Sister chromatid exchanges in lymphocytes of petroleum retailers

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Abstract

Occupational exposure to petroleum vapour was assessed in workers employed in suburban petroleum retail outlets. Urinary output of thioethers provided a non-specific estimate of exposure to chemicals metabolised via a mercapturic acid pathway. Urinary d-glucaric acid (DGA) excretion was taken as an estimate of hepatic enzyme activity. Sister chromatid exchange frequency in lymphocytes was used as an indicator of genotoxic response to exposure. Workers were classified according to their employment at self service (where customers operate petrol pumps) or at driveway attended service stations (at which an employee operates the pumps), and according to exposure to cigarette smoke on the basis of urinary cotinine excretion. Prework and postwork urine samples of workers employed at driveway attended petrol stations contained more thioether than did those of self serve workers. When classified according to smoking behaviour there were no statistically significant differences, although thioether excretion tended to be higher in smokers than in non-smokers. Urinary DGA excretion was similar in the two exposure groups. Cigarette smokers excreted more DGA, however, than non-smokers. Sister chromatid exchange frequencies were higher in driveway attendants than in self serve personnel. When the influence of cigarette smoking was investigated there was a significant increase of sister chromatid exchange with combined exposure to petrol and cigarette smoking, but not with either factor alone. Correlation analysis showed that urinary cotinine concentrations were positively associated with urinary excretion of thioether and DGA, indicating that cigarette smoke induces the activity of hepatic enzymes

and acts as a source of substrates metabolised through a thioether pathway. In conclusion it seems that exposure to petroleum vapour causes increased sister chromatid exchange in circulating lymphocytes of cigarette smokers, possibly as a result of enhanced hepatic conversion of vapour components to reactive metabolites. Urinary thioether output does not clearly discriminate between workers exposed to different amounts of petroleum vapour at retail outlets.

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Petroleum fuels for vehicles are volatile, inflammable fuels made up of a mixture of hydrocarbons, together with additives such as lubricants, dyes, and rust inhibitors. The exposure of the general public during the normal course of maintaining and operating a motor car may be light, but a greater exposure may occur from occupational sources in certain industries. Exposure to petroleum products may take place at various sites during the manufacturing process and at the point of sale. The atmospheric concentrations of total hydrocarbons associated with many of these duties depend on particular operations and exposure locations.¹

Air samples have been collected from the breathing zone of service station personnel to measure exposure to petroleum and its components.^{2,3} Such ambient monitoring techniques fail, however, to take into account individual differences in exposure pattern and frequency, systemic absorption, and ultimate response. Biological monitoring of petroleum components, or their metabolites, or the measurement of indicators of biological effect such as hepatic enzyme induction or cytogenetic responses more accurately reflect individual exposure and absorption patterns.

Petroleum retailers, particularly those providing driveway services, including the operation of petrol pumps, may be at a greater risk of exposure to petroleum than other groups in this industry, due to their daily contact during fuel sales. Hepatic enzyme activity^{4,5} and the excretion of urinary thioethers⁶ (end products of metabolism via mercapturic acid pathways⁷) have previously been measured in

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petroleum retailers. Although biological monitoring has been applied to groups of workers exposed to petrol⁸⁻¹² few studies have attempted cytogenetic monitoring.¹³⁻¹⁵ These have concentrated on workers at petrochemical plants, as did Rosenberg *et al*¹⁶ in a study of sperm morphology.

This work describes studies undertaken to compare simultaneous measurements of urinary thioether and d-glucaric acid (DGA) output, and sister chromatid exchange frequencies in peripheral blood lymphocytes of workers at self serve and operator attended (driveway) retail petroleum outlets.

Methods

All reagents were obtained from Sigma Chemical Co unless otherwise indicated.

SAMPLE COLLECTION FROM PETROLEUM RETAILERS

Thirty two male and nine female employees aged 18 to 60 (mean (SEM) 34 (2)) from 18 South Australian suburban retail petrol outlets were recruited for this study. Nineteen were employed at self service petrol stations and 22 at driveway attended stations. All had worked as petrol pump attendants, garage mechanics, or both, for at least one year. During site visits, it was noted that ventilation was adequate in shop and office areas, but relied upon open doors and portable fans in workshops. Forecourt areas were generally open on three sides, with a canopy providing protection from rain.

Each subject gave informed consent and was asked to provide two urine samples during the same midweek working day; one sample before the start of the working shift and one at the end. Blood samples were collected by venepuncture on the same day as the urine sample. Urine samples were stored frozen until analysis. Duplicate lymphocyte cultures were initiated for sister chromatid exchange analysis from blood samples within five hours of collection. Prework and postwork urine samples were analysed for thioether and creatinine. Postwork samples only were analysed for DGA and cotinine. A questionnaire giving details of diet, medication, smoking, and employment was completed by each subject at the time of sample collection.

MEASUREMENT OF URINARY THIOETHER CONCENTRATIONS

Measurement of thioethers was adapted from van Doorn *et al*¹⁷ by the colorimetric method described by Ellman¹⁸ using 5-5'-dithio-bis(2-nitrobenzoic acid) (DTNB). Samples (2 ml) of urine were acidified with 50 μ l of HCl (4 M) and double extracted with 4 ml aliquots of ethyl acetate for 15 minutes. The ethyl acetate fractions were pooled and evaporated under nitrogen at 50°C, and the residue taken up in

2 ml distilled water. To 1 ml of urine extract was added 0.5 ml NaOH (4 M) and this was bubbled with nitrogen, capped firmly, and heated at 95°C for 60 minutes. After cooling on ice, 0.5 ml HCl (4 M) was added. Five minutes later, 2 ml 0.1 M phosphate buffer (pH 7.4) containing EDTA (1 mM) was added to 0.25 ml hydrolysed extract, followed by 0.3 ml 35 mM citrate buffer containing DTNB (0.4 mg/ml). The absorbance at 412 nm was read compared with distilled water within 30 minutes. Sample blanks were included to take urinary pigments into account. The absorbances were compared with those obtained from standard solutions of N-acetyl cysteine that had been taken through the preparatory steps. Endogenous thiol output was estimated using samples omitting the hydrolysis step. Concentrations of thioether ([hydrolysed]-[non-hydrolysed]) were expressed as mmol thioether/mol urinary creatinine.

MEASUREMENT OF URINARY CREATININE CONCENTRATION

Creatinine concentration was measured using the reverse phase high performance liquid chromatography (HPLC) method of Huang and Chiou¹⁹ as modified by Muirhead *et al*²⁰ in which 50 μ l of clear urine was added to 2 ml of mobile phase (NH₄H₂PO₄ (20mM):acetonitrile, 9:1). Of this, 10 μ l was injected onto a column packed with Partisil-10 SCx protected by a C₁₈ guard column. Samples were eluted at a flow rate of 1.5 ml/min and sample detection was by UV absorbance (254 nm). Peak heights were compared with those of standard solutions of creatinine.

SISTER CHROMATID EXCHANGE IN LYMPHOCYTES

Blood samples were centrifuged, cell pellets were washed three times in phosphate buffered saline (PBS) to remove chemicals that may have an effect on sister chromatid exchange in vitro and were made up to their original volume in PBS. Duplicate cultures were started by adding 0.5 ml of this to 4.5 ml of RPMI 1640 (Flow Laboratories) supplemented with 10% heat inactivated foetal calf serum (Commonwealth Serum Laboratories), additional l-glutamine (2 mM), phytohaemagglutinin M (Gibco, 50 μ g/ml), penicillin (100 IU/ml), and streptomycin sulphate (100 mg/l). Cell cultures were kept in the dark at 37°C under an atmosphere of 5% CO₂ in air for a total of 72 hours. Bromodeoxyuridine was added at 24 hours at a final concentration of 20 μ M. One drop of colcemid (0.02% w/v) was added to each culture for the final two hours. Cells were harvested, treated with hypotonic KCl for 15 minutes, and fixed in methanol/acetic acid (3/1 v/v). Cells were dropped on to slides and harlequin stained by the method of Perry and Wolff²¹ as modified by Block.²² Briefly, slides were flooded with citrate buffer and exposed to ultraviolet light (254 nm and 366 nm) for 30 minutes. Slides were rinsed with distilled water and soaked for 15

minutes in 10X standard saline citrate at 60°C. Slides were rinsed again and stained with 6% giemsa for eight to nine minutes. The staining pattern was studied by light microscopy. Sister chromatid exchanges in 20 second division metaphase cells were scored for each subject. Cells containing 40–60 chromosomes were included in the analysis and results expressed as sister chromatid exchange/chromosome.²³

MEASUREMENT OF URINARY COTININE CONCENTRATION

Urinary cotinine concentration was determined by extraction of cotinine from urine, followed by normal phase HPLC. To 3 ml urine was added 0.3 ml of HCl (4 M) and 3 ml CHCl₃. After five minutes extraction samples were centrifuged to give separate phases. After aspiration of the aqueous phase 2 ml of organic phase was transferred to a tube containing 2 ml HCl (0.1 M). Extraction was repeated and 1.5 ml of acid phase was transferred to a fresh tube containing 0.2 ml NaOH (4 M) and 1.5 ml mobile phase (CHCl₃: methanol containing 1% NH₄OH, 95:5). The aqueous phase was again removed and 25 µl was injected on to a silica column and eluted at a flow rate of 2 ml/minute. Sample detection was by UV absorbance (254 nm) with detector attenuation at 0.02 AUFS. Cotinine eluted after 2.8 minutes and nicotine after 3.6 minutes. Cotinine concentration was determined by measurement of peak height compared with standard solutions of cotinine (20–2000 ng/ml) that had been carried through the extraction procedure. Cotinine concentration was expressed as mmol cotinine/mol urinary creatinine.

MEASUREMENT OF URINARY D-GLUCARIC ACID CONCENTRATION

Urinary DGA concentration was determined by the method of Marsh²⁴ as modified by March *et al.*²⁵ and Latham.²⁶ To one of duplicate tubes containing 2 ml urine was added 0.4 ml of formate buffer (2 M, pH 3.3) and to the other was added 0.4 ml of tris buffer (1.75 M, pH 9.0). Tubes were capped and boiled for 60 minutes. After cooling on ice, formate and tris buffers were added to tubes in reverse order. To each tube was added 1.2 ml acetate buffer (2 M, pH 4.8). Aliquots (1 ml) of acid and alkali treated samples were transferred to fresh tubes containing 0.75 ml substrate (phenolphthalein glucuronide, 0.5 mM). To these were added 0.5 ml enzyme solution (β-glucuronidase, 500 IU/ml) and mixtures were incubated at 37°C for 30 minutes. Glycine buffer (3 ml at 2 M, pH 12.0) was added to each tube to stop the reaction and develop colour. Absorbance was read at 555 nm relative to water. Sample blanks were carried through the incubation step but glycine buffer was added before the addition of enzyme. Standard solutions of DGA were subjected to acid treatment only. The sigmoid standard curve was constructed

by plotting % inhibition against log DGA concentration, and was fitted to the data using a computer graphics package.²⁷ Equivalents of DGA in acid and alkali treated samples were estimated from the standard curve. Concentration of DGA in urine was obtained from the difference [acid treated]–[alkali treated], and expressed as mmol DGA/mol urinary creatinine. Because self reported smoking behaviour is liable to subjective bias or deception²⁸ volunteers in this study were classified as smokers when they had a postwork cotinine concentration of greater than 0.01 mmol/mol creatinine.

STATISTICAL ANALYSIS

The data were assessed for homogeneity of variance using Bartlett's test²⁹ followed by two way analysis of variance and Tukey's HSD. Pre and postwork comparisons were made with the Wilcoxon signed rank test and comparisons of self service and driveway groups with Student's *t* test. Relations between parameters were determined with Kendall's coefficient of rank correlation (*τ*). From individual correlation estimates were calculated partial correlations—these more reliably represent the relation between parameters—and the probability associated with these partial correlations.

Results

Figure 1 shows urinary thioether output in workers employed at self service and driveway attended service stations. There was no significant change in

Figure 1 Urinary thioether excretion of workers from self service (five non-smokers, 13 smokers) and driveway attended (seven non-smokers, 14 smokers) petroleum retailers (mean (SEM)). Open and shaded columns indicate prework and postwork values respectively.

Figure 2 Urinary d-glucuronic acid excretion of workers from self service (five non-smokers, 13 smokers) and driveway attended (seven non-smokers, 13 smokers) petroleum retailers (mean (SEM)).

Figure 3 Sister chromatid exchange frequencies in lymphocytes of workers from self service (five non-smokers, 13 smokers) and driveway attended (eight non-smokers, 14 smokers) petroleum retailers (mean (SEM)).

thioether output after compared with before work ($p > 0.05$). Both prework and postwork output of urinary thioether of driveway attendants (mean SEM) 5.55 (0.04) and 6.03 (0.85) respectively) were greater than those of self service operators (3.92 (0.50) and 4.26 (0.45) respectively). When classified according to smoking behaviour, smokers did not excrete significantly more thioethers than non-smokers ($p > 0.05$), although there was a tendency for this.

Urinary excretion of DGA (fig 2) was similar in both exposure groups (Student's t test, $p > 0.05$) with mean concentrations of 3.74 (0.37) mmol/mol creatinine in self service workers and 4.48 (0.40) mmol/mol creatinine in driveway attendants. When categorised according to smoking behaviour, urinary DGA was found to be higher in smokers than in non-smokers ($p < 0.05$) and was increased with exposure to petrol, although this was not statistically significant.

The mean sister chromatid exchange frequency in self service personnel (0.115 (0.005)) and driveway

attendants (0.141 (0.007)) were significantly different (Student's t test, $p < 0.05$). When subjects were grouped as cigarette smokers or non-smokers (fig 3) there was a significant increase of sister chromatid exchange frequency associated with the combination of exposure to petrol and with cigarette smoking although not with either factor alone ($p < 0.01$).

Correlation analysis was carried out to determine the relative effect of several parameters on sister chromatid exchange. The table lists partial correlations derived from Kendall's τ between parameters measured in these groups. It seems that urinary thioether concentrations at the end of a work shift were positively correlated with cigarette smoking ($p=0.019$), as was urinary DGA output, an index of hepatic enzyme activity, ($p=0.009$). Sister chromatid exchange correlated with age ($p=0.011$). No other correlations were significant, although there was a weak correlation between DGA output and sister chromatid exchange frequency ($p=0.096$), and a weak negative correlation between urinary cotinine concentration and age ($p=0.064$).

Partial correlations derived from Kendall's τ

	Age	Postwork thioether	DGA	Cotinine
Postwork thioether	-0.061			
DGA	-0.013	0.002		
Cotinine	-0.175	0.238*	0.274*	
Sister chromatid exchange	0.263*	0.139	0.150	0.057

* $p < 0.05$.

Discussion

This study has shown that workers exposed to petrol at driveway attended stations excreted a significantly greater amount of thioethers in urine than those at self service outlets. These results are qualitatively consistent with those of Stock and Priestly,⁶ who reported a greater output in smokers than non-smokers, together with a higher output of thioether in urine of exposed subjects after work compared with that before work. It has been noted in some industries at least, that urinary thioethers may accumulate during the working week if the thioethers are cleared slowly.³⁰ In the present study, workers collected their urine samples on a midweek working day, and morning thioether samples may have contained a significant quantity of thioether from the exposures of the previous working day. Work related increases in thioethers of exposed groups may have been masked by either the high prework thioether concentrations or the high degree of variation in the data. Also, differences in ambient temperature on sampling days, recent alterations in work practice (such as the widespread use of unleaded fuel), or day to day variations in output of thioether may account for this.

Exposure to petrol did not cause an increase in output of DGA. Previous work⁴ showed that salivary antipyrine clearance, an index of hepatic enzyme activity, was increased in petrol station attendants. This work also confirmed that exposure of rats to commercial petroleum at 5000 mg/m³ commercial leaded petrol for eight hours a day for three weeks caused an increase in microsomal enzyme activity (aminopyrine N-demethylase, ethylmorphine N-demethylase, aniline hydroxylase, and benzo(a)-pyrene hydroxylase). The reasons for this discrepancy are not clear, although cigarette smoking was found to have a profound influence on excretion of DGA. Dietary or other environmental factors may also affect output of DGA and contribute to within group variation.

The effect of induction of hepatic enzymes caused by cigarette smoking may be protective if the liver simply becomes more efficient at eliminating xenobiotics, or it may exacerbate toxicity if the liver produces more active metabolites after chemical exposure than the saturable detoxifying pathways (such as glutathione transferase) can keep pace with. In this case sister chromatid exchange frequency was unaffected by cigarette smoking or petrol exposure alone, but was increased with combined exposure. This suggests that cigarette smoking may cause a greater activity of hepatic enzymes and hence a greater degree of formation of reactive metabolites that induce sister chromatid exchange after exposure to petrol.

Correlation analysis showed that, although output of thioethers in smokers was not statistically different

from that of non-smokers, urinary cotinine concentrations were positively associated with postwork thioether concentrations. This suggests that cigarette smoking is the greater determinant of excretion of thioethers. The increase in thioether output in smokers may be due to two factors. One is that the components of cigarette smoke are excreted as thioethers. The other is that cigarette smoking induces the activity of hepatic enzymes responsible for detoxifying xenobiotics via a mercapturic acid pathway. Because urinary DGA concentrations were increased in smokers compared with non-smokers, this indicates that the induction of metabolism would at least partly explain this phenomenon. This interpretation is limited by the absence from the partial correlation analysis of a variable directly associated with exposure to petroleum, such as airborne vapour concentrations or urinary output of a specific component of petroleum, or a metabolite. It would be preferable to categorise groups as exposed or non-exposed on the basis of such an independent estimate.

Although the data on humans presented here indicate that the exposure to petroleum caused only weak effects in the thioether test (although somewhat stronger evidence of effects on sister chromatid exchange) these results were reflected more clearly in rats injected intraperitoneally with unleaded petroleum at up to 1.0 ml/mg body weight daily for five consecutive days.³¹ Dose dependent increases in both urinary thioether output and lymphocyte sister chromatid exchange frequency were found. Hepatic enzyme activity was not measured.

In conclusion, the use of the thioether test as an indicator of occupational chemical exposure is based upon its non-specific nature and the generality of its application. It may be potentially suitable as an index of exposure to a wide range of chemicals and chemical mixtures. For urinary thioether to be a suitable discriminatory test for such exposure, however, there is a need for rigorous control of sampling protocols to allow this differentiation to take place. The influence of cigarette smoking in particular must be taken into account when interpreting the thioether test. Further, the thioether test may be seen as a signal that chemical exposure *may* have taken place, and that more precise or specific techniques should be performed. Non-specific biological effect markers, such as sister chromatid exchange, may provide a more relevant estimate of health risks associated with exposure to chemicals. There remains an inference that in subjects with raised MFO activity, as a result of exposure to cigarette smoking or other xenobiotics, may be more at risk of cytogenetic damage which gives rise to sister chromatid exchange.

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