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Use of the Glycophorin A Human Mutation Assay to Study Workers Exposed to Styrene

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The glycophorin A (GPA) assay is a human mutation assay that is potentially useful for large epidemiological studies. The assay is rapid and requires a minimal amount of blood, which can be stored before analysis. The data presented here were collected from workers exposed to styrene in a boat manufacturing plant. This study was the first to apply the GPA assay to an occupationally exposed population. Subjects with a mean styrene exposure of 30 ppm had a higher frequency of GPA NΦ variant cells than subjects with mean exposure of 1 ppm, but the subjects differed in respect to smoking and age distribution. Results indicate that the original 1-W-1 version of the assay may not be suitable for studies of small numbers of exposed subjects due to variability and artifacts. The newer BR6 version, however, has much lower variability and shows promise for use in the occupational setting.

Introduction

The glycophorin A (GPA) gene loss mutation assay estimates genetic damage to humans by measuring the frequency of GPA-variant red blood cells in peripheral blood samples (*1,2*). The GPA assay has advantages for the epidemiologist over the other mutation assays currently available, namely, the hprt, HLA-A, and Hb-S assays (*3*). The GPA assay is rapid and simple compared to the hprt and HLA-A assays—the results can be obtained within 48 hr, and the assay requires a minimal (< 1 mL) blood sample that can be stored for up to 2 weeks at refrigerator temperatures. The GPA assay also presumably detects a wider spectrum of mutational mechanisms than the hprt and Hb-S assays because GPA variant cells may arise from mutations that involve chromosome-wide events or chromosomal interactions such as mitotic recombination (*2*). The latter mechanisms have been found to be involved in the loss of tumor-suppressor genes, as reviewed by Grant et al. (*4*).

There are also disadvantages to the GPA assay from the epidemiological perspective; the GPA assay requires a specific blood type (MN) and so can be performed on only about 50% of all subjects, and, in addition, it measures mutations in bone marrow cells only because circulating red blood cells lack a nucleus. The effect of exposure must therefore penetrate to bone marrow to result in a response. Elevated frequencies of GPA-variant cells have been found in subjects exposed to ionizing radiation (*5,6*), in patients receiving high doses of antineoplastic drugs (*7*), and in persons with cancer-prone syndromes (*8,9*).

This study was undertaken to evaluate the potential utility of the GPA assay in a low-exposed population more typical of environmental epidemiology than the high-dose/high-risk populations, previously studied, using the original 1-W-1 version of the GPA assay (*1*) as well as a new and more precise version of the assay (BR6) developed (*2*) during the time period of the study. The data presented here represent the first application of the GPA assay to an occupationally exposed population.

Materials and Methods

Determination of GPA Variant Cell Frequency

The GPA assay was performed essentially as described (*1,2*). Briefly, fixed red blood cells obtained from heterozygous (MN) individuals were reacted with fluorescently labeled monoclonal antibodies to M and N forms of GPA. A flow cytometer was used to measure fluorescence from each cell. Normal MN cells

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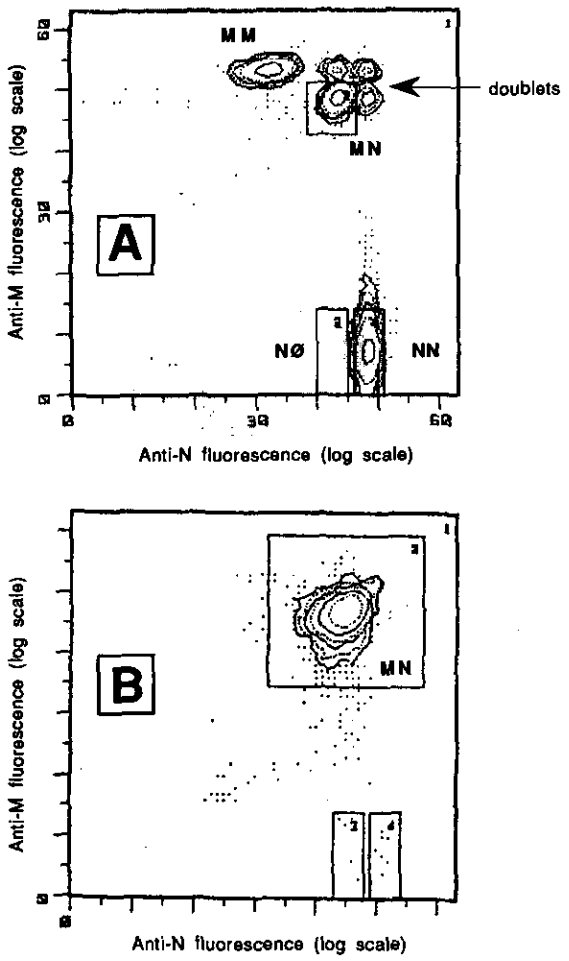

FIGURE 1. (*A*) Flow-cytometric measurements of fluorescence from mixture of MM, MN, and NN cells stained with anti-M and anti-N antibodies. (*B*) Results of BR6 assay using 1 million red blood cells from a low-exposed styrene worker. Six NΦ and nine NN GPA variant cells were found. The average of five such assays was 5.5 NΦ and 7.5 NN variant cells per million red cells.

fluoresce both red (anti-M antibody) and green (anti-N antibody), and variant cells fluoresce green only; NΦ variants (missing the M form) show the same level of green fluorescence as normal cells, and NN variants show double the normal green fluorescence level. These two classes of variants presumably arise through different mechanisms, gene inactivation and gene duplication. Data outputs are typically in the form of histograms with green fluorescence on one axis and red on the other, using counts as a third dimension. Figure 1*A* shows a histogram from the BR6 assay of a mixture of MM, MN, and NN blood cells that is used to set expectations for fluorescence of variant cells. Figure 1*B* is a histogram of 1 million cells from a worker exposed to a low level of styrene. As can be seen, variant cells contribute only a few counts per million MN red cells counted. The variant cell frequency, averaged over 5 such runs of 1 million cells each from this worker, was 5.5 NΦ variants per million and 7.5 NN variants per million.

Two versions of the GPA assay were used. The original (*1*), known as 1-W-1, used a dual-beam flow cytometer with sorter (DBS). The sorter was necessary because the antibody combinations used frequently generated artifacts, especially NN artifacts, in the fluorescence histograms, and visual confirmation of separated variant cells was necessary. However, the sorter itself introduced additional variability, and the DBS is a complex instrument requiring skilled maintenance. Obtaining a new antibody combination allowed the development of a new assay known as BR6 (*2*) based on a commercially available single-beam flow cytometer without a sorter. The BR6 assay shows improved precision, especially for NN variants, and more cells can be collected per subject, which reduces the variability due to Poisson distribution of rare events. The lower cost and ease of operation of the countertop, single-beam flow cytometer will aid in export of this assay to other laboratories.

Study Population

The population studied was a group of workers in a boat-manufacturing plant exposed to styrene during hand lamination of the boat hulls. Other plant employees with low styrene exposures, such as maintenance workers, were used as controls. Styrene oxide is a carcinogen in rodents and styrene and styrene oxide are mutagenic in human cells *in vitro*. Cytogenetic studies of people occupationally exposed to styrene vapor have found increases in chromosomal aberrations, micronuclei, single-strand DNA breaks, and unscheduled DNA synthesis in peripheral lymphocytes [reviewed in Bond (*10*), Barale (*11*), and Maki-Paakkanen et al. (*12*)].

This study was part of a larger study at University of California at Berkeley that examined multiple end points of styrene exposure: air levels, exhaled air levels, and blood levels of styrene; adducts of styrene oxide to hemoglobin, albumin, and DNA; glutathione *S*-transferase enzyme levels in blood; micronuclei frequencies and sister chromatid exchange frequencies in lymphocytes; and GPA variant frequencies in red blood cells (*13,14*). This was a longitudinal study design in which air and exhaled air levels were determined during eight survey visits over the course of 18 months, with blood samples being obtained on five of these visits. All workers were administered a confidential questionnaire to determine past exposures that could cause genotoxic damage or could interfere with the assays used.

Results

Styrene Exposure of Subjects

Overall, 61 workers participated in the study, with 37 of them having the MN blood type required for use of the GPA assay. Workers were continuously recruited to the study between surveys 1 and 5 to reach the maximum of 37 MN subjects, and enrollment declined in surveys 6, 7, and 8 due to job terminations, absences at the time of visit, or transfers to another shift. Due to the difficulty of obtaining subjects for the study, all workers willing to participate were enrolled regardless of smoking status. However, enrolled workers had to have worked in the plant for at least 1 year. Blood samples were drawn on surveys 1, 3, 5, 7, and 8. Survey 8 was made specifically to apply the then-new BR6 assay to subjects in the styrene study.

Job classification was found not to be a reliable guide to exposure classification. The workers who were originally enrolled in the study as controls on the basis that they were employed

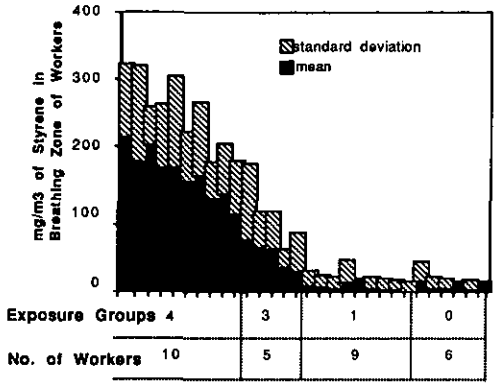

FIGURE 2. Mean and SD of styrene concentration in breathing zone, measured for each of 30 workers and averaged over the study period. Workers were assigned to exposure groups as shown. In addition to the workers shown in this figure, there were six additional workers in exposure category 0 and one additional worker in category 1 for whom no air concentrations of styrene were measured. All additional workers were assumed to have been exposed to less than 10 mg/m³ of styrene because all worked in outbuildings.

in the plant but did not work with styrene all proved to have low but measurable exposure to styrene, from 0.25 to 3.5 ppm (1–15 mg/m^3). However, time-weighted average exposures to styrene showed suprisingly little seasonal variation, so that workers could be grouped into exposure categories based on their overall mean exposure. The workers were divided into exposure categories, as shown in Figure 2. Exposure category 4 had average exposures of 170 mg/m^3 (40 ppm), and exposure category 3 averaged 55 mg/m^3 (13 ppm), whereas categories 4 and 3 combined averaged 132 mg/m^3 (32 ppm). Exposure category 0 was made up of persons who had never worked with styrene and category 1 of those not currently working with styrene but who had previously worked with styrene. The distinction between category 0 and category 1 was made because functionally immortal stem cells in the bone marrow give rise to the red blood cells measured, so that exposure to styrene in the past could cause a current elevation in variant frequencies if styrene had given rise to GPA-mutated stem cells. The mean exposure of categories 1 and 0 combined was 1.2 ppm (5 mg/m^3).

Styrene Exposure and GPA Variant Frequencies

To study the effect of styrene on GPA variant frequencies, comparisons were made of variant frequency (per million red cells) of workers in exposure category 4 (10 workers) versus category 0 (12 workers) and categories 4 and 3 combined (15 workers) versus categories 1 and 0 combined (15 workers). While categories 4 and 0 differ most in styrene exposure, differences in variant frequency are harder to detect with significance because they have smaller numbers of workers than the combined categories. In addition, comparisons made using exposure category 4 are the most compromised from the epidemiological viewpoint, because this category contains a higher proportion of older women and smokers than any other category. The GPA variant frequencies measured in this study did not fit a normal or log-normal distribution on statistical fit tests. The median values were used in all analyses so as to minimize the effects of outlier-measured variant frequencies. Due to technical difficulties encountered

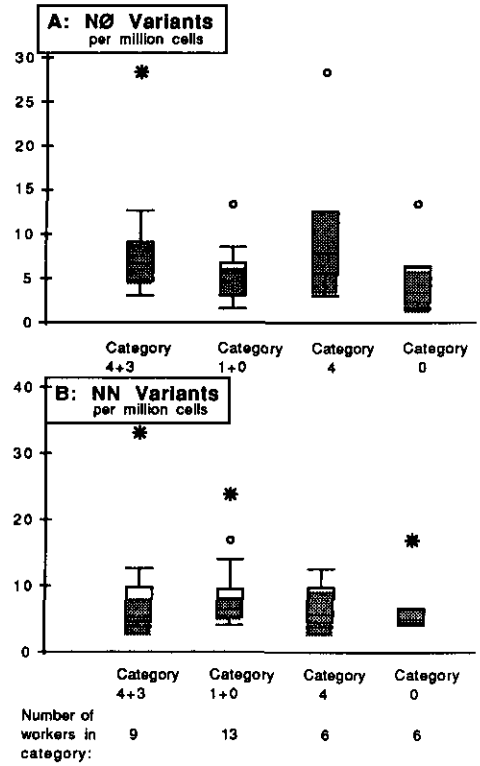

FIGURE 3. Measurements using the BR6 assay on blood collected during final survey. (*A*) NΦ and (*B*) NN GPA variant frequency, compared by worker exposure group. The data are presented using a boxplot format, a nonparametric presentation where the box encloses the middle 50% of the data and the median is shown as a line. The shaded areas represent approximately the 95% confidence interval of the median determined for that data set; (*) and (○) represent outliers.

with using the original 1-W-1 assay for the measurement of NN variants, specifically with the occurrence of artifacts that increase measured frequencies of NN variants, the decision was made that the measurements of NN variant frequencies using the 1-W-1 assay should not be used. This conclusion was also reached by Kyoizumi et al. (*15*). The median values of NΦ variant frequency (per million) measured using the 1-W-1 assay were: categories 4+3, median = 10.3 compared to categories 1+0, median = 6.9 (p = 0.028) (all probabilities given are Mann-Whitney). Category 4 had a median NΦ of 10.8 per million, as compared to 8.4 for category 0 (p = 0.088).

Two measurement runs were made on the blood from each worker in each survey, and examination of the NΦ variant frequencies suggested that the results from the second run showed a higher variability than those from the first run. The NΦ medians of categories 4+3 compared to categories 1+0 using data from the first run only are 9.64 versus 4.4, with p = 0.0164. Comparing category 4 with category 0 the medians are 8.5 versus 4.9, with p = 0.060.

Survey 8 was made to apply the new BR6 assay being developed at that time to subjects in this study. Unfortunately, it was only possible to collect blood from 9 workers in categories 4+3 (down from 15 in earlier surveys). Figure 3 shows GPA median frequencies obtained using the BR6 assay. For NΦ variant

frequencies, categories 4+3 have a median of 6.7 versus 4.7 for categories 1+0, with $p = 0.087$, whereas category 4 has a median of 8.0 versus 3.5 for category 0, with $p = 0.055$. Again, these results suggest an increase in NΦ variant frequency in the more highly exposed categories, but because the categories are not directly comparable with respect to smoking and other factors the effect could be due to exposures other than to styrene. The results do not suggest any increase in the frequency of NN variants with increasing styrene exposure.

Effect of Possible Confounding Factors

A clear separation of the effects of styrene and smoking is not possible because a greater proportion of the smokers were exposed to styrene: 50% of the smokers were exposed compared to 25% of nonsmokers. Comparing factor by factor, there is no indication of a GPA variant frequency increase with smoking, nor with a history of X-ray exposure. An effect of age (< 40 or ≥ 40 years) is suggested in the NN variant frequency, both for all exposure categories ($p = 0.089$) and for exposure categories 1+0 ($p = 0.036$). For the NN variants, females appear to have a greater variant frequency than males, both for all exposure categories combined ($p = 0.096$) and in exposure categories 1+0 ($p = 0.0188$). This effect is probably due to the greater proportion of subjects over 40 years old among females than among males. Age therefore seems to be the major confounder for GPA NN variant frequencies measured in the GPA assay (Compton-Quintana et al., manuscript in preparation.)

Performance of the GPA Assay

One great improvement that has resulted from the development of the BR6 version of the GPA assay is the reduction of the variance in measurements of GPA variant frequency. The coefficient of variation (CV) has been reported by Langlois et al.(2), using fresh blood samples from 17 selected laboratory normal donors under the most favorable laboratory conditions, to be 64% for NΦ and 132% for NN variant frequencies in the 1-W-1 assay, and 30% and 19% for NΦ and NN, respectively, in the BR6 assay. The results in the occupational setting reported here show a considerably higher variance using the 1-W-1 assay. For subjects in exposure categories 1 and 0, the CV measured using the 1-W-1 assay was 135% for NΦ variant frequencies and 137% for NN variant frequencies. These values for the CV represent what might be expected from the 1-W-1 GPA assay if blood is collected in the the occupational setting and shipped for analysis under the conditions of this study. Using the BR6 assay, the CV was 27% for NΦ variant frequencies and 24% for NN variant frequencies.

Because in this study multiple measurements were made per workers, the effect of the variance of the 1-W-1 assay was minimized and the power of the 1-W-1 assay was increased to a level similar to that of a one-time measurement using the BR6 assay. This illustrates the importance of longitudinal studies.

Discussion

One of the strengths of this study design is the longitudinal characterization of the styrene exposure of the workers, reducing the possibility that workers are wrongly classified into styrene exposure categories and also increasing the power of the study through increasing the number of blood samples per worker. However, the small number of subjects, the presence of possible confounding factors such as smoking, and the low concentrations of styrene to which the exposed workers were in fact exposed all tended to decrease the capability of the detect an effect, if present, of styrene.

The frequency of GPA NΦ type variants but not NN variants is increased in workers more highly exposed to styrene, although the level of statistical significance is not compelling. The finding, if real, that the genotoxic effect of styrene apparently increases NΦ-type GPA variants to a greater extent than than NN-type variants accords with the finding that styrene oxide and styrene +S9 are classified as mutagens in the Ames test and other assays. These results should be confirmed with a larger number of subjects in exposure categories that are better matched for age, gender, and smoking, and such a study is, in fact, being carried out (Bigbee et al., manuscript in preparation).

The original 1-W-1 version of the assay has a high variability, which would make detection of a small effect difficult. Many of the sources of variability in the GPA assay, however, have been reduced or eliminated in the newer BR6 assay. The BR6 assay therefore holds much more promise for the successful application of the GPA assay to occupationally or environmentally exposed populations. Data from this study suggest that age, and possibly sex, are confounders in the GPA assay and should be carefully controlled in future studies. GPA varant frequency may also be affected by individual susceptibility to mutation. Data from persons given chemotherapy (7) demonstrated that patients receiving a direct-acting chemotherapeutic agent had a more uniform increase in GPA variant frequency than those receiving an agent requiring metabolic activation, a process that is known to vary between individuals.

In summary, the GPA assay has been shown to have both unique advantages as well as disadvantages. It is rapid and requires a minmal amount of blood for analysis. However, it is clear that more characterization needs to be made of the sources of variability, including those present in the assay itself, those arising from natural differences in MN variant frequency in the general population, and those associated with confounders of the assay, before the GPA assay can be applied routinely for biological monitoring studies. Until such information is obtained, the most productive plan may be to obtain a pre- and post-exposure sample, to the extent feasible in the occupational or environmental setting, and use each subject as his or her own control.

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