Hyperbasophilic Foci as Sites of Neoplastic Transformation in Hepatic Parenchyma

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SUMMARY

The occurrence of hyperbasophilic foci and tumors has been investigated in livers of rats fed (a) hepatocarcinogens, (b) hepatotoxic but noncarcinogenic agents, and (c) basal control diets. Hyperbasophilic foci were found to develop exclusively in livers of animals fed hepatocarcinogens, thus suggesting a close correlation between the occurrence of such areas and tumor formation.

The properties of the hyperbasophilic foci have also been examined in sections submitted to mild RNase treatment, which selectively extracts the RNA responsible for hyperbasophilia. Some foci differed from the surrounding tissue only by their increased basophilia and showed no other apparent change. Most foci showed one or several additional changes but could still be distinguished from the tumors which, in addition, showed a displacement of the surrounding parenchyma. The hyperbasophilic foci thus differ from both the regenerating parenchyma from which they arise and from the tumors. They appear to be a transitional tissue and probably represent the sites of neoplastic transformation.

INTRODUCTION

The feeding of 4-dimethylaminoazobenzene to rats produces extensive destruction in centrolobular regions of the liver, a process which is followed by regeneration in periportal areas and the formation of hyperplastic nodules. After prolonged 4-dimethylaminoazobenzene feeding, some areas of the nodular parenchyma acquire hyperbasophilic properties due to some alteration in cytoplasmic RNA, and such foci apparently develop into hepatomas (6). The hyperbasophilic foci show changes in the cell cycle (24, 25), an increase in mitotic activity (6), extensive dedifferentiation (12), and other features typical of cancer cells (5, 12, 18, 19).

The occurrence of hyperbasophilic foci is not specific to 4-dimethylaminoazobenzene carcinogenesis. Similar areas characterized by intense RNA staining have been observed in livers of animals fed various hepatocarcinogens (Table 1). Their occurrence thus appears to be a general feature, and possibly an essential step, of liver carcinogenesis. To our knowledge, such foci have never been observed in livers of animals fed basal control diets or in animals fed hepatotoxic but noncarcinogenic agents. It must be stressed, however, that a systematic search for hyperbasophilic foci in the latter conditions has not been made. To ascertain whether hyperbasophilic areas develop exclusively in livers of animals fed carcinogenic agents, it seemed desirable to carry on such a systematic investigation. The occurrence of hyperbasophilic foci and tumors was therefore examined in livers from rats fed (a) hepatocarcinogens, (b) hepatotoxic but noncarcinogenic agents, and (c) basal control diets.

The problem of whether the hyperbasophilic foci represent a transitional tissue or bona fide neoplastic tissue has also been considered. Several authors have reported that progressive changes towards cancer can be observed in such areas, but comparison of the transformed areas with early cancers has received little attention. In this work, attempts have been made to distinguish between foci at various stages of transformation. For this purpose, studies were made on sections submitted to mild RNase treatment which extracts selectively the RNA responsible for hyperbasophilia (3).

MATERIALS AND METHODS

Material. Tissue blocks were prepared during the course of various studies by investigators from this and other laboratories on rats fed carcinogenic and noncarcinogenic diets.

Animals. The animals were male rats of the Wistar and Sprague-Dawley strains weighing 150 to 250 g. The carcinogenic agents 3'-methyl-4-dimethylaminoazobenzene and 4-dimethylaminoazobenzene were given at a concentration of 0.06% in a basal, 12% protein diet (Ref. 17, Diet 3). Diethylaminoethylsulfonimine was given at a concentration of 0.01% in drinking water to animals fed laboratory chow. The noncarcinogenic azo dyes 2-methyl-4-dimethylaminoazobenzene, 4-aminoazobenzene, and azobenzene were included in the basal, low-protein diet at a concentration of 0.06%. Animals maintained on control 6, 12, and 25% casein diets were also used.

The rats were fed these diets ad libitum and groups of animals were sacrificed at semimonthly or monthly intervals. The experiments with carcinogenic agents and noncarcinogenic

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Hyperbasophilic Agents

Foci Animals References

Table 1: Occurrence of hyperbasophilic foci in liver parenchyma of animals fed various carcinogenic and noncarcinogenic diets

<table>
<thead>
<tr>
<th>Agents</th>
<th>Hyperbasophilic foci</th>
<th>Animals</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td>Carcinogens</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Nitrosomorpholine</td>
<td>+ Rats, mice</td>
<td>2</td>
<td></td>
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<tr>
<td>Dimethylnitrosamine</td>
<td>+ Trout</td>
<td>1</td>
<td></td>
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<tr>
<td>Diethylnitrosamine</td>
<td>+ Rats</td>
<td>4, 9, 10, 23b</td>
<td></td>
</tr>
<tr>
<td>Methylallylnitrosamine</td>
<td>+ Rats</td>
<td>14</td>
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<tr>
<td>2-Acetylaminofluorenone</td>
<td>+ Rats</td>
<td>20</td>
<td></td>
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<tr>
<td>3'-Methyl-4-dimethylaminoazobenzene</td>
<td>+ Rats</td>
<td>15, b</td>
<td></td>
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<tr>
<td>4-Dimethylaminoazobenzene</td>
<td>+ Rats</td>
<td>6, 19, b</td>
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<tr>
<td>Aflatoxins</td>
<td>+ Trout</td>
<td>11, 22, 26, 27</td>
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<tr>
<td>2-Methyl-4-dimethylaminoazobenzene</td>
<td>+ Rats</td>
<td>7, 21</td>
<td></td>
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<tr>
<td>Ethionine</td>
<td>+ Rats</td>
<td>7, 8</td>
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<tr>
<td>Noncarcinogens</td>
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<tr>
<td>2-Methyl-4-dimethylaminoazobenzene</td>
<td>- Rats</td>
<td>b</td>
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<td>4-Aminoazobenzene</td>
<td>- Rats</td>
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<tr>
<td>Azobenzene</td>
<td>- Rats</td>
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<td>Basal control diets</td>
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<tr>
<td>6% casein diet</td>
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<tr>
<td>12% casein diet</td>
<td>- Rats</td>
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<tr>
<td>25% casein diet</td>
<td>- Rats</td>
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a + present; -, absent.
b Present study.

Azo dyes were pursued for periods of 3 to 4 months, i.e., until tumors developed in the former groups. Animals fed the basal control diets were sacrificed at similar time intervals, but observations were also made on rats maintained on these diets for periods up to 10 months. The livers were fixed in Carnoy's fluid or ethanol:acetic acid (3:1), and then they were dehydrated and embedded in paraffin.

Preparation of Tissue Sections. Histological sections were cut at 5μ, stained with toluidine blue (6), and mounted in balsam under a coverslip.

For studies on changes occurring in hyperbasophilic foci, liver sections were submitted to mild RNase treatment (3) prior to staining. The sections were immersed for 0.5 and 1 min in a solution containing 50 μg of RNase (bovine pancreas RNase crystallized 5 times from Sigma Chemical Company, St. Louis, Mo.) per ml of 0.2 M acetic acid:acetate buffer at pH 6.0 and maintained at 37°. This resulted in a selective extraction of the RNA responsible for hyperbasophilia, as reported by Brière (3). Slides carrying adjacent tissue sections were left in the control solution for the same period of time prior to staining while others were stained directly with toluidine blue.

RESULTS

Gross and Microscopic Changes in Livers of Animals Fed Various Diets. The livers of animals fed 4-dimethylaminoazobenzene or 3'-methyl-4-dimethylaminoazobenzene show after 5 to 8 weeks a characteristic hoboainl appearance (Fig. 1). This change is followed by the formation of small, whitish masses (Fig. 2) which develop into large, protuberant tumor masses (Fig. 3). While a period of 3 months is required for the induction of tumors in rats fed 4-dimethylaminoazobenzene, tumors can be detected after 2 months in livers of rats fed the 3'-methyl derivative.

At the microscopic level, necrosis of hepatic parenchyma is observed in centrolobular areas during the first weeks of carcinogenic azo dye feeding. This degenerative process is followed by regeneration of the hepatocytes in the periportal areas. The bile duct and connective tissue elements also proliferate and soon replace the necrotic tissue, as previously described (6). The liver then becomes organized as nodules of hepatocytes surrounded by trabeculae of bile ducts and connective tissue (Fig. 4). The nodules expand due to the proliferation of the hepatocytes producing the hoboainl appearance. The mitotic activity is relatively uniform in the various nodules, but the nodules show some variation in size which can be attributed to the subdivision of the primary nodules (6) and/or slight differences in rates of cell proliferation. Hepatomas (Fig. 5) were observed to develop from various nodules irrespective of their size or their location.

The administration of diethylnitrosamine induces extensive necrosis of hepatic tissue in periportal areas, and hepatocytes of the centrolobular areas are observed to undergo a regenerative process. They soon form nodules of relatively uniform size with negligible infiltration of the tissue by bile duct cells. Hepatomas develop from numerous sites after 2 months of feeding.

The rats fed the noncarcinogenic azo dyes occasionally showed centrolobular necrosis, and appreciable proliferation of the hepatocytes occurred in animals fed 2-methyl-4-dimethylaminoazobenzene for periods of up to 6 weeks. No significant histological change was noted, however, at later time intervals in livers of rats fed these azo dyes.

The animals maintained on basal control diets showed a
liver architecture comparable to that of normal rats even after prolonged periods of feeding.

Occurrence of Hyperbasophilic Foci. Areas of nodular parenchyma showing abnormally high RNA staining with basic dyes (Fig. 6) have been repeatedly observed in livers of rats fed 4-dimethylaminoazobenzene or 3'-methyl-4-dimethylaminoazobenzene. Such foci as well as developing tumors (Fig. 7) were found after 3 months in rats fed 4-dimethylaminoazobenzene, while both features were detected as soon as 2 months in animals fed the more potent 3'-derivative.

A similar phenomenon was noted after 2 months in rats receiving diethylnitrosamine but, in this case, the hyperbasophilic areas were more diffuse and seemed to be more widely distributed. This may be correlated with the fact that hepatomas apparently develop almost simultaneously from various regions of the liver after 2 months of diethylnitrosamine administration.

Areas of preneoplastic livers characterized by intense RNA staining have been observed by several authors (Table 1), and, as judged from the published photographs, such areas apparently exhibit the same properties in animals fed different hepatocarcinogens. In addition to their increased basophilia, these regions may show cytological changes such as an increase in mitotic activity, an increase in nucleocytoplasmic ratio, and the presence of prominent nucleoli and irregular nuclei.

The search for similar foci in livers of animals fed noncarcinogenic azo dyes for several months has been in vain. Neither hyperbasophilic foci nor tumors could be detected in the livers of animals fed 2-methyl-4-dimethylaminoazobenzene, 4-aminazobenzene, or azobenzene. The results obtained with the noncarcinogenic 2-methyl-4-dimethylaminoazobenzene may be particularly significant since this azo dye gives a high level of hepatic protein-bound dye (16) and duplicates several of the cytological changes induced by highly active hepatocarcinogens (13, 28). The animals kept on basal control diets did not show hyperbasophilic regions either, even after prolonged periods of feeding.

This study thus indicates that hyperbasophilic foci develop exclusively in livers of animals fed hepatocarcinogens. Moreover, the time necessary to induce this change corresponds to the induction period of tumors by different carcinogens, and the hyperbasophilic areas show a wider distribution in cases where tumors apparently develop from relatively large fields of tissue. It thus seems that a close correlation exists between the phenomenon of hyperbasophilia and tumor formation.

Properties of Hyperbasophilic Foci. Examination of tissue sections submitted to mild RNase treatment reveals that some foci with hyperbasophilic properties do not show any other apparent alteration. The hyperbasophilic area illustrated in Fig. 8, for instance, can be easily distinguished from the surrounding tissue in this untreated section, but after the selective extraction of the RNA responsible for hyperbasophilia (Fig. 9), the staining reaction given by the residual nucleic acids is uniform and no particular cytological feature then distinguishes the cells of such an area (Fig. 10) from the adjacent hepatocytes (Fig. 11).

Most hyperbasophilic foci show, however, one or several additional modifications. The area illustrated in Fig. 12, for instance, remains distinguishable after mild RNase treatment (Fig. 13) due to its increased nucleocytoplasmic ratio, the occurrence of prominent nucleoli, and/or the presence of irregular nuclei. The cells of this area (Fig. 14) thus differ from those of surrounding tissue (Fig. 15) in several respects. These changes probably follow the phenomenon of hyperbasophilia since hyperbasophilic foci without such cytological changes are encountered while, on the other hand, areas showing similar changes but no hyperbasophilia have never been observed.

The tumors show, in addition to the hyperbasophilic properties and the cytological changes, a displacement of the surrounding tissue. Compression of the adjacent parenchyma, i.e., elongation, thinning, and parallel curving of the adjacent cell cords, is evident in untreated (Fig. 16) or control sections. After mild RNase treatment (Fig. 17), the tumors can be recognized by both their abnormal cytological features and their effects on the surrounding hepatocytes. The cytological alterations are usually more conspicuous in hepatomas than in hyperbasophilic foci, and the tumor cells (Fig. 18) clearly differ from the hepatocytes of the adjacent tissue (Fig. 19).

**DISCUSSION**

According to the site of liver damage induced by carcinogenic azo dyes and diethylnitrosamine, the regenerative process occurs in either the periportal or the centrolobular areas. In both cases, however, the resulting nodules exhibit a relative uniformity in size and rate of cell proliferation, and terms such as "hyperplastic nodules," "hyperplastic parenchyma," "regenerating parenchyma," or "nodular parenchyma" are used indifferently to designate the parenchymal tissue in these conditions. This situation differs from that encountered under some circumstances with ethionine and other carcinogens where the larger part of the hepatic parenchyma remains more or less quiescent while some areas develop into huge nodules sharply demarcated from the surrounding parenchyma (7, 8). In such cases, the terms "hyperplastic nodules" or "hyperplastic parenchyma" are applied restrictively to the expanding nodules. While hepatomas may arise from different sites in livers where a widespread tissue regeneration is induced, they arise specifically from the developing nodules where a more localized tissue proliferation occurs, a fact suggesting that hyperplasia represents an essential step in carcinogenesis.

The phenomenon of hyperbasophilia, i.e., the intense staining of cytoplasmic RNA in islands of hepatocytes, occurred exclusively in animals fed carcinogens. The time at which the hyperbasophilic foci develop coincided with the induction period of tumors, and the distribution of such areas in hepatic parenchyma showed some similarity with the distribution of tumors in the organ. This latter point is further supported by the fact that similar areas are observed exclusively in the hyperplastic nodules giving rise to tumors in animals where the larger part of the hepatic parenchyma is maintained in a nonhyperplastic state (7, 8). These
observations thus indicate a close association between the formation of hyperbasophilic foci and the development of hepatomas. The experiments undertaken to determine whether hyperbasophilia represents a transition between hyperplasia and neoplasia have shown that hyperbasophilic foci can actually be distinguished from both the surrounding hyperplastic parenchyma and the tumors on the basis of histochemical, cytological, and histological criteria (Table 2). They differ from the adjacent parenchyma in their intense RNA staining and additional cytological changes which are also encountered in tumor cells, but they simply represent modified areas and do not result from the outgrowths of smaller foci. They differ in this latter aspect from the tumors which represent excrenceses of transformed areas and cause alterations of tissue architecture. The hyperbasophilic foci appear therefore to be a transitional tissue and probably represent the sites of neoplastic transformation. The alteration in RNA responsible for hyperbasophilia may initiate the series of changes observed in these areas, including the alterations in the control of the cell cycle which transform such foci into abnormal masses of tissue, i.e., bona fide neoplastic tissue.

Studies on the histogenesis of liver tumors thus suggest that hyperplasia is a prerequisite in tumor formation and that the islands of hyperplastic hepatocytes which acquire hyperbasophilic properties at a later stage are the actual sites of tumor development.

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Fig. 1. Liver from rat fed 3'-methyl-4-aminoazobenzene showing a hobnail appearance due to the nodular organization of the hepatic tissue.

Fig. 2. Presence of small, whitish masses of cancerous tissue (arrow) in liver from a rat fed the same carcinogen.

Fig. 3. Development of large tumor masses in rat liver after more prolonged azo dye feeding.

Fig. 4. Liver section showing the nodular organization of the hepatic parenchyma following administration of 4-dimethylaminoazobenzene. H & E, X 3.

Fig. 5. Hepatoma arising from nodular parenchyma (arrow) in a rat fed the same carcinogen. Part of a large mass developing from another lobe is shown on the right (7). H & E, X 3.

Fig. 6. Area of hepatic parenchyma showing abnormally intense RNA staining in a rat fed 4-dimethylaminoazobenzene. This hyperbasophilic focus extends over 5 adjacent nodules. Toluidine blue, X 100.

Fig. 7. Hepatoma developing amid the hepatic tissue and compressing the adjacent cell cords in an animal fed the same azo dye. Toluidine blue, X 100.

Figs. 8 to 19. Liver sections from rats fed 4-dimethylaminoazobenzene for 5 months. Toluidine blue staining.

Fig. 8. Untreated section showing part of a hyperbasophilic focus on the left. X 250.

Fig. 9. Adjacent section submitted to mild RNase treatment prior to staining. The cytoplasmic RNA responsible for hyperbasophilia is extracted selectively. The staining of the residual nucleic acids of the focus becomes comparable to that of nucleic acids in adjacent hepatocytes, and no other apparent alteration characterizes this focus. X 250.

Figs. 10 and 11. Higher magnifications of areas corresponding to hyperbasophilic focus and surrounding parenchyma, respectively, in section submitted to mild RNase treatment. No morphological differences can be observed. X 1000.

Fig. 12. Untreated section showing part of an island of hyperbasophilic cells on the left. X 250.

Fig. 13. Adjacent tissue section submitted to mild RNase treatment prior to staining. The cells in this island differ from those of the adjacent parenchyma in several respects, including increased nucleocytoplasmic ratio and occurrence of prominent nucleoli and irregular nuclei. X 250.

Figs. 14 and 15. Higher magnifications of areas corresponding to hyperbasophilic focus and adjacent tissue, respectively, in section submitted to mild RNase treatment. Several cytological differences can be observed. X 1000.

Fig. 16. Untreated section showing a portion of a hepatoma on the left. Note the compression of the surrounding parenchyma. X 250.

Fig. 17. Adjacent tissue section submitted to mild RNase treatment prior to staining. The tumor mass is recognizable on the basis of both the cytological alterations and the displacement of the adjacent parenchyma. X 250.

Figs. 18 and 19. Higher magnifications of hepatocytes in tumor and surrounding tissue after mild RNase treatment. The cancer cells show several abnormal features. X 1000.
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