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Molecular Dosimetry of DNA and Hemoglobin Adducts in Mice and Rats Exposed to Ethylene Oxide

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Experiments involving ethylene oxide (ETO) have been used to support the concept of using adducts in hemoglobin as a surrogate for DNA adducts in target tissues. The relationship between repeated exposures to ETO and the formation of *N*-(2-hydroxyethyl)valine (HEtVal) in hemoglobin and 7-(2-hydroxyethyl)guanine (7-HEG) in DNA was investigated in male rats and mice exposed by inhalation to 0, 3, 10, 33, or 100 ppm ETO for 6 hr/day for 4 weeks, or exposed to 100 ppm (mice) or 300 ppm (rats) for 1, 3, 5, 10, or 20 days (5 days/week). HEtVal was determined by Edman degradation, and 7-HEG was quantitated by HPLC separation and fluorescence detection. HEtVal formation was linear between 3 and 33 ppm ETO and increased in slope above 33 ppm. The dose–response curves for 7-HEG in rat tissues were linear between 10 and 100 ppm ETO and increased in slope above 100 ppm. In contrast, only exposures to 100 ppm ETO resulted in significant accumulation of 7-HEG in mice. Hemoglobin adducts were lost at a greater rate than predicted by normal erythrocyte life span. The loss of 7-HEG from DNA was both species and tissue dependent, with the adduct half-lives ranging from 2.9 to 5.8 days in rat tissues (brain, kidney, liver, lung, spleen, testis) and 1.0 to 2.3 days in all mouse tissues except kidney ($t_{1/2}$ = 6.9 days). The concentrations of HEtVal were similar in concurrently exposed rats and mice, whereas DNA from rats had at least 2-fold greater concentrations of 7-HEG than DNA from mice. Due to differences in formation, persistence, repair, and chemical depurination, the relationships between HEtVal and 7-HEG concentrations varied with length of exposure, interval since exposure, species, and tissue. Thus, it appears unlikely that HEtVal adducts in hemoglobin will provide accurate predictions of DNA adducts in specific tissues of humans under conditions where actual exposure scenarios are unknown.

Introduction

Ethylene oxide (ETO) is a potent mutagen and carcinogen in laboratory animals, but its potential to cause cancer in man is still uncertain. In carcinogenicity bioassays, ETO caused dose-related increases in the incidence of gliomas, peritoneal mesotheliomas, and mononuclear cell leukemias in F344 rats (*1,2*) and lymphomas and adenomas/adenocarcinomas of the lung, uterus, harderian gland, and mammary gland in B6C3F$_1$ mice (*3*). In the earliest epidemiology studies on ETO and cancer, Hogstedt et al. (*4–7*) reported excesses of leukemia and lymphatic and stomach cancers among sterilant workers and employees in a chemical production plant. More recently, two independent studies found an excess of non-Hodgkin's lymphoma in ETO workers (*7,8*). Several other epidemiological studies have not demonstrated any association between ETO exposure and cancer in workers (*10–12*). However, the sensitivity of epidemiologic studies designed to assess the potential health hazards of environmental chemicals is seriously compromised by the lack of reliable quantitative exposure data for individuals in exposed populations (*13*). Consequently, a major goal in the study of environmental carcinogens is to identify biomarkers that are suitable for determining dose–response relationships in exposed humans.

Because genetic damage and mutation are thought to play a critical role in chemical carcinogenesis, damage to DNA can be used as an internal molecular dosimeter of carcinogen exposure [for review see Swenberg et al. (*14*)]. Therefore, DNA adducts or a validated surrogate are relevant indicators of the biologically effective dose (molecular dose) of carcinogen. Evaluation of these potential biomarkers over a broad dose range in experimental animals offers a means of critically assessing which biomarkers will be useful for monitoring human exposure and for enhancing risk extrapolation across species.

A major initiative in monitoring carcinogen exposure came from Ehrenberg and his co-workers, who suggested measuring

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adducts in hemoglobin as a surrogate for the molecular dose of adducts in DNA (*15,16*). However, as noted by Wogan (*17*), information concerning interrelationships between the formation of DNA and hemoglobin adducts is very limited, and studies designed to evaluate these relationships are needed. Thus far, most animal experiments investigating these relationships have used single doses of carcinogen (*18*).

Several experiments involving ETO have been used to support the concept of using adducts in hemoglobin as a surrogate for DNA adducts in target tissues. However, previous studies investigating the formation of both hemoglobin and DNA adducts have used only single exposures of rats (*19,20*) and mice (*21*) to ETO. The data from these studies showed a constant ratio between DNA and hemoglobin alkylation over the range of doses of ETO investigated, supporting the suggestion that the determination of the hemoglobin dose *in vivo* is a valid indicator of the dose to DNA in target tissues (*20*). However, many DNA adducts can be lost by processes such as repair and chemical depurination (*14*), whereas hemoglobin adducts of ETO appear to be stable. Therefore, it is essential to examine the relationships between hemoglobin and DNA adduct formation in rats and mice over a range of exposures and times of exposure to ETO (*22–24*). The molecular dosimetry studies reviewed here demonstrate that the ratios between DNA and hemoglobin adduct concentrations change over time during repeated exposures to ETO and that the nature of the relationship between DNA and hemoglobin alkylation is both tissue and species dependent due to differences in the life span of the erythrocyte and differences in DNA repair.

Methods

The methodological details of these investigations have been published elsewhere (*22–24*). Thus, only a brief summary of the methods is given here.

Animal Exposures

Groups of 9-week-old male B6C3F$_1$ mice and F344 rats were exposed in 8-m^3 stainless-steel and glass inhalation chambers to 0, 3, 10, 33, or 100 ppm ETO for 6 hr/day for 4 weeks (5 days/week), or exposed to 100 ppm (mice) or 300 ppm (rats) ETO for 1, 3, 5, 10, or 20 days (5 days/week). In the dose–response studies, the mice and rats were exposed concurrently in the same exposure chambers. The ETO concentration inside each chamber was monitored continuously using a Miran infrared spectrophotometer. At necropsy of treated and control animals, heparinized blood was taken from each animal by cardiac puncture and red blood cells were washed with isotonic saline, frozen, and stored at −20°C until globin samples were isolated. Brains, kidneys, leukocytes (300 ppm ETO-treated rats only), livers, lungs, spleens, and testes were removed, frozen, and stored at −20°C until the DNA was isolated.

Assay for *N*-(2-Hydroxyethyl)valine

Erythrocytes from individual animals were lysed, and globin was isolated by extraction with acidic isopropanol and precipitation with ethyl acetate (*23,25*). *N*-(2-Hydroxyethyl)valine (HEtVal) in globin samples from control and treated animals was determined by a modified Edman degradation and GC–MS quantitation of its pentafluorophenylisothiocyanate derivative (*26*). Addition of a known amount of [$^2H_4$]ethylene oxide-treated globin as an internal standard, prior to Edman degradation, provided a means for quantitation of the amount of HEtVal present in the isolated globin samples. Derivatized samples were analyzed by GC–MS in the negative ion chemical ionization mode using a Finnigan 4500 GC–MS instrument equipped with an on-column injector and a DB-5 column, with methane as the reagent gas. The m/z 348 and m/z 352 were monitored for detection of the analyte and internal standard, respectively. Quantitation was based on comparison of the peak area of the analyte to that of the internal standard and comparison to a calibration curve for HEtVal in globin.

Assay for 7-(2-Hydroxyethyl)guanine

Whole tissues from individual rats or sets of four mice were homogenized, and DNA was isolated using an automated phenolic extraction procedure (*22,24*). DNA samples from rats exposed to 300 ppm ETO were analyzed for 7-(2-hydroxyethyl)-guanine (7-HEG) using neutral thermal hydrolysis and acid precipitation, cation-exchange HPLC separation, and fluorescence detection (*22*). The detection limit for the assay was 20 pmole 7-HEG/mg DNA.

The assay for 7-HEG was subsequently improved by using selective enrichment via Centricon 30 microconcentrators (Amicon, Danvers, MA) in place of acid precipitation, and by developing a new chromatography system (*24*). The improved assay was used to analyze DNA from animals in the dose–response studies and the mouse time-course study. Up to 2 mL of each thermal hydrolysate was cooled to 4°C and filtered through a Centricon 30 by centrifugation. The DNA backbone was retained on the Centricon 30 ultrafiltration membrane (30,000 molecular weight cutoff), and essentially 100% of the 7-HEG was recovered in the filtrate. The filtrates were reduced to a 1.0-mL injection volume and chromatographed using a hybrid RP-SCX column (250 × 5.6 mm, 60 Å, 5 μm, lot no. 1990055VW; ES Industries, Marlton, NJ) eluted with 80 mM ammonium formate, pH 2.8, with 50 % acetonitrile. 7-HEG was quantified by measuring fluorescence intensity (excitation at 295 nm and emission at 370 nm) and comparing peak areas to a calibration curve for 7-HEG standard. The detection limit of this assay was 2 pmole 7-HEG/mg DNA.

Results and Discussion

Analysis of globin from control animals showed background concentrations of HEtVal averaging 42 ± 8 and 58 ± 10 (SE) fmole adduct/mg globin in rats and mice, respectively, while analyis of DNA from control tissues revealed the presence of peaks equivalent to 2–5 pmole 7-HEG/mg DNA. The 7-HEG standard coeluted with these peaks, giving similar concentrations using standard additions. Comparable concentrations of HEtVal in hemoglobin (*26*) and 7-HEG in lymphocytes (*27*) have been found in control populations of humans and experimental animals without known exposure to hydroxyethylating agents or any obvious precursor.

Repeated exposures of rats and mice to ETO led to accumulation of HEtVal in hemoglobin and 7-HEG in DNA of all tissues

examined. After 4 weeks of exposure, the dose–response relationships for HEtVal and 7-HEG were nonlinear in both rats and mice. The dose–response curves for HEtVal were linear between 3 and 33 ppm ETO and increased in slope above 33 ppm (Fig. 1). The concentrations of HEtVal were similar in concurrently exposed rats and mice. A comparison of the dose–response curves for 7-HEG in DNA of both species showed that rats had significantly greater accumulations of 7-HEG than those in similarly exposed mice (Fig. 2). In rats, the dose–response curve for 7-HEG was linear between 10 and 100 ppm ETO (Fig. 2) and increased in slope above 100 ppm (see Fig. 6A). In contrast, after 4 weeks of exposure of mice to 10 and 33 ppm ETO, the concentrations of 7-HEG were similar to and just above, respectively, those concentrations found in control mouse tissues (Fig. 2). Repeated exposures to 100 ppm ETO were required to demonstrate any significant accumulation of 7-HEG in mouse tissues.

Exposures of rats and mice for 4 weeks (5 days/week) to 300 and 100 ppm ETO, respectively, led to an accumulation of HEtVal that was 14 (rats) and 15 (mice) times greater than that found after a single day of exposure (Fig. 3). After cessation of the time-course studies, HEtVal was lost more rapidly than would be predicted by the normal erythrocyte life span in rats and mice (Fig. 4). The initial phase of rapid decline in HEtVal concentrations in exposed rats (Fig. 4A) was consistent with the removal of older, more heavily alkylated populations of red blood cells, accompanied by a burst of erythropoiesis. Moreover, evaluation of the protocols used in these studies revealed that discontinuous exposures to ETO can result in complex patterns of hemoglobin adduct removal (23,28), in contrast to the predictable patterns of adduct removal observed after single exposures or exposures exceeding the life span of erythrocytes (29).

Accumulations of 7-HEG were similar in target and nontarget tissues within each species, with the exception that the

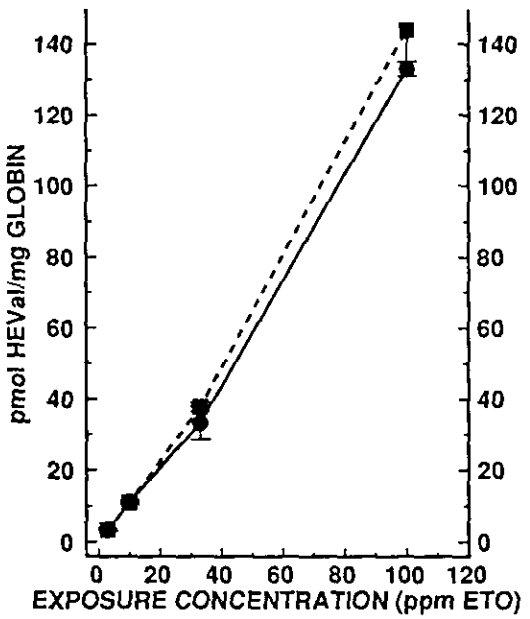

FIGURE 1. Dose response for N-(2-hydroxyethyl)valine in hemoglobin of mice (■) and rats (●) after 4 weeks (6 hr/day, 5 days/week) of exposures to ethylene oxide. Data points are means ± SE (n = 5).

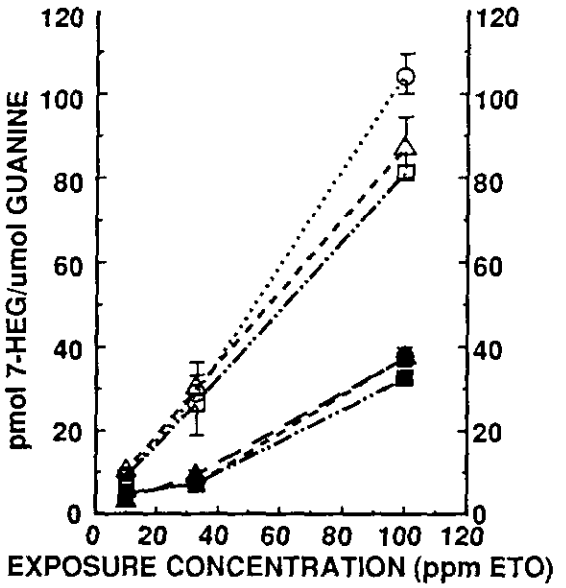

FIGURE 2. Dose response for 7-(2-hydroxyethyl)guanine in DNA of rats (△ brain, ○ lung, □ spleen) and mice (▲ brain, ● lung, ■ spleen) after 4 weeks (6 hr/day, 5 days/week) of exposures to ethylene oxide. Data points are means ± SE (n = 4).

adduct concentration in testis was 50–70% and 35–47% lower than in other rat and mouse tissues, respectively (Fig. 3). After cessation of exposures, two distinct patterns of 7-HEG persistence were apparent among the rat and mouse tissues investigated (Fig. 5). 7-HEG disappeared in a slow, steady fashion from DNA of mouse kidney ($t_{1/2}$ = 6.9 days) and rat lung, brain, and testis ($t_{1/2}$ = 4.8–5.8 days), consistent with adduct loss primarily by chemical depurination (24). In contrast, the loss of 7-HEG from other mouse ($t_{1/2}$ = 1.0–2.3 days) and rat ($t_{1/2}$ = 2.9–3.9 days) tissues was more rapid, consistent with loss by a combination of depurination and active removal of adducts by DNA repair. Finally, a comparison of the dose–response, formation, and persistence curves for each species indicated that saturation of 7-HEG repair had occurred at the concentrations of ETO used in the time-course studies. Furthermore, the occurrence of nonlinear dose–response curves suggested that repeated exposures of rats and mice to lower concentrations of ETO, matching the linear portions of the curves, would lead to species and tissue differences in 7-HEG accumulation as a result of differences in DNA repair.

DNA and Hemoglobin Adducts As Molecular Dosimeters after Repeated Intermittent Exposures to ETO

The nature of the relationships between the formation of HEtVal in hemoglobin and 7-HEG in DNA during repeated exposures of rats and mice to ETO was revealed, in part, by comparisons of the shapes of the formation curves for these adducts in each species (Fig. 3). The shape of the formation curve for HEtVal in rats exposed to 300 ppm ETO indicated that the hemoglobin adduct should accumulate well beyond 4 weeks with continued exposures to the epoxide. These results are consistent

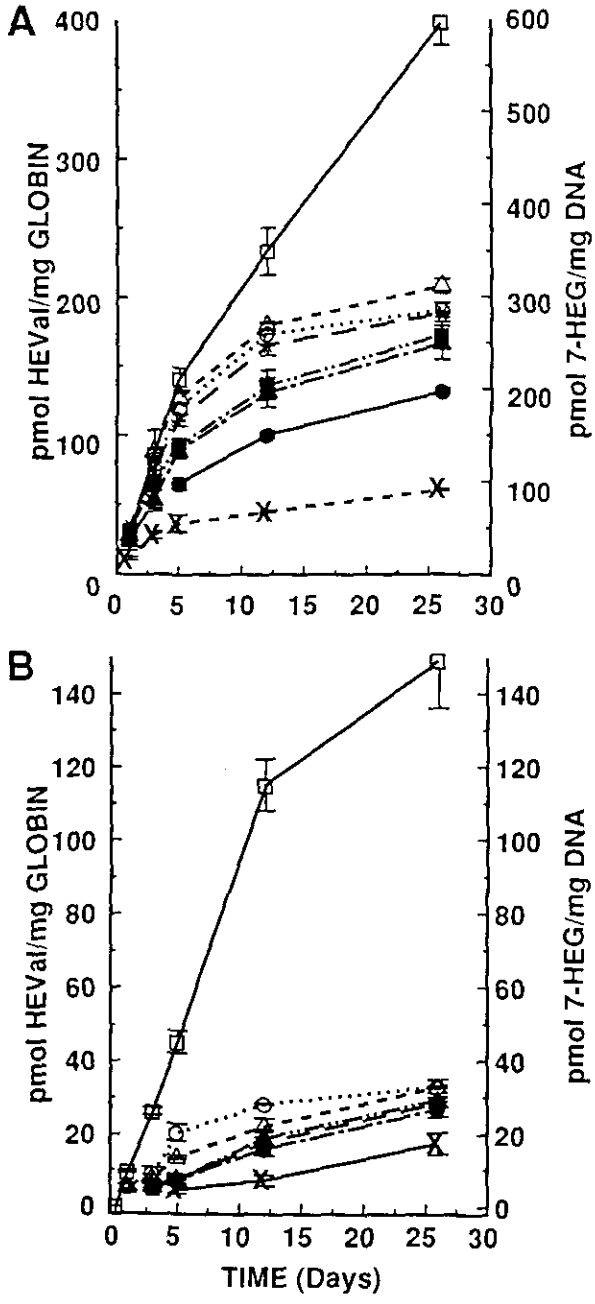

FIGURE 3. Formation of *N*-(2-hydroxyethyl)valine in hemoglobin and 7-(2-hydroxyethyl)guanine in DNA of rats (*A*) exposed to 300 ppm ethylene oxide and mice (*B*) exposed to 100 ppm ethylene oxide for up to 4 weeks (6 hr/day, 5 days/week). Globin (□), brain (△), lung (○), leukocytes (✱), spleen (■), kidney (●), liver (▲), testis (X). Data points are means ± SE (n = 4 or 5). Note that the scale of the Y-axes for *A* are adjusted so that the data points for *N*-(2-hydroxyethyl)valine and 7-(2-hydroxyethyl)guanine overlap on day 1 of exposure.

with published experiments showing that 4-aminobiphenyl hemoglobin adduct accumulated in a parabolic fashion over the life span of the erythrocyte (*30*), which is 66 days in the F344 rat (*31*). In comparison, the shapes of the formation curves for 7-HEG in rat tissues suggested that this DNA adduct should approach steady-state concentrations by 4 weeks of ETO exposure. Figure 3*A* shows that the ratio of the concentrations of HEtVal in hemoglobin and 7-HEG in DNA changed throughout repeated exposures of rats to ETO. Over 4 weeks of exposure to 300 ppm ETO, the ratio of HEtVal:7-HEG increased by a factor of 1.8–2.6, depending on the tissue. With additional exposures, the ratio should continue to increase as HEtVal accumulates over the lifetime of the rat erythrocyte. During exposures equivalent to or exceeding the RBC life span, hemoglobin adduct concentrations would reach a plateau and the HEtVal:7-HEG ratio would finally become constant.

In contrast to the curves for rats, the shapes of the formation curves for HEtVal and 7-HEG in mice exposed to 100 ppm ETO suggested that hemoglobin adduct concentrations would approach a steady state shortly after DNA adduct concentrations had plateaued in mouse lung (Fig. 3*B*). However, accurate predictions of the times to steady state for 7-HEG in other mouse tissues will require better characterization of the kinetics of DNA

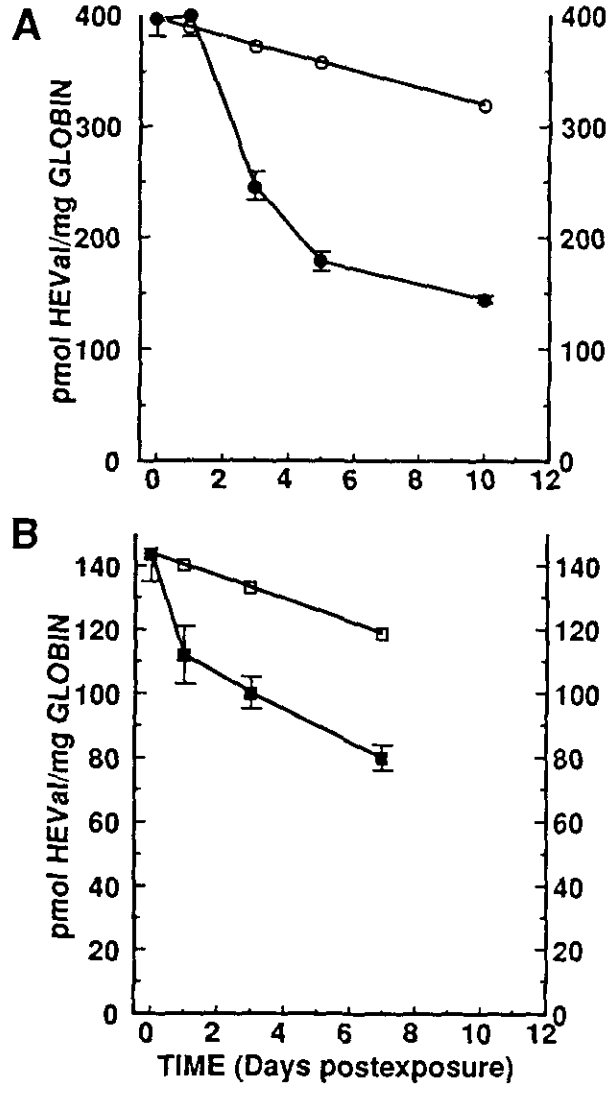

FIGURE 4. Comparison of the projected loss of *N*-(2-hydroxyethyl)valine to the observed loss of adduct after 4 weeks of exposure of rats (*A*) to 300 ppm ethylene oxide and mice (*B*) to 100 ppm ethylene oxide. Projected (○,□); observed (●,■). Data points are means ± SE (n =5). The derivation of the curves for the projected loss of *N*-(2-hydroxyethyl)valine is discussed in Walker et al. (*23*) and Fennell et al. (*28*).

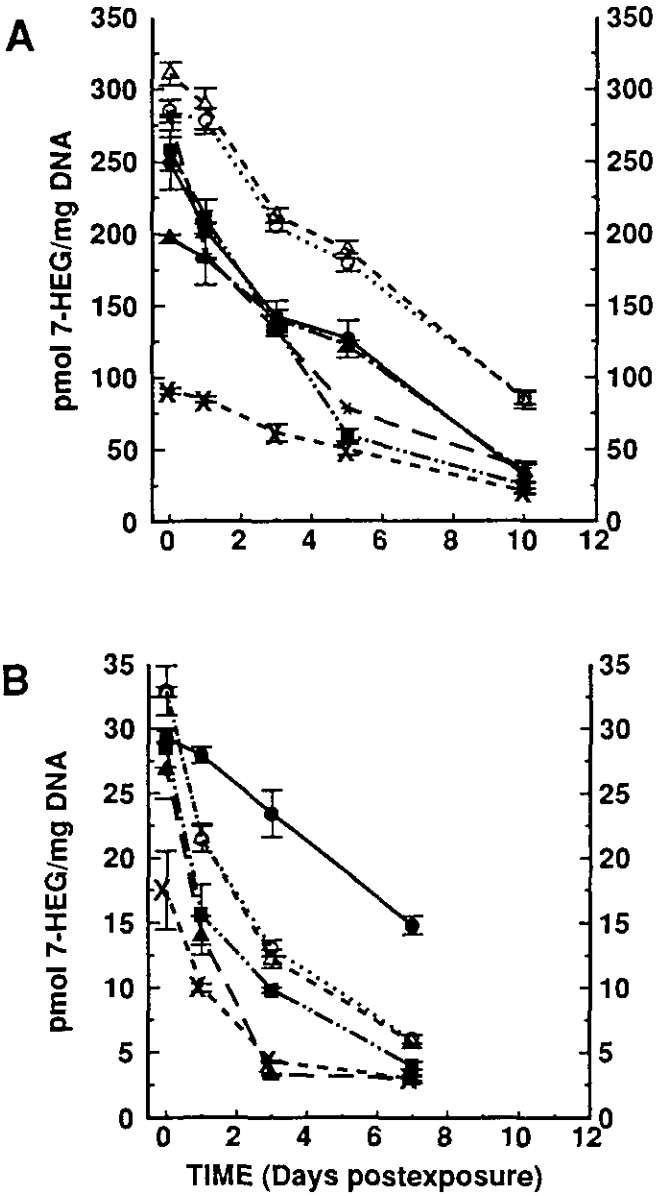

FIGURE 5. Persistence of 7-(2-hydroxyethyl)guanine in DNA after 4 weeks of exposure of rats (*A*) to 300 ppm ethylene oxide and mice (*B*) to 100 ppm ethylene oxide. Brain (△), lung (○), leukocytes (✱), spleen (■), kidney (●), liver (▲), testis (X). Data points are means ± SE (n = 3 to 5).

adduct removal during exposures of mice to lower concentrations of ETO. Nevertheless, the ratio of the concentrations of HEtVal and 7-HEG changed throughout repeated exposures of mice to 100 ppm ETO. Over the 4 weeks of exposure, the ratio of HEtVal:7-HEG increased by a factor of 2.5–4.2, depending on the tissue.

During repeated exposures to ETO, there should also be tissue-dependent differences in the relationship between HEtVal and 7-HEG due to tissue differences in DNA repair. For example, the half-lives for 7-HEG in various rat tissues (Fig. 5*A*) indicated that, *during exposures that do not saturate repair, the adduct* should reach a steady state by 2 weeks in leukocytes and spleen compared to 3–4 weeks in other tissues. As noted above, HEtVal accumulation should plateau after 9 weeks of ETO exposure.

In summary, these data demonstrate that the relationships be*tween hemoglobin and DNA adduct accumulation can change* over time during repeated or intermittent exposures to electrophiles, with the degree of change depending on such factors as the pattern and duration of exposures, cell kinetics, and the stability of the adducts under study. As illustrated by the figures comparing the formation (Fig. 3) and dose–response (Fig. 6) curves for HEtVal and 7-HEG in ETO-exposed rats and mice, the

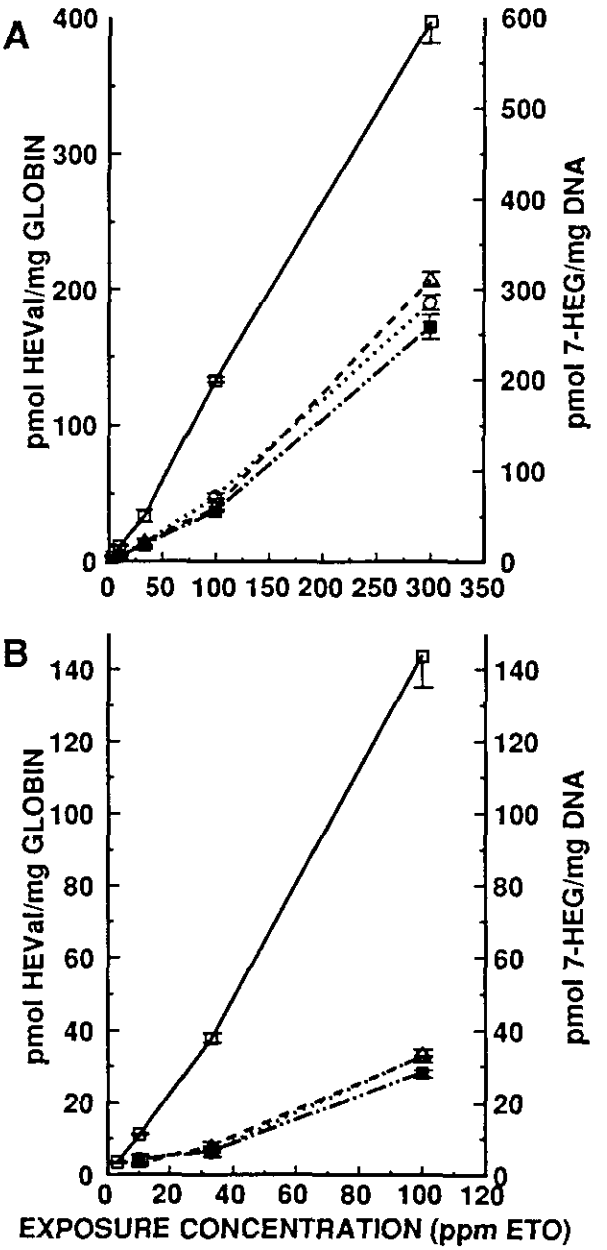

FIGURE 6. Comparison of the dose response for *N*-(2-hydroxyethyl)valine in hemoglobin and 7-(2-hydroxyethyl)guanine in DNA of rats (*A*) and mice (*B*) exposed to ethylene oxide for 4 weeks (6 hr/day, 5 days/week). Globin (□), brain (△), lung (○), spleen (■). Data points are means ± SE (n = 3 to 5). Note that the scale of the X-axis is different in *A* and *B* and that the scale of the Y-axes in *A* are adjusted so that the data points for *N*-(2-hydroxyethyl)valine and 7-(2-hydroxyethyl)guanine overlap at 3 ppm ethylene oxide.

nature of the nonconstant relationship between the hemoglobin and DNA adducts can be both tissue and species dependent.

Implications for Human Biomonitoring

In human biomonitoring, quantitative analysis of DNA and hemoglobin adducts serves two related goals, assessment of the internal dose and evaluation of the effects produced in target cells by a putative carcinogen (*29*). The results of the molecular dosimetry studies in rats and mice exposed to ETO have confirmed certain principles concerning relationships between DNA and hemoglobin adducts and their use in exposure monitoring and risk assessment (*18,29*).

First, these data demonstrate that considerable knowledge concerning the stability and repair of DNA adducts in different tissues and at different exposure concentrations is needed before any predictions can be made from DNA biomonitoring data from exposed people. Thus far, no information is available concerning the formation and removal of 7-HEG from DNA of ETO-exposed people or ETO-treated human tissues.

Second, the kinetics of hemoglobin adduct removal following intermittent and variable exposures are much more complex than those associated with single or continuous exposures (*23,28*). The pattern of hemoglobin adduct removal can be complicated further by exposures that influence erythrocyte life span or erythropoiesis. As a possible example, chronic exposure to tobacco smoke could induce mild, cumulative toxic effects in erythrocytes and explain, in part, the faster-than-expected decline in 4-aminobiphenyl hemoglobin (4ABP–Hb) adducts seen in smokers enrolled in a withdrawal program (*32*). In the first 3 weeks of withdrawal, the rate of adduct decline was most compatible with an erythrocyte life span of 80 days, compared to the normal life span of 120 days. Maclure and co-workers (*32*) suggested that some degradation of 4ABP–Hb adduct might occur over time, but it is also possible that the life span of erythrocytes was reduced in smokers due to the combined alkylating potency of such tobacco constituents as ETO, acrylonitrile, 4-aminobiphenyl, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Compensatory erythropoiesis in these smokers would certainly be sufficient to maintain normal red blood cell counts and could be evaluated by assaying for reticulocytes. Therefore, appropriate information on the pattern of exposures and red blood cell integrity should be considered essential when assessing the significance of adduct values obtained in monitoring humans exposed to hemoglobin alkylating agents.

Third, due to differences in formation, persistence, repair, and chemical depurination of 7-HEG and toxicokinetic effects on erythrocytes, the relationships between 7-HEG and HEtVal concentrations will vary with length of exposure, interval since exposure, species and tissue or even cell type. Thus, it appears unlikely that HEtVal adducts in hemoglobin will provide accurate predictions of DNA adducts in specific tissues of humans under conditions where actual exposure scenarios are unknown. If the exposure conditions are known, the HEtVal and exposure data could be incorporated into a computer model to describe the kinetics of accumulation and removal of adducts formed *in vivo* in complex exposure scenarios. Fennell and co-workers (*28*) have recently developed such a computer model for the formation and removal of hemoglobin adducts. Extension of this type of model to include delineations of DNA repair/loss kinetics of adducts in human tissues and cells could provide a means of estimating genetic damage in exposed individuals using DNA and/or hemoglobin adduct measurements. Limited data from rat studies have already been incorporated into a preliminary physiologically based pharmacokinetic model for simulation of ETO adduct concentrations in the rat (*33*).

Little is currently known about the relationship between 7-HEG and the induction of mutations and cancer by ETO. Although similar concentrations of 7-HEG have been found in target and nontarget tissues of rats and mice after single ETO exposures (*19–21*) and multiple exposures that saturated DNA repair (*22,24*), the tissue-dependent variation in the half-life for this adduct indicates that there will be species, tissue, and even cell differences in the extent of 7-HEG accumulation during multiple exposures to lower concentrations of ETO. However, other critical factors appear to be involved in the species and tissue specificity for tumor induction by this chemical. For instance, the location of lesions in the genome and tissue susceptibility or resistance genes represent important determinants that could quantitatively affect the dose–response relationship for ETO-induced tumorigenesis. Identification of these events and their relationships to 7-HEG or HEtVal will require additional research.

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