (2.80)	NA
After shift		43.9 (31.5)***	22.3 (27.3)*	17.5 (21.4)	1240 (641)	3.9 (6.9)	11.5 (16.8)	0.83 (0.73)**	9.2 (4.6)
Smokers:	17								
Before shift		10.0 (13.0)	12.6 (25.1)	11.6 (15.0)	1290 (1044)	4.5 (7.5)***	4.5 (4.4)*	1.17 (3.34)	NA
After shift		49.4 (34.7)	22.2 (30.9)	15.3 (28.2)	1377 (706)	2.9 (6.1)	12.8 (16.4)	0.90 (0.83)	9.7 (5.5)
Non-smokers:	8								
Before shift		20.3 (29.5)	6.6 (8.6)	14.0 (7.1)	1224 (752)	0.7 (1.4)***	1.2 (2.7)*	0.16 (0.13)	NA
After shift		32.4 (19.0)	22.6 (17.4)	22.2 (23.1)	949 (319)	6.2 (8.1)	8.5 (17.3)	0.68 (0.42)	8.0 (0.6)
Non-exposed controls:									
Total group:	25								
Before shift		5.5 (9.5)	2.8 (10.1)	9.8 (12.3)	1314 (1213)	0.9 (2.1)	1.5 (3.1)	0.22 (0.26)	NA
After shift		4.3 (8.6)	0.5 (2.5)	30.3 (32.8)	1018 (610)	0.7 (1.3)	3.5 (8.0)	0.48 (0.66)	7.5 (2.9)
Smokers:	13								
Before shift		6.3 (10.9)	ND	13.6 (14.5)	1518 (1389)	1.1 (2.7)	1.6 (2.0)	0.25 (0.22)	NA
After shift		5.6 (10.4)	ND	25.2 (23.9)	1107 (548)	0.6 (1.0)	3.9 (8.6)	0.53 (0.54)	7.6 (2.8)
Non-smokers:	12								
Before shift		4.6 (7.1)	5.8 (14.0)	5.6 (7.2)	1094 (939)	0.6 (1.3)	1.4 (4.0)	0.18 (0.29)	NA
After shift		2.8 (5.6)	1.1 (3.6)	35.7 (39.6)	922 (657)	0.8 (1.6)	3.0 (7.2)	0.42 (0.76)	7.6 (2.8)

*P < 0.05; **P < 0.01; ***P < 0.001; paired differences between exposed and unexposed group for individual aromatic hydrocarbon metabolites by Mann-Whitney rank sum test.

ND = not detected; NA = not analysed.

ethylbenzene 1.51 (3.42) ppm; benzene 0.24 (0.24) ppm) than were controls (all <0.004 (0.02) ppm). Toluene exposure, however, was higher in the controls (1.31 (2.68) ppm) than in the exposed workers (0.79 (1.97) ppm). Individual xylene isomers were not determined.

BIOMONITORING OF EXHALED AROMATIC HYDROCARBONS

Analysis of exhaled air after the shift confirmed that workers in the production areas were exposed to significantly ($P \leq 0.001$) higher concentrations of all aromatic hydrocarbons except for xylenes (table 2). In the control group, smoking had a significant confounding effect on exhaled benzene ($P = 0.007$) and toluene ($P = 0.021$), but not on the concentrations of other aromatic hydrocarbons in the exhaled air of exposed workers.

A significant positive correlation between personal air monitoring and exhaled benzene (Pearson's coefficient $r = 0.51$; $P = 0.01$) was found. Non-significant or negative correlations were obtained for styrene ($r = 0.31$), ethylbenzene ($r = 0.23$), and toluene ($r = -0.13$).

BIOMONITORING OF AROMATIC HYDROCARBON METABOLITES IN URINE

In comparison with the control workers (table 3), metabolite concentrations in urine sampled after the shift in the exposed workers

showed significantly higher exposure to styrene and ethylbenzene (mandelic acid $P < 0.001$; phenylglyoxylic acid $P = 0.03$) and benzene (trans,trans-muconic acid $P = 0.003$), but not *o*-, *m*-, or *p*-xylenes (*o*-, *m*-, or *p*-methylhippuric acids). Comparison of urine samples before and after the shift also showed a significant exposure of workers to styrene and ethylbenzene (mandelic acid $P < 0.001$; phenylglyoxylic acid $P = 0.06$), but not to benzene, toluene, and xylenes. No significant confounding effect due to smoking was evident. The urinary excretion of *m*- and *p*-methylhippuric acid was higher before the shift in smokers than in non-smokers in the exposed group. This was only just significant ($P = 0.049$).

DNA ADDUCTS IN MONOCYTES

No significant increase in total or segregated monocyte DNA adducts (figure), measured as relative adduct concentrations, were present in the exposed workers (table 4). Considerable variability between individual workers, but no exposure related or confounding effects due to smoking, was detected.

LYMPHOCYTE MICRONUCLEUS ASSAY

The frequency of micronucleated lymphocytes was not significantly different in exposed and control workers (table 5). Smokers in both groups showed lower but not significantly

Table 4 DNA adducts in peripheral monocytes

Exposure group	Subjects n	Monocyte DNA adducts or relative adduct concentration (mean (SD))				
		Area A	Area B	Area C	Area D	Total
Exposed workers:						
Total group	25	4.17 (2.76)	13.67 (8.74)	15.13 (9.93)	41.78 (24.61)	74.7 (42.8)
Smokers	17	4.45 (3.04)	14.67 (9.82)	16.22 (10.84)	41.11 (26.60)	76.4 (46.6)
Non-smokers	8	3.52 (2.06)	11.38 (5.52)	12.64 (7.60)	43.32 (21.16)	70.9 (35.7)
Control workers:						
Total group	25	3.76 (2.39)	11.90 (7.36)	10.38 (6.8)	36.75 (25.4)	62.8 (38.9)
Smokers	13	3.47 (2.06)	10.67 (5.56)	10.06 (6.33)	35.57 (25.34)	59.8 (37.7)
Non-smokers	12	4.08 (2.77)	13.24 (8.98)	10.74 (7.55)	38.04 (26.54)	66.1 (41.7)

Table 5 Micronucleus assay in lymphocytes

Exposure group	Subjects n	Binucleate cells with micronuclei (%) (mean (SD))	Kinetochore positive micronuclei (%) (mean (SD))
Exposed workers			
Total group	25	1.90 (0.78)	39.43 (10.22)**
Smokers	17	1.84 (0.61)	38.25 (11.26)*
Non-smokers	8	2.03 (1.11)	42.14 (7.36)*
Control workers:			
Total group	25	1.87 (0.71)	31.79 (8.15)
Smokers	13	1.61 (0.62)	30.25 (7.85)
Non-smokers	12	2.15 (0.71)	33.33 (8.49)

*P < 0.05; **P < 0.01 paired differences between exposed and unexposed group (kinetochore positive micronuclei only) by *t* test.

Table 6 Sister chromatid exchange in lymphocytes

Exposure group	Subjects n	Sister chromatid exchange/cell (mean (SD))
Exposed workers:		
Total group	25	9.27 (1.24)
Smokers	17	9.38 (1.37)
Non-smokers	8	9.04 (0.99)
Control workers:		
Total group	25	9.24 (1.24)
Smokers	13	9.67 (1.36)
Non-smokers	12	8.87 (0.96)

different frequencies of micronuclei than their non-smoking counterparts. Significantly higher proportions of kinetochore positive micronuclei were found in the total exposed working group ($P = 0.007$), exposed smokers ($P = 0.045$), and exposed non-smokers ($P = 0.035$). The proportion of kinetochore positive micronuclei was also lower in smokers in both the exposed and control groups when compared with their non-smoking counterparts. This difference was not significant.

SISTER CHROMATID EXCHANGE IN LYMPHOCYTES

The frequency of sister chromatid exchange was almost identical in the exposed workers and controls (table 6). Slight, but not significant, increases were found in smokers compared with non-smokers in both the exposed and control groups.

DNA SINGLE STRAND BREAKS IN LYMPHOCYTES

No significant differences in DNA single strand breaks in lymphocytes were found between the exposed and control workers (table 7), and smoking had no effect. Significant repair of DNA single strand breaks

($P < 0.003$) occurred when lymphocytes from both the exposed and control workers were kept for an additional 12 hours in culture. The repair capacity was not reduced in smokers when compared with non-smokers.

Discussion

Personal monitoring showed that exposure to all aromatic hydrocarbons with the exception of toluene and xylenes (not determined) was higher in the exposed workers. Comparison of exhaled air after the shift confirmed that the exposed workers were exposed to significantly higher concentrations of styrene, ethylbenzene, benzene, and toluene (all $P < 0.001$). As in other studies,³⁹⁻⁴¹ smoking had a significant confounding effect in the control group on the exhaled concentrations of benzene (non-smokers 3.2 (2.0) $\mu\text{g}/\text{m}^3$; smokers 7.6 (3.8) $\mu\text{g}/\text{m}^3$; $P = 0.007$) and toluene (non-smokers 10.6 (2.7) $\mu\text{g}/\text{m}^3$; smokers 17.8 (14.5) $\mu\text{g}/\text{m}^3$; $P = 0.021$), but not in the exposed group. As analysis of exhaled air is a sensitive biomonitoring method for toluene and xylene,⁴² it is evident (table 2) that the workers had little or no exposure to xylenes above background concentrations.

In the exposed working group, urine samples taken after the shift, corrected for creatinine, contained significantly higher concentrations of phenylglyoxylic acid ($P = 0.03$), mandelic acid ($P < 0.001$), and trans,trans-muconic acid ($P = 0.003$) providing further confirmation of combined occupational exposure to styrene, ethylbenzene, and benzene. The increase found in trans,trans-muconic acid is only partially in agreement with other studies in which both trans,trans-muconic acid and phenol are reported to be unsuitable for biomonitoring low benzene exposures (<1 ppm).^{27 43 44}

Considerable variability between individual workers, but no increase in DNA adducts related to exposures was detected in peripheral blood monocytes (figure), which suggests that sources unrelated to the low occupational exposure to aromatic hydrocarbons may have been responsible for the adduct concentrations found (table 4). Monocytes have a biological half life of eight hours in peripheral blood⁴⁵ and are metabolically competent to activate aromatic xenobiotics.⁴⁶ Consequently, relative adduct labelling in monocytes represents recent rather than historical exposure. None of the detected adducts corresponded to DNA adducts formed in vitro by incubation of calf thymus DNA with hydroquinone or benzoquinone—two DNA reactive metabolites of benzene. No relation was found between the relative adduct concentrations and exposure to individual or total aromatic hydrocarbons.

No significant differences were found in the frequency of micronuclei in lymphocytes between the exposed and control workers (table 5). The only difference found was a significant increase in the proportion of kinetochore positive micronuclei in the total exposed working group ($P = 0.007$), exposed

Table 7 DNA single strand breaks in lymphocytes

Exposure group	Subjects n	DNA single strand breaks (cpm, relative) mean (SD)	
		5 h after sampling	17 h after sampling
Exposed workers:			
Total group	25	2370 (1063)***	1137 (441)***
Smokers	17	2185 (882)***	1160 (498)***
Non-smokers	8	2763 (1358)**	1089 (311)**
Control workers:			
Total group	25	2550 (988)	1247 (393)
Smokers	13	2530 (987)	1255 (362)
Non-smokers	12	2572 (1034)	1240 (441)

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ paired differences between exposed and unexposed group (5 h and 17 h after sampling) by *t* test.

smokers ($P = 0.045$), and exposed non-smokers ($P = 0.035$). In the cytokinesis-block method³⁶ that we used, blastogenesis of T lymphocytes stimulated by phytohaemagglutinin provides a means of assessing cytogenetic effects due to long term (historical) exposure as T cells are not always found in the peripheral vasculature, have a half life in excess of four years, and may last in extravascular tissue compartments for extended periods. Micronuclei represent small, additional nuclei formed by the exclusion of chromosome fragments (kinetochore negative micronuclei) or whole chromosomes (kinetochore positive micronuclei) lagging at mitosis. The CREST staining technique is highly effective in distinguishing agents that induce aneuploidy from clastogens in human lymphocytes.³⁷ As the frequency of total micronuclei and kinetochore positive micronuclei was lower in smokers than in non-smokers in the control group, although not significantly (total micronuclei $P = 0.056$; kinetochore positive micronuclei $P = 0.366$), it can only be concluded that the increase in the proportion of kinetochore positive micronuclei in the exposed workers is directly related to occupational exposure to either aromatic hydrocarbons or some other unidentified agent that induces aneuploidy. Although styrene and styrene-7,8-oxide induce micronuclei in human lymphocytes,⁴⁷ the increased proportion of kinetochore positive micronuclei is more consistent with an indirect genotoxic effect due to benzene metabolites.⁴⁸ In contrast with most studies in which structural chromosomal aberrations have been found in workers with current benzene exposure, studies that detected aneuploidy have generally been performed on people with diagnosed bone marrow toxicity some time after benzene exposure had ceased.^{49, 50} This is the first reported use of the CREST technique to study kinetochore positive micronuclei in occupationally exposed workers. This reported increase in kinetochore positive micronuclei is particularly significant as very recent studies have shown that both hydroquinone⁵¹ and 1,2,4-trihydroxybenzene,⁵² two metabolites of benzene, induce aneuploidy, a chromosomal defect often detected in leukaemia.⁵³

The frequency of sister chromatid exchange in lymphocytes was almost identical in the exposed and control workers (table 6). Previous investigations on exposure to low concentrations of benzene and other aromatic hydrocarbons have produced conflicting results in which either an increase,^{54, 55} or no change in the frequency of sister chromatid exchange has been reported.^{9, 22, 56, 57}

No significant differences in DNA single strand breaks in lymphocytes were found in the exposed and control workers (table 7), as DNA is modified at an estimated rate of 10^5 bases per cell per day in the absence of exogenous damage.⁵⁸ In contrast with conventional techniques to detect DNA single strand breaks, the nick translation assay allows the detection of in situ DNA single strand breaks

in intact cells.³⁸ Numbers of DNA single strand breaks were first determined five hours after blood sampling during which time repair of some of the lesions occurred. This was evident from lymphocytes that were kept in culture for a further 12 hours before performing the nick translation assay. Five to 17 hours after drawing the blood samples, significant ($P < 0.003$) repair of lesions was still evident. With conventional alkaline elution techniques to detect DNA single strand breaks, exposure to styrene (11.2 (0.9) ppm),⁹ benzene (4.2 (4.2) ppm), and toluene (70 (66) ppm)¹⁹ results in significant increases in DNA single strand breaks in lymphocytes. Both these studies did not investigate possible repair effects. Our results indicate that DNA lesions are not induced by exposure to low concentrations of aromatic hydrocarbons and that lesions present are subject to repair. Furthermore, the capacity for repair is not compromised in smokers.

In summary, low exposure to volatile aromatic hydrocarbons was determined in workers who produce styrene and was compared with a non-exposed control group by air monitoring and biomonitoring techniques. Short term genotoxic effects of exposure were not detected in the form of DNA adducts in peripheral blood monocytes, DNA single strand breaks, and sister chromatid exchanges in lymphocytes. A long term increase in kinetochore positive micronuclei, but not total micronuclei (related to exposure) was found in peripheral lymphocytes, which is consistent with exposure to benzene. Smoking had no confounding effect on any of the measured cytogenetic variables.

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