Sister Chromatid Exchanges and Micronuclei in Peripheral Lymphocytes of Shoe Factory Workers Exposed to Solvents

Marià Pitarque,[1] Alexander Vaglenov,[1,2] Maria Nosko,[3] Sonya Pavlova,[4] Vera Petkova,[4] Ari Hirvonen,[5] Amadeu Creus,[1] Hannu Norppa,[5] and Ricard Marcos[1]

[1]Grup de Mutagènesi, Departament de Genètica i de Microbiologia, Edifici Cn, Universitat Autònoma de Barcelona, Bellaterra, Spain; [2]National Centre of Radiobiology and Radiation Protection, Sofia, Bulgaria; [3]Department of Hygiene and Medical Ecology, Medical University, Sofia, Bulgaria; [4]Clinical Centre of Occupational Diseases, Sofia, Bulgaria; [5]Laboratory of Molecular and Cellular Toxicology, Department of Industrial Hygiene and Toxicology, Finnish Institute of Occupational Health, Helsinki, Finland

We examined sister chromatid exchanges (SCEs) and micronuclei (MN; cytokinesis-block method) in cultured peripheral lymphocytes from 52 female workers of two shoe factories and from 36 unexposed age- and sex-matched referents. The factory workers showed an elevated level of urinary hippuric acid, a biomarker of toluene exposure, and workplace air contained high concentrations of various organic solvents such as toluene, gasoline, acetone, and (in one of the plants only) ethylacetate and methylenediphenyl diisocyanate. The shoe factory workers showed a statistically significant higher frequency of micronucleated binucleate lymphocytes in comparison with the referents. This finding agreed with three preliminary MN determinations (each comprising 27–32 shoe workers and 16–20 controls) performed in one of the plants 2–5 years earlier. The shoe factory workers also had a lower average level of blood hemoglobin than the referents. In contrast, no difference was found between the groups in SCE analysis. Smokers showed significantly higher mean frequencies of SCEs per cell and high frequency cells (HFC) than nonsmokers. Aging was associated with increased MN rates and reduced cell proliferation. Polymorphism of the glutathione *S*-transferase M1 gene (*GSTM1*) did not affect the individual level of SCEs; but in smoking shoe workers an effect of the occupational exposure on the frequency of micronucleated cells could be seen only in *GSTM1* null subjects. The low prevalence of the glutathione *S*-transferase T1 (*GSTT1*) null genotype precluded the evaluation of the influence of *GSTT1* polymorphism. Our results show that the shoe factory workers have experienced genotoxic exposure, which is manifest as an increase in the frequency of MN, but not of SCEs, in peripheral lymphocytes. The exposures responsible for the MN induction could not be identified with certainty, but exposure to benzene in gasoline and methylenediphenyl diisocyanate may explain some of the findings. *Key words:* glutathione *S*-transferase M1, glutathione *S*-transferase T1, micronuclei assays, shoe factory workers, sister chromatid exchange assays, solvent exposure. *Environ Health Perspect* 110:399–404 (2002). [Online 11 March 2002]
http://ehpnet1.niehs.nih.gov/docs/2002/110p399-404pitarque/abstract.html

During the last few years, genotoxicity biomarkers have received considerable interest as tools for detecting human genotoxic exposure and effects, especially in health surveillance programs dealing with occupational exposure to chemical carcinogens. Currently, only cytogenetic end points in peripheral blood lymphocytes allow a reasonable epidemiologic evaluation of cancer predictivity. The largest databases are available for chromosomal aberrations (CA); high CA level has been associated with increased cancer risk (*1,2*). Evaluations have also been performed for lymphocyte sister chromatid exchanges (SCEs) and micronuclei (MN), but thus far these biomarkers have not been shown to predict cancer (*1,2*). These findings may reflect the relatively small databases, the comparatively young cohorts, and variation in techniques, which have made it difficult to standardize individual SCE and MN values obtained from different sources. In a few years, accumulation of more uniform data will make it possible to reassess the value of both SCEs and MN. In the meantime, SCEs and MN are used as biomarkers of genotoxic exposure, as simpler alternatives to CA analysis. In the present study, we applied these methods to monitor solvent-exposed workers from two Bulgarian shoe factories.

People employed in the shoemaking industry are at an increased risk of leukemia and nasal cancer (*3*), and an excess of mortality due to other types of cancer has also been reported (*4–6*). Workers in shoe and boot factories are exposed to a mixture of organic solvents, among which toluene and acetone are usually the most common. Neither of these solvents is considered a genotoxin or a carcinogen; the weight of evidence from human *in vivo* studies suggests that exposure to toluene does not cause somatic cell genotoxic damage (*7*), although this view has been questioned by recent studies of rotogravure printers (*8,9*).

The glues and gasoline used in shoe manufacture may contain benzene, which could be responsible for some of the cancers found in shoe workers. Benzene is a well-known clastogen that requires metabolic activation to be mutagenic. The genotoxic metabolites are also thought to play an important role in benzene myelotoxicity and leukemogenesis. The quinone metabolites of benzene can break chromosomes by inducing reactive oxygen species but may also act as aneuploidogens, causing microtubule disruption (*10,11*). Recent investigations have indicated that structural CA are increased in shoe factory workers exposed to benzene and toluene (*12,13*).

In a preliminary study of the genotoxic effects of occupational exposure to organic solvents, using the alkaline Comet assay, we did not detect any increase in DNA damage in cryopreserved peripheral blood mononuclear leukocytes from a group of women employed in the two Bulgarian shoe factories (*14*).

Here we report results of lymphocyte SCE and MN analyses in a larger group of shoe workers from the same plants. We also present MN data from three other cross-sectional studies conducted 2–5 years earlier than the last sampling. In all cases, the concentrations of toluene, gasoline, acetone, ethylacetate, and methylenediphenyl diisocyanate (MDI) in workplace air and the concentration of hippuric acid (HA) in the urine were determined. To evaluate whether genetically determined individual variations in xenobiotic metabolizing capacity modified individual susceptibility to the possible genotoxic effects of the occupational exposure, we examined the subjects for their glutathione

Address correspondence to R. Marcos, Grup de Mutagènesi, Departament de Genètica i de Microbiologia, Edifici Cn, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain. Telephone: +34-93-581-20-52. Fax: +34-93-581-23-87. E-mail: rmd@cc.uab.es

We thank T. Amador, A. Corral, and G. Umbert for their expert technical assistance. The help and advice of K. Mitrunen, S. Saarikoski, and H. Wikman in the genotype analysis are much appreciated.

This investigation was supported in part by the European Union (EV5V-CT92-0221), the Spanish Ministry of Education and Science (SAF95-0813; PM98-0179), and the Generalitat de Catalunya (SGR95-00512). We are grateful to the Universitat Autònoma de Barcelona for supporting M. Pitarque's stay at the Finnish Institute of Occupational Health and to the Spanish Ministry of Education and Culture, which granted a visiting research position for A. Vaglenov (SAB 1995-0689).

Received 2 November 2000; accepted 30 October 2001.

S-transferase M1 (*GSTM1*) and T1 (*GSTT1*) genotypes.

Materials and Methods

Subjects studied. We conducted four independent biomonitoring studies of Bulgarian shoe factory workers in 1992, 1993, 1995, and 1997. In the first three years, only workers from plant B (Gabrovo) were examined; the last survey also included subjects from plant A (Sofia). In 1997, we examined 33 workers from plant A and 19 from plant B. The control group consisted of 36 unexposed women from the administrative staff of these plants. The subjects represented about half of the staff in each factory and were chosen randomly. In general, the workers participated in the study only once, except for a few individuals who were included in two or three of the samplings. All subjects gave written informed consent and were interviewed for personal data, duration of employment, and smoking habits. The characteristics of the studied groups are summarized in Table 1. The subjects were all women, except eight control subjects and three exposed workers enrolled in 1992. In all cases, the numbers of smokers among the controls and the exposed subjects were adequately matched, except in the 1993 study, where the number of smokers was higher among the controls than among the exposed. In the shoe factory workers, the total duration of occupational exposure gradually increased during the follow-up period; the difference in the average time of employment in the plant between the first and the last sampling was 3 years.

Exposure measurements. We used special diffusive monitors (Hygitest Co., Sofia, Bulgaria) placed at breath level to obtain workplace vapor samples. The average concentrations of gasoline and methylenediphenyl diisocyanate (MDI) were measured by express linear-colorimetric method. We measured environmental levels of toluene, acetone, and ethylacetate with an infrared gas chromatography analyzer Miran 1B2 (Foxboro, MA, USA). We monitored these substances in workplace air according to the Bulgarian analytic protocols (*15*) and Bulgarian Health Ministry's Order (*16*). Because the administrative staff was expected to be exposed to very low environmental levels of organic solvents, compared with the exposed workers, and given the results of earlier studies (*17–19*), exposure values for control subjects included in this study were taken to be zero. The mean workplace levels (milligrams per cubic meter) of some organic solvents in the shoe factories are shown in Table 2, expressed as time-weighted average (8-hr TWA) values. It is evident that the shoe workers were exposed to a complex mixture of solvents. In most cases, the concentrations of these solvents clearly exceeded the limits recommended by European and American standards (*20–22*).

As a biomarker of toluene exposure, urine samples were collected in the end of the working day, also from the controls, and were analyzed for HA, according to a method previously described (*23*). As can be seen from Table 3, the shoe factory workers showed a marked increase in the mean HA levels, with a statistically significant difference from controls [$p < 0.001$; analysis of variance (ANOVA)] in each sampling. A significant relationship between HA concentrations in urine and toluene air levels was found in the entire population studied ($\beta = 0.651$, $p < 0.001$). The presence of phenol, the main urinary metabolite of benzene, was analyzed (*24*) in urine samples from 29 shoe factory workers and 16 controls included in the 1992 study. As there were no differences between these groups in urinary phenol levels (exposed: 0.12 ± 0.03 mmol/L; controls: 0.10 ± 0.02 mmol/L; detailed data not shown), the workers did not appear to be exposed to high levels of benzene. However, urinary phenol may not reliably indicate lower benzene exposures from gasoline, but these benzene levels may have genotoxic effects.

We analyzed the glues used in these plants in 1997 for benzene content, using a gas chromatograph with a mass-spectrometric detector (Hewlett-Packard 6890/5972A, Wilmington, DE, USA). We detected no benzene traces in these glues.

Blood collection. Heparinized venous blood samples were drawn from each donor, and hemoglobin values were measured in Bulgaria by standard methods. In the 1992, 1993, and 1995 studies, the blood samples were processed in Bulgaria, and blood cultures were set up in a few hours after sampling. To carry out the 1997 study, the blood samples were sent to Barcelona and were put in culture within 48 hr after the collection. Briefly, the blood samples were centrifuged at 170 *g* for 5 min, the plasma was removed, and RPMI 1640 culture medium (GIBCO, Eragny, France) was added to each blood pellet to attain its initial volume.

Sister chromatid exchanges. We performed the SCE assay only for blood samples obtained in 1997, following standard methodology (*25*). Briefly, two whole-blood lymphocyte cultures were set up for each donor, and the cultures were incubated covered from light for 72 hr in the presence of 5-bromodeoxyuridine (BrdU; final concentration 8 μg/mL). The cultures were treated with colcemid for the last 2 hr, and the cells were then harvested, treated with hypotonic solution (0.075 M KCl) for 20 min, and fixed three times with methanol-glacial acetic acid. Microscopic slides were prepared from the fixed cells by air drying, and the slides were stained by the fluorescence-plus-Giemsa procedure (*26*). We purchased BrdU and Hoechst 33258 from Eastman Kodak

Table 1. Characteristics of the exposed shoe workers and control subjects studied.

Year, group	No.	Age, years (mean ± SD)	Duration of employment, years (mean ± SD)	No. of smokers (%)
1992				
Controls	16	35.3 ± 7.9	—	8 (50.0)
Exposed	32	38.8 ± 9.4	9.7 ± 9.5	15 (46.9)
1993				
Controls	19	40.2 ± 7.8	—	12 (63.2)
Exposed	27	38.2 ± 8.0	11.1 ± 10.1	9 (33.3)
1995				
Controls	20	38.4 ± 10.3	—	10 (50.0)
Exposed	27	38.4 ± 8.3	12.3 ± 9.7	16 (59.3)
1997				
Controls	36	43.7 ± 8.2	—	16 (44.4)
Exposed	52	40.7 ± 8.8	12.8 ± 10.5	29 (55.8)

Table 2. Concentrations (milligrams per cubic meter) of some solvents in workplace air of two shoe factories.[a]

Year	Plant	Toluene	Gasoline	Acetone	Ethylacetate	Diisocyanate
1992	B	275 ± 123 (100–410)	746 ± 430 (422–1,477)	628 ± 363 (159–1,003)	—[b]	—[b]
1993	B	235 ± 64 (68–618)	1,334 ± 247 (450–2,800)	539 ± 252 (24–1,744)	—[b]	—[b]
1995	B	148 ± 104 (18–320)	300 ± 126 (150–600)	960 ± 955 (55–2,900)	93 ± 82 (7–360)	0.21 ± 0.07 (0.06–0.29)
1997	A	76 ± 11 (66–96)	457 ± 45 (392–526)	374 ± 17 (341–389)	—[b]	—[b]
	B	236 ± 125 (137–412)	468 ± 201 (283–723)	764 ± 189 (523–927)	246 ± 105 (164–400)	0.23 ± 0.08 (0.15–0.34)

[a]Measured during an 8-hr workshift, except for diisocyanate, which was measured during a 15-min exposure. Values shown are mean ± SD (range). [b]Not used.

(Rochester, NY, USA), and Giemsa from Merck (Darmstadt, Germany).

For each donor, we analyzed 50 metaphases (25/replicate) on coded slides to determine the frequency of SCEs per cell. The analysis was conducted by two scorers, one per replicate. We estimated the percentage of high frequency cells (HFC) for each individual using the pooled distribution of all SCE measurements. We defined HFC as cells with SCE number over the 95th percentile of the distribution of SCEs per cell in the whole population. We examined 100 metaphases to calculate the proliferation rate index (PRI) (*27*).

Micronuclei. Whole blood cultures for the cytokinesis-block MN test were established according to Surrallés et al. (*28*), with some minor adaptations to the specific conditions of the Sofia and Barcelona laboratories. In both cases, the cultures were incubated at 37°C for 72 hr and, at 44 hr after initiation of the cultures, cytochalasin-B (Sigma Chemicals, St. Louis, MO, USA) was added at a concentration of 6 μg/mL. The cells were harvested, treated with a mild hypotonic solution (0.075 M KCl) for 2–3 min, and gently fixed three times using methanol/acetic acid (5:1) solution. Air-dried preparations were made, and the slides were stained in a 10% (v/v) solution of Giemsa in phosphate buffer (pH 6.8) for 20 min.

We examined 1,000 binucleate cells for each subject on coded slides (the same scorer in Barcelona and Bulgaria), and the total numbers of MN and the frequency of binucleate cells with MN (BNMN) were scored. We scored 500 lymphocytes to evaluate the percentage of cells with 1, 2, 3, or 4 nuclei, and we calculated the cytokinesis-block proliferation index (CBPI) according to Surrallés et al. (*29*).

Genotype analysis. We used genomic DNA extracted from leukocytes by standard methods as a template in the *GSTM1* and *GSTT1* genotype analyses, as described elsewhere (*30*). Briefly, the *GSTM1* and *GSTT1* genotypes were determined simultaneously in a multiplex polymerase chain reaction (PCR) approach using β-globin specific primers in addition to the *GSTM1* and *GSTT1* specific primer pairs. The presence of the β-globin specific signal verified the proper functioning of the PCR reaction, whereas the absence of G*STM1* and *GSTT1* specific amplification products revealed the corresponding null genotypes.

Statistical analysis. For the cross-sectional studies performed in 1992–1997, all variables analyzed were compared with the normal distribution by the Kolmogorov-Smirnov test of goodness of fit. The distributions of our data did not significantly depart from normality ($p > 0.05$) in any case, so parametric tests were adequate for the statistical analysis.

For each independent study, the effects of various factors on HA, hemoglobin, BNMN, and SCEs were simultaneously assessed by the ANOVA. These factors were occupational exposure, smoking, and, if available, GST genotypes, and we considered the age of the subjects as a covariate. To determine the relationship between solvent exposure levels and both HA and hemoglobin data, we applied a multiple regression analysis in the whole population. To this effect, from those subjects sampled several times, only the latest results were included in the analysis. Because of the low prevalence of the *GSTT1* null genotype, *GSTT1* genotype was not included as an independent variable in the analysis. The results were analyzed using the CSS: STATISTICA/W (StatSoft, Tulsa, OK, USA) statistical package.

Results

As shown in Table 3, the hemoglobin values of shoe factory workers were reduced compared those of nonexposed women. This difference, apparently stemming from the occupational exposure, reached statistical significance ($p < 0.001$) in the ANOVA.

In 1992–1995, we used the MN assay to assess whether the occupational exposure of the shoe factory workers could exert some genotoxic effects. We observed significant increases in the mean frequency of BNMN of the exposed groups each year in comparison with the respective control groups (Figure 1). Especially noteworthy was the constant increase in the mean frequency of BNMN in the exposed groups from 1992 (38.00, SD 20.18) to 1995 (55.2, SD 17.1), whereas control subjects consistently showed similar basal mean values (from 18.6, SD 6.3 to 22.0, SD 10.0).

In the last study, carried out in 1997 and involving both factories, the MN assay confirmed our previous positive findings. Table 4 shows the MN and SCE results, separately for nonsmokers and smokers, among the controls and exposed workers. In the multiple ANOVA, occupational exposure (plant A, plant B, and controls) constituted a statistically significant ($p < 0.001$) source of variation for the BNMN values. The *post hoc* least squares difference test showed that workers of plant B had a clearly higher BNMN frequency ($p < 0.001$) than controls or plant A workers, but also plant A workers differed significantly ($p = 0.035$) from the controls. We detected no effect on cell-cycle kinetics, measured as CBPI, as a consequence of the solvent exposure.

In the SCE assay (Table 4), we were not able to detect any genotoxic effect related to occupational exposure. We found no correlation between MN and SCEs. The occupational exposure was associated with a slight decrease in PRI ($p = 0.048$).

Age was a significant covariate ($p = 0.006$) in the multiple analysis of variance of the 1997 BNMN data, reflecting an

Table 3. Urinary hippuric acid and blood hemoglobin levels in exposed shoe workers and control subjects (means ± SD).

Year, group (plant)	No.	Hippuric acid	Hemoglobin
1992			
Controls	16	0.53 ± 0.28	150.50 ± 9.97
Exposed (plant B)	32	3.05 ± 2.14	123.00 ± 9.56
1993			
Controls	14	0.91 ± 0.64	131.83 ± 13.59
Exposed (plant B)	26	2.97 ± 1.51	124.38 ± 11.72
1995			
Controls	20	0.34 ± 0.16	141.80 ± 8.76
Exposed (plant B)	27	1.56 ± 1.55	115.04 ± 8.99
1997			
Controls	36	0.48 ± 0.31	139.42 ± 12.44
Exposed (total plants A and B)	52	2.26 ± 1.63	124.06 ± 13.52
Exposed (plant A)	33	1.90 ± 1.05	128.64 ± 14.02
Exposed (plant B)	19	2.87 ± 2.22	116.11 ± 7.94

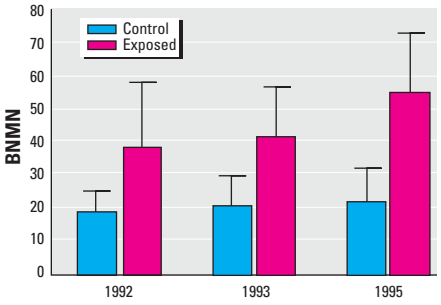

Figure 1. Mean (+ SD) frequencies of binucleate lymphocytes with BNMN in shoe factory workers of plant B and in unexposed controls (first three samplings). In the multiple analysis of variance, occupational exposure was a statistically significant ($p < 0.001$) source of variation for each sampling. In the 1995 collection, age ($p = 0.024$) and smoking ($p = 0.021$) were also significant variables. The numbers of subjects studied in 1992, 1993, and 1995 were, respectively, 32, 27, and 27 for the shoe factory workers and 16, 19, and 20 for the controls.

age-dependent increase in BNMN frequency. Age appeared to be partially associated with a decreased cell proliferation, as indicated by its significant effect on PRI (p = 0.001); CBPI was not affected by age. In the same analysis, smoking did not significantly affect BNMN frequencies in the 1997 study. However, both the mean frequency of SCEs per cell (p = 0.006) and the mean number of HFC (p = 0.004) were increased by smoking. This smoking-related effect was stronger among the controls than among plant A workers ($p < 0.05$ in both cases; t-test), but it did not attain statistical significance among plant B workers.

In agreement with reported frequencies for Caucasian populations, the prevalence of *GSTM1* and *GSTT1* null subjects in the 1997 study was 56.8% and 13.6%, respectively (Table 5). When the few *GSTT1* null subjects were removed for a subsequent ANOVA (including occupational exposure, smoking, and *GSTM1* genotype as independent factors and age as a covariate), neither the MN nor the SCE assay outcome was significantly affected by the *GSTM1* genotype. However, considering plant A and B workers together, the *post hoc* least squares difference test indicated a higher BNMN frequency in the exposed than the controls in *GSTM1* null subjects among both smokers (p = 0.006) and nonsmokers (p = 0.003), but in *GSTM1* positive subjects only among nonsmokers (p = 0.017). Tables 6 and 7 show the average of BNMN, CBPI, SCEs, HFC, and PRI values for *GSTM1* positive and *GSTM1* null subjects among the different subgroups.

Discussion

The present study showed an increased frequency of MN in peripheral lymphocytes of shoe factory workers exposed to a mixture of organic solvents. In plant B, where lymphocyte MN frequencies were followed up over 5 years, all four samplings yielded the same result. Although the shoe factory workers did not show any effects in the frequency of SCEs, HFC, or DNA breakage, as measured earlier by the Comet assay (*14*), our findings indicate the presence of genotoxic exposure at the workplace. This would appear to act via an aneugenic or clastogenic mechanism that does not involve DNA breakage readily detectable by the Comet assay and that does not efficiently lead to SCE formation.

In interpreting these findings, one must consider that although there are several examples of MN induction in human binucleated lymphocytes by known clastogenic exposures *in vivo*, evidence that the *in vivo* effects of an aneugen can be expressed in the first *in vitro* division of a binucleated cell is scanty (*31,32*). The binucleated cells that are exclusively analyzed in the cytokinesis-block MN assay have, by definition, completed their first *in vitro* mitosis, and most MN observed in such cells have been formed in the culture. Thus, an *in vivo* MN inducer would have to be able to initiate its effect in the resting G_0 lymphocyte. Further studies of the shoe factory workers using CA analysis or an MN assay incorporating pancentromeric fluorescence *in situ* hybridization or antikinetochore antibodies to identify the contents of the MN will be useful in identifying the nature of the exposure that produced the excess MN.

Although the glues used in the factories did not contain measurable amounts of benzene, there probably was benzene in the gasoline used as solvent. The air concentrations of gasoline were high, and benzene may well be the principal agent responsible for the observed genotoxic effects. In factory B, the levels of MDI (measured as diisocyanate) were very high and could explain some of the genotoxic effects. MDI is highly reactive and induced CA in human lymphocytes *in vitro* (*33*). MN frequencies were clearly higher in plant B than in plant A, and this could be caused by MDI, since diisocyanate was not found at all in plant A. Even in plant B, diisocyanate was not discovered during the first three sample collections. Thus, although it may not be possible to point out a single genotoxic agent at the workplace, benzene in gasoline and MDI may explain some of the findings. A possible indication of the heavy organic solvent exposure of the workers was the consistently lowered blood hemoglobin level, which we also observed in the previous study (*14*). Anemia and other hematologic abnormalities have been associated with organic solvent exposure in Bulgarian petroleum workers (*34*).

Several investigations have suggested an increased sensitivity of the *GSTM1* null genotype to genotoxicity induced by various exposures, including tobacco smoke (*35–47*). Such an association was not, however, unequivocally demonstrated in most of the few studies where SCEs or MN were used as biomarkers (*48–58*). Nevertheless, our MN results appear to support the increased sensitivity of the *GSTM1* null

Table 4. Mean (± SD) frequencies of BNMN and SCEs in lymphocytes of shoe factory workers of plants A and B and unexposed controls, according to smoking habit (last sampling).

	Micronucleus assay[a]			SCE assay[b]			
Group	No.	No. BNMN/1,000 cells	CBPI	No.	No. SCEs/cell	Percent HFC	PRI
Controls							
Nonsmokers	20	24.15 ± 7.49	1.89 ± 0.16	15	7.83 ± 1.04	2.67 ± 2.47	2.26 ± 0.24
Smokers	16	22.19 ± 8.45	1.87 ± 0.11	13	9.05 ± 1.73	8.37 ± 9.26	2.32 ± 0.12
Plant A workers							
Nonsmokers	15	31.13 ± 10.01	1.93 ± 0.08	15	7.59 ± 0.86	2.53 ± 2.45	2.15 ± 0.16
Smokers	18	25.28 ± 12.13	1.89 ± 0.09	18	8.47 ± 1.10	6.11 ± 5.29	2.28 ± 0.28
Plant B workers							
Nonsmokers	8	47.25 ± 9.10	1.85 ± 0.20	8	7.77 ± 0.80	4.25 ± 3.62	2.29 ± 0.10
Smokers	11	43.45 ± 13.47	1.95 ± 0.17	11	8.15 ± 1.34	5.17 ± 4.98	2.38 ± 0.10
Total							
Controls	36	23.28 ± 7.87	1.88 ± 0.14	28	8.40 ± 1.51	5.31 ± 7.05	2.29 ± 0.19
Plant A workers	33	27.94 ± 11.44	1.91 ± 0.09	33	8.07 ± 1.08	4.48 ± 4.55	2.22 ± 0.24
Plant B workers	19	45.05 ± 11.69	1.91 ± 0.19	19	7.99 ± 1.13	4.76 ± 4.37	2.34 ± 0.11

[a]BNMN/1,000 cells was affected by occupational exposure ($p < 0.001$) and age (a covariate; p = 0.006) in analysis of variance. [b]Smoking affected no. SCEs/cell (p = 0.006) and HFC (p = 0.004) in analysis of variance.

Table 5. Distribution of *GSTM1* and *GSTT1* genotypes among exposed shoe factory workers and control subjects in 1997.

		Controls		Exposed		Total	
Genotypes		No.	Percent	No.	Percent	No.	Percent
GSTM1 +	*GSTT1* +	13	36.1	19	36.5	32	36.4
GSTM1 +	*GSTT1* null	4	11.1	2	3.8	6	6.8
GSTM1 null	*GSTT1* +	16	44.5	28	53.9	44	50.0
GSTM1 null	*GSTT1* null	3	8.3	3	5.8	6	6.8

genotype, because in smokers an occupational exposure effect could be seen only among *GSTM1* null subjects. Although this finding is based on a few individuals, it could indicate, for example, that smoking induces GSTM1 activity in GSTM1-proficient individuals, which would protect them from the genotoxic effects of the occupational exposure. Smoking is not known to markedly induce GSTM1 in humans *in vivo*. At present, the possible effect of *GSTM1* genotype on MN formation is not well understood. Our previous studies suggested that the baseline level of MN is decreased in *GSTM1* null subjects (*55*).

In one investigation, the *GSTM1* null genotype was described to increase SCEs in smokers, although the difference to *GSTM1* positive smokers was quite small (0.23 SCEs/cell) and was statistically significant only in heavy smokers (0.53 SCEs/cell) (*48*). An increase of this magnitude may have been undetected in the present study. An effect of *GSTM1* polymorphism on the baseline level of SCEs in nonsmokers was reported by Cheng et al. (*59,60*), but this finding has not been reported in other studies (*52–54,57*), and it was not supported by our data.

In contrast, there is some evidence that the *GSTT1* null genotype is associated with an increased baseline frequency of SCEs (*61–64*) and possibly also CA (*54,65*). This finding does not appear to be explained by smoking or other known exposures. Unfortunately, we could not properly evaluate the effects of *GSTT1* polymorphisms because of the low frequency of the *GSTT1* null genotype.

In conclusion, our results indicate that the shoe factory workers studied have experienced genotoxic exposure, which is manifested as an increase in the frequency of MN, but not of SCEs, in peripheral lymphocytes. The workers also showed a reduction in blood hemoglobin values. Although high air concentrations of several organic solvents were found at the factories, the exposures responsible for the MN induction could not be identified with certainty. Exposure to benzene in gasoline and MDI may explain some of the findings.

Table 6. Mean (± SD) frequency of BNMN and CBPI in shoe factory workers and unexposed controls, according to smoking and *GSTM1* genotype (last sampling).

Group, smoking habit	Genotype	No.	No. BNMN/1,000 cells[a]	CBPI
Controls				
Nonsmokers	*GSTM1* null	7	22.43 ± 7.44	1.89 ± 0.21
	GSTM1 +	8	22.38 ± 4.81	1.89 ± 0.14
Smokers	*GSTM1* null	9	19.89 ± 8.34	1.84 ± 0.15
	GSTM1 +	5	25.20 ± 10.03	1.89 ± 0.03
Plant A workers				
Nonsmokers	*GSTM1* null	7	30.29 ± 11.24	1.91 ± 0.07
	GSTM1 +	7	31.29 ± 10.14	1.94 ± 0.08
Smokers	*GSTM1* null	11	27.82 ± 14.35	1.90 ± 0.07
	GSTM1 +	6	21.17 ± 7.19	1.91 ± 0.12
Plant B workers				
Nonsmokers	*GSTM1* null	4	47.50 ± 11.68	1.92 ± 0.13
	GSTM1 +	3	48.33 ± 8.62	1.73 ± 0.29
Smokers	*GSTM1* null	6	45.00 ± 13.83	2.00 ± 0.19
	GSTM1 +	3	33.33 ± 12.50	1.94 ± 0.20

[a] *Post hoc* least squares difference test indicated a higher BNMN frequency in the exposed (plants A and B together) than the controls in *GSTM1* null subjects among both smokers ($p = 0.006$) and nonsmokers ($p = 0.003$), but in *GSTM1* positive subjects only among nonsmokers ($p = 0.017$).

Table 7. Mean (± SD) frequency of SCEs, percentage of high frequency cells, and PRI in shoe factory workers and unexposed controls, according to smoking and *GSTM1* genotype (last sampling).

Group, smoking habit	Genotype	No.	No. SCEs/cell	Percent HFC	PRI
Controls					
Nonsmokers	*GSTM1* null	6	7.70 ± 1.05	3.33 ± 3.50	2.28 ± 0.17
	GSTM1 +	5	7.86 ± 1.08	2.40 ± 1.67	2.20 ± 0.38
Smokers	*GSTM1* null	7	8.89 ± 2.08	8.00 ± 11.83	2.27 ± 0.13
	GSTM1 +	4	8.76 ± 1.48	7.69 ± 7.55	2.38 ± 0.06
Plant A workers					
Nonsmokers	*GSTM1* null	7	7.49 ± 0.57	2.29 ± 2.14	2.14 ± 0.16
	GSTM1 +	7	7.84 ± 1.07	3.14 ± 2.79	2.17 ± 0.16
Smokers	*GSTM1* null	11	8.31 ± 1.01	6.00 ± 3.69	2.23 ± 0.34
	GSTM1 +	6	8.66 ± 1.38	6.33 ± 8.24	2.38 ± 0.13
Plant B workers					
Nonsmokers	*GSTM1* null	4	8.01 ± 0.86	6.00 ± 4.32	2.28 ± 0.14
	GSTM1 +	3	7.53 ± 0.94	2.67 ± 2.31	2.32 ± 0.06
Smokers	*GSTM1* null	6	8.45 ± 1.44	5.08 ± 5.71	2.38 ± 0.09
	GSTM1 +	3	7.05 ± 0.32	2.67 ± 3.06	2.31 ± 0.08

References and Notes

1. Hagmar L, Bonassi S, Strömberg U, Mikoczy Z, Lando C, Hansteen I-L, Huici A, Knudsen L, Norppa H, Reuterwall C, et al. Cancer predictive value of cytogenetic markers used in occupational health surveillance programs: a report from an ongoing study by the European Study Group on Cytogenetic Biomarkers and Health. Mutat Res 405:171–178 (1998).
2. Hagmar L, Bonassi S, Strömberg U, Brøgger A, Knudsen L, Norppa H, Reuterwall C, and the ESCH. Chromosomal aberrations in lymphocytes predict human cancer: a report from the European Study Group on Cytogenetic Biomarkers and Health (ESCH). Cancer Res 58:4117–4121 (1998).
3. IARC. Boot and shoe manufacture and repair. IARC Monogr Eval Carcinog Risks Hum Suppl 7:232–235 (1987).
4. Garabrant DH, Wegman DH. Cancer mortality among shoe and leather workers in Massachusetts. Am J Ind Med 5:303–314 (1984).
5. Walker JT, Bloom TF, Stern FB, Okun AH, Fingerhut MA, Halperin WE. Mortality of workers employed in shoe manufacturing. Scand J Work Environ Health 19:89–95 (1993).
6. Fu H, Demers PA, Seniori Costantini A, Winter P, Colin D, Kogevinas M, Boffetta P. Cancer mortality among shoe manufacturing workers: an analysis of two cohorts. Occup Environ Med 53:394–398 (1996).
7. McGregor D. The genetic toxicology of toluene. Mutat Res 317:213–228 (1994).
8. Hammer KD, Mayer N, Pfeiffer EH. Sister chromatid exchanges in rotogravure printing plant workers. Int Arch Occup Environ Health 71:138–142 (1998).
9. Pelclová D, Cerná M, Pastorková A, Vrbíková V, Procházka B, Hurychová D, Dlasková Z, Hornychová M. Study of the genotoxicity of toluene. Arch Environ Health 55:268–273 (2000).
10. Yardley-Jones A, Anderson D, Parke DV. The toxicity of benzene and its metabolism and molecular pathology in human risk assessment. Br J Ind Med 48:437–444 (1991).
11. Snyder R, Hedli CC. An overview of benzene metabolism. Environ Health Perspect 104:1165–1171 (1996).
12. Tunca BT, Egeli U. Cytogenetic findings on shoe workers exposed long-term to benzene. Environ Health Perspect 104:1313–1317 (1996).
13. Bogadi-Sare A, Brumen V, Turk R, Karacic V, Zavalic M. Genotoxic effects in workers exposed to benzene: with special reference to exposure biomarkers and confounding factors. Ind Health 35:367–373 (1997).
14. Pitarque M, Vaglenov A, Nosko M, Hirvonen A, Norppa H, Creus A, Marcos R. Evaluation of DNA damage by the Comet assay in shoe workers exposed to toluene and other organic solvents. Mutat Res 441:115–127 (1999).
15. Bulgarian Analytic Protocols. BDS 12430–74. Sofia, Bulgaria:State Agency for Standardization and Metrology, 1974.
16. Bulgarian Health Ministry. Decree 13/24.07.1992 for maximum allowable concentrations (MAC) of the pollutants in the working area. Sofia, Bulgaria:State Official Gazette 81:6–25 (1992).
17. De Bortoli M, Knoppel H, Pecchio E, Peil A, Rogora L, Schauenburg, Schlitt H, Vissers H. Measurements of indoor air quality and comparison with ambient air: a study on 15 homes in Northern Italy. EUR 9656 Environmental and Quality of Life Series. Luxembourg:Commission of the European Communities, 1985.
18. Krause C, Mailahn W, Nagel R, Schulz C, Seifert B, Ullrich D. Occurrence of volatile organic compounds in the air of 500 homes in the Federal Republic of Germany. In: Proceedings, 4th International Conference on Indoor Air Quality and Climate, Vol 1 (Seifert B, Esdorn H, Fischer M, Rüden H, Wagner J, eds). Berlin:Institute for Water, Soil, and Air Hygiene, 1987;102–106.
19. Foo SC, Phoon WO, Khoo NY. Toluene in blood after exposure to toluene. Am Ind Hyg Assoc J 49:255–258 (1988).
20. ACGIH. 1994–1995 Threshold Limit Values and Biological Exposure Indices. Cincinnati, OH: American Conference of Governmental Industrial Hygienists,1994.
21. Deutsche Forschungsgemeinschaft. Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area. List of MAK and BAT values 1995. Weinheim, Germany:VCH, 1995.

22. Widowo A. DEC and SCG Basis for an Occupational Health Standard. Gasoline. Arbete och Hälsa 7:65 (1992).
23. Pagnotto LD, Liberman LM. Urinary hippuric acid excretion as an index of toluene exposure. Am Ind Hyg Assoc J 28:129–134 (1967).
24. Bardodej Z. Benzteilung der Benzol und Phenol Gerärdung mit dem Phenol test. Arbeitsmed Socialmed Arbeitshyg 5:141–142 (1968).
25. Pitarque M, Carbonell E, Lapeña N, Marsá M, Valbuena A, Creus A, Marcos R. SCE analysis in peripheral blood lymphocytes of a group of filling station attendants. Mutat Res 390:153–159 (1997).
26. Perry P, Wolff S. New Giemsa method for the differential staining of sister chromatids. Nature 251:156–158 (1974).
27. Lamberti L, Bigatti Ponzetto P, Ardito G. Cell kinetics and sister-chromatid exchange frequency in human lymphocytes. Mutat Res 120:193–199 (1983).
28. Surrallés, J. Carbonell E, Marcos R, Degrassi F, Antoccia A, Tanzarella C. A collaborative study on the improvement of the micronucleus test in cultured human lymphocytes. Mutagenesis 7:407–410 (1992).
29. Surrallés J, Xamena N, Creus A, Catalán J, Norppa H, Marcos R. Induction of micronuclei by five pyrethroid insecticides in whole-blood and isolated human lymphocyte cultures. Mutat Res 341:169–184 (1995).
30. Hirvonen A, Saarikoski ST, Linnainmaa K, Koskinen K, Husgafvel-Pursiainen K, Mattson K, Vainio H. Glutathione *S*-transferase and *N*-acetyltransferase genotypes and asbestos-associated pulmonary disorders. J Natl Cancer Inst 88:1853–1856 (1996).
31. Norppa H, Luomahaara S, Heikanen H, Roth S, Sorsa M, Renzi L, Lindholm C. Micronucleus assay in lymphocytes as a tool to biomonitor human exposure to aneuploidogens and clastogens. Environ Health Perspect 101 (suppl 3):139–143 (1992).
32. Luomahaara S, Norppa H. Induction of micronuclei in cultured human lymphocytes treated with vinblastine before and after mitogen stimulation. Mutat Res 324: 29–34 (1994).
33. Mäki-Paakkanen J, Norppa H. Chromosome aberrations and sister chromatid exchanges induced by toluene diisocyanate and methylenediphenyl diisocyanate in cultured human lymphocytes. Toxicol Lett 36:41–45 (1987).
34. Hritseva-Mirtcheva V. Changes in the peripheral blood of workers with occupational exposure to aromatic hydrocarbons. Int Arch Occup Environ Health 71:S81–83 (1998).
35. Hirvonen A, Nylund L, Kociba P, Husgafvel-Pursiainen K, Vainio H. Modulation of urinary mutagenicity by genetically determined carcinogen metabolism in smokers. Carcinogenesis 15:813–815 (1994).
36. Grinberg-Funes R, Singh VN, Perera FP, Bell DA, Young TL, Dickey C, Wang LW, Santella R. Polycyclic aromatic hydrocarbon-DNA adducts in smokers and their relationship to micronutrient levels and the glutathione *S*-transferase genotype. Carcinogenesis 15:2449–2454 (1994).
37. Lambert B, Bastlova T, Österlund A-M, Hou S-M. Analysis of mutation at the *hprt* locus in human T lymphocytes. Toxicol Lett 82/83:323–333 (1995).
38. Hou S-M, Lambert B, Hemminki K. Relationship between *hprt* mutant frequency, aromatic DNA adducts and genotypes for *GSTM1* and *NAT2* in bus maintenance workers. Carcinogenesis 16:1913–1917 (1995).
39. Binková B, Lewtas J, Misková I, Rössner P, Cerná M, Mrácková G, Peterková K, Mumford J, Meyer Srám R. Biomarker studies in Northern Bohemia. Environ Health Perspect 104 (suppl 3):591–597 (1996).
40. Gabbani G, Hou SM, Nardini B, Marchioro M, Lambert B, Clonfero E. *GSTM1* and *NAT2* genotypes and urinary mutagens in coke oven workers. Carcinogenesis 17:1677–1681 (1996).
41. Scarpato R, Hirvonen A, Migliore L, Falck G, Norppa H. Influence of GSTM1 and GSTT1 polymorphism on the frequency of chromosome aberrations in lymphocytes of smokers and pesticide-exposed greenhouse workers. Mutat Res 389:227–235 (1997).
42. Topinka J, Binková B, Mrácková G, Stávková Z, Peterka V, Benes I, Dejmek J, Lenícek J, Pilcík T, Srám RJ. Influence of *GSTM1* and *NAT2* genotypes on placental DNA adducts in an environmentally exposed population. Environ Mol Mutagen 30:184–195 (1997).
43. Topinka J, Binková B, Mrácková G, Stávková Z, Benes I, Dejmek J, Lenícek J, Srám RJ. DNA adducts in human placenta as related to air pollution and to GSTM1 genotype. Mutat Res 390:59–68 (1998).
44. Butkiewicz D, Grybowska E, Hemminki K, Øvrebø S, Haugen A, Motykewicz G, Chorazy M. Modulation of DNA adduct levels in human mononuclear white blood cells and granulocytes by *CYP1A1*, *CYP2D6* and *GSTM1* genetic polymorphisms. Mutat Res 415:97–108 (1998).
45. Rojas M, Alexandrov K, Cascorbi I, Brockmöller J, Likhachev A, Pozharisski K, Bouvier G, Auburtin G, Mayer L, Kopp-Schneider A, et al. High benzo[*a*]pyrene diol-epoxide DNA adduct levels in lung and blood cells from individuals with combined *CYP1A1* MspI/MspI-*GSTM1**0/*0 genotypes. Pharmacogenetics 8:109–118 (1998).
46. Knudsen L, Norppa H, Gamborg MO, Nielsen PS, Okkels H, Soll-Johanning H, Raffn E, Järventaus H, Autrup H. Chromosomal aberrations induced by urban air pollution in humans: Influence of DNA repair and polymorphisms of glutathione *S*-transferase M1 and *N*-acetyltransferase 2. Cancer Epidemiol Biomarkers Prev 8:303–310 (1999).
47. Viezzer C, Norppa H, Clonfero F, Gabbani G, Mastrangelo G, Hirvonen A, Celotti L. Influence of *GSTM1*, *GSTT1*, *GSTP1* and *EPHX* gene polymorphisms on DNA adduct level and HPRT mutant frequency in coke-oven workers. Mutat Res 431:259–269 (1999).
48. van Poppel G, Verhagen H, van´t Veer P, van Bladeren PJ. Markers for cytogenetic damage in smokers: association with plasma antioxidants and glutathione *S*-transferase µ. Cancer Epidemiol Biomarkers Prev 2:441–447 (1993).
49. Carstensen U, Alexandrie A-K, Högstedt B, Rannug A, Bratt I, Hagmar L. B- and T-lymphocyte micronuclei in chimney sweeps with respect to genetic polymorphism for CYP1A1 and GST1 (class Mu). Mutat Res 289:187–195 (1993).
50. Ichiba M, Hagmar L, Rannug A, Högstedt B, Alexandrie A-K, Carstensen U, Hemminki K. Aromatic DNA adducts, micronuclei and genetic polymorphism for CYP1A1 and GST1 in chimney sweeps. Carcinogenesis 15:1347–1352 (1994).
51. Cheng T-J, Christiani DC, Xu X, Wain JC, Wiencke JK, Kelsey KT. Increased micronucleus frequency in lymphocytes from smokers with lung cancer. Mutat Res 349:43–50 (1996).
52. Scarpato R, Migliore L, Hirvonen A, Falck G, Norppa H. Cytogenetic monitoring of occupational exposure to pesticides: characterization of *GSTM1*, *GSTT1* and *NAT2* genotypes. Environ Mol Mutagen 27:263–269 (1996).
53. Sorsa M, Osterman-Golkar S, Peltonen K, Saarikoski Sram R. Assessment for exposure to butadiene in the process industry. Toxicology 113:77–83 (1996).
54. Srám RJ, Rössner P, Peltonen K, Podrazilová K, Mrácková G, Demopoulos NA, Stephanou G, Vlachodimitropoulos D, Darroudi F, Tates AD. Chromosomal aberrations, sister-chromatid exchanges, cells with high frequency of SCE, micronuclei and comet assay parameters in 1,3-butadiene-exposed workers. Mutat Res 419:145–154 (1998).
55. Falck G, Hirvonen A, Scarpato R, Saarikoski ST, Migliore L, Norppa H. Micronuclei in blood lymphocytes and genetic polymorphism for *GSTM1*, *GSTT1* and *NAT2* in pesticide-exposed greenhouse workers. Mutat Res 441:225–237 (1999).
56. Lucero L, Pastor S, Suárez S, Durbán R, Gómez C, Parrón T, Creus A, Marcos R. Cytogenetic biomonitoring of Spanish greenhouse workers exposed to pesticides: micronuclei analysis in peripheral blood lymphocytes and buccal epithelial cells. Mutat Res 464:255–262 (2000).
57. van Delf JHM, Steenwinkel M-JST, van Asten JG, de Vogel N, Bruijntjes-Rozier TCDM, Scouten T, Cramers P, Maas L, van Herwijnen MH, van Schooten F-J, Hopmans PMJ. Biological monitoring the exposure to polycyclic aromatic hydrocarbons of coke oven workers in relation to smoking and genetic polymorphisms for *GSTM1* and *GSTT1*. Ann Occup Hyg 45:395–408 (2001).
58. Yong LC, Schulte PA, Wiencke JK, Boeniger MF, Connally LB, Walker JT, Whelan EA, Ward EM. Hemoglobin adducts and sister chromatid exchanges in hospital workers exposed to ethylene oxide: effects of glutathione *S*-transferases T1 and M1 genotypes. Cancer Epidemiol Biomarkers Prev 10:539–550 (2001).
59. Cheng T-J, Christiani DC, Xu X, Wain JC, Wiencke JK, Kelsey K. Glutathione *S*-transferase: genotype, diet, and smoking as determinants of sister chromatid exchange frequency in lymphocytes. Cancer Epidemiol Biomarkers Prev 4:535–542 (1995).
60. Cheng T-J, Christiani DC, Wiencke JK, Wain JC, Xu X, Kelsey K. Comparison of sister chromatid exchange frequency in peripheral lymphocytes in lung cancer cases and controls. Mutat Res 348:75–82 (1995).
61. Kelsey KT, Wiencke JK, Ward J, Bechtold W, Fajen J. Sister-chromatid exchanges, glutathione *S*-transferase theta deletion and cytogenetic sensitivity to diepoxybutane in lymphocytes from butadiene monomer production workers. Mutat Res 335:267–273 (1995).
62. Norppa H, Hirvonen A, Järventaus H, Uusküla M, Tasa G, Ojajärvi A, Sorsa M. Role of GSTT1 and GSTM1 genotypes in determining individual sensitivity to sister chromatid exchange induction by diepoxybutane in cultured human lymphocytes. Carcinogenesis 16:1261–1264 (1995).
63. Schröder R, Wiebel FA, Reich S, Dannappel D, Bolt HM, Hallier E. Glutathione-*S*-transferase (GST) theta polymorphism influences background SCE rate. Arch Toxicol 69:505–507 (1995).
64. Wiencke JK, Pemble B, Ketterer B, Kelsey KT. Gene detection of glutathione *S*-transferase θ: Correlation with induced genetic damage and potential role in endogenous mutagenesis. Cancer Epidemiol Biomarkers Prev 4:253–259 (1995).
65. Landi S, Norppa H, Frenzilli G, Cipollini G, Ponzanelli I, Barale R, Hirvonen A. Individual sensitivity to cytogenetic effects of 1,2:3,4-diepoxybutane in cultured human lymphocytes: influence of glutathione *S*-transferase M1, P1 and T1 genotypes. Pharmacogenesis 8:461–471 (1998).