Biological Monitoring of Genotoxic Hazard in Workers of the Rubber Industry

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Biological monitoring of genotoxic hazard in the rubber industry was performed in 19 male workers and 20 age-matched controls in a local health unit in northern Italy. Peripheral blood lymphocytes were analyzed for the presence of DNA damage (single-cell microgel-electrophoresis, or comet assay) and for cytogenetic parameters (sister chromatid exchanges and micronuclei frequency, and proliferative rate index). The following bioassays were performed in urine samples: *a*) mutagenicity test and concentration of thioethers as markers of exposure, and *b*) excretion of D-glucaric acid and 6-β-hydroxycortisol (related to 17-hydroxycorticosteroid excretion) as indicators of the inductive status of the microsomal enzyme system (phase-I). The exposed subjects showed statistically higher mean values of 17-hydroxycorticosteroids and micronuclei and lower values of 6-β-hydroxycortisol than controls, when taking cigarette smoking into account. The comet assay showed higher values for migration distance in exposed subjects than controls, although the differences were not significant at a *p*-value of 0.05. These findings suggest that industrial exposure in the rubber processing industry may cause genetic damage and may modify the activity level of some enzymes; these results should be considered with caution due to the small number of subjects enrolled. — Environ Health Perspect 104(Suppl 3):543–545 (1996)

Key words: rubber industry, biomonitoring, comet assay, cytogenetic assay, urine D-glucaric acid, urine thioethers, urine mutagenicity, genotoxicity, cytochrome P450 enzymes

Introduction

A broad spectrum of chemical compounds belonging to many structural and use categories is employed during rubber processing (vulcanization agents, accelerators, activators, colorants, solvents, etc.), usually at high temperatures and pressures (*1*). Some of these compounds, such as 1,3-butadiene, have been shown to be genotoxic or carcinogenic (*2,3*) in both *in vitro* and *in vivo* experiments and epidemiological studies. An increase in the frequency of chromosomal aberrations and sister chromatid exchanges has been demonstrated in workers in the rubber industry (*4,5*). Furthermore, epidemiological investigations have shown an increased cancer risk arising from occupational exposure during styrene–butadiene rubber manufacturing (*6,7*).

With the aim of assessing the genotoxic hazard following rubber processing, we carried out a biological monitoring study involving male workers from the rubber industry and age-matched controls living in the same area.

Peripheral blood lymphocytes of exposed and control subjects were analyzed for the presence of DNA damage (single-cell microgel-electrophoresis or comet assay) and for cytogenetic parameters (sister chromatid exchanges [SCE] and micronuclei [MN] frequency, proliferative rate index [PRI]). The use of SCE and MN as markers of early biological effects is well established in biomonitoring studies, while the comet assay is a more innovative technique.

We have applied some bioassays in urine samples such as urinary mutagenicity as an aspecific marker of exposure to genotoxic compounds and urinary thioethers excretion as a marker of exposure to electrophilic compounds. In addition, we assessed urinary excretion rates of D-glucaric acid (DGA) and 6-β-hydroxycortisol (6β-OHC), which are well established noninvasive assays to monitor the inductive status of phase I enzymes in man because they reflect the activity of different but overlapping parts of the microsomal enzyme system.

Materials and Methods

Subjects

We enrolled 19 male rubber workers at four factories in the Local Health Unit No. 12 (Lovere) in north Italy and 20 age-matched (± 5 years) blood donors who lived in the same area as controls. The controls had no history of occupational exposure to chemicals. Each person was interviewed by a trained interviewer about possible confounding factors such as smoking habits, alcohol consumption, medicine intake, medical exposure to radiation. Urine and blood samples were collected at the end of the workday; in the exposed subjects, samples were taken on Wednesday. All subjects were healthy when giving biological samples and interviews.

Thioethers Excretion

Thioethers were extracted from the urine samples with ethyl acetate and hydrolyzed at 100°C with sodium hydroxide. The thiols (-SH groups) were then determined spectrophotometrically with the Ellman's reagent (*8*).

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Abbreviations used: DGA, D-glucaric acid; 6β-OHC, 6-β-hydroxycortisol; ELISA, enzyme-linked immunoadsorbent assay; 17-OHCS, 17-hydroxycorticosteroids; MN, micronuclei; MD, migration distance; PRI, proliferative rate index; SCE, sister chromatid exchanges.

D-Glucaric Acid Excretion

DGA excretion was determined with a low-pH enzymatic assay based on the conversion of DGA into its 1,4-glucaro-lactone and measurement of inhibition of 1,4-lactone against β-glucuronidase at pH 5 (*9*). DGA is a final product of carbohydrate metabolism produced via the glucuronic pathway, and its urinary excretion is increased by some compounds known to enhance microsomal enzyme activity (mainly cytochrome P450 reactions). Due to this parallelism, DGA is used as an indirect marker of enzyme induction.

6-β-Hydroxycortisol and 17-Hydroxycorticosteroids

The urinary 6β-OHC was measured by an enzyme-linked immunoadsorbent assay (ELISA) system (*10*). The urinary elimination of 17-hydroxycorticosteroids (17-OHCS) was measured by a colorimetric method (*11*). 6β-OHC is formed from cortisol via cytochrome P450. The extent of 6β-OHC formation also depends on the daily cortisol formation, the variation of which is compensated for by referring 6β-OHC values to excretion rates of 17-OHCS. The ratio of 6β-OHC to 17-OHCS assesses the status (induction or inhibition) of cytochrome 3A4 enzymes.

Urinary Mutagenicity

Cyanopropyl-bonded silica cartridges were used to adsorb mutagens, and a microsuspension–preincubation method was used to evaluate the mutagenicity of urine extracts (*12,13*). *Salmonella typhimurium* strains TA98 and TA100 were used, either in the presence or in the absence of an added metabolic activation system (100 µl of 10% S9 mix; Aroclor 1254-induced rat liver S9) (*14*) and either in the presence or in the absence of β-glucuronidase (120 U/plate).

Sister Chromatid Exchanges and Micronuclei Test

Whole-blood 72-hr cultures were set up for cytogenetic analyses in exposed and control lymphocytes. Culture procedures, cell collection, and staining of slides were done using routine methods (*15–17*). SCE frequency was summarized as the mean SCE per cell. The PRI was calculated in a total of 100 metaphases by determining the proportion of cells that undergo one, two, three, or more divisions (*18*). The induction of MN was evaluated by scoring a total of 1,000 binucleated lymphocytes with preserved cytoplasm for each subject.

Table 1. Mean ± SD of urinary excretion values of thioethers, D-glucaric acid, and 6-β-hydroxycortisol (related to 17-hydroxycorticosteroids) in workers in the rubber industry (exposed) and control subjects.

Groups	Thioethers[a]	D-Glucaric acid[a]	6-β-hydroxycortisol		
			6β-OHC[b]	17-OHCS[c]	6β-OHC/17-OHCS
All subjects					
Exposed (n=19)	16.10 ± 3.99	4.37 ± 0.95*	0.45 ± 0.20*	7.67 ± 3.13**	0.073 ± 0.06**
Controls (n=20)	16.11 ± 9.93	3.68 ± 1.65*	0.72 ± 0.39*	3.54 ± 2.39**	0.358 ± 0.36**
Smokers					
Exposed (n=9)	16.11 ± 2.94	4.33 ± 1.09	0.46 ± 0.20	8.24 ± 3.60	0.061 ± 0.03
Controls (n=8)	16.08 ± 7.99	4.12 ± 2.53	0.62 ± 0.33	4.33 ± 2.52	0.155 ± 0.09
Nonsmokers					
Exposed (n=10)	16.09 ± 4.98	4.39 ± 0.91	0.44 ± 0.21	7.10 ± 2.65	0.084 ± 0.08
Controls (n=12)	16.14 ± 11.40	3.39 ± 0.76	0.71 ± 0.43	2.96 ± 2.24	0.561 ± 0.43

Results are expressed as [a]mmole/mole creatinine, [b]ng/ml, [c]µg/ml. *p<0.05, **p<0.001 by the Mann-Whitney test.

Determination of DNA Damage (Comet Assay)

Isolated lymphocytes were obtained from 1 ml whole blood by lysis of erythrocytes (*19*). Cell viability was determined using the fluorescein diacetate/propidium iodide assay (*20*). For determination of DNA damage by comet assay, cell embedding and lysis, electrophoresis under alkaline conditions, and staining of the liberated DNA were performed according to the original method of Singh et al. (*21*). Photographs of representative areas of the slides were taken at 500 × magnification, and the migration length (µm) of at least 50 randomly selected cells was determined on the photographs.

Statistical Methods

The biomarker data were tested regarding approximation to normal distribution using the Shapiro-Wilk test, and SCE and MN frequencies were square-root transformed to achieve a better approximation. The Mann-Whitney rank test was used for comparison between the exposed subjects (factory workers) and controls, and the Spearman's rank correlation coefficient (r_s) was used for the analysis of correlation between biomarkers. The differences in mean values of biomarkers according to occupational exposure, age, and tobacco smoking were investigated using two-factors models with fixed effects of analysis of variance (ANOVA). The ex-smokers were included among current smokers if they had stopped smoking within 12 months of the study, and among nonsmokers otherwise. All the statistical tests were two-tailed at the alpha level of 0.05.

Results and Discussion

The exposed and control subjects were relatively young, the mean age being 33.1 (SD = 7.2) and 33.8 (SD = 7.5) years, respectively. The current smokers constituted 47.4% of the exposed subjects and 40.0% of the controls, and heavy smokers (15 or more cigarettes/day) constituted 21.1% of the exposed subjects and 25.0% of the controls.

No differences in urine mutagenicity were found between the exposed subjects and controls (data not shown). No biomarker was associated with age, and no correlation was found between the biomarkers except a faint, inverse correlation between DGA and 6β-OHC (r_s = –0.49).

At univariate analysis, the mean values of urinary DGA and 17-OHCS were significantly higher and 6β-OHC was lower in the exposed subjects than in controls; no difference was found regarding thioethers excretion (Table 1). The comet assay showed higher values for migration distance (MD) in exposed subjects than in controls, although the differences were not significant at a p-value of 0.05 (Table 2). MN frequency was significantly higher in the exposed subjects than in controls, whereas no difference was found for SCE (Table 3).

The exposed subjects who had worked 10 or more years in the rubber industry when interviewed did not show higher values of biomarkers than those who had worked for less time. Significant differences

Table 2. DNA damage (comet assay) among the rubber workers and unexposed subjects (controls).

Groups	MD (µm)	
	Mean ± SD	Median
All subjects		
Exposed (n=19)	43.08 ± 12.49	37.99
Controls (n=20)	38.92 ± 8.06	33.81
Smokers		
Exposed (n=9)	41.24 ± 12.04	35.88
Controls (n=8)	38.58 ± 9.83	33.38
Nonsmokers		
Exposed (n=10)	45.62 ± 12.95	39.88
Controls (n=12)	39.15 ± 7.11	34.10

Group data are reported as the mean ± SD and median values of the comet length (migration distance, MD). No correlation observed by the Mann-Whitney test.

Table 3. Sister chromatid exchanges, proliferative rate index, and micronuclei among the rubber workers and unexposed subjects (controls).

Groups	SCE[a]	PRI[b]	Micronuclei[c]
All subjects			
Exposed (n=19)	5.51 ± 0.82	2.37 ± 0.13	22.84 ± 15.82*
Controls (n=20)	6.06 ± 1.15	2.35 ± 0.09	13.74 ± 4.42
Smokers			
Exposed (n=9)	5.66 ± 0.92	2.40 ± 0.15	26.89 ± 21.36
Controls (n=8)	6.47 ± 1.13	2.37 ± 0.09	14.33 ± 4.03
Nonsmokers			
Exposed (n=10)	5.34 ± 0.74	2.35 ± 0.11	19.20 ± 7.98
Controls (n=12)	5.72 ± 1.13	2.32 ± 0.10	13.20 ± 4.89

Group data reported as the mean ± SD. Results expressed as: [a]SCE/metaphase; [b]proliferative rate index = $(M_1 + 2M_2 + 3M_3)/100$; [c]MN/1,000 binucleated cells. *$p<0.05$ by the Mann-Whitney test.

were found among the factories: the workers (n = 4) in one factory showed significantly higher mean values of thioethers, SCE, and comet assay than those at the other factories all together.

The association of smoking status was significant with SCE (p = 0.03) and at the limit (p = 0.06) with MN frequency among controls only. No analysis could be performed regarding the number of cigarettes smoked per day or the number of years of smoking because of the small number of subjects.

Finally, a series of two-way ANOVAs was performed, including each biomarker as an outcome variable and smoking status and occupational exposure as dichotomous independent variables. The exposed subjects showed lower mean values of 6β-OHC (p = 0.02) and higher values of 17-OHCS (p = 0.001) than controls, while no effect of smoking was found. Furthermore, MN frequency was found to be higher in the exposed subjects than in controls (p = 0.02), and only a faint effect of smoking was found (p = 0.06).

The preliminary results of this biomonitoring study suggest that workers in the rubber industry are exposed to compounds able to interfere with the DNA structure; this was shown by the higher MN frequency and the higher mean values of MD in the exposed subjects than in controls in the comet assay. The presence of mutagenic/carcinogenic compounds in the working environment have also been monitored using chemical methods (nitrosamines in the air) and biological tests (Ames test in the particulate and *Tradescantial* micronuclei test in the total air samples). The results are under study.

The results regarding biomarkers to estimate the induction of the microsomal enzyme system (DGA and 6β-OHC) are not consistent and deserve further investigation.

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