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SHORT COMMUNICATIONS

Inhibition of poly(A)$^+$- and poly(A)$^-$-ribonucleic acid synthesis by a nonlethal dose of aflatoxin B_1 *in vivo*

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Since the earliest reports that *in vivo* administration of aflatoxin B_1 (AFB) to rats causes inhibition of liver nuclear RNA synthesis in intact animals [1, 2], liver slices [2] and isolated nuclei [3], major efforts have been directed toward the elucidation of the possible mechanism involved. The inhibition *in vitro* of RNA synthesis by AFB requires the presence of a microsomal fraction [4], and it is recognized that this effect is caused by a metabolite(s) of the toxin rather than by AFB itself. There is a substantial amount of information suggesting that the metabolite(s) acts at the level of the DNA template [5–9]. There is also strong evidence showing that either one or both of the nuclear RNA polymerases are affected [4, 10–12]. Studies with solubilized and DEAE-Sephadex column purified RNA polymerases, assayed in the presence or absence of α-amanitin, with endogenous or exogenous template, suggest that AFB inhibition of rat liver RNA synthesis is due to both impairment of the nucleolar DNA template function and direct inhibition of the enzyme function of RNA polymerase II [13]. Most of the studies, whether *in vivo* or *in vitro*, measured the incorporation of labeled precursors into tissue RNA as acid-insoluble radioactive materials [1–5, 7–13] or into RNA released from the polysomes and displayed by sucrose-density centrifugation [6]. No attempts were made to characterize and isolate the transcriptional products, poly(A)$^+$- and poly(A)$^-$-RNA.

It is known that during and after inhibition of protein synthesis by cycloheximide (CHI), there is an increased transcriptional activity resulting in an enhancement of poly(A)$^+$-mRNA in cells [14, 15] and tissues [16–18]. CHI does not affect the activity of rat liver nuclear RNA polymerase I and II either *in vitro* or *in vivo* [19, 20]; therefore, the CHI-induced transcriptional activity is thought to be associated with the DNA template function. In fact, an elevated level of specific mRNA for liver tyrosine aminotransferase or tryptophan pyrrolase has been identified [17, 18]. In the present study, we have examined the effect of a nonlethal dose of AFB at the transcriptional level by analyzing the *in vivo* synthesis of liver poly(A)$^+$- and poly(A)$^-$-RNA in normal as well as CHI-pretreated rats.

Male Sprague–Dawley rats (190 ± 20 g) were fed chicken diet (Taiwan Sugar Corp., Taipei, Taiwan) and water *ad lib.* Animals were divided into four groups: Group I, untreated normal; Group II, AFB-treated [AFB (Makor Chemical Co., Jerusalem, Israel), dissolved in dimethylsulfoxide–1,2-proplyeneglycol (1:1), was injected i.p. at a dose of 1 mg/kg body wt 2 hr before the animals were killed]; Group III, CHI-treated [CHI (Sigma Chemical Co., St. Louis, MO. U.S.A.), in 0.9% NaCl was injected i.p. at a dose of 2.0 mg/kg body wt 18 hr prior to killing]; and Group IV, CHI–AFB-treated [AFB was administered to CHI-pretreated animals 2 hr before the animals were killed].

In each experiment, livers of two to three animals in a group were pooled and homogenized in 6 vol. of 0.25 M sucrose/2 mM Tricine/1 mM EDTA, pH 7.6, with six strokes of a motor-driven Teflon–glass Potter–Elvehjem homogenizer. The homogenate was filtered through two layers of cheesecloth before the various determinations were made. For the isolation of cytoplasmic ribonucleoprotein complexes (RPC), the $MgCl_2$ precipitation method of Palmiter [21] was used as described previously [16]. Cytoplasmic RNA species were extracted from the RPC by the phenol-chloroform procedure outlined by Palmiter [21]. Poly(A)$^+$- and poly(A)$^-$-RNA were separated by chromatography on oligo(dT)-cellulose (Collaborative Research, Waltham, MA, U.S.A.) by the method of Aviv and Leder [22].

To measure the RNA synthesis *in vivo*, 50 μCi/100 g body wt of [^{3}H]orotic acid (24 Ci/mmole, Radiochemical Center, Amersham, U.K.) was injected i.m. 60 min before killing the animals. To measure the protein synthesis, [^{3}H]leucine (40–60 Ci/mmole, Radiochemical Center, Amersham, U.K.), 20 μCi/100 g body wt, was injected i.m. 60 min before killing. Samples containing radioactive label were precipitated with 10% trichloroacetic acid and col-

Table 1. *In vivo* effects of aflatoxin B_1 on the synthesis of liver RNA and protein in rats treated with or without cycloheximide

Group	Treatment	Liver RNA (10^5 dpm/g liver)	Ribonucleoprotein RNA (10^3 dpm/A_{260})	Liver protein (10^5 dpm/g liver)
I	Untreated control	10.3 ± 0.7* (100)	1.2 ± 0.1* (100)	3.9 ± 0.4* (100)
II	AFB-treated	4.1 ± 0.9† (40)	0.47 ± 0.1† (39)	3.8 ± 0.4 (97)
III	CHI-treated control	14.6 ± 0.2 (100)	2.15 ± 0.1 (100)	4.2 ± 0.5 (100)
IV	CHI–AFB-treated	4.2 ± 0.9† (29)	0.45 ± 0.1† (21)	3.6 ± 0.6 (86)

* Mean ± S.E.M.

† Indicates P values less than 0.05 when compared with the corresponding control group. The percentage of control value is given in parentheses.

Table 2. Content and synthesis of poly(A)$^+$- and poly(A)$^-$-RNA in animals treated with aflatoxin B_1, cycloheximide or both

Group	Treatment		Content		Synthesis	
	CHI	AFB	Poly(A$^+$)-RNA (%)	Poly(A$^-$)-RNA (%)	Poly(A$^+$)-RNA (10^3 dpm/A_{260})	Poly(A$^-$)-RNA (10^3 dpm/A_{260})
I	–	–	1.5 ± 0.4	98.4 ± 0.5	8.67 ± 1.1 (100)	1.03 ± 0.2 (100)
II	–	+	2.2 ± 0.8	97.8 ± 0.8	2.07 ± 0.6* (24)	0.31 ± 0.1* (30)
III	+	–	2.1 ± 0.4	97.9 ± 0.5	11.40 ± 1.2 (100)	1.88 ± 0.1 (100)
IV	+	+	2.5 ± 0.8	97.5 ± 0.8	3.08 ± 0.6* (27)	0.34 ± 0.1* (18)

* Indicates P values less than 0.05 when compared with the corresponding control group. The percentage of control value is given in parentheses.

lected on Millipore filter discs as described by Devlin and Ch'ih [23]. Protein was determined by the method of Lowry *et al.* [24] with crystalline bovine serum albumin (Sigma Chemical Co.) as the standard. Data presented are means ± S.E.M. of four to five experiments. Differences between means were evaluated using Student's *t*-test, and a P value of less than 0.05 was considered to be significant.

As shown in Table 1, significant inhibition of [^{3}H]orotic acid incorporation into liver RNA was observed 2 hr after the administration of a nonlethal dose of AFB. In rats without CHI pretreatment, there was 60 per cent inhibition of [^{3}H]orotate incorporation by the liver homogenate and purified RNA isolated from the RPC, results that are in agreement with the report of Gelboin *et al.* [3], who showed a 35–70 per cent inhibition of *in vitro* transcriptional activity of nuclei isolated 2 hr after the administration of AFB (1 mg/kg) to Sprague–Dawley rats weighting 200 g. The decrease in the rate of [^{3}H]orotate incorporation observed is not attributable to either a change of the precursor pool or reutilization, for it is known that orotic acid incorporation into the nucleotide pool is not affected by AFB treatment [2, 5, 6]. Furthermore, the precursor could not be reutilized to a large extent because a 1-hr labeling time is necessary for maximum radioactivity to appear in RNA associated with both free and membrane-bound polysomes in the cytoplasm [16]. The lack of inhibition of protein synthesis observed in this study (Table 1), as well as by others [5, 9], indicates that the reduced rate of RNA synthesis is not secondary to the effect of AFB on protein synthesis. In CHI-treated animals, the stimulation of RNA synthesis and the lack of inhibition on protein synthesis confirm the findings of Ch'ih *et al.* [16, 25]. When AFB was given to CHI pretreated rats, CHI-induced RNA synthesis was eliminated completely and showed a rate of RNA syntheses similar to that of the AFB-treated group. The inhibition was highly significant (P values < 0.05), indicating that the nonlethal dose of AFB (1 mg/kg) used in this study was sufficient to affect RNA synthesis *in vivo*.

To distinguish the gene product types that were affected, poly(A)$^+$- and poly(A)$^-$-RNA were isolated by oligo(dT)-cellulose column chromatography. As shown in Table 2, there were no significant differences in the relative amounts of the two RNA species in the various experiments. Poly (A)$^+$-RNA comprised about 2 per cent of the RNA isolated from the RPC, whereas poly(A)$^-$-RNA constituted 98 per cent. The higher rate of [^{3}H]orotate incorporation into poly(A)$^-$-RNA (183 per cent as the normal) than into poly(A)$^+$-RNA (131 per cent as the normal) in CHI-treated animals was similar to that reported by Ch'ih *et al.* [16], suggesting that nucleolar DNA template activity was selectively activated. The 70 per cent inhibition caused by AFB of poly(A)$^-$-RNA synthesis is similar to the inhibition observed by Yu [13] of RNA synthesis *in vitro* in isolated nuclei or nucleoli using endogenous DNA template. Because it is known that both AFB and CHI do not affect the RNA polymerase I activity [11, 13, 19, 20], the 82 per cent inhibition of poly(A)$^-$-RNA synthesis by AFB in CHI-treated animals (Table 2, Group IV) supports the theory that the specificity of the carcinogen on DNA template activity resides in nucleolar, and not nucleoplasmic, DNA.

As to the synthesis of poly(A)$^+$-RNA, 76 and 73 per cent inhibition (compared with the corresponding controls) was observed in the AFB-treated and CHI–AFB-treated animals, respectively (Table 2). In studies with purified RNA polymerase II, the inhibition caused by *in vivo* administration of AFB was shown to vary from 57 to 67 per cent [11–13]. A greater inhibition, which could not be accounted for by the inhibition of the enzyme, was observed here. This difference suggests that AFB, in addition to inhibiting RNA polymerase II activity, may have an additional effect on either the nucleoplasmic DNA template function or mRNA processing, or on both. The observed inhibition by AFB of the activities of cortisol-induced tyrosine aminotransferase and tryptophan pyrrolase [1, 2, 5, 9] strengthens this view. Nevertheless, data presented in this report clearly show that AFB impairs the function of nucleolar DNA template, resulting in the inhibition of the synthesis of poly(A)$^-$-RNA.

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REFERENCES

1. M. B. Sporn, C. W. Dingman, H. L. Phelps and G. N. Wogan, *Science* **151**, 1539 (1966).
2. J. I. Clifford and K. R. Rees, *Nature, Lond.* **209**, 312 (1966).
3. H. V. Gelboin, J. S. Wortham, R. G. Wilson, M. Friedman and G. N. Wogan, *Science* **154**, 1205 (1966).
4. E. O. Akinrimisi, B. J. Benecke and K. H. Seifert, *Eur. J. Biochem.* **42**, 333 (1974).
5. J. I. Clifford and K. R. Rees, *Biochem. J.* **102**, 65 (1967).

6. A. K. Roy, *Biochim. biophys. Acta* **169**, 206 (1968).
7. A. M. Q. King and B. H. Nicholson, *Biochem. J.* **114**, 679 (1969).
8. G. S. Edwards and G. N. Wogan, *Biochim. biophys. Acta* **224**, 597 (1970).
9. G. E. Neal, *Biochem. J.* **130**, 619 (1972).
10. R. S. Pong and G. N. Wogan, *Cancer Res.* **30**, 294 (1970).
11. F. C. Saunders, E. A. Barker and E. A. Smuckler, *Cancer Res.* **32**, 2487 (1972).
12. G. E. Neal, *Nature, Lond.* **244**, 432 (1973).
13. F. L. Yu, *J. biol. Chem.* **252**, 3245 (1977).
14. L. F. Johnson and R. Meister, *J. cell. Physiol.* **92**, 57 (1977).
15. L. E. Maroun and E. T. Miller, *J. cell. Physiol.* **92**, 375 (1977).
16. J. J. Ch'ih, L. M. Pike and T. M. Devlin, *Biochem. J.* **168**, 57 (1977).
17. M. J. Ernest, L. DeLap and P. Feigelson, *J. biol. Chem.* **253**, 2895 (1978).
18. E. Hofer and C. E. Sekeris, *Eur. J. Biochem.* **86**, 547 (1978).
19. T. Higashinakagawa and M. Muramatsu, *Biochem. biophys. Res. Commun.* **47**, 1 (1972).
20. T. J. Lindell and J. J. Duffy, *J. biol. Chem.* **254**, 1454 (1979).
21. R. D. Palmiter, *Biochemistry* **13**, 3606 (1974).
22. H. Aviv and P. Leder, *Proc. natn. Acad. Sci. U.S.A.* **69**, 1408 (1972).
23. T. M. Devlin and J. J. Ch'ih, *Archs Biochem. Biophys.* **152**, 521 (1972).
24. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
25. J. J. Ch'ih, L. S. Faulkner and T. M. Devlin, *Biochem. Pharmac.* **28**, 691 (1979).

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Single-dose tolerance to the effects of morphine on brain 3-methoxy-4-hydroxyphenylethylene glycol sulfate*

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Measurements of the concentration of 3-methoxy-4-hydroxyphenylethylene glycol sulfate (MOPEG-SO_4), the major rat brain metabolite of norepinephrine [1], have been used in several recent studies to obtain an index of the effects of various treatments on brain norepinephrine turnover [2–4]. In agreement with previous studies demonstrating an increased synthesis of brain norepinephrine from radiolabeled tyrosine [5], the acute administration of morphine has been shown to produce dose-dependent increases in MOPEG-SO_4 in whole rat brain [6] and several of its parts [7]. Recent evidence from our laboratory indicates that this observed increase in brain norepinephrine turnover is a specific opiate receptor effect [8]. Thus, morphine and related opiate agonists produce dose-dependent increases in rat brain MOPEG-SO_4 that exhibit characteristic potency differences, stereospecificity, and naloxone antagonism, and that do not occur as a result of interference with the transport of this major metabolite from brain [8].

An additional criterion for specific opiate action is the development of tolerance, and the effect of morphine on brain MOPEG-SO_4 has been shown to be attenuated following chronic administration [6]. However, significant tolerance to the analgesic effect of morphine has been demonstrated following the administration of a single dose of the opiate [9], and single-dose morphine tolerance has also been exhibited by the depletion of rat brain calcium [10]. The requirement for newly synthesized protein in tolerance development has been implicated in both of these instances by demonstrations of tolerance prevention after prior administration of protein synthesis inhibitors [10, 11]. The present study was designed to further characterize the relationships between increased brain norepinephrine turnover, opiate action, and tolerance development by evaluating the ability of morphine to induce single-dose tolerance to its effect on brain MOPEG-SO_4 and the ability of cycloheximide, an inhibitor of protein synthesis, to antagonize the development of single-dose tolerance to this effect.

Male Sprague–Dawley rats (Holtzman, 170–230 g) were injected i.p. with either isotonic sodium chloride or a 10 mg/kg dose of morphine sulfate (Mallinckrodt, Inc., St. Louis, MO). Twenty-four hours after this initial injection, one of several doses of morphine was administered, and the animals were decapitated 1 hr following the second injection at the time of the peak effect on brain MOPEG-SO_4 [8]. Whole brain MOPEG-SO_4 concentrations were determined by the fluorometric method of Meek and Neff [12]. In experiments designed to evaluate the antagonism of tolerance, a 1 or 2 mg/kg i.p. dose of cycloheximide (Aldrich Chemical Co., Milwaukee, WI) was administered 1 hr prior to the initial 10 mg/kg injection of morphine [10], and the subsequent schedule was repeated exactly as described above. The effect of cycloheximide (2 mg/kg) on the increase in brain MOPEG-SO_4 produced by single injections of morphine was determined by killing groups of cycloheximide (1 hr and 24 hr) pretreated animals 1 hr after the administration of morphine (10 mg/kg). Brain MOPEG-SO_4 levels were expressed as picomoles per gram of brain (wet weight), and appropriate statistical comparisons were made with a one-way analysis of variance and Student's t-test ($\alpha = 0.05$).

The degree of protein synthesis inhibition produced by the intraperitoneal administration of these doses of cycloheximide in rats was determined by measuring the incorporation of L-[U-^{14}C]valine (ICN Pharmaceuticals, Irvine, CA, 250 Ci/mole) into brain protein according to the procedure of Sperk *et al.* [13]. Rats were injected with saline or cycloheximide (1 or 2 mg/kg, i.p.) 1 hr before intraperitoneal administration of [^{14}C]valine and killed 1 hr after. The [^{14}C]valine was diluted with a saturated solution of cold valine (50 mg/ml) so that the final concentration of the label was 2.5 μCi/ml and each rat was given 5 μCi/100 g of body weight. Brains were homogenized in 10 ml of 10% (w/v) trichloroacetic acid (TCA). Following lipid extraction, the pellets were dried, weighed, dissolved by heating in 1 N NaOH, neutralized with glacial acetic acid, and added to 15 ml of commercial counting solution (Scintiverse, Fisher Scientific, Fair Lawn, NJ). Radioactivity was

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