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Evaluation of chromosome aberration and micronucleus frequencies in blood lymphocytes of workers exposed to low concentrations of benzene

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ABSTRACT

The frequency of chromosome aberrations (CA) and micronuclei (MN) was investigated in the peripheral lymphocytes of workers occupationally exposed to low or very low concentrations of benzene. The study included 43 exposed workers (all males), namely 19 fuel-tanker drivers and 24 filling-station attendants, and 31 male subjects with no occupational exposure to the toxicant (controls). Benzene exposure was verified by means of environmental monitoring with passive personal samplers (Radiello®), and through biological monitoring, *i.e.* by measurement of urinary *trans*,*trans*-muconic acid, *S*-phenylmercapturic acid and benzene. The frequency of CA and MN in peripheral lymphocytes was determined according to standard procedures. Exposure to benzene was found to be significantly higher for fuel-tanker drivers (median 246.6 $\mu g/m^3$) than for filling-station attendants (median 19.9 $\mu g/m^3$). Both groups had significantly higher exposure than controls (median 4.3 $\mu g/m^3$). No increased frequency of CA and MN was observed in either fuel-tanker drivers or filling-station attendants compared with controls. In all subjects examined as a single group, the frequency of MN was significantly dependent on age. Only in the fuel-tanker drivers was the frequency of MN found to depend not only on age, but also on exposure to benzene. In conclusion, the frequency of MN, but not of CA, could be influenced by exposure to benzene concentrations of up to one order of magnitude lower than the threshold limit value (time-weighted average).

1. Introduction

Benzene is a chemical agent that can provoke toxic and carcinogenic effects in man after exposure to high concentrations [1]. In view of these characteristics, its use in industrial applications has long been strongly limited in western nations. However, occupational exposure to benzene concentrations considerably below the Threshold Limit Value–Time Weighted Average (TLV–TWA) of the American Conference of Governmental Industrial Hygienists (ACGIH), *viz.* 1600 $\mu g/m^3$, is still possible among workers in oil refineries, in the chemical and petrochemical industrial sectors, in cokeries, and in the fuel-transport and filling-station sectors [1–3]. Instead, in the general environment the presence of benzene is practically ubiquitous, largely due to vehicle-exhaust fumes, although cigarette smoking contributes a fair proportion in indoor environments [4]. Urban pollution by benzene affects the general population and in particular those who work in urban traffic, such as traffic wardens and public-transport drivers [5].

Benzene can induce clastogenic effects, as shown by the presence of chromosome aberrations (CA) and micronuclei (MN) in the peripheral lymphocytes of both experimental animals and humans [6,7]. An increased frequency of CA, correlated with benzene exposure in workers with occupational exposure to concentrations of this toxicant exceeding or equal to the above-cited TLV–TWA, has been reported in many studies, while only few studies have investigated this biomarker for exposure to lower concentrations, *i.e.* up to two orders of magnitude below this limit, and some of these studies found no increased CA frequency [8–11].

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Instead, studies that focused on the frequency of MN in workers exposed to benzene have reported conflicting results for exposure to concentrations near the TLV–TWA, and to lower concentrations [8,9,12–15]. Among the latter studies, Basso et al. [16] and Angelini et al. [17] observed an increased frequency of MN in refinery workers and traffic wardens exposed to benzene concentrations two orders of magnitude below the TLV–TWA, although in neither of these cases was this finding correlated with the level of airborne benzene. According to literature reports, therefore, the exposure–response relationship for genotoxic effects after exposure to low or very low concentrations of benzene, like those currently present in working and living environments, still has not been defined.

The aim of the present research was to study the possible genotoxic effects of benzene by determining the frequency of CA and MN in workers with occupational exposure to low or very low concentrations of benzene.

2. Materials and methods

2.1. Subjects

The study included two groups of male workers with occupational exposure to benzene (exposed workers), *viz.* 19 fuel-tanker drivers responsible for loading, delivering and unloading fuel (drivers) and 24 filling-station attendants working in filling-stations located in both urban and suburban areas of the city of Bari, Italy (attendants). Controls were 31 male workers with no history of occupational exposure to benzene or other xenobiotics, resident in the same geographical area, and matched with the exposed workers for age and smoking habits. The drivers carried out fuel-loading and -unloading operations in a closed-circuit system and were wearing gloves, but no mask. The filling-station attendants used fuel pumps with functioning vapor-recovery systems, but wore no individual protection devices. All participants gave prior written informed consent to take part in the study. All subjects were administered a questionnaire enquiring about personal data, current and previous jobs, smoking history, alcohol consumption, medical history, use of medicinal drugs and exposure to ionizing radiation over the previous 12 months, as well as possible exposure to non-occupational sources of benzene. Alcohol consumption was estimated in g/day on the basis of the data collected in each questionnaire on the individual consumption of alcoholic units. Exclusion criteria were therapy with ionizing radiation or chemotherapy, radiography in the previous 12 months, chronic disease or previous neoplasia and, for exposed workers, a job duration of less than 1 year as a fuel-tanker driver or filling-station attendant.

2.2. Assessment of exposure

2.2.1. Environmental sampling

Environmental exposure to airborne benzene was monitored in the exposed workers and in the controls, by use of passive personal samplers (Radiello®) containing an active-carbon cartridge. The samplers were worn by all subjects in the respiratory zone for 8 h, *i.e.* during the entire work-shift for the exposed workers, and during the period between 8 a.m. and 5 p.m. for the controls. After sampling, the Radiello® vials were preserved at +4 °C until the time of analysis. All analyses were conducted blind.

Analysis of the Radiello® vials was performed by gas chromatography combined with flame-ionization detection (GC-FID) after desorption of benzene from the activated charcoal with benzene-free carbon disulfide according to the modified NIOSH method [18]. The detection limit of this procedure for benzene was 3 μg/m^3.

2.2.2. Urine sampling

On the day of the environmental sampling, all the exposed workers and the controls provided a urine sample at the end of the working-day, for the determination of the biomarkers of exposure to benzene. Each urine sample was divided in two aliquots: 1 of 30 ml was used for the determination of *trans*,*trans*-muconic acid (*t*,*t*-MA) and *S*-phenylmercapturic acid (SPMA); it was kept in a sterile container without the addition of preservatives or stabilizers at −20 °C until the time of analysis. The other sample (10 ml) was used for the determination of benzene; it was immediately transferred to a pre-sealed 20-ml vial containing 4 g of NaCl, and preserved at +4 °C until the time of analysis. All analyses were conducted blind.

Urinary *t*,*t*-MA analysis was carried out by use of HPLC with UV detection (264 nm), after extraction by solid-phase extraction (SPE) (SAX column-Varian), following an analytical procedure described elsewhere [19]. The limit of detection (LOD) of this method was 10 μg/L.

The analytical determination of urinary SPMA was performed according to the application described in Sabatini et al. [20]. Briefly, after SPE and liquid chromatography separation, samples were analyzed by HPLC/electro-spray tandem-mass spectrometry (HPLC–ESI-MS/MS), operated in negative-ion mode, with isotope-labeled analogs as internal standards. This method meets all the required validation criteria. Its LOD was 0.20 μg/L.

The analytical determination of urinary benzene was carried out by headspace analysis with automated solid-phase micro-extraction (SPME), according to a method previously described by Barbieri et al. [21]. A gas chromatograph equipped with a split-splitless injector and coupled to a mass-selective detector was used for GC/MS analysis. An autosampler CTC Combi PAL system was combined with the GC/MS system for the SPME process. The LOD of the procedure, estimated by calculating the standard error of the intercept on the calibration curve, was 0.02 μg/L for benzene.

Analyses of urinary creatinine were performed with the DCA 2000®+ analyzer. The creatinine assay is based on the Benedict/Behre test, and was performed on the same urine samples used for the other analyses [22].

2.3. Cytogenetic biomarkers

Analyses of the frequency of CA and MN were done on heparizined peripheral blood samples obtained from all exposed workers and controls, immediately before the start of the environmental sampling. The blood samples were coded and transported at room temperature to the laboratories where they were cultured for the cytogenetic tests within 24 h of collection.

To analyze CA, 350 μl of whole blood were incubated in 5 ml of RPMI 1640 medium supplemented with 10% fetal bovine serum, 5 μg/ml of phytohaemagglutinin, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM of L-glutamine. All cultures were set-up in duplicate and incubated at 37 °C. The cultures were maintained for 48 h and cell division was interrupted by the addition of 0.2 μg/ml of colcemid during the last 2 h. Chromosome preparations were set-up and stained according to standard procedures. For each donor, at least 100 metaphases on coded slides were counted and scored blind in accordance with the International System for Human Cytogenetic Nomenclature for CA [23]. In the subsequent analyses, the total CA were considered, as well as chromosome breaks and chromatid breaks separately, while achromatic lesions (gaps) were not counted as CA.

Analyses of MN were made with the cytokinesis-block technique [24]. Peripheral lymphocytes were isolated by gradient centrifugation on Histopaque, cultured at a density of 2×10^6 cells in 5 ml RPMI 1640 medium supplemented with 15% fetal calf serum, 1% phytohaemagglutinin, 1 mM L-glutamine, and incubated at 37 °C in 5% CO_2 for 72 h. For each study subject, two different cell cultures were set-up. After 44 h of incubation, cytochalasin B was added at a final concentration of 6 μg/ml to block cytokinesis. At the end of the incubation time, cells were treated with a mild hypotonic solution and fixed according to standard procedures. Slides were stained with conventional May–Grünwald–Giemsa and scored blind with an optical microscope in accordance with standard criteria [25]. For each subject, 2000 bi-nucleated cells with well-preserved cytoplasm were examined, and the frequency of micronuclei was expressed as the total number of micronuclei per 1000 cells analyzed. Cell-cycle parameters were evaluated by means of the nuclear division index (NDI), calculated by scoring at least 1000 cells per series of cultures. The NDI was calculated following the formula $NDI = (M1 + 2M2 + 3M3 + 4M4)/n$, where $M1 - M4$ indicate the number of cells with 1–4 nuclei and n is the total number of cells scored [26].

2.4. Statistical analyses

Statistical analyses were performed with the SPSS program (version 14.0, Chicago, IL, USA). A value corresponding to one-half of the detection limit was considered to be below analytical detection. The normal distribution of all variables was assessed by the Kolmogorov–Smirnov test. Not normally distributed variables were analyzed by parametric tests after logarithmic transformation or by non-parametric tests. The level of significance was set at $p < 0.05$.

3. Results

There were no significant differences as regards general characteristics and lifestyle habits among the fuel-tanker drivers, filling-station attendants and controls (Table 1).

Exposure to airborne benzene during the work-shift was found to be significantly higher in the drivers than in the attendants ($p < 0.001$) and higher in the latter than in the controls ($p < 0.001$). Urinary *t*,*t*-MA, SPMA and benzene were also significantly higher in the drivers than in the other two groups, whereas the NDI and the frequencies of total CA, chromosome and chromatid breaks, and MN were not different among fuel-tanker drivers, filling-station attendants and controls (Table 2).

To study the relationship between cigarette-smoking, airborne benzene, biomarkers of internal dose, and genotoxic effect biomarkers, all subjects, regardless of group, were subdivided into smokers and non-smokers (Table 3). In the smokers group, urinary *t*,*t*-MA, SPMA and benzene values were found to be significantly