DNA Synthesis and Neoplastic Transformation in Rat Liver Parenchyma

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Summary

The incorporation of tritiated thymidine into the DNA of preneoplastic liver parenchyma (hypobasophilic, basophilic, and hyperbasophilic populations), as well as in normal liver and hepatomas, was investigated by radioautographic methods in rats fed the azo dye 4-dimethylaminoazobenzene (DAB).

Quantitative determinations of the incidence of radioactive nuclei revealed that the number of radioactive nuclei in the basophilic population was higher than in normal liver. The formation of hyperbasophilic regions was accompanied by a significant increase in the number of labeled nuclei and a still higher value was obtained for the incidence of radioactive nuclei in hepatomas. The increase in DNA synthesis occurring in preneoplastic liver was found to follow closely the phenomenon in hepatomas. The increase in DNA synthesis occurring in preneoplastic liver was found to follow closely the phenomenon in hepatomas. The chromosome fragments and pyknotic granules resulting from abnormal mitoses and nuclear degeneration were found in the different populations of preneoplastic liver cells and hepatomas. The chromosome fragments and pyknotic granules present in basophilic cells were not radioactive, while those found in hyperbasophilic regions and hepatomas gave positive reactions indicating that such particles can be the site of DNA synthesis. It appears from the present study that the neoplastic transformation is associated with an increase in the number of radioactive nuclei in the basophilic population higher than in normal liver. The incorporation of tritiated thymidine into the extranuclear Feulgen-positive material can be the site of DNA formation in preneoplastic and neoplastic liver parenchyma and also with an uptake of tritiated thymidine in extranuclear chromosome fragments and pyknotic granules.

Introduction

Rats fed a diet containing the azo dye 4-dimethylaminoazobenzene (DAB) show a loss of basophilia and a degeneration of cells in centrolobular regions of the liver. These changes are followed by regeneration of the basophilic cells in perportal areas and formation of parenchymal nodules surrounded by trabeculae of bile ducts and connective tissue. As the regenerative process goes on, groups of parenchymal cells become hyperbasophilic due to a greater affinity of their cytoplasmic RNA for basic dyes. Such change is rapidly followed by an increase in mitotic activity and other cytologic changes leading to the development of hepatomas (6, 7, 22, 24, 25). The hyperbasophilic regions apparently represent the sites of tumor formation in preneoplastic liver and identification of these sites offers interesting possibilities for studying cytochemical alterations associated with the neoplastic transformation (7).

In the present work, synthesis of DNA in preneoplastic and neoplastic livers was investigated by radioautographic methods. The incorporation of tritiated thymidine into nucleic DNA was estimated in hypobasophilic, basophilic, and hyperbasophilic parenchyma of preneoplastic livers, and these results were compared with those obtained on normal and neoplastic tissues. These investigations were carried out to correlate the changes in DNA synthesis with the variations in mitotic activity observed in the different cellular populations (7), and to determine whether alterations in DNA synthesis preceede or follow the changes in cytoplasmic RNA associated with the neoplastic transformation.

Another aspect of this work was to investigate the incorporation of tritiated thymidine into the extranuclear Feulgen-positive fragments resulting from abnormal mitoses. Such fragments derive from the rupture of chromosomes bridges or from lagging chromosomes and are left in the cytoplasm when the nuclear membranes form at telophase (6, 18). They are commonly observed in the liver of animals fed carcinogens and other hepatotoxic agents (14, 15, 18, 33). The fate of such fragments has been little investigated and it was thought desirable to determine whether they can be the site of DNA synthesis in preneoplastic liver parenchyma and liver tumors. Larger pyknotic granules resulting from the degeneration of interphase nuclei or blocked metaphases are also found in the same tissues and the incorporation of tritiated thymidine in these granules has been also examined.

Thus the aim of the present study is 2-fold: (a) to obtain quantitative data on the synthesis of DNA in interphase nuclei during hepatocarcinogenesis and to determine whether alterations in DNA synthesis preceede or follow the formation of hyperbasophilic regions; and (b) to examine whether extranuclear Feulgen-positive material can be the site of DNA formation in preneoplastic and neoplastic liver parenchyma.

Materials and Methods

Adult, male, albino rats (Wistar strain) were fed a basal, low protein diet [diet 3 of Miller et al. (21)] containing the azo dye DAB at a concentration of 0.06%. The rats were sacrificed in groups of 4 at 30-day intervals during a 180-day period of feeding. The animals were inoculated s.c. with tritiated thymidine (1...
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\( \mu g/\text{gm of body weight} \) at 4:00 A.M., i.e., at time of maximum DNA synthesis (20), and sacrificed 1 hr later. Animals were exsanguinated from the abdominal aorta under ether anesthesia. The livers were excised with scissors and fixed in Carnoy's fluid for 24 hr. The fixed livers were dehydrated, cleared, and embedded in paraffin. Histologic sections were cut at 5 \( \mu \) and stained with toluidine blue (7) or by the Feulgen reaction (34). Feulgen-stained sections were covered with a photographic emulsion using the dipping technic (16, 19). After 1-, 2-, and 4-week exposures, the preparations were developed and mounted in balsam under a cover slip.

The different cellular populations in preneoplastic livers were identified in sections stained with toluidine blue, and differential counts of labeled and unlabeled nuclei were made in corresponding areas of the adjacent sections stained by the Feulgen method and covered with a photographic emulsion. A reticle delimiting a square field was placed in 1 ocul of a binocular microscope. The objective was placed at random on either hypobasophilic, basophilic, or hyperbasophilic parenchyma, and differential counts were done until 4000 nuclei of each population had been examined per animal. The results were expressed as numbers of radioactive nuclei/100 nuclei.

The same material was used to study the incorporation of tritiated thymidine in extranuclear Feulgen-positive fragments. The whole sections were examined under oil immersion for the presence of chromosome fragments and pyknotic granules resulting from abnormal mitoses and degenerating nuclei. In addition, sections from animals fed DAB for 150 days and sacrificed 10 and 20 min after i.v. injection of tritiated thymidine were used in this study. The occurrence of labeled and unlabeled fragments was investigated in the different populations of parenchymal cells, and quantitative data were obtained from 5 different hepatomas.

Results and Discussion

DETERMINATION OF PERCENTAGES OF RADIOACTIVE NUCLEI.

The number of counts necessary to constitute an adequate sample was determined from cumulative curves of percentages of labeled nuclei as function of the number of nuclei examined. Percentages of radioactive parenchymal nuclei were calculated in subsets of 200 for a total of 5200 nuclei. The cumulative curves obtained from 3 animals on DAB diet (Chart 1) show important variations in percentages for counts up to 2000 nuclei and these curves become fairly stable afterward. The range of variations after 2000 counts is of the order of 10\% and decreases to less than 5\% after 4000 observations. The latter number was adopted for estimating the percentages of radioactive nuclei in different populations of liver parenchymal cells during carcinogenesis.

PERCENTAGES OF RADIOACTIVE NUCLEI IN NORMAL LIVER PRENEOPLASTIC CELLULAR POPULATIONS AND HEPATOMAS. The incorporation of tritiated thymidine in the normal, preneoplastic and neoplastic cellular populations is shown in Figs. 1-8. In normal rat liver (Fig. 1), nuclei incorporating tritiated thymidine (Fig. 2) are rare. In animals fed the carcinogenic diet, the basophilic parenchyma (Fig. 3) shows an appreciable number of labeled nuclei (Fig. 4), and the formation of hyperbasophilic

![Chart 1. Variations in percentages of radioactive nuclei with number of counted nuclei in basophilic liver parenchyma of 3 rats fed DAB for 60 days.](image-url)
regions (Fig. 5) is accompanied by an increase in tritiated thymidine uptake (Fig. 6). Hepatomas (Fig. 7), which develop from hyperbasophilic regions, are the site of active incorporation of tritiated thymidine into nuclear DNA (Fig. 8).

Quantitative results on the same cellular populations, 1 hr after the injection of tritiated thymidine, are presented in Table 1. A low percentage of radioactive nuclei is observed in normal liver parenchyma, namely 0.63. Hypobasophilic cells, found in centrolobular regions of the liver at early stages of DAB feeding, showed no incorporation of tritiated thymidine, indicating that DNA synthesis does not occur in that cellular population. The number of labeled nuclei in basophilic parenchyma is higher than in normal tissue, i.e., 1.68%, and is relatively constant throughout the whole 180-day period of feeding. A mean value of 3.96% is obtained for hyperbasophilic regions found at later stages of DAB feeding (90–180 days). The hepatomas show a higher rate of incorporation, 8.63% of the nuclei being labeled in the neoplastic tissue. Statistical analyses indicate significant differences (P < 0.01) between the different cellular populations.

The present data on the percentages of labeled nuclei in preneoplastic liver parenchyma and liver tumors are in good agreement with previous results on the mitotic activity of these tissues (6, 7). An increase in the rate of DNA synthesis, as well as in the rate of mitoses, was noted in the hyperbasophilic regions and such results provide additional evidence supporting the view that hyperbasophilia represents a transition step in the sequence of cytologic events leading to the development of hepatomas (7, 24, 25). Increased uptake of tritiated thymidine in regions similar to hyperbasophilic regions was also observed by MacDonald (17) in animals fed 3'-methyl-4-dimethylaminoazobenzene, and by Côté et al. (5) in rats given diethylnitrosamine. It thus appears that similar alterations in nucleic acid metabolism occur in animals fed various hepatocarcinogens.

One of the objectives of the present study was to determine whether the increase in DNA synthesis precedes or follows the formation of hyperbasophilic regions. Even if a higher rate of DNA formation was measured in hyperbasophilic regions, this fact did not exclude the possibility that areas of high activity could exist in surrounding basophilic parenchyma and that increased DNA synthesis would actually precede the alterations in the basophilic properties of these sites. Systematic examinations of all sections have been made independently by the 2 authors for the presence of such areas of high DNA synthesis in basophilic parenchyma. Sites showing tritiated thymidine incorporation comparable with the one observed in hyperbasophilic and neoplastic cells could not be detected in basophilic parenchyma by either investigator. It thus seems that the increased incorporation observed in hyperbasophilic regions does not precede but accompanies or follows the alteration in basophilic properties. Further information in this regard may be obtained from examination of the rates of DNA synthesis in individual regions. About one-fourth of the hyperbasophilic regions showed values comparable with those obtained for basophilic parenchyma, and three-fourths showed higher values, while all tumors showed relatively high rates of incorporation. These results support the view that the increase in DNA synthesis actually occurs in hyperbasophilic regions and follows the alteration in basophilia.

The phenomenon of hyperbasophilia might represent a turning point in the process of carcinogenesis, such change being rapidly followed by an increase in DNA duplication and other cytologic changes leading to tumor formation. Increased DNA synthesis, on the other hand, might be the key mechanism in the transformation of hyperbasophilic regions into hepatomas.

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<th>DNA Synthesis and Neoplastic Transformation</th>
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### TABLE 1

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<th>Percentages of Radioactive Nuclei in Normal, Preneoplastic, and Neoplastic Liver Parenchyma, 1 hr after Injection of Tritiated Thymidine</th>
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<tr>
<td>Cellular population</td>
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<td>---------------------</td>
</tr>
<tr>
<td>Normal</td>
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<tr>
<td>Preneoplastic</td>
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<td>Hyperbasophilic</td>
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<td>Hepatoma</td>
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**Occurrence of extranuclear Feulgen-positive fragments in preneoplastic and neoplastic liver.** Abnormal mitoses are commonly observed in the livers of rats fed DAB and other hepatotoxic agents (14, 15, 18, 33) and, in the present work, such abnormalities were found at all stages of the carcinogenesis process in the basophilic, hyperbasophilic, and neoplastic parenchymal tissues. Figs. 9 and 10 illustrate a chromosome bridge and a lagging chromosome found in hepatomas. The abnormal mitoses can produce chromosome fragments which are left outside of the nuclei when the nuclear membranes form at telophase (Fig. 11). Such chromosome fragments in the cytoplasm of basophilic cells are shown in Figs. 12 and 13, while similar fragments in hyperbasophilic parenchyma are illustrated in Fig. 14.

**Pyknotic fragments resulting from the degeneration of interphase nuclei** and whole mitotic figures are also found in the same populations. Hyperchromatic masses in interphase nuclei and dividing cell are shown in Figs. 15 and 16, respectively. The degenerating nuclei usually break up into a few pyknotic masses (Fig. 17), while arrested mitotic figures may give rise to either a few (Fig. 18) or a large number of pyknotic granules (Fig. 19).

**Incorporation of tritiated thymidine into extranuclear Feulgen-positive fragments.** In animals sacrificed 1 hr after the injection of tritiated thymidine, the chromosome fragments and pyknotic granules present in basophilic parenchyma showed no incorporation of the radioactive precursor. Those present in hyperbasophilic regions and liver tumors, however, gave positive radioautographic reactions. An extranuclear chromosome fragment showing tritiated thymidine uptake is presented in Figs. 20 and 21. Figs. 22 and 23 illustrate a positive reaction given by pyknotic masses in a degenerating nucleus. Pyknotic granules which have incorporated the radioactive precursor are shown in Figs. 24 and 25.

**Positive reactions given by chromosomes fragments and granules in animals sacrificed 1 hr after injection of tritiated thymidine might be interpreted as indicating a de novo synthesis of DNA in these nuclear fragments or simply an incorporation of radioactive precursor occurring some time before the formation of extranuclear chromosome fragments or pyknotic granules actually takes place.** As far as chromosome fragments are concerned, at least, the latter explanation appears improbable for
the following reasons. First, both the telophase or posttelophase nuclei and the satellite fragments should be labeled if the region by fragments would result from previous incorporation into the nuclear DNA. Such pairing of reactions was not observed in our material. Second, the duration of the postsynthetic phase $G_2$ is relatively constant in mammalian cells, ranging from 0.5 to 2 hr (2, 27-29) and the length of the mitotic division extends from 0.5 to 2.5 hr (2, 27-29). An interval of at least 1 hr must therefore elapse between the end of the synthetic phase $S$ in interphase nuclei and the formation of extranuclear chromosome fragments at telophase. That this is actually the case with the present material is supported by the fact that no labeled mitotic figures were found in either population of parenchymal cells in the animals sacrificed 1 hr after injection of tritiated thymidine. Thus, an uptake of radioactive precursor during the duplication stage of the cycle could not explain the positive reactions given by chromosome fragments. The uptake of tritiated thymidine by extranuclear chromosome fragments apparently represents a de novo synthesis of DNA in these sites.

The same arguments could be used concerning the pyknotic granules resulting from the degeneration of arrested mitotic figures. As no labeled mitotic figure was found in animals sacrificed 1 hr after injection of tritiated thymidine. Thus, an uptake of radioactive precursor during the duplication stage of the cycle could not explain the positive reactions given by chromosome fragments. The uptake of tritiated thymidine by extranuclear chromosome fragments apparently represents a de novo synthesis of DNA in these sites.

Quantitative estimations carried out on 5 different hepatomas revealed that the incidence of fragments and granules was 2.1/100 nuclei, of which 3.37% were found to be labeled.

The extranuclear chromosome fragments investigated in the present work apparently correspond to similar fragments described by several authors (1, 9, 10, 14, 15, 18, 31, 33). They do differ however from the relatively large nonpyknotic micronuclei which have been the object of radioautographic studies by Banerjee (1), Das (9), and Scott and Evans (31). Such morphologically healthy nuclear fragments were commonly observed in neoplastic cells and many were found to incorporate tritiated thymidine as reported by these authors. The present study suggests that, in addition to these micronuclei, the pyknotic chromosome and nuclear fragments can incorporate tritiated thymidine.

Quantitative estimations carried out on 5 different hepatomas revealed that the incidence of fragments and granules was 2.1/100 nuclei, of which 3.37% were found to be labeled.

Thus the present results indicate that extranuclear Feulgen-positive material can be the site of DNA synthesis in hyperbasophilic regions and liver tumors. Presumably, the chromosome fragments and pyknotic granules in basophilic parenchyma are digested by DNases present in this cellular population. They would be retained, on the other hand, in hyperbasophilic regions and tumors devoided of DNase activity (6) and could be the site of de novo synthesis of DNA in these cellular populations. At any rate, it appears from the present study that the neoplastic transformation is associated with an increased uptake of tritiated thymidine in interphase nuclei and with an incorporation of the radioactive precursor in chromosome fragments and pyknotic granules.

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References


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