Ethylation of Nucleic Acids by Ethynitrosourea-1-\(^{14}\)C in the Fetal and Adult Rat\(^1\)

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SUMMARY

The monofunctional alkylating agent \(N\)-ethyl-\(N\)-nitrosourea has become known as a highly potent transplacental pulse carcinogen, specifically directed against cells associated with the nervous system. In the present study, the formation of the alklylation product 7-ethylguanine in the nucleic acids of brain and liver of both fetal and adult BD-IX rats (18th day of gestation) was demonstrated by ion-exchange radiochromatography, after administration of \(N\)-ethyl-\(N\)-nitrosourea-1-\(^{14}\)C. One hr after i.v. injection of 75 \(\mu\)g of \(N\)-ethyl-\(N\)-nitrosourea-1-\(^{14}\)C/g body weight (~30% of the 50% lethal dose), the fractions of guanine residues ethylated (7-ethylguanine : guanine) in the nucleic acids of the whole fetus were 1.7 \(\times 10^{-5}\) (DNA) and 0.6 \(\times 10^{-5}\) (RNA). The 7-ethylguanine : guanine values for the DNA of fetal brain and liver were 2.2 \(\times 10^{-5}\) and 4.4 \(\times 10^{-5}\), respectively. The corresponding values for brain and liver of the adult BD-IX rat were 2.1 \(\times 10^{-5}\) and 3.5 \(\times 10^{-5}\), respectively. The data are compared with the results or similar measurements with other \(N\)-nitroso carcinogens and discussed in relation to the relative toxicity and carcinogenicity of the respective compounds.

INTRODUCTION

Among the nitrosamides, both ENU\(^3\) and MNU are known to be highly carcinogenic (9). In particular, it has been shown that ENU is a potent single-dose transplacental carcinogen in rats (12). In addition to an intriguing specificity for cells connected with the central and peripheral nervous system (25), these compounds are characterized by their rapid heterolytic decomposition under physiological conditions ("pulse carcinogens" (21)). This property may prove to be particularly useful in studies relating to the temporal sequence of molecular and cellular events associated with the process of malignant transformation.

As in the case of other \(N\)-nitroso carcinogens, it has been shown that administration of MNU in vivo results in the alkylation of different molecular species in a variety of tissues (14, 19, 22, 23), the major site of alkylation in DNA and RNA being the N-7 position of guanine. In the following report we describe the results of experiments demonstrating the formation of 7-EG in the nucleic acids of whole rat fetuses, as well as of fetal and adult rat brain and liver after administration of ENU-1-\(^{14}\)C. Some of these data have recently been communicated in a preliminary form (10, 11).

MATERIALS AND METHODS

Animals. Female BD-IX rats (8), 234 ± 12 g, were used on the 18th day of gestation, counting the 1st day after conception (in the early evening) as Day 1 of gestation (8). The average weight of the fetuses at this stage was 0.83 ± 0.025 g; the litter size was 8 ± 1.

Administration of ENU-1-\(^{14}\)C. ENU-1-\(^{14}\)C, specific activity, 5.57 \(\mu\)Ci/mmol, was obtained from Farbwerke Hoechst AG, Frankfurt/Main, Germany. The rate of decomposition of ENU in aqueous solution is strongly pH dependent (9) and extremely high at values above pH 7.0 (Chart 1). Therefore, ENU-1-\(^{14}\)C was dissolved in citric acid : disodium phosphate buffer (20), pH 6.0, to give a 0.1 M solution of ENU-1-\(^{14}\)C and was immediately administered i.v. The applied dose was 75 \(\mu\)g of ENU-1-\(^{14}\)C per g body weight in all but 1 experiment (25 \(\mu\)g of ENU-1-\(^{14}\)C per g). Although this dose corresponds to about 30% of the 50% lethal dose for pregnant BD-IX rats (12), the survival of the offspring seemed unimpaired when the litter was fostered by untreated mothers in control experiments.

Preparation and Radiochromatography of Nucleic Acids. One hr after injection of ENU-1-\(^{14}\)C into 3 or more pregnant BD-IX rats, fetuses, brains, and livers, after a brief portal perfusion with cold 0.9% NaCl solution, were quickly removed, pooled, and frozen in liquid nitrogen. Ten animals were used for the preparation of nucleic acids from adult brain (average brain weight, 1.7 g). For the preparation of nucleic acids from fetal brain (average weight, 69 mg) and fetal liver (average weight, 58 mg), these organs were immediately excised from a total of 36 fetuses and frozen in liquid nitrogen together with a 10-fold amount of the corresponding tissues from untreated rats.

With a modified Kirby method (K. S. Kirby, personal communication, as described in Refs. 6 and 13), DNA and RNA were isolated from the same pooled tissue samples. The tissue was homogenized in a solution of 0.5% sodium naphthalene-1,5-disulfonic acid (10 ml/g tissue) and then shaken with a mixture of phenol : m-cresol : \(H_2O : \text{8-hydroxy-quinoline} (1,000:140:110:1)\). After centrifugation at 2,200 \(X\)
Alkylation of nucleic acids after in vitro (15) and in vivo (19) exposure to alkylating carcinogens has been found to occur predominantly on the N-7 position of guanine. Lower degrees of alkylation were also detected on N-1 and N-3 of adenine, N-1 of cytosine (16), and O-6 of guanine (18). The positions of the peaks of 7-methylguanine and 7-EG between those of guanine and adenine in Dowex 50 radiochromatograms of nucleic acid hydrolysates have been repeatedly described in the literature (19) and confirmed in the present experiments.

The curves for fetal (Chart 2b) and adult liver RNA (Chart 2e) clearly show the presence of a peak of $^{14}$C-labeled material corresponding to 7-EG, as indicated by the position of the nonradioactive 7-EG added as a marker. Correspondingly, the formation of 7-EG was also shown for fetal liver (Table 1). The RNA of fetal and adult brain was not analyzed. There is a distinct difference between the adult liver and the fetus with regard to the incorporation of $^{14}$C activity into adenine, N-1 of cytosine (16), and O-6 of guanine (18). The formation of 7-EG was equally evident in the case of DNA from whole fetuses (Chart 2a), fetal brain (Chart 2c), fetal liver (Table 1), adult brain (Table 1), and adult liver (Chart 2d). Additional small peaks of $^{14}$C activity appeared before chromatography. It was prepared by reacting equimolar quantities of DIMP and diethyl sulfate at pH 7.2 and 37° for 120 hr (1) and identified by UV absorption spectroscopy. In addition to the localization of the peaks of 7-EG-$^{14}$C in the radiochromatograms by cochromatography of nonradioactive carrier 7-EG, fractions assumed to contain 7-EG-$^{14}$C were chromatographed on Whatman No. 4 paper, as described by Craddock et al. (6). It could be shown that the position of the $^{14}$C activity coincided with that of 7-EG.

The fraction of ethylated guanine residues (7-EG: guanine) was determined from the integral $^{14}$C activity in the peak of 7-EG (considering its specific activity as identical with that of the injected ENU-$^{14}$C) and the amount of guanine derived from the extinction of the guanine peak (based on a molar extinction coefficient in acid of $\epsilon_{260} = 8000$). The values obtained for the degree of guanine ethylation in DNA were corrected for the RNA contamination of the respective DNA preparations.

RESULTS

Alkylation of nucleic acids after in vitro (15) and in vivo (19) exposure to alkylating carcinogens has been found to occur predominantly on the N-7 position of guanine. Lower degrees of alkylation were also detected on N-1 and N-3 of adenine, N-1 of cytosine (16), and O-6 of guanine (18). The positions of the peaks of 7-methylguanine and 7-EG between those of guanine and adenine in Dowex 50 radiochromatograms of nucleic acid hydrolysates have been repeatedly described in the literature (19) and confirmed in the present experiments.

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The formation of 7-EG was equally evident in the case of DNA from whole fetuses (Chart 2a), fetal brain (Chart 2c), fetal liver (Table 1), adult brain (Table 1), and adult liver (Chart 2d). Additional small peaks of $^{14}$C activity appeared between the peaks of guanine and 7-EG, especially in the case of adult liver DNA (Chart 2d). Although they were not further identified, the position of the peak to the left of the 7-EG peak (Chart 2d) is reminiscent of the well-known position of 7-methylguanine (6, 23).

The degrees of ethylation (expressed as the fraction of G residues ethylated, 7-EG:guanine) obtained in DNA and RNA...
are summarized in Table 1, together with the mean $^{14}C$ activity per mg of total DNA, RNA, and protein, respectively. The degree of guanine ethylation obtained for the DNA of the whole fetus, as well as for the DNA of the fetal and adult liver, exceeded that for the RNA of these tissues by factors of approximately 2.5 to 5.0 (fetal liver). The highest 7-EG:guanine values were found in the DNA of fetal and adult liver (7-EG:guanine $\sim 4 \times 10^{-5}$), followed by the DNA of fetal and adult brain and of the whole fetus (7-EG:guanine $\sim 2 \times 10^{-5}$).

In an additional experiment, the question of linearity between dose of ENU and degree of guanine ethylation was investigated for the RNA of the whole fetus and of the adult liver, with the use of a dose of 25 $\mu$g of ENU-$^{14}C$ per
Table 1
Degree of guanine ethylation in DNA and RNA of whole BD-IX rat fetuses (18th day of gestation), as well as fetal and adult BD-IX rat brain and liver, 1 hr after administration of a single i.v. dose of 75 μg of ENU-1,14C per g body weight

<table>
<thead>
<tr>
<th>Tissue</th>
<th>DNA (g residues ethylated (7-EG:guanine))</th>
<th>RNA (μCi/mg)</th>
<th>14C activity (dpm/mg X 10^-3) recovered in:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole fetus, BD-IX rat, 18th day of gestation</td>
<td>1.7 X 10^-5</td>
<td>0.6 X 10^-5</td>
<td>DNA: 2.9, RNA: 2.3, Protein: 4.1</td>
</tr>
<tr>
<td>Fetal brain, BD-IX rat, 18th day of gestation</td>
<td>2.2 X 10^-5</td>
<td>Not measured</td>
<td>DNA: 3.2, RNA: 2.9, Protein: 1.0</td>
</tr>
<tr>
<td>Fetal liver, BD-IX rat, 18th day of gestation</td>
<td>4.4 X 10^-5</td>
<td>0.9 X 10^-5</td>
<td>DNA: 9.0, RNA: 5.9, Protein: 3.8</td>
</tr>
<tr>
<td>Brain, adult female, BD-IX rat, 18th day of gestation</td>
<td>2.1 X 10^-5</td>
<td>Not measured</td>
<td>DNA: 1.1, RNA: Not measured, Protein: 0.8</td>
</tr>
<tr>
<td>Liver, adult female, BD-IX rat, 18th day of gestation</td>
<td>3.5 X 10^-5</td>
<td>1.4 X 10^-5</td>
<td>DNA: 2.7, RNA: 2.4, Protein: 10.5</td>
</tr>
</tbody>
</table>

a Mean values for ≥2 independent determinations with 3 pregnant rats each. Maximum deviation of the measured values from the means, ≤ 30%

b Mean values for the brains or livers from 36 fetuses, respectively (see "Materials and Methods").

g. Of those found after a dose of 75 μg of ENU-1,14C per g, the 7-EG:guanine values obtained were 32 and 43%, respectively. Together with recent estimates by Swann and Magee (24) for the degree of nucleic acid guanine ethylation in liver and brain of adult Wistar albino rats 2 hr after an i.v. dose of 152 μg of ENU per g, these values are consistent with the assumption that linearity between dose and degree of guanine ethylation may indeed exist over the dose range investigated.

DISCUSSION

The present results indicate that 1 hr after administration of a single i.v. dose of 75 μg of ENU per g to pregnant rats, the degree of ethylation (expressed as the fraction of guanine residues ethylated, 7-EG:guanine) in both DNA and RNA of the fetal and adult tissues is of the order of 2 X 10^-5 (Table 1). With a nuclear DNA content of ~6 X 10^9 mg (4), this degree of DNA guanine ethylation corresponds to about 4 X 10^4 ethylated DNA guanine bases per average diploid cell nucleus. Since cells associated with the central and peripheral nervous system of the embryo apparently represent the primary targets for malignant transformation in this system (12, 25), it is important to note that, in terms of its alkylating effect, ENU is equally effective when administered via the transplacental route.

The degree of nucleic acid guanine alkylation observed after in vivo application of ethylating N-nitroso carcinogens is generally lower than that obtained with their methylating counterparts (19). After recalculation for equimolar doses, assuming a linear relationship between dose and degree of alkylation, the 7-EG:guanine values obtained for ENU may be compared with the corresponding data (methylguanine: guanine for the related nitrosamide MNU reported by Swann and Magee (23). Such a comparison shows that the degrees of guanine alkylation by MNU in rat liver DNA and RNA exceed those obtained with ENU by a factor of approximately 30. A similar relationship holds for the hepatocarcinogenic nitrosamines dimethylnitrosamine (23) and diethylnitrosamine (10), where the respective factor for rat liver nucleic acids was estimated to be in the range of 60 to 100 (10).

Within both classes of carcinogenic N-nitroso compounds, there appears to be no direct correlation between the relative toxicity (in terms of the 50% lethal dose; Refs. 9 and 12) and the maximum degree of DNA or RNA guanine alkylation in rat liver. However, alkylation by N-nitroso carcinogens is not restricted to the nucleic acid bases, but occurs to an even greater extent on functional groups of other cellular macromolecules. Toxicity might, therefore, show a better correlation with other parameters, such as the degree of alkylation of cellular proteins. Furthermore, differences in the degree of alkylation in liver may not be representative of the situation in other cell systems which are more sensitive with respect to toxic effects, such as bone marrow and intestinal epithelium.

The possible role of nucleic acid alkylation in the initiation of the process of malignant transformation of mammalian cells has been repeatedly discussed during the past years (2, 17, 19). However, the present knowledge of the molecular mechanisms by which an alkylation of constituent bases of nucleic acids, and of DNA in particular, could lead to permanent alterations in the genome and in the phenotypic expression of target cells is still largely theoretical.

In the present experiments, similar degrees of guanine ethylation by ENU were observed in both fetal and adult brain DNA (7-EG:guanine ~2.2 X 10^-5 and ~2.1 X 10^-5, respectively) on the one hand, and in the DNA of the fetal and adult liver (7-EG:guanine ~4.4 X 10^-5 and ~3.5 X 10^-5, respectively) on the other (Table 1). Furthermore, the degree of DNA guanine ethylation in both fetal and adult animals was higher in the liver than in the brain by an approximate factor of 2 (Table 1). These findings indicate that the tumorigenic effect of an alkylating carcinogen is not a simple function of the degree of guanine N-alkylation produced in the nucleic acids, since the carcinogenicity of a single dose of ENU to pregnant rats is much higher in the offspring than in the mothers, and the tumors arising in the offspring are almost
exclusively associated with the nervous system, whereas liver tumors have not been observed (Refs. 12 and 25; unpublished possible to establish a correlation between the degree of the binding of β-propiolactone and some related alkylating results from this laboratory). Indeed, with the exception of must also be considered that the N-7 position of guanine may not be the critical site of alkylation associated with the nucleic acid alkylation in vivo and the proportion of target cells "initiated" by alkylating carcinogens (23). The possibility makes it must be shown whether or not methylation and ethylation of nucleic acid bases may be considered equivalent with respect to their effectiveness to initiate malignant transformation and whether, under in vivo conditions, the rates of depurination of DNA are different for ethylated versus methylated bases (10). These questions are related to the problem of nucleic acid repair mechanisms, for which the role in the process of malignant transformation has not yet been clarified. Finally, information is needed on the question of whether the sensitivity (accessibility of critical sites) of mammalian cells towards alkylating carcinogens and their capacity for the phenotypic expression of altered properties varies as a function of their proliferative and functional state. The ENU-rat fetus system may provide an interesting model for the investigation of these questions.

REFERENCES

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