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Interaction of 7H-Dibenzo[c,g]carbazole and Its Organspecific Derivatives With Hepatic Mitochondrial and Nuclear DNA in the Mouse

Odette Périn-Roussel, François Périn, Nicole Barat, Marie-José Plessis, and François Zajdela

Institut Curie-Biologie, Centre Universitaire, Orsay, France

The recent observation of a high level of adducts in mitochondrial DNA (mtDNA) of cells exposed to chemical carcinogens aroused new interest in the hypothesis that carcinogen-induced damage in mitochondria plays a role in one or more stages of carcinogenesis. In order to investigate whether differences in the metabolic activation of carcinogens have qualitative and quantitative effects on mt- and nuclear DNA (nuDNA) adduct formation, mice were exposed to the potent hepatocarcinogenic and sarcomagenic polycyclic hydrocarbon 7H-dibenzo[c,g]carbazole (DBC) and to three of its derivatives that show large differences in enzymatic activation: N-acetyl-DBC (N-AcDBC), which is carcinogenic for several tissues; 5,9-dimethyl-DBC (DiMeDBC), which is exclusively hepatocarcinogenic; and N-methyl-DBC (N-MeDBC), which is exclusively sarcomagenic. Adduct formation and toxic effects were measured over 48 hr. With a moderate 5 μmol/kg dose of DBC, the adduct level in liver 24 hr after treatment was always higher in nuDNA than in mtDNA; after 48 hr a substantial increase in the level of adducts in mtDNA was observed, with a parallel decrease in the level in nuDNA. With DiMeDBC, a 4.9-fold increase in mtDNA was seen at 48 hr, whereas, at the same dose, the non-hepatocarcinogenic N-MeDBC induced a very small number of adducts. In order to obtain a nearly identical level of adducts in nu- and mtDNA at 24 hr, the dose of DBC must be three times higher (15 μmol/kg); this and higher dose levels had a strong cytotoxic effect in liver cells. Qualitative differences in adduct distribution were observed on chromatograms of mtDNA and nuDNA, showing that the access to mtDNA is a complex process. Our results confirm that mouse liver mtDNA is a major target for DBC and its hepatocarcinogenic derivatives. The possible interference of genotoxic alterations in mtDNA with carcinogenic mechanisms is discussed.

Key words: ^{32}P-postlabeling assay, chemical carcinogenesis, dibenzo[c,g]carbazole derivatives, mitochondrial and nuclear DNA, mouse liver

INTRODUCTION

Mitochondria play an essential role in cellular economy, regulating, for instance, cellular respiration, ATP synthesis, intracellular pH, ionic homeostasis, and membrane renewal [Pedersen, 1978]. A unique feature of mitochondria is their maternally inherited genome. Defects in mitochondrial DNA (mtDNA) have been described in a number of human diseases [Ikebe et al., 1990; Rotig et al., 1990; Wallace et al., 1990], and degenerative changes in mitochondria are considered to be one of main mechanisms of aging [Miquel and Fleming, 1984]. A series of classical carcinogens tested in yeast induced unusually high levels of mitochondrial mutations [Wilkie et al., 1983]. The classical but controversial observations of Warburg [1956] on respiratory deficiency in cancer cells, and more recent observations that mtDNA is modified in some tumors [Agrawal et al., 1981; Taira et al., 1983], raise the hypothesis that carcinogen-induced damage in mitochondria plays a role in one or more stages of carcinogenesis.

Several authors have provided direct evidence that mtDNA reacts strongly with chemical carcinogens [Allen and Combs, 1980; Backer and Weinstein, 1992]. Adduct formation after administration of alkylating carcinogens [Wunderlich et al., 1970] or aflatoxin [Niranjan et al., 1982], observed over periods up to 48 hr in liver in vivo and in other cell types in culture, showed that the extent of covalent binding to mtDNA was about 3–5 times greater than to nuDNA. Differences of 50–500 times were observed when cultured cells were exposed to carcino-

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Address reprint requests to Odette Périn-Roussel, Institut Curie-Biologie, Bâtiment 112, Centre Universitaire, 91405 Orsay, France.

Abbreviations: DBC, 7H-dibenzo[c,g]carbazole; DiMeDBC, 5,9-dimethyl-7H-dibenzo[c,g]carbazole; N-AcDBC, N-acetyl-dibenzo[c,g]-carbazole; N-MeDBC, N-methyl-dibenzo[c,g]carbazole; nuDNA, nuclear DNA; mtDNA, mitochondrial DNA; PAH, polycyclic aromatic hydrocarbons; PEI, polyethyleneimine; ip, intraperitoneal; RAL, relative adduct level.

genic polycyclic aromatic hydrocarbons (PAH) or their reactive forms [Allen and Combs, 1980; Backer and Weinstein, 1992]. These results were obtained using ^{3}H-labeled carcinogens, which permit quantitative estimations of adduct formation; labeling experiments cannot, however, provide information about any qualitative differences in adduct distribution in the two organelles. The large quantitative differences in covalent binding of carcinogen to mtDNA in liver and in the other cell types tested are difficult to interpret, but may be due to factors such as organ-specific differences in xenobiotic metabolism.

The environmental, heterocyclic, aromatic carcinogen 7H-dibenzo(c,g)carbazole (DBC) is both a local and a systemic carcinogen [Boyland and Brues, 1937; Strong et al., 1938]. It is a ubiquitous pollutant, found in wood and cigarette smoke and in the sediments of industrially polluted rivers [IARC, 1972]. Schurdak and Randerath [1985] reported that DBC bound covalently to DNA of various organs of mice after subcutaneous administration, and had particular affinity for liver DNA. Parks et al. [1986] showed that DBC bound to cultured human cells. Analysis of the metabolic activation of DBC in our laboratory [Périn et al., 1981] and elsewhere [Warshawsky and Myers, 1981] showed that its sarcomagenic activity may depend on PAH-type activation, and its hepatocarcinogenic activity on activation involving the NH group. By chemically blocking the regions of the DBC molecule involved in these different types of activation, organ-specific DBC derivatives were obtained and their specific carcinogenic activities confirmed in vivo [Valéro et al., 1983, 1985; Szafarz et al., 1983]. Schurdak et al. [1987a] investigated the formation of DBC adducts in liver, skin, and other organs in mice by the ^{32}P-postlabeling assay, and the very weak adduct formation in liver by the N-methyl DBC derivative (N-MeDBC) that has no hepatocarcinogenic activity [Shurdak et al., 1987b]. According to the NH group activation hypothesis for hepatic activity, our investigation on the in vitro metabolism of this product showed the lack of demethylation by mouse liver microsomes [Périn et al., 1984].

The present study was planned to investigate whether DBC, which undergoes several types of metabolic activation in mouse liver, also forms DNA adducts at different levels in mtDNA and nuDNA, as reported previously for a number of carcinogens. The very sensitive ^{32}P-postlabeling procedure, which permits qualitative and quantitative analysis of DNA adducts, was used. We also tested some of DBCs organ-specific derivatives, synthesized in our laboratory: N-acetyl-DBC (N-AcDBC), which is sarcomagenic and hepatocarcinogenic [Szafarz et al., 1988]; 5,9-dimethylDBC (DiMeDBC), an exclusively hepatocarcinogenic derivative [Valéro et al., 1983]; and N-MeDBC, which has strong local activity but no effect on mouse liver [Perin et al., 1984; Schurdak et al., 1987b].

MATERIALS AND METHODS

Chemicals

DBC, DiMeDBC, N-AcDBC, and N-MeDBC were synthesized in our laboratory, as described elsewhere [Perin et al., 1981, 1984; Valéro et al., 1983]. Micrococcal nuclease, nuclease P1, digitonin, RNase A, RNase T1, and olive oil were purchased from Sigma; T4 polynucleotide kinase from Epicentre Technologies; and calf spleen phosphodiesterase from Boehringer. PEI-cellulose thin-layer sheets were prepared in our laboratory and ^{32}P-orthophosphate (carrier free; 300 mCi/ml) was purchased from ICN Radiochemicals.

Treatment of Animals

XVII nc/Z homozygous female mice, 3 months of age, were injected intraperitoneally with 5, 15, or 30 μmol/kg of the test chemicals dissolved in olive oil; 15 animals were used for each DNA preparation. Each animal experiment was repeated independently two or three times.

Isolation of Mitochondria and Nuclei

Livers were homogenized, nuclei were separated, and mitochondria were purified by the procedure of Jones et al. [1988]. The mitochondrial pellet obtained after centrifugation at 15,000*g* for 30 min was centrifuged over a discontinuous sucrose gradient as described by Tapper et al. [1983], and the mitochondrial layer at the interface of the two sucrose layers was recovered with a Pasteur pipette and diluted with an equal volume of buffer (10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA) in 10% (w/v) sucrose and centrifuged at 12,000*g* for 20 min. The pellet was resuspended in the same buffer and treated with digitonin (0.3 mg/mg mitochondrial protein, dissolved in the same buffer without sucrose) [Greenwalt, 1974]. Mitoplasts, i.e., mitochondria cleared of their external membrane and intermembrane material by digitonin treatment, were sedimented at 10,000*g* for 20 min after dilution with 3 volumes of buffer (30 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2.5 mM $CaCl_2$, 0.25 M sucrose).

In order to isolate nuclei, the pellet obtained after centrifugation of liver homogenate at 1,000*g* for 15 min [Jones et al., 1988] was resuspended in 2.1 M sucrose, 1 mM $MgCl_2$, 1 mM PO_4H_2K, pH 6.8, and centrifuged in a SW41 Spinco ultracentrifuge rotor for 70 min at 18,000 rev/min. The nuclei setted in the pellet with all impurities remaining in the supernatant.

Isolation of DNA

The nuclei were lysed and nuDNA extracted by the solvent procedure described by Gupta [1984]. The mitoplasts were purified and their DNA isolated as described by Jones et al. [1988]. The yield of mtDNA was reproducibly 80 to 90 μg for 15 g of liver tissue. As mtDNA represents only about 1% of total cellular DNA, it was essential to avoid all contamination with nuDNA. The purity of mtDNA was checked for nuDNA contamination by 0.7% agarose gel electrophoresis of EcoRI and Bam HI restriction endonuclease digests, using a Hind III digest of lambda phage DNA as a molecular size marker, stained with ethidium bromide, and inspected under ultraviolet light for mtDNA-specific bands. Only mtDNA found to be free of nuDNA and ribonucleoside contamination was used.

^{32}P-Postlabeling Analysis of Adducts

Aliquots of 6 μg DNA were reduced to 3′-monophosphates by digestion with micrococcal nuclease and spleen phosphodiesterase for 4 hr at 37°C. The digests were treated with nuclease P_1, as described by Reddy and Randerath [1986], and then ^{32}P-labeled using polynucleotide kinase and [γ-^{32}P]ATP, 120 μCi [Gupta et al., 1982]. ^{32}P-labeled DNA adducts were separated by development overnight on PEI-cellulose in 2.3 M sodium phosphate and resolved by contact transfer on 12.5 × 15 cm plates of PEI-cellulose [Randerath et al., 1984]; D3 development on a 12.5 cm dimension was run in 2.7 M lithium formate, 6.4 M urea, pH 3.5. D4 development (at right-angles to the previous development) was carried out in 0.5 M Tris-HCl, 0.5 M H_3BO_3, 0.01 M EDTA, 1.3 M NaCl, 7 M urea, pH 8.

The chromatograms were autoradiographed using Trimax 3M films and intensifying screens. Radioactive spots were removed from the TLC plates and the radioactivity in each was counted by Cerenkow assay. Background levels were determined by counting blank areas of each chromatogram and the value was substracted from radioactivity associated with each spot of adduct. The specific activity of [γ-^{32}P]ATP in each labeling session was measured by quantitation of ^{32}P incorporation in a known amount of dAp [Reddy and Randerath, 1986] and relative adduct levels (RAL) were calculated as described herewith and by Randerath et al. [1985]. Student's t-test was used for statistical analysis.

Histology

Livers from treated mice were fixed in Zenker-formol. Paraffin wax was used as the embedding medium and sections were cut at 2 μm. After dewaxing in toluene and removing of mercury, the sections were thoroughly rinsed with water. Nuclei were stained with Ehrlich hemalun, and mitochondria with 0.2% aqueous solution of acid fuchsin (Gurr's michrome No. 5) overnight. The excess of stain was eliminated in absolute ethanol. Immersion phase optics was used to visualize mitochondria distinctly.

RESULTS

DBC Adducts

The ^{32}P-postlabeling chromatograms of DBC adducts showed a complex pattern: 10 major quantifiable spots were detected highly reproducibly, together with weaker ones that required longer exposure. The chemical structures of DBC adducts requiring much more adduct material was not investigated. The chromatograms of mt- and nuDNA adducts elicited by 0.1 μmol DBC (5 μmol/kg) 24 hr after i.p. injection are presented in Figure 1A and B. The characteristics of the two fingerprints were close, but showed a clear qualitative difference: the prominent spot 1 in the nuDNA chromatogram (Fig. 1A) was absent from that of mtDNA (Fig. 1B). A less striking difference is the substantial reduction in the mtDNA fingerprints of the fast-migrating spots 10 and 12. The absence of spot 1 in the mtDNA chromatogram was also ascertained in DNA extracted 48 hr after DBC administration, providing additional evidence that the mtDNA was not contaminated with nuDNA. As it is often the case with the complex process of ion-exchange chromatography on PEI-cellulose TLC, small differences in the migration of a given spot were observed. For example, the spot 6, which is present in all DBC chromatograms, was migrating in some fingerprints to the vicinity of spot 9, specially when a lesser amount of labeled mixture was spotted (data not shown). Likewise, spot 11 sometimes comigrated with spot 12.

The quantitative results of DBC adduction in mouse liver are summarized in Table I. High levels of binding were observed: 24 hr after i.p. administration of 5 μmol/kg DBC, the binding level in nuDNA was 1.9 times higher than that in mtDNA. This binding level in nuDNA, 48 hr after treatment, had decreased by about 17%, but had increased in mitochondria by a factor of 1.45, so that the adduct levels in the two organelles were virtually the same. The mt- and nuDNA liver chromatograms of 3-month-old control mice (Fig. 1C and D) showed the presence of I-compounds when an appropriate exposure time, more important than for adducts, was used. I-compounds (Indigenous DNA adducts) are age-related bulky DNA modifications that are detected in organs in the absence of exogenous carcinogens. Figure 1C and D present profiles similar to that published by Gupta et al. [1990] in rat liver, and cannot be confused with DBC-DNA adducts.

DiMeDBC

This DBC derivative may be one of the most efficient hepatocarcinogens in mice. It is remarkably less toxic to liver than DBC and is a typical organ-specific carcinogen for liver regardless of route of administration. After subcutaneous injections, to give a cumulative dose of only 12 μmol, it induced multiple metastasizing hepatic tumors in 100% of mice of strains with very low spontaneous hepatoma incidences, such as XVII nc/z and C57Bl. Like other hepatocarcinogens in mice, it shows large sex-related differences [Valéro et al., 1983, 1985].

The binding of DiMeDBC 24 hr after administration was more than 5 times lower than that of DBC in nuDNA, and more than 14 times lower in mtDNA (Table I). This result may be due to steric hindrance introduced into the DBC molecule by the two methyl groups. The ^{32}P-postlabeling chromatogram of DiMeDBC was very different from that of DBC: 17 different spots could be reproducibly observed (Fig. 2A and B), and the same pattern was observed for nu- and mtDNA 24 and 48 hr after treatment. Significantly lower activity was seen in all the spots in the mtDNA fingerprints after 24 hr, and especially in spots 2 and 5 (Fig. 2B), where the activity was reduced to a negligible level. The highest binding level was attained in nuDNA 24 hr after administration of DiMeDBC (Table I), when it was five times the value observed in mtDNA. This difference evolved rapidly between 24 and 48 hr, by which time the DiMeDBC binding level in mtDNA had risen fivefold, raising the ratio of the mt:nu

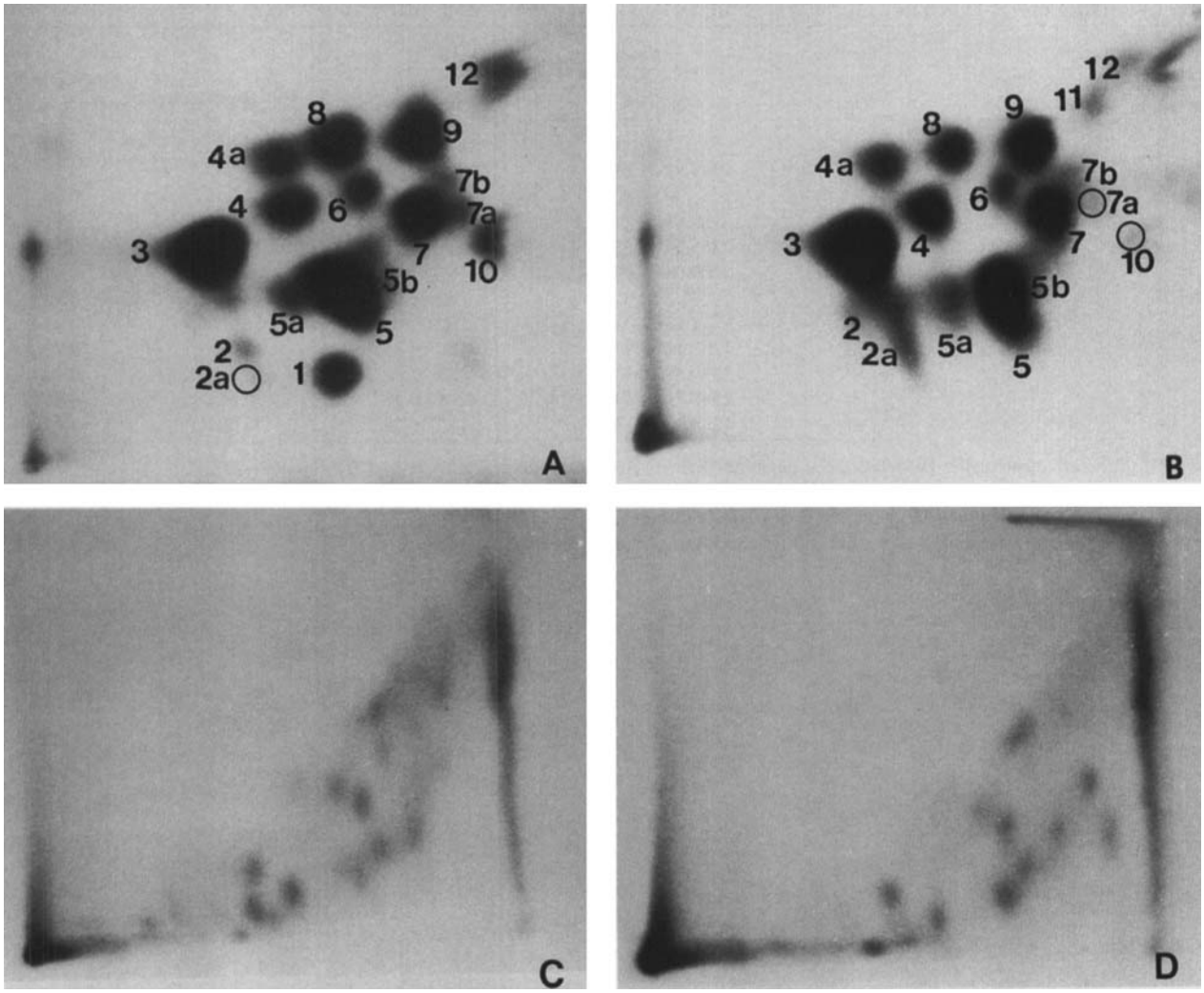

Fig. 1. Autoradiograms of PEI-cellulose thin-layer chromatography maps of ^{32}P-labeled digests of nuDNA (**A** and **C**) and mtDNA (**B** and **D**) from mouse liver treated with olive oil only (**C** and **D**) or with 5 μmol/kg of 7H-dibenzo(c,g)carbazole (24 hr) (**A** and **B**). Adducts were separated as described in Materials and Methods. Faint adducts spots are circled. Autoradiography was carried out at −70°C for 30 min (A), 1.5 hr (B), or 3.5 hr (C and D).

binding indexes from 0.2 to 1.1 (Table I). Thus, 48 hr after DiMeDBC treatment, the binding level was slightly higher in mtDNA than in nuDNA.

N-AcDBC

This DBC derivative was synthesized in an attempt to modify the high hepatic toxicity of DBC, which led to an elevated mortality in experimental animals by acute hepatic insufficiency. When tested in mice by skin painting and subcutaneous injections [Szafarz et al., 1988], N-AcDBC had the same pluri-tissular oncogenic properties as DBC, but less acute hepatic toxicity. In vitro, the acetyl group is removed immediately when in contact with liver microsomal fractions (unpublished results). The ^{32}P-post-labeling chromatogram of N-AcDBC is similar to that of DBC, with a supplementary, fast migrating adduct (spot 13, Fig. 3A and B). Spot 9, which appears as a single spot in the mtDNA chromatogram, is divided into two spots in some nuDNA fingerprints of N-AcDBC.

Qualitative differences were observed between the nu- and mtDNA chromatograms of this compound 24 and 48 hr after injection of 5 μmol/kg. The most striking difference was the absence, or sometimes in minute quantity, in mtDNA fingerprints of spot 1, which is very conspicuous in nuDNA chromatograms, as with DBC. In addition, spots 6 and 10 reproducibly showed less activity. The capacity of N-AcDBC to bind to nuDNA and mtDNA is remarkably lower than that of DBC (Table I). Fifty and 40% lower values were noted at 24 hr in nu- and mtDNA,

TABLE I. Binding of 7H-Dibenzo[c,g]carbazole and its Derivatives to nu- and mtDNA

Compound[a]	Dose (μmol/kg)	Time after treatment (hr)	Binding index RAL × 10^8[b] nuDNA	Binding index RAL × 10^8[b] mtDNA	P[c]	Ratio of mean binding index mtDNA:nuDNA
DBC	5	24	541.45 ± 73.8	281.71 ± 58.05	< 0.01	0.52
	5	48	449.57 ± 26.3	408.24 ± 36.02	N.S.	0.91
DiMeDBC	5	24	99 ± 8.2	20.2 ± 0.28	< 0.001	0.20
	5	48	88.83 ± 2.02	98.33 ± 4.28	< 0.05	1.11
N-AcDBC	5	24	265.09 ± 55.17	170.73 ± 18.17	< 0.01	0.64
	5	48	209.08 ± 10.96	176.3 ± 14.26	< 0.02	0.84
N-MeDBC	5	24	1.5 ± 0.31	0.8 ± 0.22	N.S.	0.53
	5	48	1.23 ± 0.43	1.15 ± 0.11	N.S.	0.94
DBC	15	24	1202.11 ± 94.61	1106.37 ± 85.26	N.S.	0.92
	15	48	1679.18 ± 105.34	823.26 ± 13.77	< 0.001	0.49
DBC	30	24	2887.73 ± 197.8	2340 ± 131.7	< 0.05	0.81
	30	48	2792.2 ± 138.6	635.7 ± 28.3	< 0.001	0.23

[a] DBC, 7H-dibenzo[c,g]carbazole; DiMeDBC, 5,9-dimethyl-DBC; N-AcDBC, N-acetyl-DBC; N-MeDBC, N-methyl-DBC.
[b] RAL: relative adduct level (see Materials and Methods). The results are presented as mean values ± SD of 2–3 separate animal experiments, each DNA being analysed 4 times (2 samples in 2 separate postlabeling sessions).
[c] P values from Student's t-test for binding index differences in nu- and mtDNA.

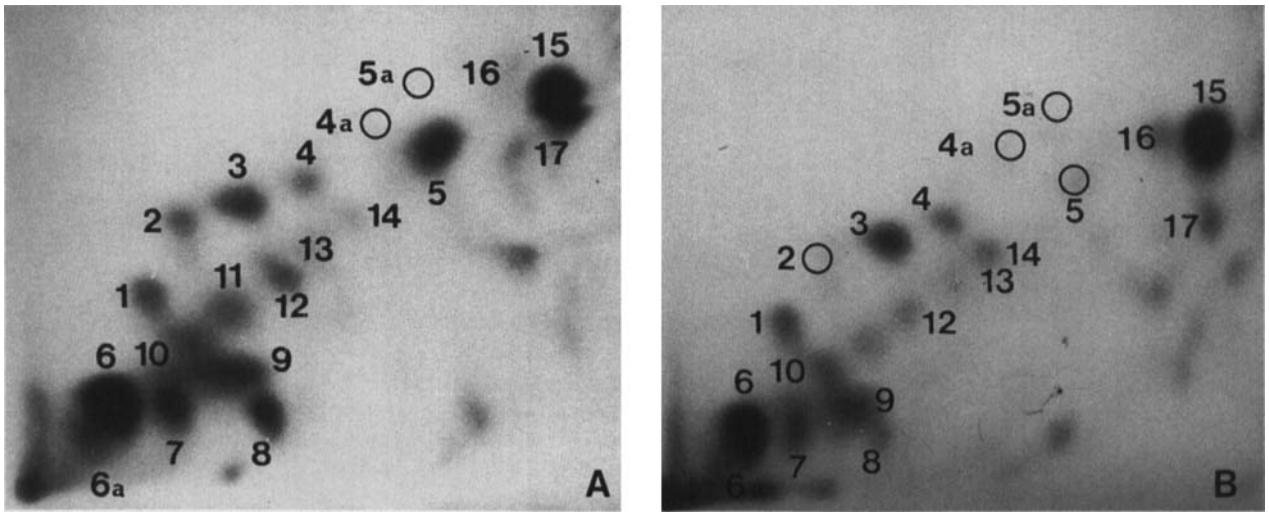

Fig. 2. Autoradiograms of PEI-cellulose thin-layer chromatography maps of ^{32}P-labeled digests from nuDNA (**A**) and mtDNA (**B**) of mouse liver treated with 5 μmol/kg of 5,9-dimethyl-7H-dibenzo(c,g)carbazole (24 hr). Adducts were separated as described in Materials and Methods. Faint adduct spots are circled. Autoradiography was carried out at −70°C for 1 hr (A) and 2.5 hr (B).

respectively. This reduction in binding capacity may explain the lower hepatotoxic activity of N-AcDBC. Quantitative binding differences and evolution of adduction are presented in Table I. The binding levels 24 and 48 hr after treatment were higher in nuDNA than in mtDNA, but a 21% decrease in nuDNA binding was observed after 48 hr, whereas that of mtDNA remained unchanged.

N-MeDBC

Removal of the hepatic toxicity and oncogenicity of DBC by N-methylation or N-ethylation was reported in 1946 by Kirby and Peacock [1946]. This DBC derivative, although innocuous for the liver, is still a potent local carcinogen, inducing epitheliomas after skin painting and sarcomas after subcutaneous injection. N-MeDBC interacts only weakly with liver-cell DNA, and an equivalent i.p. dose induced about a 360-fold lower adduct level than DBC. The same dose administered by subcutaneous injection is sufficient to elicit sarcomas in XVII nc/z mice. The binding of N-MeDBC to mtDNA 24 hr after i.p. injection is about 50% of that observed in nuDNA (Table I); a small reduction in the adduct level in nuDNA occurred after 48 hr, and a moderate increase was noted in

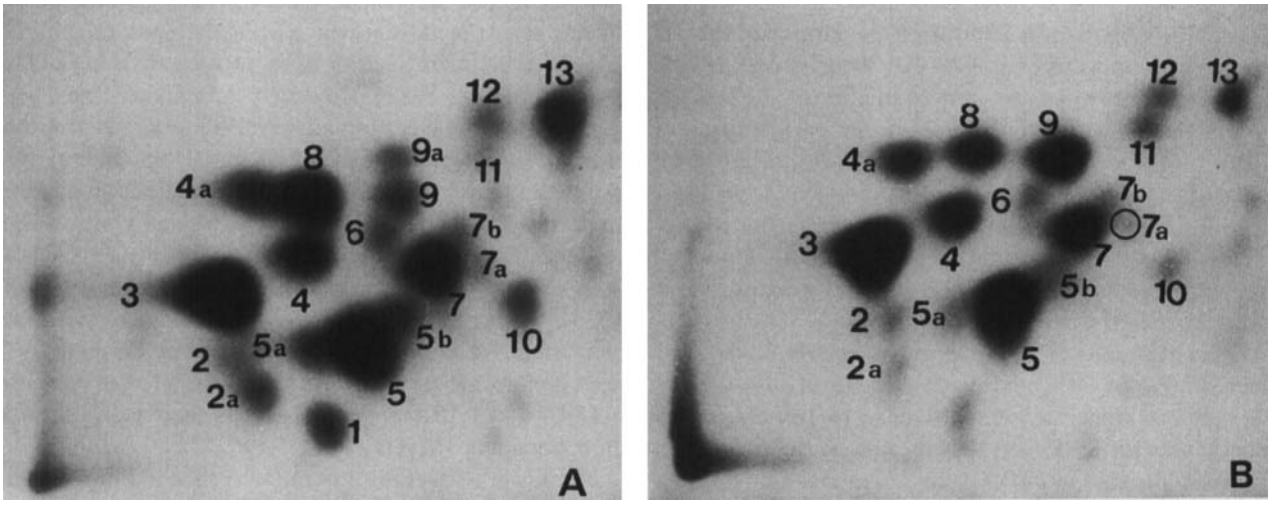

Fig. 3. Autoradiograms of PEI-cellulose thin-layer chromatography maps of ^{32}P-labeled digests from nuDNA (**A**) and mtDNA (**B**) of mouse liver treated with 5 μmol/kg of N-acetyl-7H-dibenzo(c,g)carbazole (24 hr). Adducts were separated as described in Materials and Methods. Faint adduct spots are circled. Autoradiography was carried out at −70°C for 1.5 hr (A) or 2 hr (B).

mtDNA. Qualitative differences between the two chromatograms were not analyzed because the spots seen at the 5 μmol/kg dose were inconsistent, and because I-compound spots appeared after prolonged exposure of the chromatographic plate [Gupta et al., 1990].

Dose-Effect Relationships

The high sensitivity of the ^{32}P-postlabeling assay makes it possible to use moderate doses of carcinogens and thus avoid hepatic toxicity. DBC is highly toxic for mouse liver: in our mouse strains (XVII nc/z and C57B1), a dose of 30 μmol/kg administered by i.p. injection induced transient hepatic insufficiency with loss of body weight; normality was restored after 7–10 days. In order to avoid acute liver toxicity, a dose six times smaller (5 μmol/kg) was used systematically. In order to ascertain that higher doses of DBC would not qualitatively change our results, doses of 15 and 30 μmol DBC/kg were also investigated. The spot patterns were the same as those obtained with the weaker dose. This observation confirmed the qualitative results obtained with the moderate DBC dose. Quantitatively, adduct formation was closely dose-related, as treatment with 15 and 30 μmol/kg induced 2.2 and 5.3-fold higher adduct levels in nuDNA than the 5 μmol/kg dose during the first 24 hr after injection. The augmentation was even more drastic in mtDNA, where 4 and 8-fold higher values were seen than with 5 μmol/kg. Thus, in order to attain nearly equivalent levels of adducts in nu- and mtDNA 24 hr after treatment, a dose of about 15 μmol/kg should be administered (Table I); 48 hr after the 15 μmol/kg dose, however, the adduct level in mtDNA was substantially reduced (25%), probably as a necrotoxic effect. The reduction was even higher (73%) with 30 μmol/kg. This reduction contrasts with the results obtained with moderate doses, where an increase in binding level was seen in mtDNA at 48 hr.

A histological survey of liver treated with different doses of DBC was performed. After the moderate 5 μmol/kg dose, the mitochondria were swollen and rounded, like small vacuoles; the vacuolization was more conspicuous after 48 hr, but there was no histologic evidence of cell death. After 30 μmol DBC/kg, the mitochondria were even more vacuolized at 24 hr, and the presence of very large vacuoles in many cells indicated cytoplasmic disorders. At 48 hr, the acini displayed a heterogeneous aspect, with cells at the periphery showing a necrobiotic aspect, with very large vacuoles, lipid degeneration, and sometimes nuclear pyknosis. In a number of cells with vacuolized mitochondria which were apparently intact, only moderately swollen mitochondria became visible. The mitochondrial breakdown and probable formation of new mitochondria with DNA dilution may explain the large reduction (73%) in adduct activity at 48 hr after the 30 μmol/kg dose. An intermediate situation was observed with 15 μmol/kg.

DISCUSSION

The purpose of present study was to analyse quantitatively and qualitatively the formation of adducts in mouse liver nu- and mtDNA after i.p. administration of a single dose of DBC or three of its derivatives. This was achieved by using the very sensitive nuclease P_1 modification of ^{32}P-postlabeling assay. The chemical structures and base attachment sites of DNA adducts studied here are yet

unknown, and remain to be elucidated. As the optimal level of DNA binding in liver after i.p. administration of lipophilic carcinogens seems to be 20 hr [Lutz, 1979], adduct formation was measured 24 hr after the treatment; to verify the results obtained at 24 hr and to identify any evolution of adduct levels, a second timepoint at 48 hr was analyzed. Moderate doses of carcinogens were used to avoid severe cytotoxic effects. To compare our results with previously published data, comparable high doses of DBC were tested in supplement and livers were analyzed histologically for cytotoxic effects.

Adduct formation in liver nu- and mtDNA after i.p. administration of DBC and its derivatives is dose-dependent. With a moderate standard dose (5 μmol/kg), the adduct levels for DBC, DiMeDBC, and N-AcDBC, were significantly higher in nuDNA than in mtDNA 24 hr after treatment (Table I). After 48 hr, an increase in adduct levels in mitochondria was observed for DBC ($P < 0.01$) and also for DiMeDBC, where such an evolution is particularly evident, as a 4.9-fold increase in adduct level in mtDNA was noted in three independent animal assays ($P < 0.001$). Owing to this evolution of DBC and DiMeDBC adduct formation, the binding indexes in nu- and mtDNA attained similar levels at 48 hr (Table I). Higher doses of DBC, nearer to that used in previously published experiments, i.e., 3 or 6 times higher than our standard dose, induced much higher but similar adduct levels in nu- and mtDNA after 24 hr. After 48 hr, large dose dependent decreases (25% and 73%, respectively) in adduct levels in mtDNA were observed (Table I). This reduction is very probably due to mitochondrial breakdown with selective loss of mtDNA during isolation. Possibly, modified DNA may also be diluted by formation of new mitochondria. Cytotoxic events in liver cells, with mitochondrial vacuolization and destruction, were identified after high doses of DBC (15 and 30 μmol/kg) by microscopic analysis of liver sections. These observations point out the importance of possible cytotoxic effects on adduct formation. The nonhepatocarcinogenic derivative, N-MeDBC, is devoid of hepatotropic properties and induced a marginal adduction level in nu- and mtDNA. Schurdak et al. [1987b], using the ^{32}P-postlabeling method and a much higher dose (37 μmol/kg), noted that the global binding of N-MeDBC to liver DNA was 265-fold lower than that of DBC 24 hr after an equivalent i.p. injection.

Results published previously on adduct formation in nu- and mtDNA of the liver in rodents treated with moderate doses of various carcinogens indicate ratios of binding in mtDNA:nuDNA of 5 or lower [Myers et al., 1988]. Gupta et al. [1990], using the ^{32}P-postlabeling assay to identify endogenous "I-compound" adducts in rat liver mtDNA, reported that some I-compound fractions displayed slightly higher adduct levels in mtDNA than in nuDNA. Safrole bound to the same extent to nu- and mtDNA in mouse liver [Bohr et al., 1987]. After exposure of rats to tritiated aflatoxin B_1, three to fourfold higher adduct levels were seen in liver mtDNA than in nuDNA [Niranjan et al., 1982]. However, a monooxygenase system capable of activating aflatoxin B_1 to an electrophilic reactive form which can then interact in situ with mtDNA, was identified by the authors in rat liver mitochondria. Exposure of hamsters and rats to ^{14}C-N-methylnitrosourea resulted in five times more methylation in mtDNA than in nuDNA [Wunderlich et al., 1970; Wilkinson et al., 1975]. With high doses of N-nitrosodimethylamine, hepatic mtDNA contained 1.4 times more O^6-methyl-2′-deoxyguanosine than nuDNA; but with a lower dose, the nuDNA had 1.15 times more lesions than mtDNA 18 hr after treatment [Myers et al., 1988].

The lower adduct level in mtDNA than in nuDNA after exposure to DBC and its derivatives cannot be due to rapid repair, as occurs after exposure to N-methylnitrosourea or dimethylsulfate [Le Doux et al., 1992, 1993], because the adduct level in response to a moderate dose increases between 24 and 48 hr after treatment in all cases. The repair system of mitochondria, however, is limited to methyl transferase-like reactions and base excision. The nucleotide excision system necessary to repair bulky DNA lesions, like ultraviolet induced pyrimidine dimers and large polycyclic type adducts, is missing [Le Doux et al., 1992, 1993]. For this reason, the large reduction in mtDNA adduct level 48 hr after a high DBC dose cannot be ascribed to repair.

The large variations in mtDNA adduct levels reported in the literature may be due in part to differences in methodology and to the chemical diversity of the carcinogens used, and also to the complexity of the permeability processes in mitochondria as this appears through qualitative adduct differences observed by using the ^{32}P-postlabeling method. Mitochondria are poorly protected against liposoluble carcinogens; their lipid-rich external membrane facilitates the entry and sequestration of liposoluble substances by preferential partitioning, as observed by fluorescent microscopy as early as 1940 by Graffi.

The importance of liposolubility for preferential entry into mitochondria was observed in our study, because the level of DBC adducts increased much more rapidly in mtDNA than in nuDNA when higher doses were used (Table I). Other factors may also affect access to mtDNA both quantitatively and qualitatively, including membrane proteins and their alterations by reactive metabolites, special transport systems committed to the transport of polar DNA reactive components, and the very short half-life of some reactive species, etc. In this view, differences between nuclei and mitochondria were shown by our study. The conspicuous spot 1 present in nuDNA in DBC and N-AcDBC fingerprints is absent or in minute quantity in mtDNA chromatograms, even after the highest doses of

DBC and 48 hr latency. The same was true of spot 2 in DiMeDBC chromatograms. The fastest migrating nuDNA DBC spots, 10 and 12, were clearly and reproducibly reduced in mtDNA chromatograms. In contrast, the results with low doses, specially in the case of DiMeDBC, did not confirm a better entry and sequestration of compounds in mitochondria. Equivalent adduct levels in nu- and mtDNA were only reached after 48 hr, whereas two or five times lower levels in mtDNA were registered at 24 hr. The reasons for these qualitative and quantitative differences remain unclear. One reason could be a differentiating behaviour of nuclear and mitochondrial membranes towards variable physico-chemical properties of reactive metabolites.

The significance of genotoxic alterations in mtDNA to the carcinogenic process remains controversial. Mutations in yeast mtDNA give rise to suppression of inducible respiratory enzyme systems and alteration of the cell surface. Several mutations in tRNA genes, as well as gene overexpression and rearrangement, were identified in chemically induced hepatomas [Taira et al., 1983; Corral et al., 1988]. The information contained in mtDNA principally concerns the synthesis of certain polypeptides of the inner membrane, which, in coordination with proteins coded in the nucleus, are responsible for cellular respiration, ATP synthesis, and a poorly understood system of intervention in the renewal of cell surface antigens [Smith et al., 1983]. The most plausible phenotypic expression of mtDNA mutations may thus correspond to an alteration of respiratory activity. Although alterations in energy metabolism were studied extensively between 1920 and 1960, their role in carcinogenesis remains controversial [Wilkie et al., 1983]. The same is true for many other abnormalities in tumor mitochondria described over the last 30 years. Deleterious alterations in cell membrane structure that are probably more important in the selection of cancer-prone cells have been studied only in yeast [Wilkie and Evans, 1982]. mtDNA is present in thousands of copies in a cell, and only some of it undergoes mutagenic alteration; mixtures of mutant and wild-type mtDNA, known as heteroplasmy, were identified in several hepatomas [Corral et al., 1990]. The large number of wild-type molecules may be sufficient to maintain normal functions in the cell. A large series of mitotic divisions would be necessary to segregate out cells with homogeneous mutant mitochondrial populations, but the process may be accelerated by the rapid renewal of mitochondria that is independent of the mitotic cycle [Neubert et al., 1975]. Experimental data obtained by cell fusion and cybrid formation are inconclusive. Our results confirm that mouse liver mitochondria are a direct, major cellular target for DBC and its carcinogenic derivatives, as was shown for some other hepatocarcinogens. The potential transformation of DBC-mtDNA adducts in permanent DNA lesions, as well as their significance for mouse liver hepatocarcinogenesis, ask for additional investigations.

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K. Dearfield