DNA Adducts and Related Biomarkers in Populations Exposed to Environmental Carcinogens

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Prevention of environmentally related cancer will be enhanced by the availability of sensitive early warning systems and by improvements in quantitative assessment of human risks. Accordingly, we have carried out a series of molecular epidemiologic studies aimed at validating a panel of biologic markers, including carcinogen-DNA and -protein adducts, sister chromatid exchange, micronucleus formation, DNA strand breaks, and DNA repair capacity. Results from three such studies illustrate the usefulness of these biomarkers in elucidating low-dose–response relationships, correlations between biomarkers, and the range of variation in biomarkers between individuals exposed to similar concentrations of carcinogens. Low-level workplace or ambient exposures to styrene, ethylene oxide, and polycyclic aromatic hydrocarbons (PAH) were associated with significant increases in both molecular dose of carcinogens (adducts) and various markers of preclinical effects. Correlations between biomarkers varied by exposure. For example, in the styrene study, sister chromatid exchange frequency was not correlated with any of the markers, in contrast to the studies of ethylene oxide and PAH. Significant molecular effects were observed not only in occupationally exposed people but also in residents of an area in Poland characterized by high levels of air pollution. For example, the mean PAH-DNA level in exposed residents (winter sample) was 30.4 adducts per 108 nucleotides. This level was significantly higher than that of adducts seen in summer samples from the same area (4.2/108), or in winter samples from residents of a rural area (1.0/108). Significant seasonal variation in PAH-DNA adduct formation in this group was consistent with recorded fluctuations in air pollution levels. Striking interindividual variation was observed in all three exposed populations.

Introduction

Recent studies of "model" human exposures to environmental carcinogens have demonstrated the value of biologic markers in elucidating low-dose–response relationships, correlations between molecular dose and molecular effects of carcinogens, and the extent of interindividual variation in molecular response to carcinogens. These three pieces of information are of great value in estimating the magnitude of human risk resulting from environmental exposures.

Recent Research on Styrene

The first investigation we discuss concerns workplace exposure to styrene (1). Styrene is used increasingly in the production of plastics, resins, and synthetic rubbers. The most extensive exposures occur through inhalation at workplaces where fiberglass-reinforced plastic products, such as boat hulls and bath enclosures, are manufactured. Current U.S. Occupational Safety and Health Administration regulations were recently lowered from a permissible 8-hr time-weighted average (TWA) exposure of 100 to 50 ppm.

Styrene and its principal metabolite, styrene oxide, have been shown to be mutagenic and toxic to animals and humans. Binding of styrene to proteins, especially the
N-terminal valine of hemoglobin (Hb), has been measured in both animals and humans (2,3). Both styrene and its oxide are animal carcinogens, and there is preliminary evidence associating occupational exposure to styrene with increased incidences of lymphomas and leukemias (4,5).

In our study of exposed workers and controls, exposure assessment included personal monitoring to measure average ambient exposure to styrene in air (TWA-ppm) and analysis of end-of-shift urine samples to determine levels of mandelic acid, the principal styrene metabolite. The panel of biomarkers included a chemical-specific marker (styrene–Hb adducts) that could serve as a surrogate for styrene–DNA binding as well as several nonspecific markers: sister chromatid exchange (SCE), micronucleus formation (MN), and single-strand breaks (SSB). The panel was chosen to reflect the internal dose of styrene mandelic acid, its biologically effective dose (styrene–Hb adducts), and certain biologic effects of exposure (SCE, MN, SSB). A measure of susceptibility to DNA damage [N-acetoxy-2-acetylaminofluorene (NA-AF) binding] was also included. To our knowledge, this is the first use of gas chromatography–mass spectrometry to measure styrene–Hb adducts in humans. Although all of the other assays used in this study had previously been employed with positive results in at least one other study involving human populations exposed to ambient styrene, no prior study had evaluated all of these markers in the same cohort. [See Brenner et al. (1) for additional background and references.]

The 14 male styrene-exposed fiberglass-reinforced plastics workers were employed at a New England plant where small motor and sailboat hulls were manufactured. Controls were five male and four female library workers at a university in New York State. At both work sites, a phlebotomist drew 45-mL blood samples using heparinized Vacutainer tubes. All samples were drawn at the same time of day (early afternoon) during 2 consecutive days and coded to assure confidentiality. Coded samples were assayed for the five biomarkers as described (1).

At the time of blood sample collection, all participants were interviewed to determine age, educational attainment as a measure of socioeconomic status, total number of years worked in current occupation, length of time at current job site, typical or most common job performed during the 4 months preceding the study, use of protective clothing or equipment, and other environmental exposures and health-related variables that could influence the levels of biomarkers studied.

Breathing zone concentrations of ambient styrene were measured by personal sampling with 3M Organic Vapor Monitor 3620 (passive diffusion badges). Urine samples were collected at the end of the work shift from a subset of the workers and analyzed for mandelic acid and creatinine. Additional personal air samples were collected from this same subset using charcoal tubes.

With the exception of the method for analysis of SCE in high-frequency cells (SCP Hari), in which the chi-square test was used, the nonparametric Wilcoxon rank-sum test was used to compare differences in geometric mean levels of biomarkers found among exposed workers and controls. Analyses of variance and covariance were applied to determine relationships between the dependent and independent variables.

Personal monitoring data showed that the fiberglass-reinforced plastics workers were exposed to a geometric mean TWA level of 11.2 ppm (± 0.9) styrene, with individual TWA exposures ranging from 1 to 44 ppm. This was equivalent to an arithmetic mean of 17.2 (± 15.2) ppm. Estimated styrene exposure, extrapolated from mandelic acid levels, averaged 15.2 ppm (± 0.9) (243.5 mg mandelic acid/g creatinine) and ranged from 6 to 156 ppm.

The results of laboratory assays are shown in Tables 1 and 2. Even at the relatively low exposure levels encountered in this study, four of the five biomarkers showed elevated levels in styrene-exposed workers compared to controls (styrene–Hb adducts, MN, SSB, and NA-AF binding). For three of these (MN, SSB, and NA-AF binding), the mean differences were significant; there was no significant difference between exposed workers and controls with respect to mean levels of SCE or of cells with high SCE frequencies.

Dose–response relationships were not observed when continuous measurements of current or cumulative exposure were used (TWA-ppm, mandelic acid in urine, or ppm-years). When, however, we used the trichotomized variable, "exposure category," derived from an individual's "usual job assignment" during the 4 months preceding the study, we saw dose–response relationships with three of the biomarkers: styrene–Hb adducts, MN, and NA-AF binding.

There was significant interindividual variation in levels of styrene–Hb adducts among exposed workers. For example, the marked increase in mean adduct level among the exposed workers was due largely to a reproducibly high level measured in one worker who was a laminator in the high-exposure category. This individual also had the highest levels of urinary mandelic acid found in the study. This finding of an extremely elevated styrene–Hb value in one highly exposed individual may reflect an interaction between exposure to styrene and biologic sensitivity. The range of adduct values for workers was on the order of 8-fold (excluding the individual with the highest reading) or > 5000-fold (including this individual). The levels of MN, SSB, and NA-AF binding varied by approximately 2- to 3-fold.

| Table 1. Styrene–hemoglobin adducts a in blood samples from fiberglass-reinforced plastics workers and controls. |
|---|---|---|---|
| Subjects | Geometric mean ± SE | Range | n |
| All exposed | 9.5 ± 2.2 | 2.0–11.159 | 14 |
| | 5.5 ± 0.4 b | 2.0–15.3 b | 14 b |
| High exposure | 34.5 ± 11.6 | 5.3–11.159 | 5 |
| | 8.1 ± 0.8 b | 5.3–15.3 b | 4 b |
| Medium/low exposure | 4.7 ± 0.7 | 2.0–14.6 | 9 |
| Controls | 2.2 ± 0.8 | 0.002–24 | 8 |

aRatio of N'-(1-hydroxy-2-phenylethyl) valine to D4-(hydroxyethyl) valine standard.

bExcluding one individual with a high level of adducts.
Significant correlations were seen between styrene–Hb adduct levels and MN, between MN and SSB, and between NA-AAF binding and MN (p < 0.05). NA-AAF binding was also correlated with SSB (p < 0.05).

Recent Research on Ethylene Oxide

Ethylene oxide (EtO) is an alkylating agent and a model directly acting mutagen and carcinogen. In a recent study, we evaluated a panel of biologic markers including EtO–Hb adducts, SCE, MN, chromosomal aberrations, SSBs, and an index of DNA repair (the ratio of unscheduled DNA synthesis to NA-AAF DNA binding) in the peripheral blood cells of 34 workers at a sterilization unit of a large university hospital and 23 controls working in the university library (6). Comprehensive environmental histories were obtained for each subject, including detailed occupational and smoking histories. Industrial hygiene data obtained prior to the study and personal monitoring during the 8 years preceding the study showed that workers were subject to low-level exposure near or below the current Occupational Safety and Health Administration standard of 1 ppm (TWA).

Coded blood samples were analyzed as described previously (6). The results are shown in Table 3. After adjusting for smoking, EtO exposure was significantly (p < 0.001) associated with EtO–Hb adduct levels and two measures of SCE [the average number of SCEs/cell (SCE50) and SCEHFC]; a marked interaction was seen between smoking and workplace exposure for both meas-

Table 3. Biologic markers in ethylene oxide-exposed workers and controls stratified by smoking status: means ± SE.

| Biomarkers | Workers | Controls | Two-way ANOVA* |
|------------|---------|----------|----------------|
|            | Nonsmokers | Smokers | Nonsmokers | Smokers | Smoking | Exp. | Interaction |
| EtO-Hb      | 138.79 ± 12.21 (19) | 234.34 ± 39.56 (9) | 45.30 ± 3.12 (16) | 149.74 ± 38.52 (4) | 0.000* | 0.000* | 0.019* |
| SCE50       | 1.67 ± 0.51 (18) | 6.17 ± 1.18 (9) | 1.00 ± 0.52 (6) | 2.00 ± 0.71 (16) | 0.001* | 0.008* | 0.070* |
| SCEHFC      | 1.75 ± 0.46 (20) | 6.90 ± 1.30 (9) | 1.22 ± 0.38 (18) | 4.00 ± 2.35 (4) | 0.002* | 0.006* | 0.218* |
| SCE         | 10.17 ± 0.33 (19) | 12.51 ± 0.71 (10) | 9.49 ± 0.60 (6) | 10.22 ± 0.39 (16) | 0.000* | 0.164* | 0.060* |
| SSB         | 10.22 ± 0.31 (21) | 12.96 ± 0.81 (8) | 9.75 ± 0.33 (18) | 11.61 ± 0.71 (4) | 0.177 | 0.655 | 0.598 |
| Micronuclei | 12.53 ± 1.44 (19) | 15.25 ± 1.72 (12) | 13.50 ± 1.93 (6) | 14.65 ± 1.09 (17) | 0.107 | 0.215 | 0.915 |
| Micronuclei | 12.62 ± 1.34 (21) | 15.50 ± 1.96 (10) | 13.90 ± 1.02 (19) | 16.50 ± 2.26 (4) | 0.081 | 0.993 | 0.382 |
| CA          | 2.65 ± 0.44 (20) | 2.08 ± 0.51 (12) | 4.17 ± 0.43 (6) | 2.07 ± 0.51 (15) | 0.134 | 0.451 | 0.157 |
| CA          | 2.48 ± 0.40 (23) | 2.33 ± 0.65 (9) | 2.89 ± 0.54 (18) | 1.33 ± 0.33 (3) | 0.232 | 0.782 | 0.125 |
| SSB         | 0.17 ± 0.02 (19) | 0.13 ± 0.01 (13) | 0.18 ± 0.01 (6) | 0.14 ± 0.01 (17) | 0.030* | 0.263 | 0.938 |
| SSB         | 0.17 ± 0.02 (21) | 0.12 ± 0.01 (11) | 0.16 ± 0.01 (6) | 0.10 ± 0.01 (19) | 0.749 | 0.125 | 0.956 |
| Repair index| 2.11 ± 0.15 (21) | 2.17 ± 0.20 (13) | 2.35 ± 0.15 (6) | 3.08 ± 0.67 (15) | 0.389 | 0.066 | 0.404 |

Abbreviations: EtO, ethylene oxide; Hb, hemoglobin; SCE, sister chromatid exchange; HFC, high frequency cells; CA, chromosomal aberrations; SSB, single-strand breaks.

*Two-way analysis of variance, based on ln-transformed data.

Smokers include current smokers only; nonsmokers include former smokers.

*Smokers include current and former smokers.

*p < 0.05.
ures of SCE (p < 0.004). There was an apparent suppression of DNA repair capacity in EtO-exposed individuals as measured by the DNA repair index (p < 0.01); no association was found between DNA repair index and smoking.

EtO-Hb adduct levels were highly correlated with the frequency of SCE_HFC (p < 0.01) and SCE (p < 0.02); this observation provides evidence of a direct link between a marker of biologically effective dose and markers of genotoxic response. In contrast, the frequencies of MN, chromosomal aberrations, and SSB were not significantly elevated in the workers.

Previous studies of small numbers of sterilization workers showed increases in Hb alkylation (7,8), in contrast to the results of an investigation by Van Sittert et al. (9), involving workers with very low-level exposure (generally < 0.05 ppm) in an EtO manufacturing plant (32 workers, 31 controls). In none of these studies was smoking adequately evaluated. The present findings are the first, to our knowledge, to show elevated EtO-Hb binding in a workplace with atmospheric concentrations near or below 1 TWA ppm, in which the effect of cigarette smoking was adequately taken into account. Although a body of literature demonstrates increased cytogenetic damage in EtO-exposed workers, to our knowledge, the present study is the first to evaluate exposures generally at or below 1 ppm (TWA) while accounting appropriately for cigarette smoking.

In this EtO-exposed cohort, there was considerably greater uniformity of exposure than among boatbuilders exposed to styrene. Nonetheless, interindividual variation between workers was significant for EtO-Hb adducts (> 800-fold), SCE_50, SCE_HFC, MN, chromosomal aberrations, SSB, and repair index (2- to 13-fold).

Recent Studies of Environmental Pollution in Poland

In order to evaluate the biologic dose and effects of ambient pollution, we used an enzyme-linked immunosorbent assay (ELISA) to analyze peripheral white blood cells from Polish residents for the presence of polycyclic aromatic hydrocarbons (PAH) covalently bound to DNA. These results were then compared to several other measurements in the same blood samples: carcinogen-DNA adducts using the 32P-postlabeling method (10) and SCE (G. Motykiewicz et al., in preparation). This study builds upon earlier research in the same industrial area of Poland that showed that residents of industrialized towns had adduct levels and patterns similar to those of coke-oven workers, but two to three times higher than those of residents in a relatively clean rural area (11).

Silesia is a heavily industrialized region of Poland distinguished by high ambient concentrations of airborne PAH and other pollutants. The major sources of PAH are cokeries, coal-fired power plants, and domestic coal- and wood-burning stoves. Extracts of airborne particulate matter from this region are remarkably mutagenic and clastogenic in experimental systems (12).

Peripheral blood samples were collected from residents living near a large cokerie in Gliwice, but who were not employed in PAH-generating industries. Estimated benz[a]pyrene levels in ambient air in this area range from 0.057 μg/m3 (January) to 0.015 μg/m3 (May). Controls were drawn from a relatively “clean” rural area of eastern Poland.

Blood samples were collected in winter and summer months, as shown in Table 4. The mean PAH-DNA adduct level measured by ELISA in samples collected from Gliwice residents in the winter exceeded the corresponding winter samples from the rural control group by a factor of about 2.8 (p = 0.02). For the Gliwice residents, the levels in the winter sample were approximately seven times higher than in those collected in the summer (p = 0.001). For the rural controls, the level in the winter sample exceeded that in the summer sample by a factor of 5.5 (p = 0.02). There was a clear increase in PAH-DNA adduct levels across the four groups (Gliwice residents, winter; rural controls, winter; Gliwice residents, summer; rural controls, summer), providing evidence of a dose–response relationship. These results confirm and extend our earlier finding that environmental pollution contributes significantly to the level of PAH-DNA binding (11). Here, a clear seasonal effect was observed, consistent with the increase in pollution during the winter months from combustion of coal for domestic heating.

A seasonal effect was also seen in the level of DNA adducts measured by **32P**-postlabeling (10). This observation is consistent with that of a prior study, which showed that the mutagenicity of ambient air samples from this region varied markedly by season (13).

Of the three biomarkers measured in a subset of subjects, SCE and adducts by **32P**-postlabeling were significantly correlated; however, adducts by ELISA were not correlated with either adducts by postlabeling or SCE. This finding may be explained, in part at least, by the relatively small sample size of the subgroup for all three measures.

All biomarkers showed large interindividual variation among the environmentally exposed residents: about 20-fold for adducts by **32P**-postlabeling and about 2-fold for SCE frequency (10). For PAH-DNA adducts, the interindividual variation in the same group was approximately 70-fold.

| Population          | Sampling period    | Number | Adducts/10^6 | SD  |
|---------------------|--------------------|--------|--------------|-----|
| Gliwice residents   | January–March      | 20     | 30.4         | 31.70|
| Rural controls      | March              | 21     | 11.01        | 22.6 |
| Gliwice residents   | September–October  | 21     | 4.20         | 13.50|
| Rural controls      | October            | 23     | 3.0          | 3.30 |

*Arithmetic means.*
Conclusion

These studies illustrate the advantage of using a panel of biomarkers that reflect the molecular dose of specific chemicals/agents and irreversible molecular alterations; it is a more effective means of establishing causality than conventional monitoring and questionnaires. These studies also demonstrate the high sensitivity of DNA and protein adducts, SCE, MN, SSB, and measures of susceptibility and DNA repair to various low-level carcinogenic exposures—both in the workplace and in the ambient environment.

Results in terms of dose–response relationships and correlations between biomarkers vary with exposure. For example, in the styrene study, styrene–Hb adducts, MN, and NA-AAF binding showed significant associations with exposure; SCE frequency did not. Styrene–Hb adducts, SSB, and NA-AAF binding were each correlated with MN, whereas SCE frequency was not correlated with any of the other markers. In the EtO study, however, SCE frequency was responsive to exposure, as was EtO–Hb: these markers were highly correlated. In contrast, neither MN, chromosomal aberrations or SSB was elevated in the EtO workers.

In the case of ambient PAH, the two measures of adducts (ELISA and 32P-postlabeling) and SCE all demonstrated significant associations with exposure, and all three showed seasonal variation. Of the three, PAH–DNA adduct levels by ELISA showed the clearest dose–response relationship but was not correlated with the other two markers. This review leads to the conclusion that the optimal batteries of biomarkers vary by exposure (chemical composition and pattern), and more research is needed to fully characterize the relationships between the different biomarkers.

Finally, this work indicates that biomarkers can be useful in accurately characterizing a population at risk in terms of variation in sensitivity to carcinogenic exposures. The observed variation in macromolecular binding between individuals with comparable environmental exposure to carcinogens is consistent with data on interindividual differences in carcinogen activation, detoxification, and DNA repair (14,15). Although not well characterized, variations in cellular processes (such as cell replication rates, age, diet, and immune status) related to promotion of carcinogenesis are also likely to be significant. This is important because the failure of current procedures for carcinogenic risk assessment to account explicitly for human interindividual variation may result in a significant underestimation of the carcinogenic risks of low doses, especially for the most sensitive individuals, but also for the population as a whole (16).

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