

Cytogenetic and hematological effects in plastics workers exposed to styrene

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HAGMAR L, HÖGSTEDT B, WELINDER H, KARLSSON A, RASSNER F. Cytogenetic and hematological effects in plastics workers exposed to styrene. *Scand J Work Environ Health* 1989;15:136-41. For 20 glass-reinforced plastics workers exposed to styrene and 22 unexposed referents, the frequency and size distribution of micronuclei were determined for lymphocytes stimulated with phytohemagglutinin or pokeweed mitogen, and white blood cell counts were made. Furthermore, chromosome aberrations were scored for 11 of the exposed subjects and 15 of the referents. The mean level of styrene in the breathing zone of the workers was 56 mg/m³. Workers exposed to styrene did not show an increase in any of the cytogenetic end points studied when the effect of age and smoking was allowed for in a multiple regression analysis. A significant 30 % increase in the number of peripheral monocytes was observed for the exposed workers. No correlations between the cytogenetic and hematological tests on one hand and the length of exposure time on the other could be detected.

Key terms: blood lymphocytes, chromosome aberrations, micronuclei, monocytes.

The organic solvent styrene (CAS number 100-42-5) is used mainly in the production of reinforced plastics, resins, and synthetic rubbers. Styrene has mutagenic properties, probably through its main reactive metabolite, styrene-7,8-oxide (1). Increased levels of chromosome aberrations (2-8), sister chromatid exchanges (6, 7), and micronuclei (2, 9, 10) have been found in cultured lymphocytes from styrene-exposed workers. However, in other studies, no cytogenetic effects were found (11, 12).

During the last few years we have been able to develop the micronucleus assay for lymphocytes by preserving the cytoplasm of the cells (13), which increases the precision in scoring micronuclei, and by measuring micronucleus sizes, thus separating different inducing agents (14, 15). Furthermore, we have been using both phytohemagglutinin (PHA) and pokeweed (PWM) as mitogens which probably stimulate different subgroups of lymphocytes (15, 16).

The carcinogenic organic solvent benzene induces cytogenetic effects in humans and may also, due to a toxic depression of the bone marrow, cause lymphopenia in peripheral blood (17). In contrast, exposure to other organic solvents may cause lymphocytosis (18, 19). Whether styrene exposure may affect the peripheral lymphocyte level is not known. Thus differential white blood cell counts were included in the examination.

The objectives of the present study were to investigate whether low-level exposure to styrene causes cytogenetic effects or disturbances in the peripheral white blood cell counts. Furthermore, we attempted to investigate whether styrene preferably induces larger micronuclei and whether the type of mitogen used, PHA or PWM, is of importance for styrene-induced micronuclei frequencies and size ratios.

Subjects and methods

Production and exposure classification

The exposed workers of the study came from a plant producing tanks of glass-reinforced polyester plastics. The framework of the tanks consists of cylindrical tubes (diameter 600-2 500 mm) produced in a winding machine. Gables, tube flares, and other additional parts of the tanks are made by the spraying of glass and polyester compounds onto molds. Finally, the tanks are put together in an assembly hall. This part of the work includes hand-operated application of polyester. The polyester is dissolved in styrene, which has the double function of being a solvent and a reactive monomer. A peroxide initiates the polymerization reaction.

All the workers in the plant are exposed to styrene. In addition there is low-level exposure to acetone and methylene chloride, both of which are used for the cleaning of tools and skin.

The workroom air concentrations of styrene have been determined by personal sampling since 1974. Results from a total sampling time of 703 h are given in table 1. The mean sampling time per sample was 60 h. The mean concentration during the period 1985-1986 was 56 mg/m³. As the differences in the exposure

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levels between the different kinds of work have been small and there is a large rotation of work tasks among the workers, we have not tried to classify different work operations with regard to exposure levels.

Respiratory protection devices were used during spraying operations and during work with the polyester inside the tank, otherwise not.

For 43 urine samples collected immediately after work in October 1985, the mean excretion of the styrene metabolites mandelic acid and phenylglyoxylic acid was 128 (range <6–317) mmol/mol creatinine.

Subjects and sampling

All 18 process operators and two foremen in the glass-reinforced plastics plant, employed for at least one month, participated in the study. All but one were males. Their mean age was 40.5 (range 18–64) years, and their mean time of employment was 8.1 (range 0.1–25.4) years.

Twenty-two male workers from a factory producing gelatin, without any significant chemical exposure, served as a reference group. Their mean age was 42.3 (range 23–61) years. The referents were not exposed to any industrial air pollutants. They were mainly working with internal transport, as electricians, technicians, and process supervisors.

All the subjects were interviewed by a physician (LH) with regard to occupational and medical history, especially concerning smoking habits (cigarettes per day; 1 g of pipe tobacco being considered equivalent to one cigarette), viral infections, drug intake, radiographic examinations, and exposure to ionizing radiation during the last year.

None of the referents had been occupationally exposed to styrene or any other solvents. Six out of 20 exposed workers were smokers, with a mean consumption of eight (range 3–13) cigarettes per day. Eleven out of 22 referents were smokers, with a mean consumption of 12 (range 2–20) cigarettes per day. Of the exposed subjects 13 had had radiographic examinations during the last year, and three regularly took drugs (theophyllin, terbutalin, pidolol, insulin). Two of the referents had had influenza during the last year,

12 had been examined radiographically, and five had a regular drug intake (glibenclamide, digoxin, metoprolol, clonidine, alprenolol, prazosin).

Methods

Venous blood sampling. Venous blood samples were obtained from all the examined subjects between 0800 and 0900 on Monday mornings. Samples from all the subjects could not be cultured simultaneously. Thus the samples were taken on three consecutive Monday mornings. At each sampling time about half of the subjects were referents. All blood samples were coded and immediately transferred to the laboratory for analysis.

Micronucleus assay. Buffy coat leukocytes were cultured for 80 h in Roswell Park Memorial Institute (RPMI) 1640 medium with 15 % fetal calf serum (Flow), as described by Högstedt (13). Parallel cultures were set up with either PHA (Gibco; 1 ml/100 ml) or PWM (Gibco; 1 ml/100 ml). It has earlier been shown that the appropriate incubation time is the same for both mitogens (15). The cells were prepared according to the method described by Högstedt (13).

In the first step 1 000 lymphocytes from each individual were analyzed for the presence of intracellular micronuclei. In the second step, a number of cells corresponding to 10 micronuclei were scored, and the size of the cell nuclei and the micronuclei was measured according to the method described by Högstedt & Karlsson (14). The size of the micronucleus was expressed as the ratio between the surface of the micronucleus and the corresponding main nucleus of the cell. This procedure has been found necessary as the different phases of the cell cycle cause different sizes of both cell nuclei and micronuclei. In no case was more than 3 000 lymphocytes scored in the second step. We calculated the estimated frequency of micronuclei by adding the denominators and nominators, respectively, from both steps of the analysis. All scoring was performed by one observer. The interobservational precision of scoring micronuclei was very high; the correlation coefficient for the two steps of

Table 1. Levels of styrene, acetone, and methylene chloride in the workroom air measured by personal sampling during different years.

Year(s)	Samples (N)	Total sampling time (h)	Styrene ^a (mg/m ³)		Acetone ^b (mg/m ³)		Methylene chloride ^c (mg/m ³)	
			Mean	Range	Mean	Range	Mean	Range
1974	30	140	221	52–551	..	..	..	..
1976	9	49	43	14–90	..	..	..	..
1980–1981	11	71	76	20–134	..	..	..	..
1983–1984	7	49	115	88–120	..	..	..	..
1985	33	245	49	4–145	41	17–120	12	1–35
1986	28	149	68	18–164	45	15–137	14	0–48

^a Swedish occupational exposure limit = 110 mg/m³.

^b Swedish occupational exposure limit = 600 mg/m³.

^c Swedish occupational exposure limit = 250 mg/m³.

the analysis was 0.98 for PHA-induced lymphocytes and 0.96 for PWM-induced lymphocytes ($P < 0.001$ in both cases, Spearman's rank order correlation coefficient).

Chromosome aberrations in lymphocytes. Ten drops of whole blood were incubated at 37°C with PHA in 10 ml of medium containing RPMI 1640 and 15 % fetal calf serum. The incubation time was 48 h. Colcemid (0.1 µg/ml) was added 1 h before the harvest. Hypotonic treatment was carried out with potassium chloride (0.075 mol/l) for 15 min at room temperature, and the cells were fixed in methanol/acetic acid (3:1). The chromosome preparations were stained with Giemsa. For each individual, 100 metaphases were scored according to the classification system recommended by the International System for Human Cytogenetic Nomenclature (20). The results have been presented as "gaps" and "breaks" (comprising chromatid and isochromatid breaks, pericentric inversions, rings, and dicentrics).

Due to poor preparations and mitotic inhibition, slides from nine exposed subjects and seven referents were not scored. Five of the 11 exposed subjects examined and nine of the 14 examined referents were smokers.

Differential white blood cell counts. In each blood sample 10 000 cells were analyzed by a Technicon H-6000C[®] automated flow cytochemistry cell counter (21). The precision for this technique is very good, as shown in a previous study (22).

Statistical methods. For intraindividual comparisons of the frequencies and size ratios of micronuclei, between PHA and PWM cultures, Wilcoxon matched-pairs signed-rank test and the Pearson correlation coefficient were used. For interobservational variation of the micronuclei scoring, Spearman's rank order correlation coefficient was calculated.

The styrene-exposed group and the referents were compared with regard to the biological effect variables, age and smoking habits being allowed for in the multiple regression analysis. Furthermore, in a multiple regression analysis of the biological effect variables performed only for the styrene-exposed group, the importance of employment time in the plant was tested with age and smoking being allowed for.

As the frequencies of chromosome aberrations (range 0–5/100 cells) were very low, the average square root transformation $[(\sqrt{x} + \sqrt{x+1})/2]$ was applied to these counts in order to stabilize the variance (23). Graphic methods showed that the log-transformation was suitable for both the frequencies of micronuclei and the white blood cell counts. The individual geometric mean values of the size ratios for micronuclei were analyzed untransformed.

Significance levels of 5 % or less have been considered significant. All the statistical tests were two-tailed.

Results

With PWM stimulation the mean frequency of micronuclei, for all the examined subjects, was 6.5 ‰, compared to 4.4 ‰ with PHA. This difference was statistically significant ($P < 0.0001$; Wilcoxon matched-pairs signed-rank test). The correlation coefficient between the two log-transformed scores was 0.37 (Pearson's r , $P = 0.02$).

For lymphocytes stimulated with either PHA or PWM, no statistically significant difference was found in the frequencies of micronuclei between the exposed and reference groups when allowance was made for age and smoking (table 2). For the cultures stimulated with PHA, but not for those stimulated with PWM, the micronucleus frequencies showed a significant positive association with age ($P < 0.0001$). No association with smoking habits was found.

There was no significant difference in the individual mean micronucleus size ratios between the lymphocytes stimulated with PHA or PWM ($P = 0.29$ Wilcoxon matched-pairs signed-rank test). Furthermore, there was no correlation between the two scores (Pearson's $r = 0.15$, $P = 0.4$).

The individual mean micronucleus size ratios were not associated with exposure to styrene, irrespective of the mitogen used (table 2). For the cultures stimulated with PHA, but not for those stimulated with PWM, the size ratios showed a significant association with age ($P = 0.004$). No association with smoking habits was found.

Furthermore, no association between either the micronucleus frequencies or the size ratios and the length of employment was found for the exposed group.

There was no statistically significant difference for chromosome breaks between the exposed and reference groups when allowance was made for age and smoking (table 3). On the other hand, significantly ($P = 0.02$) more gaps were observed for the referents. No association between either the chromosome breaks or gaps and the length of employment was found for the exposed group.

The number of peripheral monocytes, but not that of any other leukocytes, was increased in the exposed group when it was compared with the reference group (table 4). The mean difference was 30 % and statistically significant ($P = 0.002$). No association between any leukocyte count and length of employment was found for the exposed group.

Discussion

The main result of this study was that workers exposed to styrene did not show an increase in micronucleus frequencies or size ratios, irrespective of whether PHA or PWM was used as the mitogen, or chromosome aberrations.

Table 2. Mean number of micronuclei and the individual mean values of the size ratios for micronuclei in lymphocytes stimulated with phytohemagglutinin (PHA) or pokeweed (PWM) in 20 styrene-exposed workers and 22 referents. The unadjusted 95 % confidence interval (95 % CI) is also given. The significance level for the numbers of micronuclei refers to the difference in the means after log-transformation and for the untransformed individual mean size ratios, in both cases adjusted for age and smoking.

Micronuclei	Styrene-exposed		Referents		P	Covariates (P-value)	
	Mean	95 % CI	Mean	95 % CI		Age	Smoking
PHA							
Frequency (‰)	4.3	3.5—5.4	4.4	3.5—5.6	>0.5	<0.0001	0.4
Size ratio	0.023	0.019—0.026	0.023	0.020—0.027	>0.5	0.004	0.4
PWM							
Frequency (‰)	5.9	5.0—6.9	7.0	6.1—8.1	0.07	0.4	>0.5
Size ratio	0.020	0.018—0.023	0.022	0.020—0.025	0.19	>0.5	>0.5

Due to technical problems, the scoring of chromosome aberrations was performed for only 26 of the 42 subjects. The loss was not systematic with respect to the exposed subjects and referents and thus did not introduce any bias.

In the present study the referents had significantly more gaps than the exposed workers. However, all the scores were low and within the “normal” limits of the laboratory. As the referents were not chemically exposed, the discrepancy in the number of gaps was considered a spurious finding.

The mean exposure levels of styrene in the air were considerably higher (477 and 197 mg/m³ in some of the previous “positive” studies (4, 6) in comparison with the exposure level of 56 mg/m³ of the present study. However, an increased number of chromosome aberrations (8) and micronuclei (9, 10) has been observed also for workers exposed to styrene levels in the same range (55, 55, and 101 mg/m³) as that of the present study. On the other hand, Mäki-Paakkanen (12) could not show an increase in chromosome aberrations, sister chromatid exchanges, or micronuclei in workers exposed to a mean level of 98 mg/m³ styrene. Thus no consistent dose-response relationship between styrene exposure level and cytogenetic end points have been demonstrated so far. Furthermore, no association between cytogenetic effects and length of exposure time has been found (12). This inability to demonstrate dose-response relationships may depend on the low precision of cytogenetic methods or the problem of identifying the relevant exposure parameter. The mean level of exposure is a very insensitive parameter. High occasional exposure peaks, which probably vary in both amplitude and frequency between different plants, may be of greater importance for cytogenetic effects than the time-weighted average level. In addition the age distribution of lymphocytes in peripheral blood may differ between subjects. Thus a blood sample with many young lymphocytes probably reflects a more recent exposure than a sample with many older lymphocytes. The relevant exposure parameter may therefore vary between different exposed subjects, and this possibility contributes to the difficulties in displaying dose-response relationships.

Table 3. Mean number of gaps and chromosome breaks in 11 styrene-exposed workers and 14 referents. The standard deviation (SD) and range are also given. The significance level refers to the difference in the means after average square root [$(\sqrt{x} + \sqrt{x+1})/2$] transformation, adjusted for age and smoking.

Chromosome aberration	Styrene-exposed			Referents			P
	Mean	SD	Range	Mean	SD	Range	
Gaps (%)	0.7	1.2	0–3	1.7	1.4	0–5	0.02
Breaks (%)	1.2	1.6	0–5	1.5	2.1	0–5	0.5

Table 4. Geometric mean differential counts of the white blood cells, and unadjusted 95 % confidence interval (95 % CI), for 20 styrene-exposed workers and 22 referents. The P-value refers to the difference in the means adjusted for age and smoking habits. The calculations were based on log-transformed dependent variables.

Leukocytes	Styrene-exposed		Referents		P
	Mean	95 % CI	Mean	95 % CI	
Neutrophils (10 ⁹ /l)	3.94	3.23–4.81	4.13	3.40–5.02	0.3
Eosinophils (10 ⁹ /l)	0.18	0.14–0.23	0.14	0.11–0.17	0.17
Basophils (10 ⁹ /l)	0.06	0.05–0.08	0.06	0.04–0.07	0.4
Monocytes (10 ⁹ /l)	0.47	0.40–0.55	0.36	0.31–0.41	0.002
Lymphocytes (10 ⁹ /l)	2.17	1.93–2.45	2.04	1.72–2.41	0.4
“Large unstained cells” (10 ⁹ /l)	0.08	0.06–0.10	0.07	0.06–0.09	0.3

It has previously been shown in vitro that the size distribution of micronuclei depends on the type of inducing agent (14, 24). In an in vivo study of piperazine-exposed workers we have also observed a different size distribution between the exposed and reference groups (15). However, no such difference could be found in the present study of styrene-exposed workers.

It is well established that there is an association between age and the frequency of micronuclei in PHA-activated lymphocytes (13, 15, 22). As in the previous study of piperazine-exposed workers, there was also an age effect on the size distribution in the present study. Thus it is apparent that not only the numbers of micronuclei, but also the sizes increase with age.

This age effect on size may depend on the increased lagging of sex-linked chromosomes by age (25).

The frequencies of micronuclei induced in vitro by X-rays and mitomycin C in lymphocytes activated with PWM were found to be significantly higher than in PHA cultures (16). This was also the case in the in vivo study of piperazine-exposed workers (15). We have suggested that the two mitogens activate lymphocyte subgroups of different sensitivity to mutagens. Such an effect was, however, not observed for the styrene-exposed workers.

Regardless of exposure the micronucleus frequencies in the PWM-cultured lymphocytes were significantly higher than in the PHA-activated cells. This difference cannot be attributed to a difference in variance (table 2). Therefore, it is likely that also the "normal" level of micronuclei is higher with PWM than with PHA.

It is well known that exposure to benzene may cause lymphopenia (17). On the other hand, workers exposed to other solvents have shown peripheral lymphocytosis (18, 19). In addition workers exposed to various chemicals, including organic solvents, in a chemical plant showed lymphocytosis, and also increased levels of eosinophils and basophils but not monocytes (22). The present study does not indicate that low-level exposure to styrene affects the lymphocyte cell count. It is not obvious whether the observed, marginal increase in monocytes is a spurious finding or reflects the styrene exposure. However, it should be emphasized that using the automated cytochemistry counting technique makes it possible to detect much smaller differences in the cell counts than older techniques allowed. The intraassay variation for monocyte counts has been shown to be less than 4 % (22).

It could be questioned whether or not an extended analysis of the leukocytes ought to be performed in all cytogenetic studies, including measuring the subgroups of lymphocytes before starting the cultures. The rationale for this procedure is that not only the type of mitogen may alter the composition of lymphocytes studied, but also different types of chemical exposures may have this effect in vivo. It is interesting to note that we not only found an excess of micronuclei in a piperazine-exposed group of workers, but also an increased number of lymphocytes (15). Furthermore, in a recent study of gasoline pump repair workers, we obtained the same results (Högstedt, unpublished results). It could not be ruled out that positive cytogenetic findings could be attributed to differences in the composition of the lymphocyte subgroups studied. Moreover, the exposure-related differences in the peripheral leukocyte cell counts do not necessarily reflect increases in the production of leukocytes. They may rather be the result of an altered distribution of white blood cells between different compartments of the body.

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