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AFLATOXIN B_1—EFFECTS ON RAINBOW TROUT LIVER CHROMATIN*

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Abstract—Injection of 400 μg/kg doses of aflatoxin B_1 into the virginia strain of rainbow trout resulted in quantitative changes in the protein/DNA ratio of liver chromatin. Ammoniacal–silver staining indicated a 41 per cent loss of chromatin stainability. A 65 per cent total loss of histone and acidic proteins, relative to DNA, was observed by direct measurement of extracted and fractionated chromatin. Losses of chromatin protein were restored within 48 hr after injection.

OWING TO their potency as toxigenic and carcinogenic agents, the effects of aflatoxins on many aspects of cellular metabolism have been rigorously investigated. A considerable body of evidence suggests that carcinogenesis may result from changes in gene utilization.[1] Since gene transcription may be influenced, or perhaps governed, by the histone and non-histone proteins associated with DNA in chromatin,[2] at least one recent work has centered on the effects of aflatoxin B_1 on rat liver chromatin *in vivo*.[3] Those studies indicated that aflatoxin B_1 may limit RNA synthesis through alteration of the chromatin template rather than through direct inhibition of RNA polymerase enzymes. A number of workers have characterized the interaction of aflatoxin B_1 with component molecules of chromatin *in vitro*. Aflatoxin will bind to DNA,[4,5] and may[5] or may not[4] bind to histone depending upon the criteria used to determine interaction.

The purpose of the present studies was to determine the effects of aflatoxin B_1 on trout liver chromatin *in vivo*, with experiments being performed to determine if quantitative changes in chromatin composition occur after treatment with aflatoxin B_1.

MATERIALS AND METHODS

Rainbow trout (*Salmo gairdneri*, str. *virginia*) were obtained from the Georgia Game and Fish Commission's Lake Burton Hatchery and placed in 35-gal fiberglass tanks containing demineralized water maintained at 14°, aerated to 9 ppm oxygen, and flowing at an average rate of 2 gal/min. The fish were fed a commercial dry pellet diet which contained no measurable amounts of aflatoxins. Data were collected on

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trout 10–14 months after hatching with no changes in response during this 4-month period.

For all experiments, aflatoxin B_1 was isolated from a previously characterized mutant (No. A–18) of *Aspergillus flavis*[6] according to the method of Shotwell *et al.*[7] Using propylene glycol as a carrier, aflatoxin B_1 was injected intraperitoneally (i.p.) in doses of 350–400 μg/kg body wt (this is 50 per cent of the LD_{50}—10 days in the shasta strain of rainbow trout,[8] but less than 25 per cent of the LD_{50} in the Virginia strain). Ice was used to lower the water temperature to 4–10° to limit the mobility of the fish so they could be readily handled for injection. The aflatoxin B_1-dosed fish were placed in separate fiberglass tanks with environmental conditions identical to the controls.

Trout were sacrificed at various times after injection and their livers quickly excised. Liver chromatin was characterized histologically by ammoniacal–silver (A–S) staining of nuclei in tissue sections, and by direct measurement of the components in chromatin extracted from isolated nuclei. For A–S staining, the livers were quick-frozen and sectioned at 12 μ. After the sections were affixed to microscope slides, they were fixed in neutralized formalin for 12 hr. The fixed sections were stained according to the method of Black and Ansley.[9] A–S staining has been shown to visualize nuclear histones with the intensity of the stain being a function of the amount of histone present.[10]

Chemical analysis of the components of chromatin were made on individual trout livers. The livers were homogenized in a dense sucrose buffer (2·2 M sucrose, 2 mM $CaCl_2$, adjusted to pH 5·9 with CH_3COOH and KOH), and nuclei were isolated by centrifugation at 39,000 *g* for 30 min.[11] After the nuclei were washed twice with additional buffer, methods derived from Telia *et al.*[12] were used to extract and fractionate the chromatin proteins and DNA. The nuclear pellet was suspended in 0·14 M NaCl to solubilize albumin and globulin proteins, and again centrifuged at 1000 *g* for 10 min. This pellet containing the partially extracted nuclei was resuspended in 10 vol. of 2 M NaCl to bring the chromatin into solution. After centrifugation for 60 min at 1000 *g*, the chromatin containing supernatant was made 0·4 N with respect to HCl to precipitate acidic chromatin proteins and DNA. After centrifugation at 3500 *g* for 30 min, the supernatant, containing histones, was decanted. DNA was extracted from the pellet with 10% (v/v) $HClO_4$ at 90°. The acidic proteins which remained precipitated were solubilized in 0·075 N NaOH. Protein was estimated by the procedure of Lowry *et al.*[13] and DNA by the diphenylamine reaction.[14]

RESULTS

Following i.p. exposure to aflatoxin B_1, there was a reduction in the histone and acidic protein content, relative to DNA, of rainbow trout liver chromatin (Table 1). Within 12 hr of injection, the histone–DNA ratio decreased from 1·01 to 0·40 and the acidic protein–DNA ratio from 0·35 to 0·08. These decreases represented a 65 per cent decline in total chromatin protein relative to DNA. The relative protein content increased toward normal levels in the next 36 hr. At 48 hr post-injection, the histone–DNA ratio was 92 per cent that of the control and the acidic protein–DNA ratio was 46 per cent of control levels. No changes in the protein–DNA ratio were observed in trout injected with propylene glycol. When smaller amounts of aflatoxin B_1 were

TABLE 1. EFFECT OF AFLATOXIN B_1 (400 μg/kg) ON THE NUCLEAR PROTEIN–DNA RATIOS OF TROUT LIVER CHROMATIN

Time post-injection (hr)	Histone/ DNA	Acidic protein/ DNA	Relative protein/ DNA
0 (Control)	1·01 ± 0·07*	0·35 ± 0·09	1·00
12	0·40 ± 0·12	0·08 ± 0·03	0·35
24	0·56 ± 0·12	0·12 ± 0·07	0·50
48	0·92 ± 0·15	0·16 ± 0·08	0·79

* Values are the mean of triplicate measurement of three replicates ± standard deviation.

administered (150–200 μg/kg), no change in the chromatin protein–DNA ratio was observed.

There was a marked decrease in the intensity of A–S staining of trout liver chromatin after aflatoxin B_1 dosing (Fig. 1). Objective quantification of liver sections with a Leitz microphotometer indicated a 41 per cent increase in white light transmission 4 hr after treatment (Table 2). The deeply stained nuclei of elliptical cells in both plates (A and B) are nucleated red blood cells which act as an *in situ* control. The fact that the chromatin of these cells stains darkly after aflatoxin B_1 injection, while the chromatin of liver cells does not, provides strong evidence that the decrease in stain intensity is not an artifact.

TABLE 2. TRANSMISSION OF WHITE LIGHT BY AMMONIACAL–SILVER STAINED SECTIONS OF RAINBOW TROUT LIVER CHROMATIN

Time post-injection (hr)	Net intensity of transmitted light (fc)*	Relative increase in transmission
0	2·2 ± 0·09†	1·00
2	2·9 ± 0·10	1·32
4	3·1 ± 0·11	1·41

* Net intensity = (intensity of transmission of liver nuclei) − (intensity of transmission of red blood cell nuclei).

† Values are the mean of triplicate measurements of twelve replicates ± standard deviation.

DISCUSSION

These data indicate that there is a change in chromatin composition in a large number of liver cells after injection of 350–400 μg/kg doses of aflatoxin B_1. It is possible that losses of histone observed by measurement of isolated chromatin components are the result of proteolysis during isolation. However, it is unlikely that this could have had a significant effect in the present study since A–S staining of quick frozen liver sections, where proteolysis could not occur because of the frozen state (−30°) of the tissue, confirmed the loss of histone from chromatin.

Conversely, it is also possible that the decreases in A–S staining of chromatin could result from either (a) masking of the reactive sites of histone by phosphorylation, methylation or acetylation, or (b) a more diffuse structure of chromatin without changes in the amount of histone present. The former possibility, involving enzyme-mediated reactions, is unlikely because of the overall decrease in chromatin proteins. The latter is unlikely since the presence of histone increases the coiling of DNA, and shortens the molecule's length.[15] Thus dispersed chromatin should have less histone.

Aflatoxin B_1 is not unique in its ability to alter the components of chromatin. Stimuli such as ionizing radiation,[16,17] injection of tetanus toxoid,[9] and changes in environmental pH* have been shown to quantitatively and/or qualitatively affect chromatin. In addition, nucleohistone is completely transformed into nucleoprotamine as a result of displacement of histones by protamine during maturation of spermatids in rainbow trout.[18] Considering those observations, it is difficult to suggest that the carcinogenic effect of aflatoxin is inherently related, in a mutually inclusive manner, to its ability to cause short-term alterations in the protein–DNA balance of chromatin as observed in this study.

Edwards and Wogan[3] noted that aflatoxin reduces the template efficiency of rodent liver chromatin 30 min after aflatoxin B_1 injection. Characterization of chromatin extracted from nuclei isolated in low density sucrose buffer—0·25 M sucrose, 3 mM $CaCl_2$, 0·01 M Tris at pH 7·9—has indicated insignificant alterations in the chromatin protein–DNA ratio within 30 min after aflatoxin B_1 injection.[19] This would suggest that the decreased efficiency of chromatin as a template for RNA polymerase activity is more likely the result of qualitative rather than quantitative changes in the chromatin. Wogan and Pong[20] have suggested that aflatoxin B_1, or metabolic products, may inhibit gene transcription through direct binding to DNA. If this is the case, small changes in the protein–DNA ratio of chromatin may be of reduced importance in limiting template activity.

Several mechanisms for loss of chromatin protein after aflatoxin B_1 injections suggest themselves. Proteolytic enzymes, such as those described by Furlan and Jericijo,[21] could catabolize the chromatin proteins. Acetylation and/or phosphorylation of histones might weaken the histone DNA interaction and lead to increased susceptibility to proteolysis.[18] Additional research is being performed to determine if these events occur, and if so, their possible significances. Identification of the histone and acidic protein fractions which are lost from chromatin would also be of interest. Electrophoretic studies of these protein systems are in progress.

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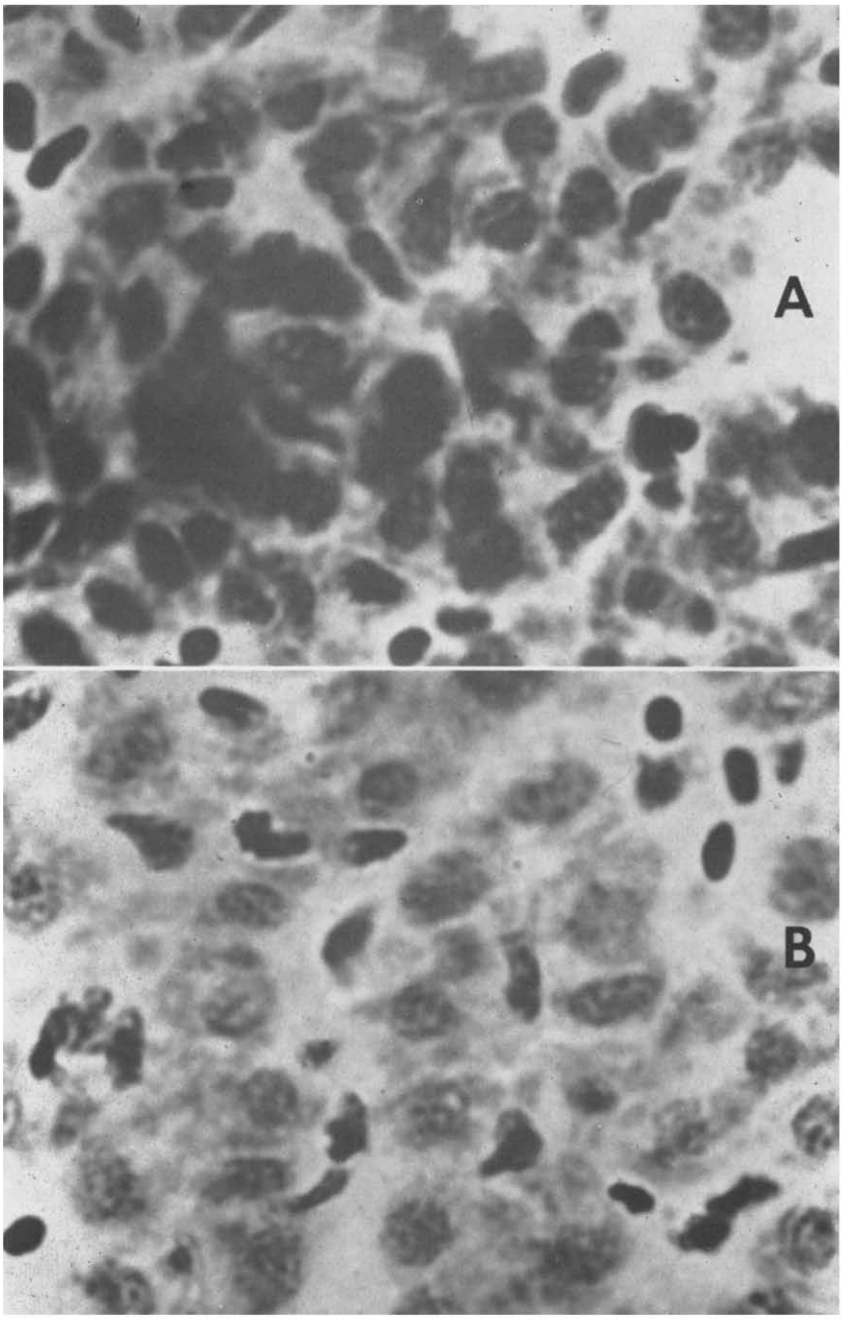

FIG. 1. Ammoniacal-silver staining of rainbow trout liver sections (12 μ). The intensity of the stain is a function of the amount of histone present in chromatin. (A) Control. (B) Four hr after injection of 400 μg/kg of aflatoxin B_1.

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