Aspects of Experimental Hepatocarcinogenesis

PART IV. CHANGES IN THE LIVER FOLLOWING CESSATION OF CARCINOGEN ADMINISTRATION

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SUMMARY

This article describes the changes in the liver which followed withdrawal of the carcinogen *p*-dimethylaminoazobenzene (*p*-DAB) after it had been fed to rats for 20 weeks. The cirrhotic changes regressed virtually completely, but glycogen storage was greatly reduced and small hyperplastic areas persisted. Several carcinomas developed in these livers as long as 5 months after carcinogen feeding was discontinued. Ultrastructural changes observed in the non-neoplastic liver included nucleolar hypertrophy, an increase in the smooth endoplasmic reticulum and abnormalities of mitochondria. The possibility that these findings may have a bearing on the occurrence of primary liver cancer in non-cirrhotic patients in high-incidence areas is considered.

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A difficult problem in evaluating the biochemical and ultrastructural effects of a carcinogen is to know whether the changes observed are essential to the process of tumour formation or whether they are simply non-specific manifestations of drug toxicity.[1] One approach to this problem is to feed the carcinogen for a given period and then to withdraw it. It may then be possible to observe that certain abnormalities persist for a long time, even with the animal on a normal diet. Such 'irreversible' changes may then perhaps be more confidently related to the mechanism of tumour formation. This article describes both light and electron microscopic changes which have been observed in the livers of rats following withdrawal of the carcinogen.

EXPERIMENTAL METHOD

Thirty male rats, weighing 100 g at the onset of the experipent, were used. *p*-Dimethylaminoazobenzene (*p*-DAB), 0,05% by weight in maize, was fed to the animals for 20 weeks and then discontinued. Animals were then placed on a normal laboratory diet. Five animals were biopsied at this stage. The rats were then observed until tumours became obvious or until they were killed at the end of the experiment. They were placed into groups (A - D) according to the duration of the recovery period. When the rats died or were killed, a full postmortem examination was done, tissue was removed from all the lobes not involved by tumour and the samples were fixed in formalin for histological study. Tissue for ultrastructural study was obtained only from the liver of animals in group D. Blocks were fixed in 2% OsO_4 in 0,1M phosphate buffer, sometimes with prior fixation in 5% glutaraldehyde in 0,1M phosphate. Tissue was embedded in Araldite and sections were stained with uranium and lead salts.

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RESULTS

The results are set out in Table I. Within 2 months of cessation of carcinogen administration, 15 animals had developed hepatic tumours (group A). The majority of these included relatively well-differentiated trabecular carcinomas but adenocarcinomas and mixed tumours were also present. All the livers showed the expected severe cirrhotic changes. The surviving 15 animals began to put on weight and their general condition improved greatly. Two animals died at 3 months (group B). Both animals had large liver tumours but the livers were now only slightly granular externally. At about 5 months, a further 6 animals had developed large, rapidly growing liver tumours (group C). In each case the tumour-bearing liver was mostly smooth, with minimal granularity. Occasional small cysts containing clear fluid were noted. The remaining 7 animals were killed after 6 months (group D). No tumours were evident and the livers were similar to those in group C.

TABLE I. RESULTS

Group	Recovery period (mo.)	Number of animals	Tumours	Livers
A	2	15	15	Cirrhotic
B	3	2	2	Moderately granular
C	5	6	6	Minimally granular
D	6	7	0	Minimally granular

LIGHT MICROSCOPIC EXAMINATION

Liver Biopsies

In each case these confirmed the existence of a cirrhosis and portion of atypical hepatocellular nodules were included in specimens.

Group A: The livers showed the complex range of changes usually associated with prolonged administration of the azo dyes.[1] All the livers were cirrhotic and the liver cells exhibited varying grades of basophilia and nuclear enlargement. Many cirrhotic nodules could be considered hyperplastic since they were composed of basophilic cells with heightened mitotic activity. Areas of cholangiofibrosis and cyst formation were common.

Group B: The livers could no longer be termed cirrhotic, though occasional irregularly shaped scars were still quite numerous. Areas of cholangiofibrosis and occasional cysts persisted. The liver cells showed far less evidence of degenerative changes, e.g. vacuolation and loss of basophilia. Cells with enlarged nuclei and basophilic cytoplasm were quite common. Glycogen was present in only about half the liver cells.

Group C: No cirrhosis was present. Occasional small scars remained in the portal or centrilobular areas (Fig. 1), and small foci of large atypical basophilic cells were suggestive of hyperplasia. Little glycogen was present in the majority of liver cells.

Group D: No tumours were found in this group but the livers were virtually identical to those in group C. Some glycogen was present in the majority of liver cells, though quantitatively it was far below that found in a normal rat liver.

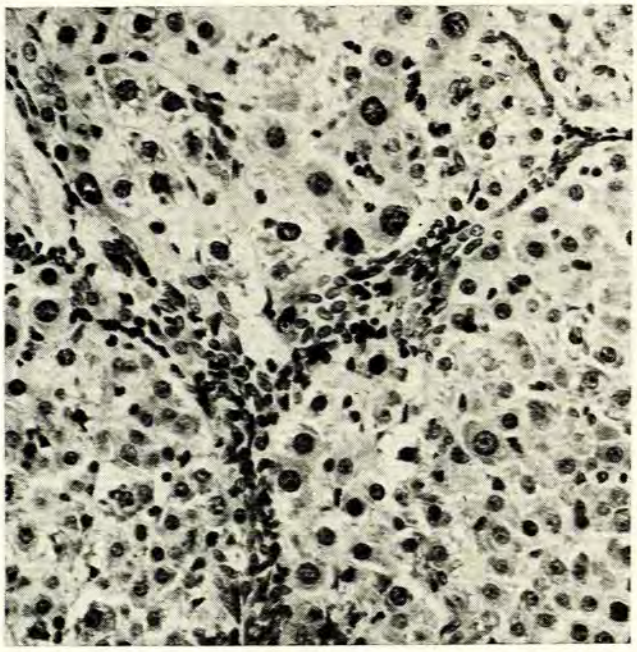

Fig. 1. Liver from animal in group C showing slight residual scarring and cellular atypicality (H. and E. × 1 000).

ELECTRON MICROSCOPY (GROUP D ONLY)

The nuclei were mostly of normal size but occasionally they were hypertrophied with enlargement of the nucleoli (Fig. 2). The latter showed a normal threadlike pattern of interlacing granular and fibrillar elements. No examples of nucleoli showing microsegregation were seen, despite a very careful search.

Fig. 2. Hypertrophied nucleolus (× 16 000).

The cytoplasm of the majority of cells showed only minor deviations from that seen in normal liver cells. However, glycogen deposits were never as prominent as those seen in the normal hepatocyte, and at most consisted of scattered glycogen rosettes intermingled with vesicular elements of the smooth endoplasmic reticulum (SER), which still appeared to permeate large areas of the cytoplasm (Fig. 3). The rough endoplasmic reticulum (RER) frequently displayed a normal stacked pattern but in some cells it was composed of numerous short, slightly dilated cisternae scattered throughout the cell. Mitochondria, though normal in perhaps the majority of cells, also showed an unusually obtrusive abnormality in the arrangement of the cristae (Figs 4 and 5). These were greatly lengthened and arranged in a parallel fashion along the outer membranes of the mitochondria. Microbodies were prominent in many cells. Autophagic vacuoles and lysosomes were rarely increased in number. Saccules of the Golgi apparatus were commonly dilated (Fig. 3).

DISCUSSION

On the one hand, it has been shown by Dunn[2] that after feeding the carcinogen ethionine to rats for periods

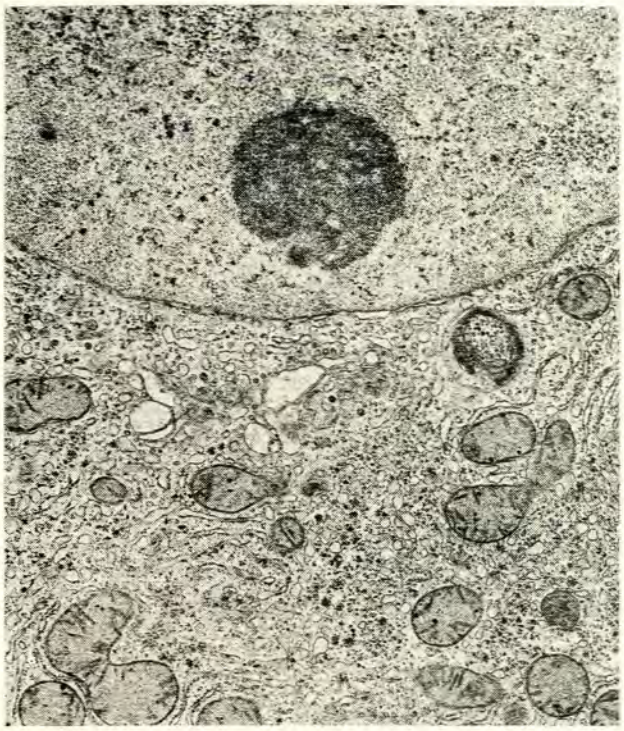

Fig. 3. Glycogen stores in this cell are below normal. Golgi vesicles are dilated and the SER is increased (× 10 000).

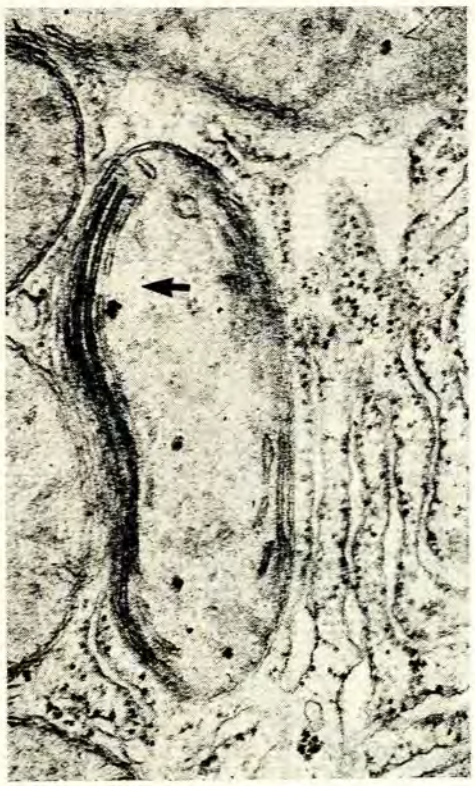

Fig. 4. Abnormal mitochondria with flattened cristae (arrow) **(× 30 000).**

Fig. 5. Abnormal mitochondria with flattened cristae (arrows) **(× 30 000).**

of 5 months and then placing them on a normal stock diet for a similar period, some cytological changes persisted in the liver cells. Large atypical cells with polyploid nuclei were found, though less commonly than while the ethionine was being given. Histochemical activity of certain enzymes, e.g. succinic dehydrogenase, ATPase and glucose-6-phosphatase also remained at subnormal levels for periods of up to 7 months. No electron microscopic studies were performed in this investigation. On the other hand, it has been pointed out by Svoboda and Higginson[1] that almost complete recovery may take place 4 - 8 weeks after stopping carcinogen feeding (the more potent carcinogen 3-methyl-dimethylaminoazobenzene was used) after 14 weeks' administration. To what extent these divergent findings may be explained on the basis of differences in animal strain, diet, carcinogenic potency or duration of feeding is not known.

The present investigation demonstrates that while many of the most severe changes, including the cirrhosis with hyperplastic nodules, regress completely with time, some light and electron microscopic changes may persist in the liver as long as 6 months after stopping carcinogen feeding; furthermore, tumours can develop in the non-cirrhotic livers. With regard to the first point, the findings suggest that some hyperplastic nodules, even those composed of atypical basophilic cells, are not necessarily irreversible and only a small percentage acquire autonomous growth characteristics and give rise to tumours. In contrast to this, Reuber[9] noted that after discontinuation of *N*-2-fluorenyl-

diacetamide feeding, the cirrhosis regressed but the hyperplastic nodules continued to grow. Different carcinogens may therefore behave differently in this respect.

The presence of large nuclei and hypertrophied nucleoli seen with the light microscope was confirmed on electron microscopy. The carcinogenic azo dyes are able to produce nucleolar abnormalities, e.g. microsegregation,[1] but it seems that these can disappear after a sufficient lapse of time. The persistence of the mitochondrial abnormalities has not previously been documented. Mitochondria of the type shown have not been seen in normal rats of the same strain and age as those used in the present series of experiments, nor have they been seen particularly frequently while the carcinogen is being administered. Whether or not such changes are accompanied by significant functional disturbances is not known. However, Svoboda and Higginson[1] have concluded that mitochondrial changes probably do not play an essential role in carcinogenesis.

A further interesting ultrastructural abnormality has been the considerable delay which has characterised the resumption of significant glycogen storage in the liver; even at the end of this experiment, this had not fully recovered. Once again the significance, if any, of this change is uncertain, since abnormalities of glycogen storage are commonly found with non-carcinogenic hepatotoxins, and such changes are consequently not easily directly related to carcinogenesis.[1] Nevertheless, it may be pointed out that glycogen storage is apparently defective in early[4] and late hyperplastic lesions[5,6] and in the majority of azo dye-induced liver cell carcinomas,[7,8] so that the full implication of defective glycogen synthesis or storage may yet have to be worked out. The SER was still undoubtedly increased in a high percentage of cells.

It is therefore clear from this and earlier work that although almost complete regression of certain gross changes does occur, a variety of cytological changes may persist for long periods after carcinogen withdrawal, but it still remains to be established whether they reflect any significant biochemical abnormality associated with tumour formation.

It may be wondered if these results have any bearing on the disease in the indigenous Black races. It is well recognised that primary cancer of the liver is usually associated with cirrhosis. However, it has also been argued that in those population groups in which there is a high incidence of the disease, a not inconsiderable percentage of cases of primary carcinoma occurs in the absence of cirrhotic changes.[9] One explanation for this may well be that such non-cirrhotic livers have at an earlier stage sustained a significant degree of hepatocellular injury, but that with time these changes largely regressed. Any lesions which persist may be mild and not easily related to the subsequent development of a carcinoma.

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