9. J. L. Biedler and H. Riehm, *Cancer Res.* **30**, 1174 (1980).
10. T. Skovsgaard, *Cancer Res.* **38**, 4722 (1978).
11. V. Ling, *Can. J. Genet. Cytol.* **17**, 503 (1975).
12. U. K. Laemmli, *Nature, Lond.* **227**, 680 (1970).
13. R. H. F. Peterson, M. B. Myers, B. A. Spengler and J. L. Biedler, *Cancer Res.* **43**, 222 (1983).
14. M. B. Meyers and J. L. Biedler, *Biochem. biophys. Res. Commun.* **99**, 228 (1981).
15. M. B. Meyers and J. L. Biedler, *J. Cell Biol.* **95**, 453a (1982).
16. R. Juliano, V. Ling and J. Graves, *J. supramolec. Struct.* **4**, 521 (1976).
17. W. T. Beck, T. J. Mueller and L. R. Tanzer, *Cancer Res.* **39**, 2070 (1979).
18. R. H. F. Peterson and J. L. Biedler, *J. supramolec. Struct.* **9**, 289 (1978).
19. N. Kartner, M. Shales, J. R. Riordan and V. Ling, *Cancer Res.* **43**, 4413 (1983).
20. Y. Sugimoto, H. Suzuki and N. Tanaka, *Biochem. biophys. Res. Commun.* **114**, 969 (1983).
21. S. A. Carsen, J. E. Till and V. Ling, *Biochim. biophys. Acta* **467**, 238 (1977).

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Effect of aflatoxin B_1 on translation in isolated hepatocytes

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Aflatoxin B_1 (AFB_1), a potent hepatocarcinogen, inhibits protein synthesis [1–4]. Investigators working with intact rats suggest that the inhibition of liver protein synthesis by the toxin is a consequence of blockage at elongation and termination [5, 6], while others working with cultured rat liver cells propose that the ribosomal site of AFB_1 inhibition is the initiation step [7]. Thus, there is still uncertainty as to the locus of AFB_1 inhibition of translation. We have approached the problem using isolated rat hepatocytes as an alternate to whole animal or cell culture studies, comparing cytoplasmic ribonucleoprotein complex (cRPC) content, proportion of polysome size classes, nascent polypeptides released from polysomes, and formation of initiation complexes in untreated and AFB_1-treated hepatocytes.

Materials and methods

Hepatocytes were prepared from male Wistar rats (250–300 g) using the procedure of Berry and Friend [8] with minor modifications [9]. The cells (1 to 1.5×10^6 cells/ml) were preincubated without or with AFB_1 (5 or 50 μM) for 15 min before [^{3}H]leucine (5 μCi/ml, Amersham Corp., Arlington Heights, IL) was added. At 60 min, hepatocytes were collected and subcellular fractions were obtained by differential centrifugation. The cRPC were isolated by the method of Palmiter [10], and various size classes of polysomes were separated [11]. Incorporation of [^{35}S]methionine (1345 μCi/mmol, Amersham Corp.) into methionyl-tRNA was determined by preincubation of hepatocytes without or with AFB_1 (50 μM) for 65 min before addition of 50 μCi [^{35}S]methionine. After 10-min labeling, 40S and 80S initiation complexes were isolated from the cRPC, and [^{35}S]methionyl-tRNA was precipitated by cetyltrimethylammonium bromide (Sigma Chemical Co., St. Louis, MO). Peptidylpuromycin was prepared by incubation of 20–25 A_{260} units of cRPC with puromycin (0.5 mM) at 37° for 30 min. Peptidylpuromycin of $M_r > 8000$ was purified as described elsewhere [11] and separated on 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate [12]. Cell viability was checked by trypan blue exclusion (0.27%). Protein was assayed by the method of Lowry *et al.* [13]. Radioactivity of trichloroacetic acid or cetyltrimethylammonium bromide insoluble material collected on glass fiber filters (Whatman GF/C) and gel slices was determined by counting in a Beckman liquid scintillation spectrometer. When counting [^{3}H], cpm were converted to dpm, while [^{35}S] was counted under a wide [^{14}C] window, and cpm were recorded. Counting errors were ±5%.

Results and discussion

Previous studies report that at 180 min of incubation, inhibition of protein synthesis measured by [^{3}H]leucine incorporation is dependent on the dose of AFB_1. The lowest dose to show significant inhibition is 5 μM, and the most inhibition is obtained with 50 μM AFB_1 [14]. Since 50 μM AFB_1 causes significant decreases in cell viability at 120 and 180 min of incubation, experiments were terminated at 60 min. This time allows the toxin to enter and act on the cells but avoids changes due to alteration of the cellular membrane. As shown in Table 1, 50 μM AFB_1 significantly inhibited [^{3}H]leucine incorporation into subcellular fractions. Specific radioactivities of cell lysate, nuclei, and cytosol were 10, 23 and 14% of the control respectively. The greater inhibition of [^{3}H]leucine incorporation into proteins of cell lysate (90%) and cytosol (84%) compared to cRPC (71%) may have been due to impairment of posttranslational processes. Since there was no change in the content of the cRPC, the decreased specific radioactivity in treated cells reflected a decrease in the rate of protein synthesis.

To determine the ribosomal site or sites affected by AFB_1, polysome size classes were examined (Table 2). In the presence of 5 μM AFB_1 there was a slight increase in the concentration of monomers accompanied by a decrease in the polysome/monomer ratio compared to the control. The changes were not significant. In the presence of 50 μM AFB_1, however, the increased concentration of monomers and decreased polysome/monomer ratio became significant. According to the scheme of Pestka [15], the accumulation of monomers suggested that the toxin blocked the initiation stage of the ribosomal cycle. Lack of complete polysome breakdown in treated cells indicated that initiation may have been only partially inhibited. To assess the extent of involvement of initiation, initiation complexes were examined (Table 3). [^{35}S]Methionine in untreated cells was associated with both 40S and 80S initiation complexes. The 80S/40S ratio of 3.3 suggested that methionyl-tRNA was present in the 40S initiation complex, allowing subsequent formation of the 80S initiation complex. The content of the complexes was similar in untreated and treated cells, but there was a greater decline in radioactivity in the 80S initiation complex in AFB_1-treated cells. The 80S/40S ratio was decreased to 1.8, similar to the inhibition of the initiation step produced by actinomycin D in CHO cells [16]. The decreased 80S/40S (Table 3) and polysome/monomer ratios (Table 2), although significant, were not sufficient to account for the 71% decrease in overall protein synthesis seen in the cRPC from AFB_1-treated cells (Table 1). The elongation stage of the ribosomal cycle was then considered as an additional target for AFB_1 action.

Analysis of [^{3}H]leucine incorporation into peptidylpuromycin (Fig. 1) indicated that labeled polypeptides of various molecular weights (M_r 10,000–160,000) were found

Table 1. Effect of aflatoxin B_1 on protein content and synthesis of various subcellular components after 60 min of incubation

Condition	Cell lysate		Nuclear pellet		Post-nuclear fractions					
					Cytosol		Ribonucleoprotein complexes		Peptidylpuromycin	
	Content	Synthesis	Content	Synthesis	Content	Synthesis	Content	Synthesis		Synthesis
Control (untreated)	169 ± 43 (18)	154 ± 18 (5)	27 ± 2 (9)	13 ± 2 (3)	127 ± 18 (9)	159 ± 5 (2)	3.3 ± 1.2 (11)	45 ± 12 (3)		38 ± 26 (3)
Treated (50 mM aflatoxin B_1)	175 ± 30 (14)	16 ± 4* (4)	26 ± 7 (5)	3 ± 1* (3)	144 ± 22 (6)	22 ± 15* (4)	3.4 ± 1.2 (8)	13 ± 8* (3)		12 ± 8* (3)

Values for protein content are expressed as mg protein/10^8 cells; values for protein synthesis are expressed as leucine incorporated (dpm × 10^{-3}/mg protein). Data are means ± S.D. with the number of experiments given in parentheses.
* $P < 0.05$, compared with controls.

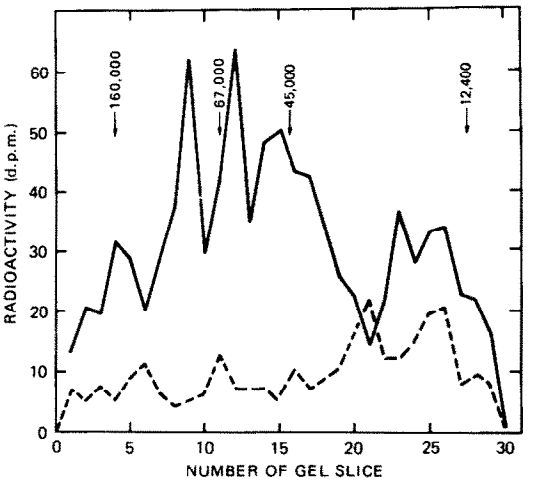

Fig. 1. Radioactivity profiles of nascent polypeptide chains separated by SDS-PAGE. *In vitro* puromycin-released polypeptides from cRPC (23 A_{260}) were isolated, concentrated, and dialyzed against 50 mM Tris–HCl (pH 8.9) containing 53 mM glycine, 0.89% 2-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride. Control, 20 μg protein (——), and AFB_1-treated, 26 μg protein (– – – –), samples were applied and electrophoresed at 2 mA/gel for 90 min in the anodal direction at room temperature. Molecular weight markers were: γ-globulin (160,000), bovine serum albumin (67,000), ovalbumin (45,000) and cytochrome *c* (12,400). Gels were sliced into 1.25 ± 0.25 mm slices, each slice was solubilized in NCS solubilizer at 50° for 2 hr, and 10 ml of OCS was added before radioactivity counting.

in both untreated and AFB_1-treated cells. Radioactivity present in the peptidylpuromycin fraction of treated cells was 32% of the control (Table 1). In polypeptides of $M_r > 45{,}000$, [^{3}H]leucine incorporation was greatly inhibited by AFB_1, exhibiting constant low levels of radioactivity whereas in polypeptides of $M_r < 45{,}000$ the incorporation was less inhibited. Polypeptides of $M_r < 8{,}000$ from treated cells contained slightly increased amounts of radioactivity when compared to controls (data not shown). This suggested a partial inhibition by the toxin at the elongation stage since [^{3}H]leucine incorporation was higher in smaller polypeptides but declined as a greater extent of elongation was required, that is, with increasing polypeptide size. If elongation was completely blocked by the toxin, [^{3}H]leucine would not be present regardless of the size of polypeptide.

Employing different but complementary approaches such as isotope labelling, polysome patterns, analysis of polypeptide chains, and formation of initiation complexes, the present study established that AFB_1-evoked inhibition of cellular protein synthesis involved translational events to a greater extent (70%) than posttranslational processes (15–20%). Lack of accumulation of larger size polysomes and newly synthesized polypeptides eliminated the possibility that the termination step had been affected by AFB_1 for, if this were the case, polysome aggregation and greatly increased radioactivity in cRPC should be seen [9, 15]. Polysome profiles exhibited moderately decreased polysome/monomer ratios, characteristic of partial inhibition of initiation and elongation. The extent of each step involved in the overall inhibition of protein synthesis was

Table 2. Effect of aflatoxin B_1 on polyribosome size classes

Aflatoxin B_1 conc (μM)	Monomers (40S + 60S + 80S)	Polyribosomes 2	3	4	5+	Polyribosome Monomer ratio
0 (8)	1.1 ± 0.1	0.7 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.9 ± 0.2	2.1 ± 0.4
5 (2)	1.2 ± 0.1	0.7 ± 0.2	0.4 ± 0.1	0.2 ± 0.1	0.8 ± 0.2	1.7 ± 0.3
50 (6)	1.5 ± 0.1*	0.6 ± 0.2	0.3 ± 0.1	0.2 ± 0.1	0.7 ± 0.3	1.2 ± 0.2*

Values for monomers and polyribosomes are expressed as mg/10^8 cells. Data are means ± S.D. with the number of experiments given in parentheses.
* $P < 0.05$, compared to controls.

Table 3. Effect of aflatoxin B_1 on the formation of initiation complexes

Conditions	40S Initiation complex Content	Radioactivity	80S Initiation complex Content	Radioactivity	80S/40S Ratio Content	Radioactivity
Control (untreated)	10	443 ± 177	90	1445 ± 517	9	3.3 ± 0.1
Aflatoxin B_1 treated (50 μM)	9	372 ± 74	91	673 ± 98*	10	1.8 ± 0.6*

Values of 40S and 80S initiation complexes are expressed as percent of total area under the peaks: values of radioactivity are expressed as cpm of acid-insoluble materials of the sucrose gradient fractions precipitated by cetyltrimethylammonium bromide. The data presented are means ± S.D. from two separate experiments.
* $P < 0.05$, compared with control.

resolved by the finding that AFB_1 inhibited 80S initiation complex formation to a greater extent than elongation. These results differ from findings reported by White and Rees [7] who, using cultured rat liver cells, suggest that initiation is the only ribosomal site affected. The difference may arise from the 4-fold higher AFB_1 concentration used in their study (192 μM) compared to our study. Investigations are in progress to assess the molecular sites of perturbation and the possible involvement of posttranslational processes.

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REFERENCES

1. P. M. Newberne and W. H. Butler, *Cancer Res.* **29**, 236 (1969).
2. E. C. Miller and J. A. Miller, *Cancer, N.Y.* **47**, 2327 (1981).
3. J. I. Clifford and K. R. Rees, *Biochem. J.* **102**, 65 (1967).
4. S. Villa-Trevino and D. D. Leaver, *Biochem. J.* **109**, 87 (1968).
5. A. Sarasin and Y. Moule, *Eur. J. Biochem.* **54**, 329 (1975).
6. A. Sarasin and Y. Moule, *Expl Cell Res.* **97**, 346 (1976).
7. J. S. White and K. R. Rees, *Chem. Biol. Interact.* **32**, 187 (1980).
8. M. N. Berry and D. S. Friend, *J. Cell Biol.* **43**, 506 (1969).
9. T. G. Helinek, T. M. Devlin and J. J. Ch'ih, *Biochem. Pharmac.* **31**, 1219 (1982).
10. R. D. Palmiter, *Biochemistry* **13**, 3606 (1974).
11. J. J. Ch'ih, L. S. Faulkner and T. M. Devlin, *Biochem. Pharmac.* **28**, 691 (1979).
12. P. J. Dehlinger and R. T. Schimke, *J. biol. Chem.* **246**, 2574 (1971).
13. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
14. J. J. Ch'ih, T. Lin and T. M. Devlin, *Biochem. biophys. Res. Commun.* **115**, 15 (1983).
15. S. Pestka, *A. Rev. Biochem.* **40**, 697 (1971).
16. N. Craig and M. Kostura, *Biochemistry* **22**, 6064 (1983).