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COMMENTARY

Quantification of Endogenous Carcinogens

THE ETHYLENE OXIDE PARADOX

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ABSTRACT. Although ethylene oxide is a proven genotoxic carcinogen in experimental animals, its human carcinogenicity is still being debated. Alkylations (hydroxyethylation) of DNA and proteins by ethylene oxide are well established. Ethylene oxide is metabolically formed from ethylene, which is a natural body constituent. Thus, endogenous sources of ethylene/ethylene oxide contribute to background alkylations of physiological macromolecules. There are now experimentally well established data sets on the background hydroxyethylations of the N-terminal valine of hemoglobin and of the 7-N position of guanine in DNA, in laboratory animals as well as in humans. A review of these data leads to the conclusion that these background levels display remarkable consistency between the different species studied and, as far as DNA adducts are concerned, also between different tissues. From the existing database it can be deduced that in rats a hemoglobin alkylation, equivalent to the level of normal background, would be caused by repetitive external atmospheric exposures to ethylene oxide (6 hr/day, 5 days/week for several weeks) of about 30 ppb. On the contrary, in the same species, a DNA alkylation, equivalent to the level of normal background, would be caused by similar repetitive exposures to ethylene oxide at about 1–2 ppm. This paradox is unresolved. It points, however, to the biological importance of endogenous DNA alkylations and questions current regulatory procedures of assessing the risk of minute doses of exogenous carcinogens. BIOCHEM PHARMACOL 52;1:1–5, 1996.

KEY WORDS. endogenous carcinogens; ethylene oxide; ethylene; DNA adducts; hemoglobin adducts; risk assessment

ENDOGENOUS CARCINOGENS

Research in chemical carcinogenesis has revealed the phenomenon of specific DNA and protein adducts of chemical carcinogens in non-exposed persons and experimental animals [1].

Marnett and Burcham [2] have pointed specifically to "physiological" backgrounds of a number of oxidized and alkylated DNA bases. By using the ^{32}P-postlabelling method, Randerath *et al.* [3] detected background DNA-adducts that were apparently age and tissue related. These adducts were called "I-compounds" because of their indigenous origin; they also were found in humans [4]. One apparent source of adducts is lipid peroxidation. For instance, DNA adducts of malonaldehyde [5] and cyclic "etheno"-adenine and "etheno"-cytosine adducts [6] have been linked with this particular process. Marnett and Burcham [2] have pointed out that "the presence of endogenous adducts in cellular DNA at levels approaching 1 per 10^6 base pairs raises provocative questions about the significance of the formation of adducts from exogenous carcinogens."

Törnqvist *et al.* [7, 8] have reported about "endogenous" levels of modifications of the N-terminal valine of hemoglobin. Average background levels were 200 pmol/g globin of *N*-methyl-valine and 20 pmol/g globin of *N*6-(2-hydroxyethyl) valine. Moreover, a number of adducts from aldehydes (e.g. formaldehyde, acetaldehyde, glycolaldehyde, and malondialdehyde) were found related to intermediary metabolism, dietary factors, or lipid peroxidation.

Very recently, it has been recognized that the endogenous hydrocarbon isoprene (2-methyl-1,3-butadiene) is carcinogenic in B6C3F1 mice, inducing liver, lung, Harderian gland, and forestomach tumors [9], its carcinogenic potency being about one order of magnitude less than that of the structurally related 1,3-butadiene. Isoprene is an endogenous product of intermediary metabolism which, in biologically activated form, is involved *inter alia* in the biosynthesis of cholesterol, steroids, bile acids, and modifications of K-vitamins. The compound has been quantitated in expired air of rats, and a toxicokinetic model has been published [10]. The diepoxide, 2-methyl-2,2′-bioxirane, is formed from isoprene by cytochrome P450 [11] and is mutagenic in *Salmonella typhimurium* TA 100 [12]. Hence, future risk assessments of exogenous isoprene and, by quantitative comparison, also of 1,3-butadiene exposures may well be related to "physiological" background risk factors.

It is therefore challenging to put data on background levels of specific macromolecular adducts and of the quan-

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titative toxicokinetic behaviour of endogenous and exogenous genotoxins producing these adducts into a combined perspective. This could finally result in a "scaling" of risks of exogenous genotoxic carcinogens, along with the unavoidable background loads of endogenous carcinogenic risk factors. Such attempts require: (i) quantitative data on background levels of specific DNA adducts, (ii) quantitative toxicokinetic data of the compound(s) in question, (iii) knowledge of the relation between external dose of carcinogen (genotoxin) and DNA adduct levels, and (iv) quantitative data of the carcinogenic potency of the compound(s) under review. At the present time, the only case where such a data set is nearly completely at hand is ethylene oxide.

THE CASE OF ETHYLENE OXIDE

Ethylene oxide is directly reactive and genotoxic. Long-term inhalation studies performed in mice and rats at exposure levels between 10 and 100 ppm ethylene oxide have resulted in development of malignant tumors at multiple sites (for review, see ECETOC [13] and IARC [14, 15]).

In humans, occupational exposures to ethylene oxide have increased levels of chromosomal aberrations, sister chromatid exchanges, and micronuclei in blood cells [14]. Hogstedt *et al.* [16] had reported about increased risks of leukemia and of stomach cancer in persons exposed to ethylene oxide. However, this was not confirmed in subsequent studies [17–22]. Hence, there is still some discrepancy between the very clearcut carcinogenic effects of ethylene oxide in experimental animals and observations in exposed humans. This is remarkable as ethylene oxide is a directly alkylating and genotoxic agent and is well distributed within the entire organism [23].

Ethylene oxide is also formed from its precursor, ethylene, in laboratory animals [23–26] as well as in humans [7, 27]. Ethylene is a normal body constituent, the endogenous formation of which has been deduced from its exhalation in untreated rats [28–30] and in non-exposed humans [27, 30, 31]. Consequently, its immediate metabolite, ethylene oxide, is also a normal body constituent.

Possible endogenous sources of ethylene/ethylene oxide are lipid peroxidation [8, 29, 32, 33], oxidation of free methionine [32, 33], oxidation of hemin in hemoglobin [34], and metabolism of intestinal bacteria [8]. These endogenous sources of ethylene/ethylene oxide contribute to a background alkylation (2-hydroxyethylation) of physiological macromolecules such as hemoglobin [7, 27, 35, 36], albumin [37], and DNA [38, 39]. An analysis of the available data, however, reveals inconsistencies between the backgrounds of hydroxyethylated hemoglobin and DNA.

PHYSIOLOGICAL BACKGROUND OF *N*-(2-HYDROXYETHYL)-VALINE IN HEMOGLOBIN

There is a well-founded data set on the physiological background of 2-hydroxyethylated N-terminal valine in hemoglobin. Investigations in mice [36], rats [36], and humans [22] have used the method of Törnqvist *et al.* [35, 40] of monitoring this adduct by a modified Edman degradation method. The differences across species appear to be minimal as the background levels of N-terminal *N*-(2-hydroxyethyl)valine in hemoglobin were found to be 58 ± 10 fmol/mg globin in mice, 42 ± 8 fmol/mg globin in rats [36], and about 20 fmol/mg globin in (non-smoking) humans [22].

A calculation of the quantity of a subchronic or chronic external ethylene oxide exposure that would lead, in the theoretical absence of endogenous ethylene oxide production, to a hemoglobin alkylation of this magnitude has been published [41]; based on the dose-alkylation data of Walker *et al.* [36] who had exposed rats and mice to 3, 10, 33, 100, or 300 (rats only) ppm ethylene oxide for 6 hr/day, 5 days/week, over 4 weeks, it was inferred that a repetitive inhalation regimen of this type, at an external exposure of *27 ppb ethylene oxide*, would lead to a level of hemoglobin hydroxyethylation in rats which is actually found as the background. A value in this order of magnitude seems plausible, and we have used the endogenous and, therefore, unavoidable levels of hemoglobin alkylation for argumentations concerning regulatory consequences [41]. However, this must be opposed to several recent quantitative findings regarding DNA adducts of ethylene oxide.

PHYSIOLOGICAL BACKGROUND OF *N7*-(2-HYDROXYETHYL)GUANINE IN DNA

The present database of physiological hydroxyethylation of guanine in DNA also gives a very uniform and consistent picture across species.

- (i) In a pilot study, Föst *et al.* [38] used a thermal depurination step of DNA obtained from human and rat lymphocytes, and GC/MS analysis after derivatization (MSTFA). From rat (N = 3) lymphocytes, the release of *N7*-(2-hydroxyethyl)guanine was 5.6 ± 3.0 pmol/mg DNA. From human (N = 8) lymphocytes, the corresponding figure was 8.5 ± 5.7 pmol/mg DNA.
- (ii) Walker *et al.* [39] studied the formation and persistence of *N7*-(2-hydroxyethyl)guanine in DNA following repeated exposures of rats and mice to ethylene oxide. They used, also after thermal hydrolysis, HPLC separation and detection of fluorescence of this particular adduct. In this study, they reported that "analysis of DNA from control mouse and rat tissues revealed the presence of peaks equivalent to 2–6 pmol/mg DNA, using both a calibration curve and a standard addition technique for adduct determinations" and claimed that this was generally consistent with the preceding observations of Föst *et al.* [38].
- (iii) Cushnir *et al.* [42] used tandem mass spectrometry to assess the excretion rates of several alkylguanines in human urine. For *N7*-(2-hydroxyethyl)guanine, they found physiological excretions of about 2 μg/day. On the basis of these data, Farmer has estimated a normal level of 5 of

these adducts per 10^6 DNA bases; this is again about 2 pmol/mg DNA.*

- (iv) Leutbecher [43] used acid hydrolysis of human lymphocyte DNA and subsequent HPLC analysis with fluorescent derivatization; a normal range of about 2 pmol *N*7(2-hydroxyethyl)guanine/mg DNA was found.

This means that use of entirely different types of analytical methodology, by different authors and research groups, has resulted in a normal range of a few picomoles (lower range at about 2 pmol) of specific ethylene oxide adduct per milligram DNA, in different organs and in different species (mice, rats, humans). As for the hemoglobin adduct, this quantity of adduct may be related to equivalent quantities of external ethylene oxide exposure. As the quantitative figures for rats, mice, and humans cover the same range of magnitude, a quantitative comparison ought to be based on the data set in rats, especially as specific dose-adduct studies are available in this species [39, 44, 45].

Walker *et al.* [39] have studied the dose-dependence of *N*7-(2-hydroxyethyl)guanine in several organs of rats exposed *subacutely* for 4 weeks (6 hr/day; 5 days/week) to 10, 33, or 100 ppm ethylene oxide. There was obvious linearity of the dose-adduct curve between 0 and 100 ppm of external inhalation exposure. In rat liver, the 100 ppm exposure regimen resulted in a DNA adduct level of 49 ± 1 pmol *N*7(2-hydroxyethyl)guanine/μmol guanine, equivalent to about 66 pmol/mg DNA. On the basis of an endogenous DNA adduct level of 2 pmol/mg DNA (*vide supra*) this would mean that a subacute external inhalation exposure (using the regimen of 6 hr/day, 6 days/week) to about *1.1 ppm ethylene oxide* would create a DNA-adduct level (guanine hydroxyethylation) that is observed as the actual background value. In this context, it should be noted that time–course studies of the ethylene-oxide-derived *N*7(2-hydroxyethyl)guanine adduct in DNA of rats have shown that after 4 weeks of repetitive ethylene oxide exposure, a plateau is reached which is about a one-decimal magnitude higher than the adduct level resulting from a single exposure.

A second set of dose-adduct data in rats is available from two independent *acute* studies using single [^{14}C]ethylene oxide inhalations of different types (6-hr inhalation of 1, 10, or 33 ppm [44] vs single doses in a closed recirculated system [45]). Both studies are consistent with each other when the different toxicokinetic conditions are properly taken into account (see Ref. 45). In order not to interfere with quantitation of the physiological background, both studies used exposures to [^{14}C]ethylene oxide and quantitated *N*7-(2-hydroxyethyl)guanine in hepatic DNA, after chromatographic separation of the adduct, by means of liquid scintillation counting. According to these data, an adduct level of 2 pmol/mg DNA in rat liver would be caused by uptake of about 0.2 mmol of a single ethylene oxide dose [45] or by a single 6-hr exposure to about 20 ppm ethylene oxide [44]. Again, these data are consistent with those of Walker *et al.* [39] if one considers the differences between acute (single) and subacute (multiple) dosing, as far as accumulation of the adduct is concerned.

CONCLUSIONS AND OPEN QUESTIONS

"Endogenous" macromolecular adducts may provide a new basis for risk assessment of exogenous genotoxic compounds. On the basis of overwhelming experimental evidence on ethylene oxide, it appears that:

- (i) There are significant background adduct levels of protein hydroxyethylation (mostly studied as N-terminal valine adducts of hemoglobin) and of hydroxyethylated DNA [the most abundant adduct being *N*7-(2-hydroxyethyl)guanine].
- (ii) These background levels display remarkable consistency between the species studied and, as far as DNA adducts are concerned, also between different tissues.
- (iii) There is a good and consistent database of dose-adduct studies on the quantity of adducts to both hemoglobin and DNA caused by external ethylene oxide exposures of rats. From this database it can be deduced that in rats a hemoglobin alkylation, equivalent to the actual level of the normal background, would be caused by repetitive exposures to ethylene oxide (6 hr/day, 5 days/week for several weeks) of about 30 ppb.
- (iv) On the contrary, in the same species a DNA alkylation, equivalent to the actual level of the normal background, would be caused by repetitive exposures (same regimen) to ethylene oxide at about 1–2 ppm.

This is an obvious paradox that urgently needs further clarification. Obviously, *endogenous ethylene oxide* (formed from ethylene) hits much more the nuclear DNA target than is resembled by the hydroxyethylation of blood proteins, which is used for biomonitoring of *exogenous ethylene oxide*. This is corroborated by recent findings [46] that the ratios of *N*7-(2-hydroxyethyl)guanine in DNA of several tissues over hydroxyethyl-valine in hemoglobin, in rats exposed to ethylene, were greater than these ratios determined in ethylene oxide-exposed rats. A possible explanation could be that the supposed uniform distribution of ethylene oxide within the organism [23] does not pertain to intracellular distributions. Differences in the intracellular distribution patterns between exogenously administered and endogenously produced ethylene oxide could, for instance, be caused by production of ethylene oxide in the nucleus.

It is also of interest that glutathione-*S*-transferase (GSTT1) polymorphism influences the background sister chromatid exchange (SCE) rate in human lymphocytes [47]. This particular GST isoenzyme is involved in the inactivation of ethylene oxide [48, 49], and the individual genetic state (GSTT1 +/−) influences the SCE response of

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human lymphocytes towards exogenous ethylene oxide [50]. Observed individual differences in the "alkaline elution" patterns of mononuclear blood cells from workers exposed to ethylene oxide [51] might, at least partly, be connected with this feature. The influence of the GSTT1 status on the background SCE rate can be viewed as an indication for a biological significance of the endogenous (physiological) ethylene oxide for a physiological background genotoxicity. Such an idea would call for reevaluation of risk assessment for ethylene oxide and related compounds. On the other hand, it calls for further research on possible, heretofore unknown, adaptive mechanisms against low levels of ethylene oxide and probably other endogenously produced carcinogens.

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