Glycophorin A Somatic Cell Mutation Frequencies in Finnish Reinforced Plastics Workers Exposed to Styrene


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Abstract

We have used the glycophorin A (GPA) in vivo somatic cell mutation assay to assess the genotoxic potential of styrene exposure in 47 reinforced plastics workers occupationally exposed to styrene and 47 unexposed controls matched for age, gender, and active smoking status. GPA variant erythrocyte frequencies (VF), reflecting GPA allele loss (VN/VN) and allele loss and duplication (VN/N) somatic mutations arising in vivo in the erythroid progenitor cells of individuals of GPA M/N heterozygous genotype, were flow cytometrically determined in peripheral blood samples from these subjects. Measurements of styrene exposure of the workers at the time of blood sampling showed a mean 8-h time-weighted average TWA8,8 concentration of styrene in the breathing zone of 285 mg/m³ (20 ppm; Finnish threshold limit value). Women in this high exposure group showed especially elevated VN/VN (adjusted geometric mean 8.5 versus 5.3 in control women; P = 0.020); this elevation was also significant if urinary MA+PGA of ≥1.2 mmol/liter was used as the basis of classification (adjusted geometric mean, 8.3; P = 0.030). The occupational exposure could not be shown to influence VN/VF. Cigarette smoking was associated with significantly elevated GPA VF among active smokers (P = 0.042 for VN/N and P = 0.028 for VN/N) and among active and ex-smokers combined (P = 0.014 for VN/N). Its influence on VN/VF was especially clear among active smokers in the control group (P = 0.005). An effect of smoking, nearly statistically significant, was also observed for the VN/VF of control ex-smokers (P = 0.055) and of all active and ex-smokers combined (P = 0.050). Thus, the two characterized chemical exposures experienced by this group of workers and controls appear to produce differential effects on the two independent classes of GPA variants enumerated in the assay. This result suggests that the genotoxicity of these agents is mediated, at least in part, by different genetic mechanisms. Styrene exposure is associated with a specific increase in GPA VN/N VF; these allele loss and duplication variants reflect predominantly somatic recombination mechanisms in erythroid progenitor cells. Tobacco smoke exposure in active and ex-smokers is also associated not only with an increase in VN/N VF but also with an increase in VN/N VF, reflecting the induction of GPA gene-inactivating mutations, including point mutations and deletions. This finding is consistent with a broad mechanistic spectrum of tobacco smoke genotoxicity associated with this complex mixture of chemical mutagens. Finally, there was no detectable effect of age on VN/VF; however, a highly significant (P = 0.0002) increase in VN/N VF with age, even after adjustment for other variables, was observed.

Introduction

Styrene is an important industrial chemical used as a plastic monomer in the manufacture of various elastomers, polymers, and resins (1). This compound is regarded as an indirect genotoxin and presumptive human carcinogen, exerting its action through metabolic conversion into styrene-7,8-oxide, a mutagenic epoxide (2). The clearest experimental indications of the genotoxicity of styrene derive from cytogenetic studies in human lymphocytes in vitro (3–5) and from in vivo experiments in rodents showing a slight but consistent induction of sister chromatid exchanges in various tissues (2, 6–8). The highest occupational exposures to styrene have been recorded in the reinforced plastics industry, where styrene is used as a reactive solvent for unsaturated polyester resins (1).
In this industry, styrene is the major air contaminant, although there are usually associated exposures to acetone, organic peroxides, glass fiber, and dust. Exposure to styrene occurs mainly via inhalation and, to a lesser extent, by absorption through the skin (9).

Styrene exposure in the reinforced plastics industry has been the focus of numerous genotoxicological biomonitoring studies, and many papers have been published on cytogenetic alterations in the lymphocytes of reinforced plastics workers. Positive findings have been reported mainly for chromosome aberrations, although several studies have reported negative results (2, 8, 10–13). The levels of sister chromatid exchanges and micronuclei in peripheral lymphocytes of reinforced plastics workers have usually not been elevated, with the exception of a few reports (14).

In addition to cytogenetic end points, a limited number of other biomarkers of genotoxic exposure have been studied in relation to human styrene exposure. Reinforced plastics workers have shown elevated levels of DNA single strand breaks (alkaline-labile sites; Refs. 15–18), unscheduled DNA synthesis (19), and styrene-7,8-oxide hemoglobin and N-guanine DNA adducts in their peripheral blood erythrocytes and lymphocytes (15, 18, 20–23). Two studies of in vivo somatic mutation at the HPRT locus in the lymphocytes of reinforced plastics workers have been reported; the first was considered to be inconclusive (12), and the most recent found significantly elevated levels of HPRT mutant frequencies in laminators compared to matched laboratory controls but not factory administrative employees (18).

In this study, we have used the GPA in vivo somatic cell mutation assay to investigate the potential genotoxicity to bone marrow cells of workers occupationally exposed to styrene. This assay is based on the autosomal GPA locus that codes for the erythroid lineage-specific cell surface sialoglycoprotein responsible for the M,N blood group. It uses immunolabeling and flow cytometry to enumerate, in peripheral blood samples, erythrocytes of GPA allele-loss phenotypes that reflect GPA locus mutations that have occurred in erythroid progenitors and pluripotent stem cells in the bone marrow (24–26). These events at the GPA locus presumably reflect a spectrum of mutational mechanisms, including point mutations, deletions/insertions/rearrangements, or gene inactivation as well as chromosomal events including loss and duplication and mitotic recombination. As we have reviewed previously (27), these somatic mutational mechanisms that give rise to the erythrocyte variants detectable in the assay are operative in the activation of proto-oncogenes and the inactivation of tumor suppressor and DNA repair genes involved in the molecular etiology of human cancer.

Previous studies have demonstrated elevated levels of GPA variants in cancer patients receiving chemotherapy with mutagenic agents (28–33), in individuals exposed to ionizing radiation (34–38), and in workers occupationally exposed to polycyclic aromatic hydrocarbons (39) and benzene (40). These studies have established that the GPA-based assay functions as an in vivo effect biomarker that responds to human exposures to chemical and physical mutagens (27).

The GPA-based assay offers several advantages in its application as a biological effect marker in epidemiological studies compared to lymphocyte-based cytogenetic or somatic mutation assays that require cell culture. Since GPA variants are directly enumerated from peripheral blood samples, the assay is relatively rapid and inexpensive to perform, thus permitting the analysis of the large numbers of samples required for population-based studies. It is also sensitive to a wide spectrum of genetic mechanisms leading to loss of expression of the gene (27) and, thus, should be responsive to a diversity of agents that exert their genotoxic effects through different mechanisms. The assay ultimately measures inactivating somatic mutations at the GPA locus in long-lived erythroid progenitor cells in the bone marrow; to the extent that these fixed mutations are persistent in these cells, the assay can provide a measure of cumulative genotoxic exposures. The inherent limitations of the assay result first from red blood biology; erythrocytes are terminally differentiated cells lacking a nucleus. Hence, variant erythrocytes cannot be clonally expanded or analyzed at the DNA level to molecularly characterize the nature of the mutation. Finally, since the assay requires M/N heterozygosity at the GPA locus, only about one-half of the individuals in a selected population can be analyzed using this method.

In a previous report, we reported an association between styrene exposure category and an elevation in GPA erythrocyte variants in a group of U.S. reinforced plastics workers (41). The interpretation of the results was, however, difficult because the worker categories were not directly comparable with respect to age, gender, and smoking history. The present report describes a second, larger study on the frequencies of GPA variants in a population of Finnish reinforced plastics workers and controls matched for these potentially confounding demographic variables.

Materials and Methods

Subject Selection and Blood Sampling. The exposed subjects were recruited from employee populations at 10 reinforced plastics plants in Finland (Table I). The plants ranged from small workshops of a few employees (plant E) to fairly large facilities (plant J). Items manufactured consisted of boats (plants A–J) and large containers, pipes, and boards (plant I). Work tasks included carpentry, centrifugal molding, cleaning and waxing, fastening metal parts on boat decks, glass cutting, manual lamination, low-pressure sack lamination, machine assembly, parts assembly and cleaning, resin mixing, rolling, and spray lamination. Individual information regarding age, gender, smoking history, health status, and employment history were obtained by questionnaire.

Control individuals, matched to the workers with respect to age, gender, and active smoking status, were recruited from an employee population at a research institute and at a university in Finland. Most of the controls were office workers, some worked in the health care organization or in the maintenance department at the institute, or were university students. Others were employed in a biological laboratory. Workers and controls reporting a history of therapeutic radiation or chemotherapy were excluded from the study, and care was taken not to include persons among the controls who might have been exposed to known genotoxic agents in their work. The study was conducted with the approval of the Institutional Review Boards of the Finnish Institute of Health and the University of California LLNL. All subjects provided informed consent prior to their participation in the study and collection of blood samples.

Peripheral blood samples were obtained concurrently over a 10-month period from 47 workers and 47 matched controls of M/N blood type required for the GPA assay. The samples were
collected by standard venipuncture into sodium heparin anticoagulant and stored at 4°C. The samples were encoded to assure individual anonymity and to provide for a blind study and then shipped in six lots in insulated containers with cold packs to maintain the samples at 4°C to LLNL (Livermore, CA) within one week of collection. Included in each lot was a fresh blood sample from a standard normal donor so as to provide a quality control check for each shipment as well as an internal control for the stability of the GPA assay during the course of the study.

Assessment of Exposure to Styrene and Other Chemicals. To assess exposure of the reinforced plastics workers to styrene, styrene concentrations in the breathing zone were determined for a total of 66 of the workers, of whom 42 participated in the study. Air samples were collected with diffusive samplers (3500 Organic Vapor Monitor; 3M, St. Paul, MN) and SKC charcoal tubes (SKC, Inc., Eighty Four, PA). Styrene was eluted from the sorbents with 2 ml of a mixture of carbon disulfide (30%) and dichloromethane (70%) containing 0.1 mg/ml 4-tert-butylcatechol as a stabilizer. Both the samples and external styrene standards (Fluka AG, Buchs, Switzerland) were assayed by capillary gas chromatography with flame ionization detection. The detection limit of air samples was 0.001 ppm (20 liters).

In addition, urine samples were assayed for the styrene metabolites MA and the sum of MA and PGA (MA + PGA) as described previously (9, 42). MA was determined from the PM urine samples of 83 workers, of whom 35 participated in the study. Air samples were collected with diffusive samplers (3500 Organic Vapor Monitor; 3M, St. Paul, MN) and SKC charcoal tubes (SKC, Inc., Eighty Four, PA). Styrene was eluted from the sorbents with 2 ml of a mixture of carbon disulfide (30%) and dichloromethane (70%) containing 0.1 mg/ml 4-tert-butylcatechol as a stabilizer. Both the samples and external styrene standards (Fluka AG, Buchs, Switzerland) were assayed by capillary gas chromatography with flame ionization detection. The detection limit of air samples was 0.001 ppm (20 liters).

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Concentrations of styrene-7,8-oxide were analyzed from the same breathing zone air samples as the styrene concentrations. The detection limit was 0.001 ppm (20 liters). The amount of methylketone peroxide added to the resin was 1% in hand lamination and 2% in spray application. Sampling for the measurement of the peroxide (43) was performed with aluminum oxide tubes (Alumina Tubes; SKC, Inc.). After elution with a solution containing 4-tert-butylcatechol in glacial acetic acid (30 mg/ml), the peroxide was determined by UV spectrometry. External standards were prepared from commercial methylketone peroxide solution in dimethylphthalate (33% Butanox 50; Akzo Chemical Ltd., Amersfoot, the Netherlands). The detection limit for the peroxide in air was 0.03 cm³/m³ (60 liters).

Acetone was used for the cleaning of lamination devices. The sampling and analysis of acetone vapors was done with charcoal tubes in a similar way as the sampling of styrene. Methylene chloride was not used in the facilities studied. Dust samples were collected as stationary samplings at the different work phases of the reinforced plastics production.

GPA Assay. Upon arrival at LLNL, the heterozygous GPA M/N phenotype of the samples was confirmed using commercial anti-M and anti-N typing sera (Ortho Diagnostics, Raritan, NJ). These samples were assayed within 1 week, and the analyses were performed on mixed batches of coded samples that were blinded with regard to worker/control status and exposure assessment.

The blood samples were fixed, immunolabeled, and assayed on a Becton Dickinson FACScan single-laser flow cytometer using the improved version of the GPA assay as described previously (25). Briefly, the samples were fixed using formaldehyde and SDS to yield formalin-fixed spherical erythrocytes. The cells were immunolabeled with fluoresceinated BRIC157, a GPAM-specific monoclonal antibody, with avidin-R phycoerythrin (CalTag Laboratories, South San Francisco, CA) to yield two-color (green and orange) fluorescence of normal cells. The cell populations were then analyzed on the cytometer for the frequency GPA M allele-loss variant erythrocytes. A rectangular gate in the forward scatter versus log side scatter distribution was used to restrict the fluorescence analysis to erythrocyte singlets. Cells were analyzed at a rate of 3000–4000 per second; a total of 5 × 10⁶ were examined for each analysis. Two classes of GPA variants are enumerated in the assay (24, 27); those of hemizygous 0/N phenotype (indicative of allele-loss mutations) defined as cells which display <1% of normal 6A7 fluorescence and BRIC157 fluorescence characteristic of a single GPA M allele, and those of homozygous N/N phenotype (indicative of allele-loss and duplication mutations) which have <1% of normal 6A7 fluorescence but display BRIC157 fluorescence at twice the level of the normal M/N cells in the sample and equal to that of N/N controls cells. The frequency per million, Vf, of each class of variant was calculated as the total number of events falling within each defined class divided by the total number of events in the sample.

Table I: Description of reinforced plastics plants and styrene exposure determinations for workers

<table>
<thead>
<tr>
<th>Plant</th>
<th>Annual resin consumption (metric tons)</th>
<th>No. of employees</th>
<th>Air styrene concentration mg/m³ (TWA, h)</th>
<th>Urinary metabolites (mmole/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean SD N</td>
<td>MA (PM)</td>
</tr>
<tr>
<td>A</td>
<td>80.0</td>
<td>23 (4)</td>
<td>183 60 6 6.6 1.9 4 4.7 1.4 4</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>ND†</td>
<td>13 (1)</td>
<td>148 106 6 3.0 2.9 6 1.8 2.8 6</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>26.5</td>
<td>20 (5)</td>
<td>215 177 5 6.6 7.0 5 2.0 1.9 5</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>110.0</td>
<td>55 (7)</td>
<td>146 54 13 3.1 1.5 12 1.6 0.8 12</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>3.3</td>
<td>3 (1)</td>
<td>405 69 3 0.4 ND ND 0 6.2 2.8 3</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>180.0</td>
<td>26 (8)</td>
<td>275 112 13 10.0 6.3 13 4.1 2.1 12</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>19.0</td>
<td>67 (3)</td>
<td>71 35 12 2.9 2.1 15 0.9 0.6 16</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>61.5</td>
<td>14 (1)</td>
<td>29 21 8 0.4 0.1 4 0.4 0.1 8</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>140.0</td>
<td>70 (10)</td>
<td>60 39 21 0.6 0.3 12 0.6 0.4 21</td>
<td></td>
</tr>
<tr>
<td>J</td>
<td>125.0</td>
<td>90 (7)</td>
<td>134 88 13 3.1 2.3 12 1.4 1.2 13</td>
<td></td>
</tr>
</tbody>
</table>

* Threshold limit value (TLV) for styrene in Finland is 85 mg/m³ (20 ppm; TWA₄₈₉₄).
† Urinary MA and MA + PGA reference value for unexposed persons in Finland is 0.2 mmol/liter action limit values, corresponding to the TLV, are 2.9 mmol/liter for MA and 1.2 mmol/liter for MA + PGA.
‡ In parentheses, the number of workers included in the present study.
§ ND, not determined.
region of the histogram divided by the total number of singlet cells analyzed.

The results of the GPA assay obtained for the six sequential blood samples from the standard donor indicated that each of the shipments reached LLNL in satisfactory condition and that the assay produced stable and reproducible data over the course of the study. The O/N and N/N V determined on the six samples ranged from 3.0 to 8.6 (median, 7.1; mean, 6.5; SD, 2.2) and from 6.6 to 13.0 (median, 9.3; mean, 9.5; SD, 2.1), respectively. These data are typical of the performance of the assay when applied to freshly drawn replicate samples from the same donor (25).

**Statistical Analyses.** Models of the effects of the individual variables of age, gender, smoking status, and styrene exposure on GPA O/N and N/N V were developed using linear regression and multivariate covariance analysis. In the analysis of covariance, adjusted least squares means were computed to balance the design with all covariates at their mean value (44). In the models, for the five cases of missing measurements of air styrene levels, values equal to the means determined for similar work tasks were used. Because the distributions of O/N and N/N V were skewed, geometric means were calculated on the basis of logarithmically transformed test variables O/N LN = ln (O/N V - 1.6) (the constant 1.6 was subtracted to normalize the distribution of the test variable) and N/N LN = ln (N/N V). The approximate normality of the univariate distributions and residuals were checked by Shapiro-Wilk statistics and plotting, using SAS univariate procedures. For the transformed N/N V, the residuals were not distributed normally, due to a single exceptionally low value (0.4) in an exposed woman. This single observation was omitted from the final analysis of ln (N/N V), which resulted in approximate normality of the residuals. Two-tailed Ps of \( p \leq 0.05 \) were considered as statistically significant. The statistical analyses were performed using SAS, PROC GLM (V. 6.05).

**Results**

The study population comprised 23 men and 24 women employed in the reinforced plastics industry with mean (± SD) durations of styrene exposure of 9.0 (8.1) years (range, 0.5–29 years) and 8.1 (5.3) years (range, 0.7–21 years), respectively (Table 2). Control subjects included 23 men and 24 women matched to the workers on the basis of age and smoking status, since these factors are known, or are suspected, to affect GPA V. These demographic characteristics were very similar in both groups and are summarized in Table 2.

The number of workers studied, the 8-h time weighted average (TWA) of styrene in the breathing zone, mean levels of urinary styrene metabolites MA and the sum of MA and PGA (MA+PGA) in each plant are summarized in Table 1. These styrene exposure and metabolite measurements were obtained for 42 of the 47 workers in the study. Among these workers, TWA, concentrations of styrene in the breathing zone ranged from 25.6 to 479 mg/m³ (6 to 114 ppm) with a median of 136 mg/m³ (32 ppm) and an arithmetic mean of 155 (SD, 117) mg/m³ (37 ppm; SD 28; Table 2). The concentrations of MA ranged from 0.4 to 16.9 mmol/liter with a median of 2.9 and an arithmetic mean of 4.4 (SD, 4.1) mmol/liter (Table 2), and the concentrations of MA+PGA ranged from 0.3 to 9.4 mmol/liter with a median of 1.3 and an arithmetic mean of 2.1 (SD, 2.1) mmol/liter (Table 2). These median and mean values of styrene exposure and metabolite measurements were very similar for the male and female workers (Table 2). The different measures of styrene exposure were highly correlated in the individual workers included in the study. The following linear correlation coefficients were obtained: styrene in versus MA (PM; \( r = 0.95, P < 0.0001 \)), styrene in versus MA + PGA (AM; \( r = 0.93, P < 0.0001 \)), and MA (PM) versus MA + PGA (AM); \( r = 0.86, P < 0.0001 \).

The airborne concentrations of styrene-7,8-oxide correlated with those of styrene but were approximately 1/100 of the corresponding styrene concentrations, ranging from 26 to 580 µg/m³ (5 to 118 ppb). Airborne peroxide concentrations varied from <5 µg/m³ in hand laminating to 560 µg/m³ in spray applications. Airborne acetone concentrations in tool cleaning varied from 8 to 1345 mg/m³. The concentrations of dust were generally low, but there were specific tasks where the occupational reference value of 10 mg/m³ was exceeded. For glass fiber mat cutting, the dust concentrations ranged from 0.1 to 0.3 mg/m³; for carpeting, from 0.4 to 1.7 mg/m³; for sawing, grinding, and sanding, from 0.2 to 13.9 mg/m³; for assembly, from 1.0 to 2.5 mg/m³; and for turning work, from 0.3 to 6.5 mg/m³. According to electron micrographs, the diameter of angular particles was 0.05–10 µm. Glass fibers were not observed in the dust samples.

The GPA O/N and N/N V determined in the 47 workers and matched controls are summarized in Table 3. No statistically significant differences were observed between the workers' and the controls' GPA V. There was, however, an effect of borderline significance for N/N V among all workers (\( P = 0.058 \)) and among laminators (\( P = 0.061 \)) compared to controls.
In a multivariate analysis of covariance model in which styrene exposure, gender, and smoking status (ever versus never) were treated as discrete independent variables and age as a continuous variable (Model 1, Table 4), no significant effect of exposure on \( \text{GPA}_{0/N} \) or \( \text{N/N}_{V_f} \) was seen overall among female or male workers. However, when the workers were classified into low- or high-exposure groups according to measured concentrations of urinary MA+PGA (0.1–1.1 mmol/liter versus ≥1.2 mmol/liter; multivariate Model 2, Table 4) or styrene in the breathing zone (1–84 mg/m\(^3\) versus ≥85 mg/m\(^3\); multivariate Model 3, Table 4), the multivariate analysis of covariance showed an elevated \( \text{N/N}_{V_f} \) in the high-exposure group, with a statistically significant effect (\( P = 0.036 \)) if the classification was based on styrene in the breathing zone. This statistical inference was stronger for women workers alone, classified either by urinary MA+PGA concentrations (adjusted geometric mean \( \text{N/N}_{V_f} \) of 8.3 versus 5.3 in control women; \( P = 0.03 \)) or styrene concentration (adjusted geometric mean \( \text{N/N}_{V_f} \) of 8.5; \( P = 0.02 \)). No statistically significant differences could be shown among men.

Styrene-7,8-oxide measurements were not available for all of the workers, but with the data available, no clear association of \( \text{GPA} \) variant cell frequencies with airborne styrene-7,8-oxide levels could be observed.
When never smokers, ex-smokers, and active smokers were examined separately, there appeared to be a styrene exposure-associated increase in mean N/N V1 among active smokers and in mean O/N and N/N V1 among never smokers and an exposure-associated decrease in mean O/N among active smokers (Table 5). In the multivariate analysis, these findings were not, however, statistically significant.

Cigarette smoking was a significant confounder in the study. As shown in Tables 4 and 5, there was a consistent trend toward higher O/N and N/N V1 in smokers and ex-smokers compared with never smokers. In the multivariate Model 1, when age, gender, and styrene exposure were controlled simultaneously, smoking (active smokers and ex-smokers combined) was a significant variable influencing O/N and N/N V1 (Table 4). Active smokers (controls and exposed combined) showed significantly elevated O/N and N/N V1 in comparison with never smokers (Table 5). An especially clear effect of smoking on O/N V1 was observed for active smokers in the control group (Table 5). When active smokers were classified in three categories according to the average number of cigarettes smoked daily (Table 4), an increase in N/N V1 was only observed for heavy smokers (> 20 cigarettes/day), while O/N V1 was significantly elevated among moderate smokers (10–19 cigarettes/day). The adjusted mean V1 of ex-smokers were similar to those of active smokers, but as ex-smokers were fewer in number than active smokers, statistically significant effects associated with previous smoking could not be demonstrated; O/N V1 of control ex-smokers were nearly significantly elevated (P = 0.055) over those of the control never smokers. Among smokers and ex-smokers, there were more unusually high O/N and N/N V1 than among never smokers (Fig. 1). No significant interactions were observed between styrene exposure and cigarette smoking (included in Model 3, Table 4, P = 0.23).

In the multivariate Model 1, subject age was shown to be a significant independent variable influencing N/N V1 (exponential increase, 1.3 per 10 years; P = 0.0002) but not O/N V1 (Table 4). Among women, the probability for a high N/N V1 increased at ages above 40; among men aged less than 30 years, N/N V1 was typically very low. Consistent with this multivariate model result, regression analysis of O/N and N/N V1 for all subjects in the study revealed age-related increases in N/N V1.

The results of this study, showing an increase of GPA N/N variant erythrocytes in peripheral blood samples of reinforced plastics workers confirm and extend our earlier results of styrene exposure-associated effects detectable with this assay (41) and provide further evidence for genotoxic exposure in this industry. Such exposure has been suggested by earlier studies demonstrating increased levels of chromosome aberrations.
DNA damage, and HPRT locus mutations among reinforced plastics workers with styrene exposure levels similar to those in the present study (2, 15–18). Styrene, an indirect genotoxin and the main air contaminant in reinforced plastics plants, has been proposed as the agent responsible for these observed genotoxic effects. The role of styrene is also supported by the styrene-7,8-oxide adducts observed in peripheral erythrocyte hemoglobin and leukocyte DNA of reinforced plastics workers (15, 18, 20–23). The statistically significant elevation of N/N V₃ was observed in workers who were, at the time of sampling, exposed to styrene levels at or above the present Finnish threshold limit value. Such an association to concurrently measured styrene levels suggests that workers in the high-exposure group, in comparison with the low-exposure group, had also experienced higher exposures during the time period critical for the formation of N/N mutations. Some of the effects could also have been due to direct exposure to styrene-7,8-oxide known to be generated from styrene when peroxides are added to the polyester resin in the presence of oxygen and light. The independent effect of styrene oxide could not be reliably demonstrated because airborne styrene-7,8-oxide levels followed the levels of styrene. As airborne styrene-7,8-oxide levels in the work places studied were typically only 1/1000 of those found concurrently for styrene (1), the influence of styrene-7,8-oxide on GPA V₃ was probably negligible, also considering that this reactive chemical may not have efficiently reached bone marrow cells. Recently, other contaminants, especially methylene chloride, have been suggested to be responsible for the genotoxic effects observed in reinforced plastics workers (12, 45). However, methylene chloride has not been used in the Finnish reinforced plastics industry and cannot, therefore, explain our results.

The selective increase of GPA N/N V₃, but not 0/N V₃, observed in this study of styrene-exposed workers, has implications regarding the genotoxic mechanisms associated with this exposure. This phenotypic class of allele-loss and duplication GPA variants presumably arises from the contributions of several chromosomal genetic mechanisms including mitotic recombination, chromosome loss and duplication, and gene conversion affecting the autosomal GPA locus (27). The molecular analysis of human peripheral blood T-lymphocyte HLA-A locus mutants arising in vivo has shown that allele-loss and duplication mutations at this autosomal locus arise predominantly by the mechanism of mitotic recombination (46). If this result can be extrapolated to the GPA locus, these data suggest that styrene or its active in vivo metabolites are recombinogenic in bone marrow erythroid progenitor cells. In this respect, these results mirror the recent findings of Rothman et al. (40), who reported a specific association of GPA N/N V₃ with lifetime cumulative occupational exposure to benzene. These results are of interest in light of the fact that benzene is weakly or non-mutagenic in most mutation testing systems but induces chromosomal aberrations in human lymphocytes in vitro and in vivo and has been associated with leukemia in occupationally exposed populations (47). Together these studies demonstrate that mutation assay systems based on autosomal loci, like GPA, are important tools in monitoring human exposures to genotoxic agents that act through these chromosomal mechanisms.

The 94 GPA M/N subjects of the present study were drawn from a larger population of 172 styrene-exposed workers and 125 controls who have been studied previously for various cytogenetic end points. In this larger group, with mean TWAₚₘₜ concentrations of styrene in the breathing zone of 162 mg/m³ (39 ppm) among laminators and 58 mg/m³ (14 ppm) among other workers and mean PM urinary MA concentrations of 4.3 and 1.7 mmol/liter, respectively, no significant effects of styrene exposure were apparent on the frequency of chromosome aberrations, sister chromatid exchanges, or micronuclei in peripheral blood lymphocytes (48, 49). The positive GPA locus mutation results obtained in the present study of a subset of these workers suggests that this somatic mutational end point may be more sensitive to the genotoxic effects of this exposure than are these cytogenetic end points. In comparison with lymphocyte cytogenetics, GPA mutations, some of which are probably formed in long-lived bone marrow stem cells, may reflect cumulative effects of exposure acquired over a longer period of time. In parallel with the findings of the present GPA-based study, in the larger population cigarette smoking increased the levels of chromosome aberrations and sister chromatid exchanges, whereas micronuclei, scored only among nonsmokers, increased significantly with age.

The data obtained in the present study provides the first evidence for increased GPA O/N and N/N V₃ in smokers. This finding is consistent with the demonstration of elevated HPRT locus mutation frequencies measured in peripheral blood T-lymphocytes in some, but not all, studies of smoking populations. Seven studies have reported significant increases in HPRT mutant frequencies in cigarette smokers (50–56), whereas three others have shown no effect (57–59). For N/N V₃, the impact of smoking could be seen particularly among heavy smokers, suggesting a dose-related effect.

The cigarette smoking-associated increase in both classes of GPA variants suggests that exposure to the complex mixture of tobacco smoke mutagens results in the induction of somatic mutations by a wide spectrum of locus-specific gene-inactivating and chromosomal allele-loss and duplication mechanisms in erythroid progenitor cells. The levels of GPA V₃ observed among ex-smokers in this study were as high as those seen in the active smokers of the population, suggesting the mutational effects of tobacco smoke exposure in this population may be persistent. This finding is in accordance with a recent observation of ours showing elevated levels of chromatid breaks in lymphocytes of ex-smokers in comparison with never smokers (60). Thus, cigarette smoke exposure appears to produce long-lasting genotoxic effects detectable with these biomarkers, which may partly reflect the fact that exposure to the components of cigarette tar in the lungs continues for some time, even after smoking cessation. The chromosome aberration findings could also be explained by long-lived circulating lymphocytes in the peripheral blood. A probable explanation for the persistent elevation of GPA V₃ is that mutations have been stably induced in long-lived bone marrow stem cells.

Caution should be exercised however in ascribing a direct causal relationship between tobacco smoke exposure and induction of these biomarkers. Tobacco use was self-reported in the subjects of this study, and these active and ex-smokers may both differ from never smokers in other important lifestyle factors that could affect the level of these end points. Population surveys have demonstrated correlated lifestyle/demographic differences between smokers and nonsmokers; it has been documented, for example, that smokers have lower intakes of vitamins A and C and folate, consume more alcohol, and tend to be of lower socioeconomic status than nonsmokers (61).

Finally, the age-related increase of GPA N/N V₃ seen in this population is similar to that we have observed in studies of unexposed populations of newborns and adults (25, 62). A smaller effect of age on 0/N V₃ was observed in these studies.
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as well; this effect was not apparent in the present study, most likely due to the smaller size and more restricted age range of this population. These increases observed at the GPA locus of erythrocytes are similar to the age-related increases in mutant frequencies at the HPRT and HLA-A loci in peripheral blood T lymphocytes (46, 50, 52, 55, 56, 63).

As in the application of other genotoxicological end points, the results of this study illustrate that the use of the GPA assay requires careful matching of exposed and control groups with respect to age and smoking status, and proper statistical analysis to provide maximum power to detect subtle elevations of V, resulting from low-level occupational or environmental exposures. In the present study, the careful matching achieved was partly broken by the finding of the effect of previous smoking. Statistical analyses are complicated by the fact that the distributions of untransformed GPA V are skewed, and exceptionally low and high “outlier” V are occasionally observed. To achieve normal distributions, logarithmic transformation was applied in the present study. The models based on this transformation explained, at their best, 25% of the variation in the results. For N/N V, a smoking 46-year-old female laminator showed an exceptionally low value; significance testing by multiple regression could be performed properly only by omitting this single value. When this observation was included in the analysis, the Ps obtained were higher, and the effect of exposure and smoking could not be shown to be significant, although only small changes occurred in the adjusted mean V. Our conclusions on N/N V were further supported by multiple logistic regression, which showed significant associations of N/N V with the occupational exposure and smoking, when N/N V was classified in two or three levels, according to the 90th percentile (emphasis on high values) or upper and lower quartiles, respectively.

The statistical power of the GPA-based mutation biomarker to distinguish a population of exposed subjects from unexposed matched controls is fundamentally determined by the magnitude of the effect of the given exposure on the level of GPA erythrocyte variants and the population variability in GPA V, contributed by confounders within the exposed and control groups. The relationship between the variation within the exposed and control groups and the mean difference in GPA V between them can be approximately modeled for required group sample size for a given study power and level of significance, assuming a log-normal distribution of V in each group and applying parametric statistics. With the present approach, statistical power to detect a 20% increase in mean logarithmically transformed N/N V, as observed among the workers with high styrene concentrations in breathing zone, was 50% (t test, unequal group sizes, two-sided confidence interval; Ref. 64). To obtain 80% power, 70+70 samples would have been needed. Assuming a binomial distribution for untransformed GPA V in the logistic regression models could possibly have offered a better modeling technique. However, the very high V in some exposed subjects and smokers would have required fitting of random effects; the programs available did not allow this with such rare events as GPA variants.

In summary, the present study supports previous concerns that occupational exposure to styrene in the reinforced plastics industry has mutagenic effects. Besides styrene exposure, smoking and age were shown to influence GPA V. Compared to standard cytogenetic end points, the GPA assay is a rapid and economical method for screening populations with potential environmental or occupational exposure to chemical or physical mutagens (27) and, based on the results of this study, may offer similar or improved sensitivity in comparison with lymphocyte-based somatic mutation and cytogenetic methods. In addition, the use of the GPA assay alone, or in combination with lymphocyte-based in vivo mutation assays at the HPRT and HLA-A loci, permits the measurement of the frequency and mechanistic/molecular spectrum of in vivo mutations induced by exposure to mutagens.

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