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Inhibition of Mitochondrial Protein Synthesis during Early Stages of Aflatoxin B₁-induced Hepatocarcinogenesis¹

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ABSTRACT

Experiments were designed to determine the *in vivo* effects of a single 6-mg/kg dose aflatoxin B₁ on rat liver mitochondrial transcription and translation processes. With the use of intact hepatocytes and also a highly active mitoplast system for incorporation, it was observed that both mitochondrial transcription and translation activities are inhibited progressively even after 24 hr of carcinogen administration. Electrophoretic patterns of mitochondrial translation products show some qualitative changes during early periods of carcinogen administration. At later stages (>12 hr), however, there is a general inhibition of many of the products, although by this time there is a qualitative and quantitative recovery in the synthesis of mitochondrial proteins imported from the cytoplasm. These results, along with the data showing considerably high levels of aflatoxin B₁ binding to mitochondrial DNA suggest that mitochondrial genetic system is one of the direct targets during experimental carcinogenesis.

INTRODUCTION

The role of mitochondria in the production and maintenance of cancer phenotype has been the subject of argument ever since the pioneering work of Warburg (27), which showed fundamental changes in the metabolic patterns of tumor tissues. Over the years, a number of studies have demonstrated altered mitochondrial content (8, 13), structure, and function (11, 12, 28) in a variety of tumor cells. It has been shown that mitochondria from tumor cells have altered ultrastructural organization, membrane composition, abnormal ion transport, and altered biochemical properties (for a discussion, see Ref. 22). Leukemic leukocytes and several solid tumors have been shown to contain unusual types of oligomeric and catenated forms of mitochondrial DNA (9, 18, 29). Another line of investigation suggests that mitochondria may be one of the cellular targets of attack by carcinogenic agents (22). Substantial amounts of carcinogens administered to experimental animals (2, 31) or incubated with tissue culture cells (4) not only are localized in the mitochondrial fraction but also are covalently bound to mitochondrial nucleic acids. Wunderlich *et al.* (31) found that hepatic carcinogen such as *N*-nitrosodimethylamine preferentially methylates mitochondrial DNA under both *in vivo* and *in vitro* conditions. Furthermore, experiments of Baker and Weinstein (4) showed that the dihydrodiol-epoxide derivative of benzo(a)pyrene covalently modifies mitochondrial DNA at efficiencies 40 to 90 times greater than those for the nuclear

DNA. Recent experiments in our laboratory showed the presence of a unique monooxygenase system, different from the microsomal activity in rat liver mitochondria, which can activate hepatic carcinogen AFB₁⁴ into electrophilic reactive forms (19). The activated components bind to mitochondrial DNA, RNA, and proteins (19, 20). All of these results taken together suggest that the mitochondrial genetic system may be the direct and possibly important target of carcinogens. In an attempt to verify this possibility, we have now studied the extent of AFB₁ attack on the mitochondrial genome and the resultant effects on mitochondrial transcription and translation processes under *in vivo* conditions.

MATERIALS AND METHODS

Materials. Amino acids, nucleotide triphosphates, chloramphenicol, cycloheximide, D-mannitol, and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, Mo. RNase-free sucrose and electrophoresis grade SDS were from Polysciences Inc., Warrington, Pa. Electrophoresis grade acrylamide, bisacrylamide and other reagents used for electrophoresis were from Bio-Rad, Richmond, Calif. AFB₁ was from Calbiochem/Behring Corp., La Jolla, Calif. Tissue culture media and reagents were from Grand Island Biological Co., Grand Island, N.Y. [³⁵S]Methionine (1200 Ci/mmol) was from Amersham Radiochemicals Co. [³H]GTP (15 Ci/mmol) and [³H]CTP (20 Ci/mmol) were from New England Nuclear, Boston, Mass. All other chemicals used were of analytical grade.

Animals and Carcinogen Administration. In all the experiments, 5- to 7-week-old male Sprague-Dawley rats (150 to 200 g) were used. AFB₁ dissolved in dimethyl sulfoxide was injected i.p. at a dose of 6 mg/kg. The control animals received equal volumes of dimethyl sulfoxide. The treated and untreated animals were anesthetized with ether and sacrificed by cervical fracture. The livers were either removed and used for isolating mitochondria or perfused *in situ* for preparing hepatocytes.

Preparation of Mitochondria. Livers were washed free of blood clots with ice-cold 0.15 M NaCl and minced with a scissor into 2- to 3-mm slices. The tissue slices were homogenized in mitochondrial isolation buffer (2 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5-0.22 M D-mannitol-0.07 M sucrose-1 mM EDTA-bovine serum albumin, 0.5 mg/ml) with 3 strokes of a loosely fitted (0.02-cm clearance) Teflon homogenizer at 1000 to 1200 rpm. The homogenate was made to 10% (w/v) with mitochondrial isolation buffer. Mitochondria were separated by the differential centrifugation method described before (3). The mitoplasts were prepared by the digitonin method as described before (25), washed once with mitochondrial isolation buffer, and used for RNA or protein synthesis.

Protein Synthesis with Mitoplasts. The details of the method used for protein synthesis with isolated mitoplasts have been described elsewhere (6, 7). Briefly, mitoplasts were suspended in protein synthesis buffer (15 mM Tris-HCl, pH 7.4; 65 mM KCl; 6 mM Mg(CH₃COO)₂; 5 mM 2-mercaptoethanol; 4 mM potassium phosphate buffer, pH 7.4; and 0.14 M sucrose at a concentration of 10 mg protein per ml and

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⁴ The abbreviations used are: AFB₁, aflatoxin B₁; SDS, sodium dodecyl sulfate.

supplemented with 2 mM ATP; 2 mM GTP; 5 mM creatine phosphate; 4 mM pyruvate; creatine phosphokinase, 0.2 mg/ml; and 100 mM concentrations each of 19 L-amino acids except methionine and cycloheximide, 300 μ g/ml. The mixture was incubated for 5 min at 35° with or without added chloramphenicol. Protein synthesis was initiated with [³⁵S]methionine (500 μ Ci/ml; 1000 Ci/mol), and the labeling was continued for the required length of time. Aliquots of 10 μ l were removed at intervals and used for determining the hot CCl₃COOH-insoluble counts (6). At the end of incubation, remaining mitoplasts were pelleted at 10,000 \times g for 10 min, washed once with mitochondrial isolation buffer and kept frozen at -70° in the presence of 3 μ g each of leupeptin and pepstatin as protease inhibitors.

RNA Synthesis with Mitoplasts. The method for RNA synthesis was modified from Avadhani *et al.* (1). Mitoplasts were suspended in protein synthesis buffer at a concentration of 3 mg protein per ml and supplemented with 2 mM ATP, 2 mM UTP, 0.5 mM GTP, 0.5 mM CTP, 5 mM malate, 5 mM creatine, creatine phosphokinase (400 μ g/ml), spermidine (20 μ g/ml), and 100 μ M concentrations each of 20 amino acids. The mixture was preincubated at 37° for 3 min, and RNA synthesis was initiated with 20- μ Ci/ml concentrations each of [³H]GTP and [³H]CTP. The incorporation was continued for 40 min. At intervals, 10- μ l samples were withdrawn and used for determining cold trichloroacetic acid-insoluble counts (1).

Protein Synthesis with Hepatocytes. Hepatocytes were prepared by perfusion with collagenase buffer, as described by Williamson *et al.* (30), except that Hanks' medium was used for perfusion as well as for releasing the cells (10). The details of protein synthesis using [³⁵S]methionine were as described elsewhere (20). Unless otherwise mentioned, protein synthesis was carried out for 120 min in either the presence or the absence of added inhibitors such as chloramphenicol and cycloheximide. Aliquots (10 μ l) were taken out at intervals and used for determining the hot CCl₃COOH-insoluble cpm as in the case of mitoplasts (6).

Electrophoretic Analysis of Proteins. Mitochondrial samples were dissolved in a lysis buffer containing 4% SDS and 10% 2-mercaptoethanol and dissociated by heating at 90° for 2 min (6). Electrophoresis was carried out on 8 to 16% gradient polyacrylamide slab gels containing 0.2% SDS, using the buffer system of Laemmli (14). The gels were stained with Coomassie blue, destained in 30% methanol-10% acetic acid, and processed for fluorography with En³hance (New England Nuclear).

Other Procedures. Protein was estimated by the method of Lowry *et al.* (15). For radioactivity determinations, the samples were counted with a 10-ml Cab-o-Sil scintillation mixture in an Intertechnique SL-4000 counter.

RESULTS

To determine the direct and indirect effects of AFB₁ on mitochondrial biosynthetic and genetic processes, we have carried out experiments with intact hepatocytes and with isolated mitochondrial particles. As shown in Chart 1, the hepatocyte system used in these studies is highly active in protein synthesis. Addition of cycloheximide (300 μ g/ml) inhibits the [³⁵S]methionine incorporation by about 87%. Addition of cycloheximide and chloramphenicol together results in >96% inhibition. In separate experiments reported elsewhere (7), we have shown that the cycloheximide-resistant and chloramphenicol-sensitive incorporation, corresponding to about 8 to 12% of total cell synthesis in rat hepatocytes, represents true mitochondrial-specific protein synthesis.

The system for RNA and protein synthesis with isolated mitochondria involves the incubation of digitonin-washed mitoplasts in a hypoosmotic medium, which permits efficient incorporation (6). The kinetics of [³⁵S]methionine incorporation

by mitoplasts isolated from control liver has been presented in Chart 2A. Addition of chloramphenicol, a specific inhibitor of mitochondrial protein synthesis, results in a near-total inhibition of incorporation. Similarly, the system incorporates [³H]GTP and [³H]CTP linearly up to about 40 to 60 min (Chart 2B), and the incorporation is sensitive to low amounts (0.5 μ g/ml) of ethidium bromide as reported for other mitochondrial systems (17, 23).

The patterns of mitochondrial-specific translation and transcription after various time intervals of AFB₁ (6 mg/kg) are presented in Chart 3. Experiments with isolated mitoplasts show a progressive inhibition of mitochondrial translation up to 60 hr after AFB₁ administration. The extent of translational inhibition observed with the mitoplast system is nearly identical to the cycloheximide-resistant incorporation, which is characteristic of mitochondrial-specific translation in intact hepatocytes (Chart 3). Similarly, the mitochondrial transcription activity as estimated by [³H]GTP and [³H]CTP incorporation by isolated mitoplasts resembles the pattern of translation inhibition following AFB₁ administration. A previous report from this laboratory showed that nuclear transcription and cytoplasmic

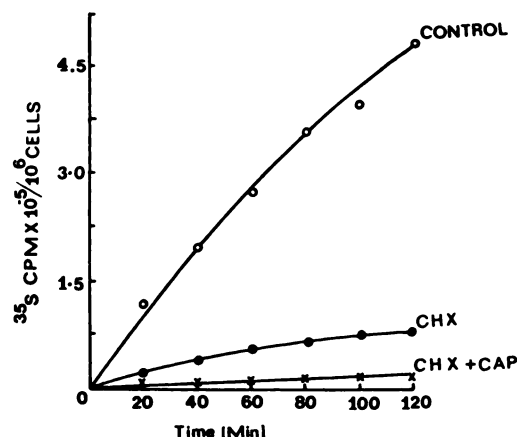


Chart 1. Kinetics of [³⁵S]methionine incorporation by isolated hepatocytes. Hepatocytes prepared from control rats were used for incorporation using [³⁵S]methionine (100 μ Ci/ml) as described in "Materials and Methods." ○, control without added inhibitor; ●, cycloheximide (CHX) (300 μ g/ml); ×, cycloheximide (300 μ g/ml) + chloramphenicol (CAP) (500 μ g/ml).

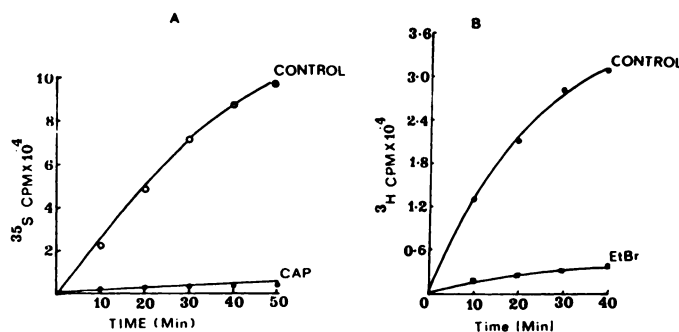


Chart 2. Protein and RNA synthesis with isolated mitoplasts. Mitoplasts were prepared from control rat liver as described in "Materials and Methods." In A, mitoplasts were suspended in protein synthesis buffer at a final concentration of 10 mg protein per ml and incubated with [³⁵S]methionine (150 μ Ci/ml). Ten- μ l samples were withdrawn at each time point for determining hot CCl₃COOH-insoluble cpm. Chloramphenicol (CAP) when added was at 500 μ g/ml. B, RNA synthesis was carried out with 20- μ Ci/ml doses each of [³H]GTP and [³H]CTP. The final concentration of mitoplasts in the reaction mixture was 3 mg/ml. Points represent cold CCl₃COOH-insoluble cpm in 10- μ l aliquots. Ethidium bromide (EtBr) when added was at 0.5 μ g/ml.

translation activities in intact hepatocytes following a drug dose of 6 mg/kg are progressively inhibited up to about 6 to 9 hr (10). It was also observed that both of these activities are recovered to control level by about 12 hr and reach a near 150 to 220% level by 24 hr (10). Thus, in contrast to the recovery process seen in the nuclear and cytoplasmic activities between 9 and 24 hr, the mitochondrial biosynthesis processes remain inhibited at least up to 60 hr after carcinogen administration.

To determine the nature of AFB₁-induced inhibition, the ³⁵S-labeled mitochondrial translation products were analyzed by electrophoresis on 8 to 16% polyacrylamide gels under dissociating conditions. The autoradiogram presented in Fig. 1 shows that rat liver mitochondria synthesize about 20 polypeptides in the size range of about 8 × 10³ to 13 × 10⁴ daltons. The pattern of mitochondrial translation, 6 hr after AFB₁ administration, shows several qualitative differences. Some unusual products in the size range of 10, 4.5, and 1.8 × 10⁴ daltons are noticeable. The 12-hr pattern shows marked inhibition of several products, and the inhibition appears to be general.

To determine if the AFB₁-induced changes in mitochondrial translation pattern are due to extramitochondrial effects or direct effects on mitochondrial genetic and biosynthetic systems, we have carried out 2 types of experiments.

In the first set of experiments, the effects of AFB₁ on the labeling of mitochondrial polypeptides contributed by the cytoplasmic translation system were studied. As shown in Fig. 2, hepatocytes from rats treated with AFB₁ (6 mg/kg) were labeled with [³⁵S]methionine in the presence of chloramphenicol, and the mitochondrial particles isolated from these cells were electrophoresed on 8 to 16% gradient gels under highly dissociating conditions. In agreement with our previous report on the pattern of cytoplasmic translation inhibition by AFB₁ (10), there is a marked inhibition of cytoplasmically imported mitochondrial proteins after 3 hr of drug treatment (Fig. 2A). This

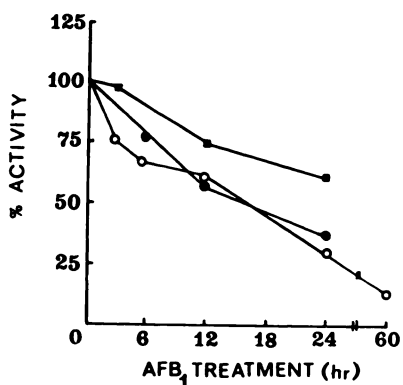


Chart 3. The time course of inhibition of mitochondrial transcription and translation by a single dose of AFB₁. Livers from AFB₁-treated animals were used for preparing mitoplasts or hepatocytes as described in "Materials and Methods." ●, mitochondrial protein synthesis with intact hepatocytes. Hepatocytes were labeled with [³⁵S]methionine for 120 min in the presence of cycloheximide (300 μg/ml) to suppress cytoplasmic translation. Other details were as described in Chart 1. ○, mitochondrial translation with mitoplasts. Mitoplasts were labeled for 60 min with [³⁵S]methionine as described in A. The values obtained with control mitoplasts were regarded as 100% activity. ■, mitochondrial RNA synthesis with isolated mitoplasts. Mitoplasts were labeled for 40 min with 20-μCi/ml amounts each of [³H]GTP and [³H]CTP as described in Chart 2B. Values obtained with control mitoplasts (Chart 2B) were considered as 100% activity.

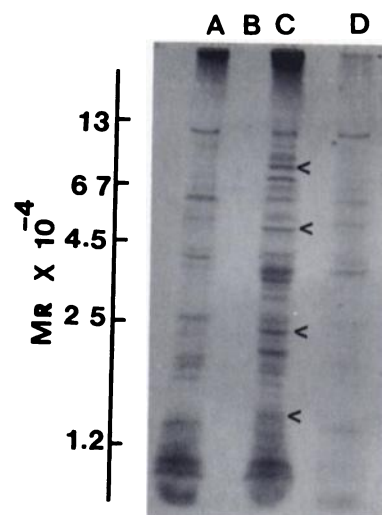


Fig. 1. Effects of AFB₁ on mitochondrial translation products. Isolated mitoplasts were labeled with [³⁵S]methionine for 60 min as described in "Materials and Methods." Samples containing about 10⁶ cpm were dissociated by heating at 90° for 2 min in the presence of 10% 2-mercaptoethanol and 4% SDS and electrophoresed on 8 to 16% gradient polyacrylamide gels. In order to account for the AFB₁-induced inhibition of incorporation, the amount of protein used in AFB₁-treated samples (160 μg) was roughly twice as much as the control (80 μg). A, control mitoplasts; B, control mitoplasts labeled in the presence of chloramphenicol (500 μg/ml); C, mitoplasts from rats treated with a single dose of AFB₁ (6 mg/kg) for 6 hr; D, mitoplasts from rats treated with a single dose of AFB₁ (6 mg/kg) for 12 hr. Arrows in C, qualitative changes in mitochondrial translation products. Details of fluorography and autoradiography were as described in "Materials and Methods."

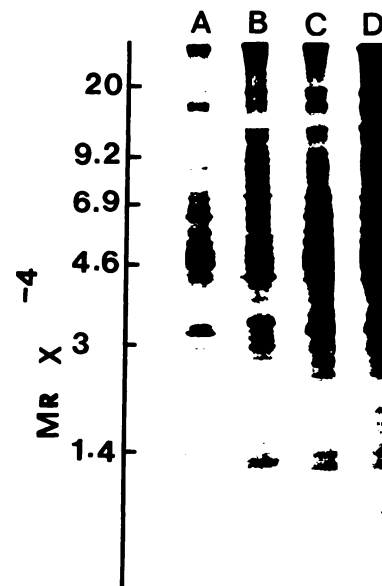


Fig. 2. Effects of AFB₁ on mitochondrial protein imported from the cytoplasm. Hepatocytes were prepared from control and AFB₁-treated animals and labeled with [³⁵S]methionine for 120 min in the presence of chloramphenicol (500 μg/ml). Mitoplasts were prepared and electrophoresed on 8 to 16% gradient gels after dissociation in the presence of 10% 2-mercaptoethanol and 4% SDS as described in Fig. 1. A, 3-hr AFB₁; B, 12-hr AFB₁; C, 24-hr AFB₁; D, control. In each case, 50 μg protein were used for electrophoresis.

inhibitory effect appears to be recovered by 12 hr (Fig. 2B), and the 24-hr pattern (Fig. 2C) compares with the control pattern (Fig. 2D) both qualitatively and quantitatively.

In the second set of experiments, we have determined the levels of bound AFB₁ to mitochondrial and nuclear DNA at

various times after the carcinogen administration. As shown in Table 1, nuclear DNA contains about 3 AFB₁ adducts/10⁷ daltons after 3 hr, whereas the mitochondrial genome at this time period contains about 9 adducts/10⁷ daltons DNA. The number of AFB₁ adducts in the nuclear DNA declines steadily to reach about 0.3/10⁷ daltons DNA at 24 hr. In contrast, the level of bound AFB₁ in the mitochondrial DNA remains nearly the same even after 24 hr. Results of these experiments together suggest that prolonged inhibition of mitochondrial translation and altered mitochondrial polypeptide pattern may result from the direct effects of AFB₁ on the mitochondrial genetic system, although some indirect effects involving the nuclear gene products cannot be overruled.

DISCUSSION

Recent studies in our laboratory showed that nuclear transcription and cytoplasmic translation activities following a single dose of AFB₁ exhibit typical inhibitory and recovery patterns (10). After nearly 80% inhibition of both of the processes at 6 to 9 hr, the activities reach a peak of about 220% level at 24 hr after AFB₁ administration (10). In view of this, the patterns of mitochondrial transcription and translation activities following AFB₁ administration are markedly different (Chart 3), in that both of these activities remain inhibited without any apparent recovery up to about 24 to 60 hr.

The analysis of mitochondrial translation products on gradient SDS-polyacrylamide gels under highly dissociating conditions shows that control mitochondria synthesize about 20 to 22 polypeptides including some in the range of 13 × 10⁴ daltons. Recently, we have shown that all of these polypeptide species represent true mitochondrial translation products, although some may be the precursors of smaller polypeptides (6). The polypeptides synthesized after 6 hr of AFB₁ treatment contain several unusual species not seen in the control samples (Fig. 1C). These polypeptides may be originated from altered processing of mitochondrial transcripts or are probably due to defective processing of the precursor polypeptides. At longer duration of AFB₁ treatment, however, there is increasing inhibition of several products. The inhibition appears to be general and may result from the inhibition of mitochondrial transcription process.

It is now reasonably well established that most of the mitochondrial proteins are imported from the cytoplasm (24). These

imported proteins are believed to be coded by the nuclear genes (24, 26). The mitochondrial genetic system, on the other hand, contributes to only <10% of the proteins, which are highly essential for the assembly of mitochondrial membranes and their function (24, 26). Although not conclusively proven, it is widely believed that most, if not all, of mitochondrially translated proteins are coded for by the mitochondrial genome (26). The observed inhibition of mitochondrial translation products may therefore be due to the direct attack on mitochondrial genetic system. Alternatively, the inhibition may be due to decreased cytoplasmic synthesis of mitochondrial biosynthetic enzymes, which are known to be imported from the cytoplasm (24). Continued inhibition of mitochondrial activity even after full recovery of cytoplasmic translation at 24 hr (Chart 3; Ref. 10, 21) suggests that this is a less likely possibility. This view is further supported by experiments showing a qualitative and quantitative recovery in the labeling patterns of cytoplasmically imported mitochondrial proteins at 24 hr after carcinogen administration (Fig. 2, C and D). Support for the direct involvement of mitochondrial genetic system comes from experiments on the levels of binding of AFB₁ to nuclear and mitochondrial DNA (Table 1). As shown for a number of carcinogens (16), the level of bound AFB₁ in the nuclear DNA reduces gradually to reach 0.3 adduct/10⁷ daltons DNA at 24 hr. Thus, the recovery in the nuclear transcription and cytoplasmic translation may reflect upon the removal of AFB₁ adducts and the repair of DNA (15). Contrary to this, the level of bound AFB₁ in the mitochondrial genome remains nearly constant even up to 24 hr, which is possibly due to the lack of repair in this organelle system (4). It should also be noted that the number of adducts per 10⁷ daltons mitochondrial DNA is about 3 to 4 times higher than the level observed for nuclear DNA. These results are consistent with previous reports showing relatively higher levels of binding of various carcinogens to mitochondrial DNA (4, 31). Thus, although it is possible that effects of AFB₁ on the nuclear genes may somehow contribute to altered mitochondrial activity, persistent inhibition of mitochondrial transcription and translation activities beyond 12 to 24 hr of carcinogen administration appears to be largely due to the direct attack on the organelle genetic system.

In summary, we have presented data which demonstrate that *in vivo* administration of AFB₁ leads to a prolonged inhibition of mitochondrial biosynthetic activities. These observations confirm and extend the view that mitochondrial DNA is a direct target for attack by chemical carcinogens (4, 19, 22, 31). It has already been shown that AFB₁ causes a long-term inhibition of various mitochondrial functions including the oxidative phosphorylation (5, 20). Although the precise role of mitochondrial genes in the carcinogenic process remains unknown, it is possible that altered mitochondrial biosynthetic processes affecting mitochondrial oxidative function and varied metabolic activities of the cell may directly or indirectly contribute to the neoplastic outcome.

ACKNOWLEDGMENTS

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Table 1

Relative levels of binding of AFB₁ to nuclear and mitochondrial DNA

Rats were given i.p. injections of 6 mg of [³H]AFB₁ per kg (30 μCi/2.9 μmol). After indicated time periods, livers were removed and used for isolating the mitoplasts (19) and nuclei (10) as described before. Mitochondrial-specific circular DNA was isolated by density banding in CsCl-ethidium bromide gradients (9, 19). Nuclear DNA was isolated by the phenol-cresol method and was further purified by banding in CsCl-ethidium bromide gradients (9). The DNA samples were freed of ethidium bromide and CsCl (19), and bound radioactivity in the DNA was estimated by counting aliquots with 10-ml Cab-o-Sil scintillation mixture as before (19). The values were derived from 3 estimates.

Single dose AFB ₁ treatment (hr)	No. of adducts/10 ⁷ daltons DNA	
	Nuclear DNA	Mitochondrial DNA
3	3.2 ± 0.4 ^a	10.2 ± 1.3
6	2.4 ± 0.6	11.1 ± 1.5
12	1.9 ± 0.5	9.8 ± 0.6
24	0.3 ± 0.2	10.1 ± 1.4

^a Mean ± S.E.

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