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Action of Phenobarbital Given to Rats Together with Diethylnitrosamine on the O^6-Ethylguanine Content of Liver DNA

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Abstract—*When rats are fed diethylnitrosamine* (10 mg/kg/day), *no O^6-ethylguanine is found in liver DNA after 2 weeks, but a considerable amount accumulates after 4 weeks. On the other hand, a 2-week feeding of diethylnitrosamine is not sufficient to induce liver cancers, whereas a 4-week treatment leads to hepatocarcinomas in 50% of the animals.*

Administration of phenobarbital (75 mg/kg/*day*) *together with diethylnitrosamine during 4 weeks prevents the formation of liver cancers. It also prevents accumulation of O^6-ethylguanine in liver DNA.*

Phenobarbital does not change the amount of O^6-ethylguanine repair activity present in liver chromatin after 2 or 4 weeks of treatment with diethylnitrosamine. It is thus concluded that, by inducing the development of the endoplasmic reticulum, phenobarbital decreases the equilibrium concentration of the ultimate carcinogen derived from this indirect alkylating agent.

INTRODUCTION

DIETHYLNITROSAMINE (DENA) is an indirect alkylating agent that, after enzymatic activation in liver, generates the carbocation CH_3-CH_2^+ which reacts with nucleophiles in DNA. Several studies have suggested that alkylation of guanine O-6 can lead to mutation and cancer [1–4]. Barbason and Betz [5] have shown that continuous administration of DENA to rats (10 mg/kg/day) for 2 weeks induces preneoplastic foci, but is not sufficient to produce neoplastic nodules; these nodules appear only when the DENA administration has lasted for 4 weeks or more.

When phenobarbital (PhB) is given after the DENA administration, it shortens the latency period between the appearance of preneoplastic foci and their malignant transformation [6]; this promoter effect of PhB given after an initiator has been found in numerous studies [7–9].

By contrast, PhB given simultaneously with DENA reduces liver cancer frequency [10, 11]. Barbason *et al.* [12] observed that the number and size of preneoplastic lesions are lower when a 4-week DENA treatment is associated with the administration of PhB; moreover not a single cancer develops whereas, when DENA is given alone, 50% of the rats show hepatocarcinomas at *post mortem* examination.

Since there might be a correlation between liver cancer and O^6-alkylation of guanine, we have hypothesized that PhB given together with DENA would decrease the O^6-ethylguanine (O^6-etG) content of liver DNA. The O^6-etG content of DNA depends on two parameters: (i) the concentration–time integral of CH_3-CH_2^+ in the cell; (ii) the rate of O^6-ctG repair. Renard *et al.* [13] observed the disappearance of O^6-etG from DNA in isolated rat liver nuclei. The repair mechanism is a transethylation from O^6-etG onto a cysteine of a chromatin protein which can be used only once (suicide reaction) [14, 15].

In this work, we have measured the O^6-etG content of liver DNA after administration to rats of DENA alone or together with PhB. We have also determined the amount of repair protein in liver chromatin in both animal groups.

MATERIALS AND METHODS

Wistar male rats of about 180 g received DENA and/or PhB in drinking water for 2 or 4 weeks. The daily doses were about 10 mg/kg for DENA, and 75 mg/kg for PhB. The animals were sacrificed after a 18-h fast.

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Preparation of liver DNA

The livers were homogenized in 1 M NaCl, 30 mM *N*-lauroyl-sarcosine (5 ml/g liver). Water-saturated phenol, 70 mM 8-hydroxyquinoleine, was added to the homogenate and the mixture shaken for 30 min at room temperature. After centrifugation, the aqueous phase containing DNA was recovered and submitted to another deproteinization with an equal volume of chloroform/isoamyl alcohol (24:1; v:v). The mixture was centrifuged and the upper phase collected. This solution was submitted to two successive digestions of 30 min at 37°C, the first with pancreatic ribonuclease (40 μg/g liver) and the second with proteinase K (200 μg/g liver). After a last treatment with chloroform/isoamyl alcohol, DNA was precipitated from the solution with an equal volume of ethoxyethanol, and the pellet redissolved in 0.15 M NaCl, 0.015 M Na citrate, pH 7.0. The DNA solution was dialyzed against 10 mM Tris–HCl, 1 mM EDTA, pH 8.0, then concentrated on polyethylene glycol 6000.

The amount of DNA per ml was determined by the diphenylamine method [16]. Samples containing 1.5 mg DNA were depurinated in 0.1 M HCl at 70°C for 50 min. After addition of 1 M K_2HPO_4 to reach pH 4.0 and lyophilization, 1 ml 50 mM ammonium phosphate, pH 4.0, was added. The samples were centrifuged 10 min at 15,000 ***g*** and the supernatants taken for HPLC analysis.

Purine analysis

The samples (250 μl) were chromatographed on an Altex ODS-10 ultrasphere column (25 × 1 cm) in an HPLC Altex 332. The elution was carried out at a rate of 2 ml/min with 50 mM ammonium phosphate, pH 4.0/acetonitrile (85:15; v:v). The O^6-etG retention time was 11 min.

Preparation of chromatin proteins

Cell nuclei and chromatin were prepared from rat liver according to Thibodeau and Verly [17]; non-histone proteins were extracted from chromatin with heparin-ultrogel as described by Renard and Verly [18]. This extract was dialyzed against 50 mM Tris–HCl, 1 mM EDTA, 2 mM dithiothreitol, pH 8.0. The amount of protein was determined by the method of Bradford [19].

Transalkylase assay

The enzyme activity was measured as described by Renard and Verly [18] and Lemaître *et al.* [20].

Calf thymus DNA was alkylated with [^{3}H]ethylnitrosourea (4.5 Ci/mmol; IRE, Fleurus, Belgium), and stored at a concentration of 1 mg/ml in 50 mM Tris–HCl, 1 mM EDTA, 2 mM dithiothreitol, pH 8.0, at 4°C.

Three volumes of chromatin protein solution were incubated with one volume of [^{3}H]ethylated DNA solution for 60 min at 37°C. To 200-μl aliquots (50 μg DNA containing 285 fmol O^6-etG; 25 μg protein) were added 5 μg non-radioactive O^6-etG in 10 mM HCl, and 33 μl 1M HCl. The mixture was heated at 70°C for 50 min. After addition of 35 μl 1M K_2HPO_4 and 57 μl acetonitrile, and centrifugation, 250-μl samples were taken from the supernatant for HPLC analysis.

The O^6-etG-containing fraction is collected after 11 min and its radioactivity measured. Knowing the chromatographic yield (from the non-radioactive O^6-etG internal standard) and the specific radioactivity of the alkylating agent, the amount of O^6-etG in the DNA submitted to depurination can be calculated.

RESULTS

Accumulation of O^6-etG in liver DNA

Male rats of the same age were randomly distributed between two groups. Animals of the first group were treated for 2 weeks, and those of the second group for 4 weeks. Each group was divided in two: rats of one subgroup received DENA alone; rats of the other subgroup received DENA and PhB. At the end of the treatments and at regular intervals for 6 weeks afterwards, three rats were sacrificed in each subgroup. The livers were pooled and the DNA extracted; 1.5 mg DNA was depurinated and the purines separated by HPLC.

The amount of O^6-etG in the DNA sample could be estimated by reference to a calibration curve obtained by injecting increasing amounts of synthetic O^6-etG in the HPLC apparatus. Knowing that 24.5% of the bases in rat DNA are guanines, the O^6-etG/10^6 G ratio in the analyzed DNA could be calculated.

Liver DNA did not contain significant amounts of O^6-etG after 2 weeks of DENA treatment alone, or associated with PhB. This means that the transalkylase removed the ethyl group from O^6-etG as soon as this adduct appeared in the cell DNA.

After 4 weeks of treatment, the results are very different in the two subgroups. The rats that received DENA alone had accumulated 1600 O^6-etG/10^6G in their DNA at the end of the treatment. This indicates that the repair capacity had been overidden. By contrast, the rats that received DENA and PhB had accumulated only 15 O^6-etG/10^6 G. After the end of the treatment, the O^6-etG progressively disappeared from liver DNA in both subgroups (Table 1); this is due to a residual repair activity (see later) and also likely to the synthesis of new transalkylase molecules.

Repair activity

Two groups of rats, treated for 2 and 4 weeks respectively, were divided into subgroups. The animals of the first subgroup were kept as a control;

Table 1. Action of phenobarbital on the O^6-ethylguanine content of liver DNA in rats treated with diethylnitrosamine

Treatment	Day 28	32	35	42
DENA	1600	50	5	1
DENA + PhB	15	3	1	0

Rats received *per os* DENA (10 mg/kg/day) with or without PhB (75 mg/kg/day) for 4 weeks. They were sacrificed in lots of three at the end of the treatment (day 28) or later. The O^6-etG content of liver DNA was measured; the results are expressed as O^6-etG/10^6G ratios.

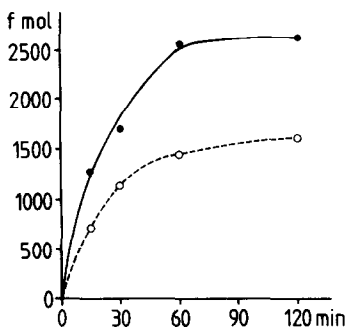

Fig. 1. In vitro repair of O^6-ethylguanine in DNA by chromatin proteins. Chromatin non-histone proteins, prepared from livers of rats treated 2 weeks (●—●) or 4 weeks (○--○) with DENA, were incubated with DNA alkylated with ethylnitrosourea. The amount of O^6-etG that disappeared from DNA was followed as a function of time; the results are expressed as fmol *O^6-etG repaired by the protein from an amount of chromatin containing* 1 mg *DNA.*

the animals of the second received DENA alone; the animals of the third received PhB alone; the animals of the fourth received DENA and PhB. At the end of the treatment, rats and their livers were weighed; the non-histone proteins were extracted from the liver chromatin of the four rats constituting each subgroup.

Livers from rats receiving PhB were enlarged, less however when they were fed DENA simultaneously.

Three volumes of chromatin-protein solution (200 μg/ml) were incubated at 37°C with one volume of [^{3}H]ethylated DNA (DNA alkylated with [^{3}H]ethylnitrosourea) solution (1 mg/ml). Three 200-μl aliquots were taken after 15, 30, 60 and 120 min. After depurination and HPLC analysis, the radioactivity in the O^6-etG peak was measured. Samples of [^{3}H]ethylated DNA were incubated without chromatin proteins to measure the amount of O^6-etG loss in absence of repair activity. The results, corrected for this control, enable to calculate the amount of O^6-etG that disappeared from the [^{3}H]ethylated DNA due to repair processes; the results are expressed in fmol O^6-etG removed from the [^{3}H]ethylated DNA by the chromatin proteins corresponding to 1 mg liver DNA.

Figure 1 shows that the amount of O^6-etG repair reached a plateau after 60 min. We recall that the repair of O^6-etG is a suicide reaction: the amount of O^6-etG that had disappeared when the reaction stopped (fmol) is equal to the amount of transalkylase in the medium. The results are presented in Table 2.

Table 2. Amounts of transalkylase in chromatin of rats fed diethylnitrosamine and/or phenobarbital

Treatment	fmol/mg liver DNA 2 weeks	4 weeks
Control	342	375
DENA	2570	1460
PhB	473	566
DENA + PhB	2226	1301

Rats were fed DENA and/or PhB for 2 or 4 weeks. Non-histone proteins were extracted from liver chromatin and incubated with DNA alkylated with ethylnitrosourea during 60 min at 37°C. The amount (fmol) of O^6-etG that disappeared from DNA is equal to the amount of transalkylase. The results are given as fmol of transalkylase in a chromatin amount containing 1 mg DNA.

We shall first comment on the situation after 2 weeks of treatment. The residual repair activity in the chromatin of rats which were fed DENA is seven times higher than the activity found in the control rats. We have seen, in the previous section, that this increased activity was sufficient to mop up the O^6-etG lesions as soon as they appeared. More pertinent to our subject is the observation that PhB administration did not change significantly the repair activity whether DENA was simultaneously given or not.

The situation after 4 weeks again shows that PhB feeding had no significant effect. On the other hand, the rats that received DENA had a repair activity higher than the controls, but which was however lower than that observed after 2 weeks, suggesting a progressive exhaustion of the repair capacity likely explaining the accumulation of O^6-etG in liver DNA of rats fed DENA alone.

DISCUSSION

We show that, when 10 mg DENA/kg/day is continuously fed to rats for 2 weeks, no O^6-etG can be found in liver DNA; the repair activity is sufficient to eliminate the adduct as soon as it is formed. This result correlates well with the observation that a 2-week DENA treatment is not sufficient to induce liver cancers [6].

When the DENA feeding is prolonged for 4 weeks, a large amount of O^6-etG (1600/10^6 G) has accumulated in liver DNA. Thus a 4-week DENA treatment seems to override the repair capacity so that some O^6-etG is continuously present in liver DNA during the last days; it, however, disappears after the end of the treatment. On the other hand,

50% of the rats die with liver cancers after about 12 months [6].

When, during the 4 weeks, PhB (75 mg/kg/day) is given together with DENA, the amount of O^6-etG found in liver DNA at the end of the treatment is 100-times smaller. The addition of PhB to DENA also prevents the appearance of liver cancers [12].

Because of the rapid disappearance of O^6-etG from rat liver DNA, Scherer *et al.* [21] have argued that O^2- and O^4-ethylthymine, which are minor alkylation products but which persist longer in liver DNA, might be responsible for liver cancers due to DENA rather than O^6-etG. Without denying a role for these O-alkylated pyrimidines, our results rather favor O^6-ctG as the main carcinogenic lesion since liver cancer seems to be induced only when the repair system is exhausted and O^6-ctG begins to accumulate in liver DNA.

To investigate the mechanism by which PhB prevents the accumulation of O^6-etG in liver DNA between 2 and 4 weeks of DENA feeding, we have measured the repair activity of the chromatin proteins. We found that the DENA treatment increased the amount of transalkylase in liver chromatin, confirming results obtained by Margison *et al.* [22] and Renard *et al.* [23]. On the other hand, the PhB treatment did not change the repair activity, expressed per mg DNA, in liver chromatin (constitutive activity for rats not receiving DENA, or induced activity for rats fed DENA).

If PhB does not change the repair activity, the lower amount of O^6-etG found after a 4-week DENA feeding if PhB is simultaneously given can only be explained by an action of PhB on the concentration of the ultimate carcinogen derived from DENA in liver cells. This ultimate carcinogen is believed to be the carbocation CH_3-CH_2^+; it is produced and destroyed by enzymes located in the endoplasmic reticulum. PhB induces an important development of the endoplasmic reticulum; one must suppose that the increase of the microsomal enzyme activities leads to a new dynamic equilibrium with a lower concentration of the ultimate carcinogen. The repair activity is then sufficient to eliminate rapidly the O^6-etG that appears in liver DNA even when the DENA treatment lasts 4 weeks.

It must be underscored that the final interpretation of the PhB protective effect (a lower CH_3-CH_2^+ concentration) explains the decreased incidence of cancers independently of the fact that the oncogenic lesion is O^6-etG or another ethylated base.

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REFERENCES

1. Loveless A. Possible relevance of O^6-alkylation of deoxyguanosine to the mutagenicity and carcinogenicity of nitrosamines and nitrosamides. *Nature* 1969, **223**, 206–207.
2. Goth R, Rajewski M. Molecular and cellular mechanism associated with pulse-carcinogenesis in the rat nervous system by ENU. *Z Krebsforsch* 1974, **82**, 37–64.
3. Goth R, Rajewski M. Persistence of O^6-ethylguanine in rat brain DNA: correlation with nervous system specific carcinogenesis by ENU. *Proc Natl Acad Sci USA* 1974, **71**, 639–643.
4. Abbott P, Saffhill R. DNA synthesis with methylated poly (dC-dG) templates. Evidence for a competitive nature to miscoding by O^6-methylguanine. *Biochim Biophys Acta* 1979, **562**, 51–61.
5. Barbason H, Betz EH. Proliferation of preneoplastic lesions after discontinuation of chronic DEN feeding in the development of hepatomas in rat. *Br J Cancer* 1981, **44**, 561–566.
6. Barbason H, Rasenfosse C, Betz EH. Promotion mechanism of phenobarbital and partial hepatectomy in DENA hepatocarcinogenesis cell kinetics effect. *Br J Cancer* 1983, **47**, 517–525.
7. Peraino C, Fry RJM, Staffeldt E, Christopher JP. Comparative enhancing effects of phenobarbital, amobarbital, diphenylhydantoin, and dichlorodiphenyltrichloroethane on 2-acetylaminofluorene-induced hepatic tumorigenesis in the rat. *Cancer Res* 1975, **35**, 2884–2890.
8. Kitagawa T, Sugano H. Enhancing effect of phenobarbital on the development of enzyme-altered islands and hepatocellular carcinomas initiated by 3′-methyl-4-(dimethylamino)azobenzene or diethylnitrosamine. *Gann* 1978, **69**, 679–687.
9. Schulte-Hermann R, Timmerman-Trosiener I, Schuppler J. Promotion of spontaneous preneoplastic cells in rat liver as a possible explanation of tumor production by nonmutagenic compounds. *Cancer Res* 1983, **43**, 839–844.
10. Kunz W, Schaude G, Thomas C. Die Beeinflussung der nitrosamincarcinogenese durch Phenobarbital und Halogenkohlenwasserstoffe. *Z Krebsforsch* 1969, **72**, 291–304.
11. Weisburger JH, Madison RM, Ward JM, Viguera C, Weisburger EK. Modification of diethylnitrosamine liver carcinogenesis with phenobarbital but not immunosuppression. *J Natl Cancer Inst* 1975, **54**, 1185–1188.
12. Barbason H, Mormont C, Massart S, Bouzahzah B. Anti-carcinogenic action of phenobarbital given simultaneously with DENA in the rat. *Eur J Cancer Clin Oncol* 1986, **22**, 1073–1078.
13. Renard A, Thibodeau L, Verly WG. O^6-Ethylguanine excision from DNA of liver nuclei

treated with ENU. *Fed Proc* 1978, **37**, 1412.
14. Renard A, Verly WG, Mehta JR, Ludlum DB. Repair of O^6-ethylguanine in DNA by a chromatin factor from rat liver. *Fed Proc* 1981, **40**, 1763.
15. Mehta JR, Ludlum DB, Renard A, Verly WG. Repair of O^6-ethylguanine in DNA by a chromatin fraction from rat liver: transfer of the ethyl group to an acceptor protein. *Proc Natl Acad Sci USA* 1981, **78**, 6766–6770.
16. Burton K. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem J* 1956, **62**, 315–323.
17. Thibodeau L, Verly WG. Cellular localisation of the apurinic/apyrimidinic endodeoxyribonuclease in rat liver. *Eur J Biochem* 1980, **107**, 555–563.
18. Renard A, Verly WG. A chromatin factor in rat liver which destroys O^6-ethylguanine in DNA. *FEBS Lett* 1980, **114**, 98–102.
19. Bradford MR. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dyes binding. *Anal Biochem* 1976, **72**, 248–254.
20. Lemaître M, Renard A, Verly WG. A common chromatin factor involved in the repair of O^6-methylguanine and O^6-ethylguanine lesions in DNA. *FEBS Lett* 1982, **144**, 242–246.
21. Scherer E, Timmer AP, Emmelot P. Formation by diethylnitrosamine and persistence of O^4-ethylthymidine in rat liver DNA *in vivo*. *Cancer Lett* 1980, **10**, 1–6.
22. Margison GP, Curtin NJ, Snell K, Craig AW. Effect of chronic *N*,*N*-diethylnitrosamine on the excision of O^6-ethylguanine from rat liver DNA. *Br J Cancer* 1979, **40**, 809–813.
23. Renard A, Verly WG, Robaye B, Goutier R. Adaptation and rat liver chromatin factor removing O^6-ethylguanine from DNA. *Fed Proc* 1980, **39**, 1738.