The Effects of Prolonged Feeding with Aflatoxin B₁ on Adult Rat Liver

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SUMMARY

The effects of feeding adult rats for 6 weeks with a carcinogenic regimen of aflatoxin-contaminated diet are described. Effects on the histological appearance of liver sections are related to changes observed in nuclear separations carried out using zonal centrifugation. Changes in the levels of nuclear RNA and DNA synthesis have been studied in the populations of hepatic nuclei separated in the zonal rotor. The first 3 weeks of the feeding period was accompanied by continuing inhibitions of nucleic acid synthesis, terminating in a loss of the majority of the tetraploid hepatocyte nuclear population. The subsequent 3 weeks of feeding was predominantly a period of proliferation, restoration of the lobular architecture, and recovery of nucleic acid-synthetic activity. The possible bases of these two opposite effects, inhibition followed by stimulation, which occurred sequentially during the continued feeding of the toxic diet, are discussed.

INTRODUCTION

In a previous communication (11) the results of preliminary experiments on the effect of aflatoxin B₁ on hepatic nuclei, separated into subpopulations by zonal centrifugation, were reported. Feeding weanling animals with an aflatoxin-contaminated diet prevented the development of a predominantly tetraploid hepatocyte nuclear population during maturation. That this effect was not due solely to a prevention of the development of tetraploid nuclei was indicated by the results of similar feeding experiments using adult rats, in which a tetraploid population was already present at the commencement of feeding the toxic diet. At the end of a 6-week carcinogenic feeding regimen for these adult animals, it was observed in zonal centrifugation studies that the tetraploid peak was depressed and that increased populations of nuclei, sedimenting in the diploid and octaploid areas of the zonal separations, were present. The process of development of these changes in nuclear profile observed in zonal centrifugation studies using nuclei isolated from adult rats has subsequently been studied in greater detail. The progressive changes in nucleic acid-synthesizing capacities of the nuclear populations, separated during the period of the toxic feeding, have also been assayed. The present communication deals with the results of this study.

MATERIALS AND METHODS

Animals

Adult male Fischer rats weighing approximately 280 g at the commencement of the experimental period, were used. Experimental feeding consisted of a 50:50 mixture of peanut meal and powdered MRC 41B diet. In the case of the toxic diet, the peanut meal was naturally contaminated with aflatoxin B₁, the final diet containing 4 ppm aflatoxin B₁. The aflatoxin-containing peanut meal (MP meal) was obtained from the Central Veterinary Laboratory, Weybridge, England. In the case of the control diet, an uncontaminated peanut meal (nurse meal), shown to be free of aflatoxin B₁, was used. Groups of animals received the diets for a maximum of 6 weeks. Animals receiving the MP meal for 6 weeks developed hepatocarcinomas, with 100% incidence, during the following 40 to 50 weeks. At weekly intervals animals were killed between 9:30 and 10:30 a.m. by decapitation and were exsanguinated, and the livers were rapidly removed to ice. Samples were removed for histological examination.

Chemicals

[methyl-³H]Thymidine (specific activity, 21 Ci/m mole) was used to label DNA in vivo, each animal received 20 µCi i.p. (in 1 ml 0.9% NaCl) 24 hr before sacrificing. [5-³H]Uridine 5'-triphosphate sodium salt (specific activity, 1 Ci/m mole) was used in the in vitro RNA polymerase assays. Both of these radiochemicals were obtained from the Radiochemical Centre, Amersham, England. ATP, UTP, CTP, and GTP were obtained from Koch Light Ltd., Colnbrook, England. Bovine pancreatic RNase type A was obtained from Sigma Chemical Co., London, England. α-Amanitin was a generous gift from Dr. W. I. P. Mainwaring, I.C.R.F., London, England.

Histology and Microdensitometry

Samples of liver were fixed in formal alcohol, and paraffin sections were stained with Harris's hematoxylin and eosin. Aliquots of the suspensions of nuclei before placement in
the zonal rotor and aliquots of the separated fractions obtained following zonal centrifugation were smeared onto microscope slides and allowed to dry at room temperature. The slides were cautiously heated over a Bunsen burner flame for heat fixation of the nuclei and then were placed in distilled water for removal of sucrose. Control slides were placed in a 0.1% solution of RNase for 90 min at 37° and then were rinsed in distilled water. Paired control and test slides were placed in cold 1 N HCl for 30 sec, transferred to 1 N HCl at 60° for 8 min, and then rinsed in distilled water and placed in Schiff's reagent at 20° for 30 min. Preliminary experiments indicated that these conditions were optimal for staining the nuclei. The slides were rinsed in 3 changes of a 0.5% solution of potassium metabisulfite in 0.05 N HCl, each rinse being approximately 30 sec, and then passed through 2 changes of distilled water, dehydrated in 70% ethanol, given 3 washes in absolute ethanol, cleared in xylene, and finally mounted in Canada balsam. Measurements of absorbance of the Feulgen stain by microphotometry were carried out using a Reichert Zetaplan research microscope fitted with a microphotometer with special control unit of increased sensitivity obtained from C. Reichert, British and American Optical Co., Slough, England. The microscope was set up for Kohler illumination and the nuclei were examined under an oil immersion objective with the photometer at 507 nm. The photometer was operated according to the instructions of the manufacturer, stop sizes used being varied according to the size of nuclei measured. Results are expressed as total absorbance of individual nuclei. The absorbance of 100 nuclei was measured in the case of each sample.

Isolation and Fractionation of Nuclei

Nuclear fractions were isolated from 16 g pooled liver, obtained from 2 rats essentially by the method of Widnell and Tata (14). Estimations of recoveries of nuclei, based on a comparison of the DNA content of the original homogenate and final nuclear pellet, were routinely carried out. Zonal centrifugation separations were carried out as previously reported (11) with the exception that the underlay solution was 50% sucrose/1 mM MgCl₂ in place of 55% sucrose/1 mM MgCl₂. This modification facilitated pumping because of its lower viscosity.

Biochemical Assays

Nuclear Counting. Nuclear counting was carried out on the nuclear suspensions in 15% sucrose/1 mM MgCl₂ prior to loading it into the zonal rotor, usually following a 10-times dilution with 0.32 M sucrose/1 mM MgCl₂. An “Improved Neubauer” hemacytometer slide (A. R. Horwell Ltd., London, England) was used to count unstained nuclei by phase contrast illumination.

DNA Synthesis. DNA synthesis was estimated by determining the incorporation of label into an acid-insoluble form in nuclei 24 hr after the injection of 20 μCi [³H]thymidine. Aliquots (10 ml) of the nuclear fractions (20 ml) collected from the zonal rotor were placed in 10-ml conical centrifuge tubes and the nuclei were sedimented at 2000 rpm in a MSE 4L Mistral centrifuge at 4°. The nuclei were washed 3 times with 3 ml ice-cold 5% trichloroacetic acid and finally were hydrolyzed in 1 ml 10% HClO₄, at 90° for 15 min. The DNA content of the hydrolysate was estimated by the method of Burton (1) as modified by Giles and Myers (4), and the radioactive content was estimated by adding aliquots of the hydrolysate to 10 ml Instagel scintillator (Packard Instrument Ltd., Caversham, England) followed by counting in a Philips Automatic Liquid Scintillation Analyzer PW 4510/01. Conversion of cpm to dpm was computed by the spectrometer from quench curve data using the external standards ratio.

DNA Synthesis. In vitro assays of RNA polymerase activity were carried out on the separated nuclear populations. The 1st 20 ml of the diploid nuclei-containing peak (Chart 1, Peak A), which contained a mixture of diploid nonparenchymal and parenchymal nuclei (7, 11), is referred to as fraction DS. The 20-ml fraction commencing at the top of the diploid peak, which contained almost exclusively diploid parenchymal nuclei, is referred to as fraction DP. It must be stressed that the subdivision of Peak A (Chart 1) into DS and DP represented only an enrichment of these subpopulations of nuclei, and no absolute resolution of diploid nonparenchymal from diploid parenchymal nuclei was achieved in the present study. Ten-ml fractions were collected as the tetraploid nuclei-containing zone (Chart 1, Peak B) emerged from the rotor, and the 3 fractions that spanned the peak were combined and are referred to as fraction TP. Five-ml aliquots out of each of these fractions (DS, DP, and TP) were placed in 10-ml conical centrifuge tubes and centrifuged at 2000 rpm for 20 min in a MSE 4L centrifuge set at 4°. After decantation of the supernatant, the nuclear pellets were resuspended in 0.1 ml 0.21 M sucrose/1 mM MgCl₂ and were used directly for RNA polymerase assays in the 10-ml conical centrifuge tubes without transfer. Assays were carried out in duplicate or triplicate, using the technique of Widnell and Tata (15) for both Mg²⁺- and Mn²⁺-(NH₄)₂SO₄-activated systems. Incubations were carried out for 10 min in the case of the Mg²⁺-activated system and for 45 min for the Mn²⁺-(NH₄)₂SO₄-activated system (following a 10-min preincubation in the absence of [³H]UTP). α-Amanitin (1 μg) was added to certain assay tubes.

RESULTS

The sequential events observed during the course of feeding adult rats for 6 weeks with the aflatoxin-contaminated MP peanut meal were as follows.

Initial Controls. The histological sections of the livers and the unfraccionated nuclear suspensions presented a normal appearance, as illustrated in Fig. 1, a and b. The separations of the hepatic nuclei in the zonal rotor (Chart 1a) indicated a preponderance of tetraploid hepatocyte nuclei, which is the pattern previously reported (7, 11) for animals of this size. That the separation of the nuclei was into diploid (Chart 1, Peak A) and tetraploid (Chart 1, Peak B) subpopulations is illustrated by the average DNA content of the separated populations as determined colorimetrically (Table 1) and by microdensitometry of Feulgen-stained nuclei (Chart 2A). The distribution of the differing ploidies within the control liver lobule was determined by microden-
Aflatoxin and Adult Rat Liver

Total labeling was naturally highest in the areas of the zonal profiles that contained the greatest numbers of nuclei. RNA synthesis in the nuclear populations separated by zonal centrifugation, pooled as indicated in "Materials and Methods," was assayed. Chart 3 shows that the levels of Mg$^{2+}$ (nucleolar)- and Mn$^{2+}$-(NH$_4$)$_2$SO$_4$ (nonnucleolar)-activated RNA polymerase activities in DP and TP nuclei were similar. This is in contrast to the results of Johnston et al. (8), who, using Norwegian rats of this size, reported a much greater RNA polymerase activity (10-fold) in TP compared with DP nuclei. In the present study we found that DS nuclei had somewhat lower levels of both RNA polymerase activities than did the DP and TP nuclei (approximately 50%). This result is in contrast to that of Gonzalez-Mujica and Mathias (5), who reported that nonparenchymal diploid nuclei, sep-

Table 1

DNA recovery and content of nuclear fractions isolated during 6 weeks of feeding toxic diet

<table>
<thead>
<tr>
<th>Time on diet (wk)</th>
<th>Fraction A</th>
<th>Fraction B</th>
<th>DNA recovery$^*$</th>
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<tr>
<td>0</td>
<td>8</td>
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</tr>
<tr>
<td>6</td>
<td>8</td>
<td>15</td>
<td>55</td>
</tr>
</tbody>
</table>

$^*$ Percentage of DNA content of homogenate recovered in final nuclear pellet.

Chromatography of Feulgen-stained sections and indicated a predominance of the polypliod nuclei in the periportal areas (H. L. Arora, G. Jones, and G. E. Neal, unpublished observations). The data obtained following the separation of nuclei 24 hr after a pulse labeling with [H]thymidine (Chart 1a) revealed an almost uniform specific activity ([$^3$H]dpm per mg DNA) across the profile of nuclei separated in the zonal rotor. The 24-hr labeling period is sufficiently long to permit nuclei, originally labeled in S phase, to have subsequently undergone mitosis and to sediment in the position of the principal nuclear peaks (7, 11). This uniformity of the specific activities of labeling of the DNA indicates that the level of DNA replication and mitosis among the nuclear populations was in proportion to their relative numbers.

Chart 1. Zonal nuclear profiles and [H]thymidine incorporation into nuclei during 6 weeks of feeding toxic diet. a, controls; b, 1 week of feeding; c, 2 weeks of feeding; d, 3 weeks of feeding; e, 4 weeks of feeding; f, 5 weeks of feeding; g, 6 weeks of feeding. O, [H] incorporated (24 hr after [H]thymidine injection); ---, light scattering at 254 nm. Redrawn from original traces. Scale indicated in d (a, b, c, e, f, and g are drawn to the same scale). Peaks A and B indicated in a. Earlier part of the elution profile (including membrane fraction) omitted from traces (11). For experimental details, see "Materials and Methods."

Chart 2. Microdensitometry of Feulgen-stained nuclear fractions obtained from livers of control rats and rats fed the toxic diet for 3 or 6 weeks. Fraction 0, unfractionated nuclear suspension. Fractions A and B, obtained following zonal centrifugation (see Chart 1). Numbers of nuclei present in peaks ($\times 10^6$/ml) of combined fraction containing Peak A or Peak B. Control: Fraction A, 2.7; Fraction B, 3.9. Three weeks of feeding: Fraction A, 2.2; Fraction B, 1.2. Six weeks of feeding: Fraction A, 2.6; Fraction B, 2.2. Means of triplicate nuclear counts, variation <5%.

Chart 3. RNA synthesis in vitro in subpopulations of nuclei isolated during the course of 6 weeks of feeding toxic diet. O, Mn$^{2+}$-(NH$_4$)$_2$SO$_4$-primed activity; a, Mg$^{2+}$-primed activity. a, fraction DS; b, fraction DP; c, fraction TP. For details, see "Materials and Methods."
arated from control young adult rats, were almost devoid of RNA polymerase activity in vitro.

One Week of Toxic Feeding. The histological sections indicated a minimal proliferation of biliary or oval cells occurring around the small portal tracts. No evidence of parenchymal cell necrosis or mitotic activity was observed. The absence of any large changes in the morphology of the liver was paralleled by the unchanged zonal centrifugation profiles obtained (Chart 1b). There were also apparently unchanged average DNA contents of the separated nuclear fractions (Table 1). The [3H]thymidine incorporation data (Chart 1b), however, indicated that DNA replication was strongly inhibited in the tetraploid nuclei and also in the DP portion of Peak A (Chart 1b). In agreement with the limited proliferation of biliary and oval cells observed in the sections, DNA synthesis in the DS portion of Peak A (Chart 1b) was depressed to a much smaller extent than in DP and TP. Chart 3 shows that, like DNA synthesis, RNA synthesis in the DP and TP nuclei was strongly inhibited. The inhibition was most marked in the case of the Mnsup2+ -(NH4)2SO4 (nucleolar)-primed activity, nucleolar RNA polymerase activity being inhibited to a much smaller extent. In the DS nuclei, however, Mg2+-primed RNA polymerase activity was unchanged and Mnsup2+ -(NH4)2SO4-primed activity possibly exhibited a small increase, again in accordance with the limited proliferation of nonparenchymal cells.

Two Weeks of Toxic Feeding. The histology showed changes similar to those seen after 1 week of feeding, with proliferating oval cells being the most prominent feature. In addition, eosinophilia of the parenchymal cells and a few small foci of necrosis were present adjacent to the portal tract. A small increase in the area of the diploid nuclei-containing peak compared to the area of the tetraploid nuclei-containing peak was observed in the zonal profile (Chart 1c), which presumably reflected the limited focal necrosis of polyplid cells in the periportal zone observed in the sections, and the accompanying proliferation of diploid nonparenchymal cells. The DNA contents of nuclear fractions present in Peaks A and B of Chart 1c (Table 1) indicated that the average DNA content of nuclei present in the diploid peak remained unchanged, whereas the average DNA content of nuclei sedimenting in Peak B (Chart 1c) was reduced. The incorporation of [3H]thymidine (Chart 1c) indicated that the strong inhibition of DNA replication observed the previous week continued in both the diploid and tetraploid parenchymal nuclei but replication was proceeding in the diploid nonparenchymal nuclear area, again in accordance with the oval cell proliferation observed in the histology. Little change was observed in the level of RNA synthesis in the diploid nonparenchymal nuclei (Chart 3). In the diploid and tetraploid parenchymal nuclei, nucleolar RNA synthesis was increasingly inhibited but the inhibition of the nonnucleolar RNA synthesis was little changed from that observed in the sample of the previous week.

Three Weeks of Toxic Feeding. Histologically, considerable changes had occurred during this week (Fig. 2a). Periportal necrosis, extensive proliferation of oval cells, variation in cell and nuclear size of the remaining parenchymal cells, and an increase in the number of mitotic figures were prominent features of the lesion. Microscopic foci of large parenchymal cells, with basophilic feathery cytoplasm containing many mitotic figures, could be identified in the centrilobular region at this time. The appearance of the unfractonated nuclear suspension is given in Fig. 2b, which shows an increased proportion of nonparenchymal nuclei compared with the control (Fig. 1b). The zonal centrifugation profile (Chart 1d) revealed a sharp drop in the proportion of tetraploid nuclei present. This was in agreement with the periportal necrosis observed histologically, the polyploid nuclei being situated predominantly in the periportal region of the lobule. The average DNA content of the diploid parenchymal nuclei remained unchanged (Table 1), but in the tetraploid peak the average DNA content was again lower than in the controls. Microdensitometry of Feulgen-stained nuclei indicated that an increased proportion of diploid nuclei was present in the tetraploid zone (Chart 2b).

The level of DNA synthesis in the parenchymal cells had increased compared with the previous week (Chart 1d), which was in agreement with the increase in mitotic figures observed in histology. DNA replication was still highest in the nonparenchymal area of the zonal profile. In the parenchymal nuclear areas of the profile the peaks of labeling appeared in those areas of the gradient that would normally be expected to contain a high percentage of S-phase nuclei, despite the fact that labeling with [3H]thymidine had been carried out 24 hr previously and therefore all labeled nuclei would have been expected to have subsequently undergone mitosis. Nucleolar RNA synthesis in all classes of nuclei was little changed from the previous week, but the nonnucleolar RNA synthesis in all classes of nuclei had dramatically decreased (Chart 3).

Four Weeks of Toxic Feeding. The morphology of the liver showed the greatest change between the 3rd and 4th week of feeding the toxic diet. Rapid proliferation of both oval and parenchymal cell populations was evident from the frequency of mitotic figures. However, the parenchymal cell population was reduced in number and confined to a narrow band around the central vein. Periportal necrosis was extensive. Many of the remaining viable parenchymal cells contained large nuclei with multiple nucleoli and the nodules of proliferating parenchymal cells in the centrilobular zone had increased in size, several compressing the surrounding tissue. The nuclear distribution pattern observed on zonal centrifugation (Chart 1e) also showed a profound change. A greatly increased diploid peak was present, with the tetraploid peak reduced to a shoulder. The DNA content of nuclei present in the diploid area was unchanged, but nuclei present in the tetraploid area had an average DNA content similar to that of the diploid (Table 1). The tetraploid parenchymal nuclear peak always contained a number of diploid nuclei even in the controls (Chart 2a), and the loss of tetraploid parenchymal cells from this peak (Chart 1e) accounted for the increased proportion of diploid nuclei in this area of the zonal profile. The lowering of the average DNA content of the nuclei was due to this rather than to a change in the sedimentation characteristics of some of the diploid nuclei. In agreement with the proliferation of oval and parenchymal cells noted in the histology, incorporation of [3H]thymidine had increased 10-fold across the spectrum of nuclei separated in the zonal rotor with the highest spe-
cific activity again being present in the nonparenchymal area of the profile (Chart 1f). Concerning RNA synthesis (Chart 3), the principal feature was a recovery of the nonnucleolar activity, which had doubled from the level recorded the previous week. The nucleolar activity was either unchanged or perhaps even showed a slight increase in the level of inhibition.

**Five Weeks of Toxic Feeding.** The histology indicated that, although necrosis of some individual lesions was still occurring in the perportal area, the main feature was repair of the lesion. The parenchymal cells had increased in number and the oval cells were confined to a narrow band connecting the portal tracts. Mitotic figures and large nuclei with multiple nucleoli were a prominent feature of the parenchymal cells. The proliferating focal lesions were now of 2 types, one consisting of small cells with a uniform nuclear size and the other consisting of larger cells with vacuolated cytoplasm and bizarre nuclei. The zonal profile (Chart 1f) showed a partial restoration of the tetraploid nuclear peak compared with the situation after 4 weeks of feeding. The presence of an increased proportion of tetraploid nuclei in Peak B (Chart 1f) was indicated by the higher average DNA content of the nuclei present in this peak compared with the previous week (Table 1). DNA synthesis was still very much elevated throughout the zonal profile (Chart 1f). Nonnucleolar RNA synthesis (Chart 3) continued to increase in all classes of nuclei and no further inhibition of nucleolar RNA synthesis had occurred.

**Six Weeks of Toxic Feeding.** Histological observation (Fig. 3a) showed that the lesion had further regressed with restoration of the lobular architecture in most areas. The nodular lesions referred to above and increased proliferation of the parenchymal cells remained. The presence of abnormal, large nuclei was observed in the unfractionated nuclear suspension (Fig. 3b). The zonal profile (Chart 1g) showed, in accordance with the histology, that the tetraploid parenchymal peak was much developed over that seen at the 5-week stage. The average DNA content (Table 1) and the densitometric determinations (Chart 2c) confirmed the reemergence of a high proportion of nuclei in Peak B (Chart 1g) having a DNA content characteristic of tetraploid nuclei. DNA replication (Chart 1g) in agreement with the reemergence of the tetraploid population was elevated in this region of the zonal profile. Overall, however, the level of replication was reduced from that seen after 5 weeks of feeding, although it had not decreased to the level observed in the control animals (Chart 1a). Synthesis of both nonnucleolar and nucleolar RNA had increased considerably in all the nuclear subfractions and was approaching the level present in control nuclei (Chart 3).

The sensitivity of Mn²⁺-(NH₄)₂SO₄-primed RNA synthesis to α-amanitin in vitro throughout the feeding period (Table 2) confirmed that the strong inhibition of this activity that occurred after 3 to 4 weeks of feeding the toxic diet could be ascribed to inhibition of nucleoplasmic RNA synthesis. On the same basis, the Mg²⁺-primed activity could largely be equated to nucleolar RNA synthesis (13).

On another occasion a similar series of experiments was carried out using a different batch of adult male Fischer rats but the same aflatoxin-contaminated diet (MP meal). The zonal profiles observed during the 6-week feeding period are given in Chart 4. Histologically, and from the evidence of the zonal centrifugation profiles, the pattern of changes that occurred was similar to that seen in the experiment recorded in Figs. 1, 2, and 3 and Chart 1. There was again a progressive perportal necrosis, proliferation of oval biliary cells, development of large nuclei, and finally regression of the lesion and restoration of the lobular pattern. There were, however, certain differences between the 2 experiments that appear to be of some interest. In the 2nd experiment, the extent of the lesion was much more restricted, maximal parenchymal cell necrosis and oval cell proliferation occurred at 5 weeks rather than at 4 weeks as in the previous experiment, and only the immediate perportal area was affected. This is reflected in the less dramatic changes in the zonal profiles in Chart 4 compared with those seen in Chart 1. Another point of difference between

<table>
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<th>Assay system</th>
<th>α-Amanitin added (1 μg)</th>
<th>Fraction A</th>
<th>Fraction B</th>
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<tr>
<td>Controls</td>
<td>Mn²⁺</td>
<td>370</td>
<td>337</td>
</tr>
<tr>
<td></td>
<td>Mg²⁺</td>
<td>+</td>
<td>71 (81)²</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>129</td>
</tr>
<tr>
<td>3-wk toxic diet</td>
<td>Mn²⁺</td>
<td>75</td>
<td>71</td>
</tr>
<tr>
<td></td>
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<tr>
<td>6-wk toxic diet</td>
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<td>Mg²⁺</td>
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<td>26 (87)²</td>
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<tr>
<td></td>
<td></td>
<td>+</td>
<td>94</td>
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</table>

² Numbers in parentheses, percentage of inhibition.
the 2 experiments is in the number of large cells and nuclei seen. These were far more numerous in the 2nd experiment. This is borne out by the presence of a very prominent octaploid peak that developed during the later stages of the toxic feeding, as can be seen from the zonal profiles given in Chart 4.

We have observed, as a general rule, that when feeding aflatoxin-contaminated diet to Fischer rats, the less overtly toxic the effects on the liver, the more numerous are the larger cells and nuclei that develop subsequent to the injury.

DISCUSSION

The complex sequence of events recorded above occurred during feeding adult male Fischer rats with an aflatoxin-contaminated meal for 6 weeks, which results in a 100% incidence of hepatocellular carcinoma about 40 weeks later. Three weeks of feeding the toxic diet results in only a negligible incidence of hepatocarcinoma (W. H. Butler, personal communication). The histologically observable liver damage in these experiments that resulted from feeding the MP toxic meal was much more extensive than that previously reported to result from feeding Rossetti meal to Fischer rats (2), although the aflatoxin B1 content of the 2 diets was similar. Examination of the MP meal did not reveal the presence of aflatoxins other than aflatoxin B1, and related mycotoxins, e.g., Sterigmatocystin, were not detected in the meal. The reason for the difference in response by the animals to the 2 toxic meals is unknown, but seasonal variations in sensitivity of the Fischer rats to aflatoxin have been noted (G. E. Neal, unpublished results) and may have been involved in the differences noted in this work. Differences in the constituents of the MRC 41B could also possibly have been involved; the composition of the diet has been shown to have profound effect on the toxicity of aflatoxin B1 (9). The more extensive changes induced by the MP meal in the present study, however, have enabled the histological and biochemical disturbances to be more readily identified. It is intended to repeat this study using Rossetti meal, to see if a greater sensitivity of the animals at present being used could account for the more extensive response.

The overall pattern that emerged during the present study was that, during the 1st 3 weeks of feeding the toxic diet, acutely toxic phenomena were observed. Inhibition of DNA and RNA synthesis and the development of cell necrosis were prominent. Inhibition of DNA and RNA synthesis by aflatoxin B1 is well documented (3). On the evidence of the zonal centrifugation studies, these toxic effects seemed to affect predominantly those hepatocytes that contained tetraploid nuclei and eventually caused this population of hepatocytes largely to disappear. These hepatocytes are apparently predominantly situated in the perportal region of the lobule, and the accompanying histological observation was necrosis of the perportal region. The reason for these cells dying after 3 weeks of feeding the toxic diet is unknown, but their disappearance coincided not with an inhibition of DNA replication, which was evident from the commencement of feeding the toxic diet, nor with a sudden increase in the inhibition of nucleolar RNA synthesis, which was not greatly inhibited in any of the subpopulations of nuclei, but with the development of a large inhibition of the Mn2+-(NH4)2SO4-activated (nonnucleolar) RNA synthesis. The fact that it was the nonnucleolar RNA synthesis that was affected was supported by the data obtained using a-amanitin. This finding could indicate, among other possibilities, that a lack of synthesis of essential mRNA species was involved in the death and disappearance of the tetraploid parenchymal cell population.

The 2nd 3-week period of feeding the toxic diet was characterized by regeneration and reestablishment of a hepatocyte population, many of which were tetraploid or even higher ploidy in nature. As reported previously, however (11), the tetraploid population did not attain the proportion typical of an adult animal of this age. An extremely high level of DNA replication involving all classes of nuclei accompanied the regeneration process. This proliferation took place in the presence of a continuing supply of the toxin, which, in the 1st 3 weeks had induced widespread hepatotoxic effects. It is possible that this sequence of events may be a fundamental feature of hepatocarcinogenesis and not one that is applicable solely to the action of aflatoxin. Hughes (6) has reported that, when the hepatocarcinogen 4-methylaminoazobenzene is fed to rats at the 0.06% level, 3 weeks of feeding results in a 0% incidence of hepatocarcinoma, whereas 6 weeks of feeding results in a 100% incidence. Again, as in the present study, proliferation occurred between 3 and 5 weeks, with a peak after 4 weeks of feeding. This indicates that, using these 2 dissimilar carcinogens, proliferation in the presence of the agent is necessary to provoke the carcinogenic response. Using these 2 dissimilar carcinogens, 4-methylaminoazobenzene and aflatoxin, a 3-week feeding period is required to induce cellular proliferation.

The reason for the subsequent immunity of liver cells to inhibition by aflatoxin after 3 weeks of feeding is of considerable interest, particularly as it is at this stage that the carcinogenic response is evoked. Two explanations for the emergence of a resistant population of cells would appear to be possible: the toxin could transform certain of the parenchymal cells into a resistant strain or a preexisting resistant population of cells could proliferate to take the place of the susceptible population as it necroses. In connection with the latter possibility, the nonparenchymal nuclei exhibited a considerable degree of resistance to the toxic activity throughout the whole experimental period and proliferation occurred in this nuclear subpopulation considerably in advance of that seen in the parenchymal population. It is not now known whether or not there is any connection between this early proliferation of partially resistant nonparenchymal cells and the later emergence of a resistant parenchymal population. However, Ogawa et al. (12), as a result of their experiments in feeding rats dimethylaminoazobenzene, have suggested that bile duct-like cells, which proliferate in response to the death of hepatocytes caused by the toxin, themselves later mature into functional hepatocytes.

The particular sensitivity of the tetraploid nuclei to the
toxic effect of feeding the MP meal, seen in the present study, is of interest in view of the finding that aflatoxin needs to be metabolized to an active form in order to inhibit RNA synthesis in rat liver (10). It could be that this metabolic activation takes place predominantly in hepatocytes containing tetraploid nuclei and that, in the resistant hepatocyte population, subsequently present, this metabolic activation is less or lacking. The mechanism of the resistance of these cells to the toxic action is at present being investigated.

ACKNOWLEDGMENTS

We wish to acknowledge the assistance of S. Preston with the photography and microdensitometry.

REFERENCES


Fig. 1. Histological section and nuclear suspension prior to zonal separation prepared from the livers of control rats. a, liver lobule illustrating the uniform size of the parenchymal cells and nuclei. H & E, × 160. b, nuclear suspension containing nuclei of uniform morphology. Phase contrast, × 370.
Fig. 2. Histological section and nuclear suspension prior to zonal separation prepared from the livers of rats fed the toxic diet for 3 weeks. a, necrosis of the parenchymal cells in the periportal zone and replacement by proliferating oval cells can be seen. Viable parenchymal cells, some with large nuclei, remain in the centrilobular area. H & E, × 160. b, nuclear suspension containing nuclei of varying morphology reflecting the lesion observed in the histological section. Phase contrast, × 370.
Fig. 3. Histological section and nuclear suspension prior to zonal separation prepared from the livers of rats fed the toxic diet for 6 weeks. a, bizarre, large nuclei in the parenchymal cells are a prominent feature of the repairing lesion. H & E, × 160. b, large nuclei are readily identified in the nuclear suspension. Phase contrast, × 307.
The Effects of Prolonged Feeding with Aflatoxin B₁ on Adult Rat Liver

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