The Effect of Aflatoxin B\textsubscript{1} on the Hepatic Structure and RNA Synthesis in Rats Fed a Diet Marginally Deficient in Choline

W. H. Butler and G. E. Neal

Medical Research Council, Toxicology Unit. Medical Research Council Laboratories, Woodmansterne Road, Carshalton, Surrey, United Kingdom

SUMMARY

Aflatoxin, a potent hepatotoxin, causes rapid inhibition of RNA polymerase activity in isolated rat liver nuclei. Its hepatotoxicity is accompanied by an injury of periportal parenchymal cells. Rats fed a diet marginally deficient in choline are protected against the acutely toxic action of aflatoxin B\textsubscript{1}. This diet does not prevent the rapid inhibition of RNA polymerase or the changes in nucleolar morphology following the administration of aflatoxin B\textsubscript{1}. The diet does however largely prevent the proliferation of smooth endoplasmic reticulum and the necrosis of periportal parenchymal cells. The diet appears to offer the possibility of separating those changes in the liver associated with hepatotoxicity from those associated with hepatocarcinogenicity.

INTRODUCTION

The hepatotoxicity of aflatoxin B\textsubscript{1} has been studied extensively by both morphological and biochemical methods. Morphologically, its primary action in rat liver is to injure periportal hepatic parenchymal cells (3). Biochemical examinations have revealed that the RNA mechanisms are rapidly inhibited (7, 9). This inhibition appears to be due to the metabolism of aflatoxin B\textsubscript{1} by microsomes to an activated form and results from the interaction of this metabolite with either the polymerase enzymes (13) or the chromatin template (8, 14). The biochemical observations have been correlated with the morphological evidence, in that the inhibitions of RNA and protein synthesis are accompanied by damage to the nucleolus and RER\textsuperscript{1} (4, 5, 15). Furthermore, in common with other hepatotoxic compounds, there is an increase in the amount of SER (5) subsequent to the initial injury.

Rogers and Newberne (17) have demonstrated that a diet marginally deficient in choline protects against the acutely lethal action of a single dose of aflatoxin B\textsubscript{1} but leaves the carcinogenic activity of repeated low-level doses either unaffected or even enhanced. This feeding regimen therefore appears to offer the possibility of providing further insight as to those biochemical and morphological processes related to the toxic rather than carcinogenic action of the toxin. A comparative study was made of the disturbance by aflatoxin B\textsubscript{1} of hepatic RNA synthesis and the accompanying morphological lesions in rats fed a choline-deficient or normal diet.

MATERIALS AND METHODS

Male inbred Fischer rats were fed a diet marginally deficient in choline (18) from weaning for 10 days. Normal-diet animals were fed Medical Research Council Diet 41B for the same time period. The rats' daily intake of choline-deficient diet was approximately one-half that of the 41B-fed animals. Growth was less on the choline-deficient diet—on an average, only 29% that of the control diet-fed animals. At the end of the experimental feeding period, both choline-deficient and normal-diet rats were given aflatoxin B\textsubscript{1} (4 mg/kg) i.p. with a maximum of 0.1 ml dimethyl sulfoxide (special for spectroscopy grade). In the acute toxicity tests, groups were also dosed with 8 mg/kg. Control animals, those fed either the choline-deficient or normal diet, were given similar amounts of dimethyl sulfoxide alone. Groups of animals were killed by decapitation 2, 6, 16, 24, and 48 hr after receiving the injections. All animals received normal diet after the injections were performed.

For examination by light microscopy, portions of the left median lobe of the liver were taken from all animals used. Livers were fixed in 10% formyl alcohol, and paraffin sections, prepared in the usual fashion, were stained with Harris's hematoxylin and eosin. For electron microscopy, at each time interval liver samples were taken from 2 choline-deficient, aflatoxin-treated rats; 1 normal diet, aflatoxin-treated rats; 1 choline-deficient, dimethyl sulfoxide-treated rat; and 1 normal diet, dimethyl sulfoxide-treated rat. Portions of the left median lobe were diced in Veronal-buffered 2% osmium tetroxide and fixed for 2 hr at 4°. Following rinsing, the tissue was dehydrated by passage through graded alcohols and embedded in Epon 812 (11). Sections (1 μm) were cut and stained with toluidine blue or basic fuchsine for orientation. Ultrathin sections, cut with glass knives on either an LBK III or a Porter-Blum MT2 microtome, were prepared from both periportal and centrilobular areas of the liver. The sections, stained in all cases with 3% aqueous uranyl acetate and lead citrate, were examined in either a Zeiss EM 9 or an AEI 801 electron microscope.

RNA Polymerase Assays. At each time interval, pooled samples of liver (approximately 2 g, fresh weight, from each liver) were taken for determination of the level of RNA polymerase activity in the hepatic nuclei. Immediately upon

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\textsuperscript{1}The abbreviations used are: RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum.

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excision, the liver samples were placed in ice, and all subsequent operations were carried out at 0 to 4°. Nuclei were prepared essentially by the method of Widnell and Tata (22). Nuclear preparations were always checked by phase-contrast microscopy for integrity of the nuclei and for freedom from gross contamination with other subcellular material. Both Mg++- and Mn++-(NH₄)₂SO₄-stimulated RNA polymerase activities present in the nuclei (approximately 200 µg DNA/assay) were assayed by the method of Widnell and Tata (23) using UTP-³H (0.5 µCi/20 nmoles). All assays were carried out on freshly prepared nuclei. Incubations at 37° were carried out for 20 min for Mg++-stimulated activities and for 30 min (following 15 min preincubation in the absence of UTP-³H) for Mn++-(NH₄)₂SO₄-stimulated activities. Radioactive counting was carried out in a Packard Tri-Carb liquid scintillation spectrometer. The counting efficiency for ³H was 15%, as determined by internal standardization with a dioxane-based scintillator (1). Determinations of the DNA content were carried out by the method of Burton (2).

In Vitro Incubations. Livers slices (approximately 0.3 mm thick) were cut from freshly excised livers with a hand microtome (19). Slices (200 mg, fresh weight) were incubated in 3 ml Krebs-Ringer phosphate medium plus 0.27% glucose at 37° in a shaking incubator bath at approximately 100 oscillations/min under an atmosphere of O₂. One µCi of orotic acid-¹⁴C (60.8 mCi/mmol) was added to the incubation flasks and the tissue was incubated for 1 hr. The specific activity of the RNA was determined spectroscopically after hydrolysis with 1 N KOH, assuming an absorbance at 260 nm of 34.2/mg RNA/ml/cm light path. Radioactive counting was carried out in a dioxane-based scintillator, as described above, with an efficiency for ¹⁴C of 70%.

Hexobarbitone "Sleeping Times." Rats were given i.p. injections of hexobarbitone sodium (150 mg/kg) (a gift from May & Baker Ltd., Dagenham, United Kingdom). The sleeping time was taken as the time elapsing between the onset of anesthesia and the regaining of the righting reflex.

RESULTS

Acute Toxicity Experiments

The effect of feeding the marginally choline-deficient diet to rats on the acutely toxic effect of a single i.p. injection of aflatoxin B₁ was determined. The results of one of the experiments are given in Table 1, from which the protective effect of the choline-deficient diet is clearly evident. Hexobarbitone sleeping times were also determined during a parallel experiment. No significant difference between the sleeping times of the 2 groups was observed.

Morphological Examinations

Dimethyl sulfoxide-treated Normal and Choline-deficient Diet-fed Rats. No evidence indicating that administration of dimethyl sulfoxide to normal-diet animals resulted in any changes in hepatic structure was observed by light or electron microscopy. At the level of choline added to the diet (0.25%) there was a slight but consistent increase in fat, and enlarged mitochondria with sparse cristae were present. The parenchymal cell nuclei were of normal appearance.

Aflatoxin-treated Normal and Choline-deficient Diet-fed Rats. Examination of liver sections under the light microscope 48 hr after the injection of aflatoxin B₁ revealed a typical periportal zone necrosis in animals fed control diet (Fig. 1), while the animals fed the choline-deficient diet showed no lesion (Fig. 2). The development and extent of the lesion in rats fed the normal diet have been described in detail (4, 5). Briefly, the earliest changes, observed after 2 hr, were a progressive degranulation and dilation of the RER of periportal parenchymal cells and random formation of segregated nucleoli. A progressive disruption of the periportal cells up to 48 hr (Fig. 3) and a centrilobular increase of SER were described (Fig. 4).

In the rats fed the choline-deficient diet, the periportal parenchymal cells at 2 hr showed similar changes, with irregular dilation of the RER cisternae and disaggregation of ribosomes. Other cytoplasmic organelles appeared to be unchanged (Fig. 5). Segregated nucleoli which were present showed no zonal distribution. However, instead of showing a progressive change, by 16 and 24 hr the cytoplasmic organelles of the central and midzonal parenchymal cells were normal (Fig. 6). A few periportal cells had multiple large autophagic vacuoles (Fig. 7) and some increase of the SER. Throughout the lobule many nuclei still showed segregated nucleoli. At 48 hr, by which time in the normal-diet animals there was complete disruption of the periportal parenchymal cells (Fig. 3), only an occasional necrotic cell (Fig. 9) was seen in the choline-deficient animals. Within the parenchymal cells immediately adjacent to these necrotic cells there was abundant SER (Fig. 9). However, in the remainder of the liver this increase of SER was not seen (Fig. 8). While many of the nuclei had a normal configuration of the nucleolam 48 hr, others showed either a persistent segregation or a dense nucleolam and sparse ribosomal components suggestive of reforming nucleolus (5).

RNA Synthesis

RNA polymerase activities present in nuclei isolated from livers excised at each time interval after aflatoxin...
treatment were assayed, and the results for Mg$^{++}$-stimulated activities are given in Chart 1, expressed as percentages of the activities observed in the corresponding dimethyl sulfoxide-treated controls. The Mn$^{++}$-(NH$_4$)$_2$SO$_4$-stimulated activities yielded results similar to those shown in Chart 1. A rapid inhibition of the polymerase activity was observed in nuclei isolated from the livers of both normal and choline-deficient rats. The recovery of activity however was more rapid in nuclei isolated from choline-deficient animals. The results given in Table 2 show that the levels of inhibition observed when polymerase activity was assayed in isolated nuclei correlated with the incorporation of orotic acid-$^{14}$C into RNA of liver slices incubated in vitro. The difference in specific activities of the RNA extracted from the livers of animals fed the control or choline-deficient diet after incubation with labeled orotic acid (Table 2) was a consistent feature of these experiments. Despite the fact that the liver obtained from choline-deficient animals had only 50 to 60% of the RNA content, on a fresh-weight basis, of the control tissues, a much lower incorporation of orotic acid in the former resulted in the lower specific activity of the RNA seen in Table 2. When the incorporation of leucine-$^{14}$C into protein was studied under conditions similar to those of the orotic acid-incorporation experiments (W. H. Butler and G. E. Neal, unpublished results) no differences in the specific activities of the protein were observed between the livers of animals fed the control or choline-deficient diet.

**DISCUSSION**

These experiments have confirmed the observations of Rogers and Newberne (17) that a diet marginally deficient in choline protects against the lethal action of a single dose of aflatoxin $B_1$. We have found that this protection is accompanied by prevention of the widespread hepatotoxic action of the compound. This protection is therefore different from that which has been reported when the administration of dimethylnitrosamine was preceded by feeding with a protein-depleted diet (12) since, in that case, although the deficient diet afforded protection against the lethal action of the dimethylnitrosamine, it did not modify its hepatotoxic action (6). In this study it was only in the Epon-embedded material that it was possible to demonstrate the presence of an occasional necrotic hepatic parenchymal cell following aflatoxin treatment of choline-deficient animals.

That the animals were made choline deficient was clearly demonstrated by the increase of lipid throughout the liver and the presence of large mitochondria which reportedly is a feature of choline deficiency (20, 21). The presence of early periportal cytoplasmic changes in the choline-deficient aflatoxin-treated animals and the nucleolar segregation throughout the liver indicated that the aflatoxin reached the hepatic cells. The initial morphologic lesion observable 2 hr after treatment with aflatoxin $B_1$ was the same in the choline-deficient and normal-diet animals. These results parallel the observations on the RNA polymerase levels which in the initial stages fell rapidly in both groups. However, the rate of recovery of the polymerase activity was slower and less complete in the normal-diet than in the choline-depleted livers. This may reflect the development of necrosis in the former group. These results indicate that the rapid inhibition of RNA synthesis does not necessarily indicate the later development of hepatic necrosis. The lack of effect of the choline-deficient diet on the hexobarbitone
sleeping time suggests that the modification of toxicity by the diet did not involve alterations in the levels of drug-metabolizing systems involving hexobarbitone. However, Rogers and Newberne (18) have reported that feeding the choline-deficient diet reduces the p-nitroanisole demethylase and benzpyrene hydroxylase activities of isolated microsomes.

Except in the immediate vicinity of the occasional necrotic cell seen in the choline-deficient group, there was no significant increase in SER, while in the rats fed the normal diet and given injections of aflatoxin there was an abundant increase in SER in the centrilobular parenchymal cells. It is not known whether the failure of SER proliferation in choline-deficient rats represents an inability to synthesize membrane or an absence of the necessary stimulus. It is of interest that 24 hr after the administration of aflatoxin B1 to choline-deficient animals, although the RNA polymerase activity had returned to >75% of the control level, nucleolar abnormality was still widespread. The relationship between nucleolar segregation and inhibition of RNA polymerase is now being studied.

In summary, it appears that the choline-deficient diet protects against both the acutely lethal action of aflatoxin B1, and the development of periporal liver necrosis. The initial inhibition of RNA synthesis that results from aflatoxin B1 administration remains unchanged, as does the carcinogenic action of the toxin (16). The possible correlation between these observations is being further investigated.

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REFERENCES

Fig. 1. Light micrograph of liver from a normal-diet rat given aflatoxin B₁ (4 mg/kg) and killed at 48 hr. There is a periportal zone of necrosis (N). H & E, × 160.

Fig. 2. Light micrograph of liver from a choline-deficient rat given aflatoxin B₁ (4 mg/kg) and killed at 48 hr. The structure of the hepatic lobule is normal with no necrosis. H & E, × 140.

Fig. 3. Electron micrograph of the periportal zone of a normal-diet rat 48 hr after administration of aflatoxin B₁ (4 mg/kg). There is considerable disruption of the parenchymal cells. × 7,250.

Fig. 4. Electron micrograph of a centrilobular hepatic parenchymal cell of a normal-diet rat 48 hr after administration of aflatoxin B₁ (4 mg/kg). Extensive SER (S) is seen throughout the cytoplasm. × 6,500.

Fig. 5. Part of a periportal parenchymal cell of a choline-deficient rat killed 2 hr after administration of aflatoxin B₁ (4 mg/kg). The cytoplasm shows some disruption of the RER cisternae, and the nucleolus is segregated. × 16,500.

Fig. 6. Part of the centrilobular parenchymal cell of a choline-deficient rat killed 16 hr after administration of aflatoxin B₁ (4 mg/kg). The cytoplasmic organelles are normal. × 12,800.

Fig. 7. The cytoplasm of a periportal parenchymal cell of a choline-deficient rat killed 24 hr after administration of aflatoxin B₁ (4 mg/kg). Multiple autophagic vacuoles (V) are present. × 12,000.

Fig. 8. A centrilobular parenchymal cell from a choline-deficient rat killed 48 hr after administration of aflatoxin B₁ (4 mg/kg). The cytoplasmic organelles are within normal limits. No increase of SER is seen. × 10,600.

Fig. 9. Survey of the periportal zone from a choline-deficient rat killed 48 hr after administration of aflatoxin B₁ (4 mg/kg). A few necrotic cells (N) are present, and the SER is abundant in the cells adjacent to the necrosis. × 3,500.
Effect of Aflatoxin on Choline-deficient Rats
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[Images of cells and structures labeled with V and N]

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