Biochemical Pharmacology, Vol. 24, pp. 1851–1854. Pergamon Press, 1975. Printed in Great Britain.

OPPOSITE EFFECTS OF PHENOBARBITAL PRETREATMENT ON AFLATOXIN B_1-INDUCED INHIBITION OF TRANSCRIPTION IN RAT AND MOUSE LIVER

YVONNE MOULÉ, VÉRONIQUE LESAGE, NICOLE DARRACQ and NICOLLE ROUSSEAU

Institut de Recherches Scientifiques sur le Cancer, Boîte Postale n° 8, 94800-Villejuif, France

(*Received* 13 *January* 1975; *accepted* 19 *March* 1975)

Abstract—The present paper reports the action of phenobarbital pretreatment on the inhibition of RNA synthesis in the liver of two animal species showing different susceptibility to aflatoxin: rats, which are very susceptible to the toxic and carcinogenic effects of the toxin, and mice, which appear much more resistant. Phenobarbital treatment before aflatoxin dosing decreased RNA synthesis inhibition in rat liver but significantly enhanced transcription inhibition in mouse liver. Thus, a potentiation of aflatoxin-induced toxic effects occurs in certain species. The implication of these results in connection with the growing number of drugs found in the human and animal environment is discussed.

Aflatoxin B_1, a mycotoxin synthesized by *Aspergillus flavus*, is one of the most, if not the most potent hepatocarcinogen so far identified [1, 2]. In short-term experiments, a single dose of toxin induces metabolic alterations in the liver, e.g. in transcriptional and translational mechanisms (see the review by Wogan [3]). The relationship between these early toxic events and the carcinogenic process is not completely understood.

Like many carcinogens and drugs [4, 5], aflatoxin B_1 must be activated before interfering with cell metabolism [6, 7]. Its conversion to an active form is achieved by inducible microsomal enzymes responsible for drug transformation in the liver. Of special interest from a physiological point of view is the extent to which the quantitative and qualitative variations in drug metabolizing enzyme activities may influence the hepatotoxic response to aflatoxin administration.

Phenobarbital is a very effective inducer of these enzymes in rodents [8]. The present paper reports the action of phenobarbital pretreatment on the inhibition of RNA synthesis in the liver of two animal species showing different susceptibility to aflatoxin: rats, which are very susceptible to the toxic and carcinogenic effects of the toxin, and mice, which appear much more resistant [9]. Phenobarbital treatment before aflatoxin dosing decreased RNA synthesis inhibition in rat liver but significantly enhanced transcription inhibition in mouse liver. Thus, a potentiation of aflatoxin-induced toxic effects occurs in certain species. The implication of these results in connection with the growing number of drugs found in the human and animal environment is discussed.

MATERIALS AND METHODS

Animals. Male Wistar rats (Commentry strain) weighing 260–280 g and male Swiss mice weighing 26–30 g were starved overnight before being killed by decapitation.

Sodium phenobarbital (Specia Rhône-Poulenc, Paris, France) was injected intraperitoneally into rats (75 mg/kg in 0·2 ml saline) for 3 consecutive days. For mice, sodium phenobarbital was administered either by intraperitoneal injections (75 mg/kg) for 3 days or as a 0·1% solution in drinking water for at least 9 consecutive days. The two modes of phenobarbital administration to mice gave similar results.

Crystalline aflatoxin B_1 (Makor, Jerusalem, Israel) dissolved in dimethyl sulfoxide (DMSO) (Rhône Poulec, Paris, France), was injected intraperitoneally 3 hr before killing the animals (1 mg/kg for rats and 60 mg/kg for mice unless otherwise stated). Control animals received an equivalent amount of the vehicle alone. For determination of *in vivo* RNA synthesis, [6-^{14}C]orotic acid (Cea, Saclay, France), was injected intraperitoneally (5 μCi for rats and 2 μCi for mice) 30 min before killing the animals.

Each experiment consisted of four groups (4 rats or mice per group) corresponding respectively to DMSO, aflatoxin B_1, phenobarbital + DMSO, phenobarbital + aflatoxin-treated animals. The number of experiments are indicated in the tables.

Isolation of liver nuclei. After decapitation of the animals, the livers were quickly removed, chilled, rinsed, and then homogenized in 9 vol. cold 2·2 M sucrose containing 0·5 mM $MgCl_2$. The homogenate was centrifuged for 80 min at 48,000 *g* at 4° in a Beckman J 21 centrifuge according to Chauveau *et al.* [10]. The pellets of nuclei were usually kept at −50° before being tested.

Determination of in vivo *RNA synthesis in liver.* Nuclei isolated from the livers of mice and rats injected with [6-^{14}C]orotic acid 30 min before killing, were resuspended in cold 5% $HClO_4$. The precipitate was washed five times with $HClO_4$, extracted with cold ethyl alcohol and dissolved in 0·5 N NaOH at 56°; $HClO_4$ was added to the alkaline digest at a final concentration of 5%. The precipitate of proteins and DNA was centrifuged off.

RNA in the supernatant was determined by a modification [11] of the Mejbaum technique [12] and the radioactivity was measured in a liquid scintillation spectrometer (Intertechnique, Paris, France). In

B.P. 24/20—B

Table 1. Effects of aflatoxin B_1 on *in vivo* RNA synthesis in rat and mouse liver

Aflatoxin B_1 treatment	% inhibition	
Rat		
1 mg/kg	83	(16)
Mouse		
15 mg/kg	none	(4)
30 mg/kg	9·5	(4)
40 mg/kg	30	(4)
60 mg/kg	35·8	(8)

Animals were killed 3 hr after aflatoxin dosing. Thirty minutes before killing, they received [6-^{14}C]orotic acid (5 μCi per rat and 2 μCi per mouse). Liver nuclei were isolated and the relative sp. act. of nuclear RNA was determined as mentioned in Materials and Methods.

Numbers in brackets refer to the number of animals.

order to minimize individual variations in precursor uptake in the acid soluble pool, the results were expressed in terms of the relative sp. act. [(dis/min per μg RNA. P)/(dis/min of acid soluble pool)].

Determination of RNA polymerase activity in isolated nuclei. Liver nuclei were isolated from rats and mice that had not received labelled orotic acid and were resuspended in 0·01 M Tris–HCl buffer pH 7·8. They were assayed for RNA polymerase activity as previously described [13] (except that treatment with RNase was omitted) in the presence of three unlabelled nucleoside-5′-triphosphates and one [^{14}C]nucleoside-5′-triphosphate. Four groups of assays were simultaneously carried out where the [^{14}C]nucleoside-5′-triphosphate was respectively [8-^{14}C]ATP, [8-^{14}C]GTP, [2-^{14}C]CTP and [2-^{14}C]UTP. The RNA polymerase activity was expressed in pmoles of labelled (ATP + GTP + CTP + UTP) incorporated per mg DNA. P. DNA in the nuclear suspension was measured according to the Schmidt and Thannhauser technique [14].

Table 2. Effect of phenobarbital pretreatment on aflatoxin B_1-induced inhibition of RNA synthesis in rat and mouse liver

	(*a*/*b*)*	
Rat	0·55 ± 0·12	(3)
Mouse	4·68 ± 2·1	(8)

* *a*/*b* = aflatoxin-induced inhibition in phenobarbital groups/aflatoxin-induced inhibition in untreated groups.

Rats received 1 mg/kg and mice 60 mg/kg of aflatoxin B_1 in DMSO and were killed 3 hr later. Thirty minutes before killing, they were injected with [6-^{14}C]orotic acid. Liver nuclei were isolated and the incorporation of labelled precursor into RNA was determined as indicated in Materials and Methods. Results are expressed as the mean ± S.E.M. with the number of experiments in brackets. Each experiment corresponds to 4 groups of 4 animals each.

RESULTS

The differences in susceptibility of the rat and mouse to the toxic and carcinogenic properties of aflatoxin B_1 are well known [9]. The LD_{50} is about 7 mg/kg for Wistar rats and 60 mg/kg for Swiss mice [15]. The early and marked inhibition of RNA synthesis which consistently occurs in rat liver nuclei is less apparent in mouse liver (Table 1), in agreement with the results of Akao *et al.* [16] and Neal [17]. Moreover, the segregation of the morphological components which alters the ultrastructure of rat liver nucleoli as early as one hour after aflatoxin B_1 dosing [18], is extremely slight in mouse liver. Kinetic studies showed that the weak response in mouse nuclei was not due to a delay in aflatoxin action in this species (Results to be published).

For each experiment, the effects of a drug-metabolizing enzyme inducer on transcription in aflatoxin-treated rats and mice were expressed in terms of the ratio *a*/*b* where: a = % inhibition induced by aflatoxin B_1 in phenobarbital treated animals, and b = % inhibition induced by aflatoxin B_1 in untreated animals. A value greater than 1 corresponds to a potentiation of aflatoxin action by the inducer whereas a value less than 1 represents a lessening of its toxic effects.

Pretreatment of the rats with phenobarbital decreased the inhibition produced by aflatoxin B_1 on *in vivo* RNA synthesis in liver nuclei (Table 2) which confirmed previous results [17, 19]. In mice, however, phenobarbital significantly enhanced the inhibition due to mycotoxin administration (Table 2).

Modifications in the morphological nucleolar changes correlate with the biochemical results. Phenobarbital decreased the incidence of nucleolar segregation induced in rats by aflatoxin, whereas it produced more pronounced modifications in mouse nucleoli (Results to be published).

The effect of phenobarbital pretreatment on aflatoxin B_1-induced inhibition of RNA polymerase activity confirms the foregoing data. In rats, the inhibition produced by the toxin was decreased by phenobarbital pretreatment while in mice it was increased (Table 3).

Table 3. Effect of phenobarbital pretreatment on aflatoxin B_1-induced inhibition of the RNA polymerase activity of isolated nuclei from rat and mouse liver

	(*a*/*b*)* Low ionic strength		High ionic strength	
Rat	0·64 ± 0·3	(4)	0·46 ± 0·3	(4)
Mouse	1·45 ± 0·2	(2)	1·73	(1)

* *a*/*b* = aflatoxin-induced inhibition in phenobarbital groups/aflatoxin-induced inhibition in untreated groups.

Liver nuclei from rats and mice were isolated as indicated in Table 2 except that the injection of [6-^{14}C]orotic acid was omitted. The RNA polymerase standard assay contained in 0·25 ml: 40 μmoles Tris–HCl buffer pH 7·9, 2 μmoles $MgSO_4$, 0·25 μmoles of each of the four nucleoside-5′-triphosphates (one of which was ^{14}C-labelled). Incubations were run 10 min at 37°.

Results are expressed as mean ± S.E.M. with the number of experiments in brackets. Each experiment was composed of 4 groups of 4 animals each.

Table 4. Effect of methylcholanthrene pretreatment on aflatoxin B_1-induced inhibition of *in vitro* and *in vivo* transcription in rat and mouse liver nuclei

	(*a*/*b*)*		
	RNA synthesis *in vivo*	RNA polymerase activity Low ionic strength	High ionic strength
Rat	0·95 ± 0·02 (3)	1·24 ± 0·19 (3)	1·12 ± 0·11 (3)
Mouse	0·2 ± (1)	0·04 ± (1)	

* *a*/*b* = aflatoxin-induced inhibition in methylcholanthrene groups/aflatoxin-induced inhibition in untreated groups.

Animals received 2 intraperitoneal injections of methylcholanthrene dissolved in olive oil (20 mg/kg on the first day and 10 mg/kg on the second day). Control groups received an equal amount of olive oil.

The analytical procedures are similar to those described in the legends of Tables 2 and 3.

The drug-induced modulations of aflatoxin action in mouse and rat liver is not a general property of any drug. Methylcholanthrene, another well-known microsomal enzyme inducer, failed to modify significantly the inhibition of transcription in aflatoxin-treated rats; however, this drug leads to an increased resistance of mice to aflatoxin-induced transcriptional impairment (Table 4). Such results clearly demonstrate the complexity of drug metabolizing systems present in microsomal membranes.

DISCUSSION

In vivo metabolism of aflatoxin B_1 proceeds by several alternative pathways functioning simultaneously in liver. Along one pathway, the toxin is activated with transient formation of highly reactive intermediates, some of which bind to cellular macromolecules. The biological effects of aflatoxin for an animal species seem to depend on the relative activities of the different enzyme pathways. Furthermore, any factor that modifies their equilibrium may vary the effects induced in this species.

The present results from the studies using rat liver support previous work showing decreased short-term effects induced by aflatoxin B_1 after phenobarbital treatment [17, 19]. Thus, the equilibrium between drug metabolizing enzymes is displaced in favor of effective detoxication processes. In mouse liver, however, toxic effects increased after phenobarbital administration. These data demonstrate that mouse liver has a potential ability of activating aflatoxin; its resistance is contingent and not determined at the genetic level. Moreover, the fact that transcription in mouse kidney is inhibited by aflatoxin in untreated animals fits well these conclusions [16].

Opposite effects of phenobarbital pretreatment on drug toxicity have been observed. Phenobarbital decreased the hepatotoxic effects induced by retrorsine in mice [20] but enhanced those promoted by carbon tetrachloride in rats [21–23] and dogs [24], and by monocrotaline [25], 1.1.1- and 1.1.2-trichlorethane in rats [26].

The consequences of the association of phenobarbital with a drug are impossible to predict; they are revealed by short-term (i.e. toxic) or long-term effects. Phenobarbital is thus capable of modulating carcinogenic incidence. In some cases, it tends to reduce the potency of a carcinogen as shown by McLean and Marshall [27] with aflatoxin B_1-treated rats; in others, it may amplify the malignant process: hepatic tumorigenesis in rats fed 2-acetylaminofluorene is enhanced by phenobarbital [28].

The repeated ingestion by man of barbituric derivatives combined with a growing number of drugs encountered in the environment must therefore be seriously considered for short- and long-term action.

Acknowledgements—This work was supported by Contract No. 73–75 from the Ministère de la Protection de la Nature et de l'Environnement, section Contamination des chaînes biologiques, and a grant from the Commissariat à l'Energie Atomique.

REFERENCES

1. L. A. Goldblatt, *Aflatoxin.* Academic Press, New York (1969).
2. G. N. Wogan, in *Methods in Cancer Research* (Ed. H. Busch), Vol. 7, p. 309. Academic Press, New York (1973).
3. G. N. Wogan and R. S. Pong, *Ann. N.Y. Acad. Sci.* **174**, 623 (1970).
4. B. N. La Du, H. G. Mandel and E. L. Way, *Fundamentals of Drug Metabolism and Drug Disposition.* Academic Press, New York (1971).
5. J. A. Miller and E. C. Miller, *Progr. exp. Tumor Res.* **11**, 273 (1969).
6. Y. Moulé and C. Frayssinet, *FEBS Lett.* **25**, 93 (1972).
7. A. Sarasin and Y. Moulé, *FEBS Lett.* **32**, 347 (1973).
8. H. Remmer, *Arch. exp. Path. Pharmak.* **235**, 279 (1959).
9. N. Platonov, *Vet. Rec.* **76**, 589 (1964).
10. J. Chauveau, Y. Moulé and C. Rouiller, *Exp. Cell Res.* **11**, 317 (1956).
11. Y. Moulé, *Arch. Sci. Physiol.* **7**, 161 (1953).
12. W. Mejbaum, *Hoppe Zeyler Zeits.* **258**, 117 (1939).
13. Y. Moulé, *Eur. J. Biochem.* **17**, 544 (1970).
14. G. Schmidt and S. J. Thannhauser, *J. biol. Chem.* **161**, 83 (1945).
15. G. N. Wogan, *Progr. exp. Tumor Res.* **11**, 134 (1969).
16. M. Akao, K. Kuroda and G. N. Wogan, *Life Sci.* **10**, 495 (1971).
17. G. E. Neal, *Biochem. Pharmac.* **21**, 3023 (1972).
18. W. Bernhard, C. Frayssinet, C. Lafarge et E. Le Breton, *C.R. Acad. Sci.* **261**, 1785 (1965).
19. M. R. Gumbmann and S. N. Williams, *Biochem. Pharmac.* **19**, 2861 (1970).
20. J. N. H. White, A. R. Mattocks and W. H. Butler, *Chem.-Biol. Interact.* **6**, 207 (1973).

21. R. C. Garner and A. E. M. McLean, *Biochem. Pharmac.* **18,** 645 (1969).
22. E. S. Reynolds, H. J. Ree and M. T. Moslen, *Lab. Invest.* **26,** 290 (1972).
23. P. Pani, M. V. Torrelli, L. Gabriel and E. Gravela, *Exp. molec. Path.* **19,** 15 (1973).
24. C. L. Litterst, T. M. Farber and E. J. Van Loon, *Toxic. appl. Pharmac.* **23,** 470 (1972).
25. J. R. Allen, C. F. Chesney and W. J. Frazer, *Toxic. appl. Pharmac.* **23,** 470 (1972).
26. G. P. Carlson, *Life Sci.* **13,** 67 (1973).
27. A. E. M. McLean and A. Marshall, *Br. J. exp. Path.* **52,** 322 (1971).
28. C. Peraino, R. J. M. Fry, E. Staffeldt and W. E. Kisieleski, *Cancer Res.* **33,** 2701 (1973).