Quantitative Analysis of the Time-dependent Development of Glucose-6-phosphatase-deficient Foci in the Livers of Mice Treated Neonatally with Diethylnitrosamine

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ABSTRACT

The development of glucose-6-phosphatase (G-6-Pase)-deficient hyperbasophilic foci was analyzed at 4-week intervals in the livers of CD-1 and C57BL/6J X C3H/HeJ F1 (hereafter called B6C3F1) mice given a single i.p. injection of diethylnitrosamine (DEN) (0.1, 0.2, or 0.4 μmol/g body weight) within 24 hr after birth. Transections of G-6-Pase-deficient foci of hepatocytes were readily discernible in liver sections of DEN-treated mice of either sex at 8 weeks of age. The size and number of these foci per liver increased with time. The occurrence of G-6-Pase-deficient focus transections with diameters as large as 1 mm coincided with the gross appearance of 1-mm gray-white nodules in the livers of male B6C3F1 mice at 16 weeks of age and in females at 32 weeks of age. Transections of all grossly visible hepatic nodules from male and female mice were G-6-Pase deficient and hyperbasophilic; the great majority were diagnosed as mouse hepatomas type A.

A single neonatal dose of DEN, the number and rate of growth of the G-6-Pase-deficient foci and the incidence and rate of appearance of gross hepatomas were greater in the livers of male than in those of female mice. In contrast, the average numbers of G-6-Pase-deficient foci in the livers of male and androgenized female B6C3F1 mice at 36 weeks of age were approximately equal and about twice that observed for the livers of DEN-treated female controls.

Quantitation of carcinogen-induced histochemically detectable foci and hepatomas as a function of time provides a useful tool for the analysis of initiation and promotion in the mouse liver.

INTRODUCTION

Starting with the early investigations on the induction of hepatic tumors in rats and mice by the long-term administration of the aminonitrosamines, numerous studies have examined the sequence of lesions associated with tumor development in rat liver (reviewed in Ref. 14). The early studies were complicated by the extensive liver damage that resulted from the administration of one or a few doses of a number of chemical carcinogens (36, 44, 45). Although most of the assays for hepatic carcinogenicity in the mouse have utilized long-term administration of the carcinogens, the administration of one or a few doses of a number of chemical carcinogens during the latter part of gestation or soon after birth will induce high incidences of hepatic tumors which may become evident within 1 year (13, 17, 26, 36, 45), as well as microscopic foci of hepatic cells showing increased storage of glycogen (2, 3). We have used the latter system with DEN as the carcinogen for an examination of the rate of development and number of G-6-Pase-deficient hepatic foci in relation to the occurrence of hepatic tumors.

MATERIALS AND METHODS

Care and Treatment of Mice. CD-1 random-bred and B6C3F1 mice were obtained by breeding animals purchased from the Charles River Breeding Labs, Inc., Wilmington, Mass. and The Jackson Laboratory, Bar Harbor, Maine, respectively. All of the animals were housed in plastic cages on hardwood shavings (Betta-Chip, Northeastern Products Corp., Warrensburg, N. Y.) and fed Wayne Breeder Blox pellets (Allied Mills, Inc., Chicago, Ill.) and tap water ad libitum.

Except for the untreated controls, each mouse was given a single i.p. injection of 0.01 ml/g body weight within 24 hr after birth of either sterile trioctanoin (Sigma Chemical Co., St. Louis, Mo.) or the same solvent containing DEN (Eastman Kodak Co., Rochester, N. Y.). All mice in each litter received the same treatment, and the litters were assigned to treatment groups in rotation. At 4 weeks of age, the mice were weaned, segregated by sex, and housed in groups of 5. Beginning at 4 weeks of

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2 The abbreviations used are: DEN, diethylnitrosamine; G-6-Pase, glucose-6-phosphatase; H & E, hematoxylin and eosin.

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1585

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age and continuing at designated intervals thereafter, randomly selected mice from each group were killed by cervical dislocation. Each animal was subjected to a gross routine autopsy in which particular attention was paid to the liver. Each liver was prepared for histological examination as described below.

There were 4 experiments. In Experiment 1, groups of 5 male CD-1 mice, each of which was given a single injection of 0.4 µmol of DEN per g body weight, were killed at 4-week intervals up to 48 weeks. In Experiment 2, 5 CD-1 mice of each sex that received either 0.1 or 0.4 µmol of DEN per g body weight were killed at 4-week intervals up to 24 weeks. For Experiment 3, a dose of 0.2 µmol of DEN per g body weight was administered to male and female B6C3F1 mice, and 5 or, in a few cases, 10 mice of each sex were killed at 4-week intervals up to 36 weeks. In each of these 3 experiments, equal numbers of control mice that had received no injection or had been given an injection of triocotanoin only were killed on the same schedule as the DEN-treated mice.

Experiment 4 was designed to study the effect of a modification of the sex hormonal environment. A group of 25 DEN-treated female B6C3F1 mice (0.2 µmol/g body weight) were ovariectomized at 24 weeks of age and, starting 2 weeks later, were given s.c. injections 3 times weekly of testosterone propionate (0.15 mg/0.05 ml of triocotanoin per mouse; Sigma) (6). The mice in 2 other groups, each consisting of 25 DEN-treated females, were left untreated or were subjected to a sham ovariectomy and subsequent injections of only triocotanoin in place of the testosterone propionate. All of these mice were killed at 36 weeks of age. Since this experiment was carried out concurrently with Experiment 3, the solvent-injected controls and the mice receiving no injection of Experiment 3 also served as controls for Experiment 4.

The incidence and multiplicity of gross hepatic tumors in the livers of at least 20 mice from each experimental group of B6C3F1 mice in Experiment 3 were determined at 36 weeks. For this purpose, each lobe of each liver was serially sectioned at 2-mm intervals, so that nearly all nodules of at least 1-mm diameter were visualized and enumerated. The H & E-stained sections from each liver of each group were examined for the presence of hepatomas. In addition, all hyperbasophilic areas in H & E-stained, formalin-fixed sections of each lobe from the livers of at least 20 mice from each experimental group of B6C3F1 mice in Experiment 3 were examined in more detail.

Preparation of Liver Sections. Each of the median, left, and right lobes of each liver was bisected; one of the 2 sections from each caudal lobe and the middle section from each of the other lobes were fixed in buffered 10% formalin and stained with H & E. For each liver, the remaining sections from all of the lobes were juxtaposed on a filter paper square to form a single tissue block, which was immediately frozen on Dry Ice. Two consecutive 6-µm sections with areas of approximately 2 sq cm were cut from the frozen tissues; one was stained with H & E, and the other was stained for G-6-Pase (46, 47).

Analysis of G-6-Pase-deficient Foci. Throughout this paper, reference is made to both the 2-dimensional focus transections seen in liver sections and to their 3-dimensional precursors in the liver. To avoid confusion, the terms “focus transection” and “transection” are used for the 2-dimensional structure, and the term “focus” is used for the 3-dimensional structure throughout this paper. For each frozen liver section, the number of G-6-Pase-deficient focus transections, as well as the minimum and maximum diameter of each focus transection, was determined at x100. The minimum diameters were, on an average, 75% of the maximum diameters. The microscope was equipped with a micrometer reticle, such that each reticle unit equalled 0.01 mm. The area of each liver section was determined by planimetry (Keuffel and Esser planimeter; Hoboken, N. J.). Histological characterization of the transections was determined from the serial section stained with H & E.

Most comparisons of the relative sizes of the foci were made on the basis of the average diameters of the focus transections. However, for some cases, the mean focus diameter for each liver was estimated from the inverses of the transection diameters according to the formula

\[ \bar{d}_{\text{focus}} = \frac{\pi}{2\bar{d}_{\text{transect}}} \]

where \( \bar{d}_{\text{focus}} \) is the mean diameter of the focus in a liver, and \( \bar{d}_{\text{transect}} \) is the mean of the inverses of the diameters of the focus transections (10, 16). The ratios of the transections can also be used to calculate the average number of foci per cu cm of liver from the formula

\[ N = \left( \frac{1}{r_1} + \frac{1}{r_2} + \frac{1}{r_3} + \cdots + \frac{1}{r_n} \right) \pi A \]

where \( N \) is the number of foci per cu cm; \( r_1, r_2, r_3, \ldots , r_n \) are the radii in cm of the observed focus transections; and A is the area in sq cm of the liver section evaluated (10, 16). Although this method assumes that the foci are spherical, it is approximately correct for ellipsoidal foci. For spheroids which have transections with minimum and maximum radii within 10% of the mean, \( \pi \) in the above equation would be replaced by constants between 2.7 and 3.4 (8, 9). The number of foci per liver was calculated by multiplying the number of foci per cu cm by the weight of the liver in g.

Time trends for the number of foci or the average transection diameters were analyzed by the methods of Theil (19) and Sen (40). For the analysis of sex or dose dependence, the data for all time points were analyzed jointly by means of a 2-way layout analogous to the median test of Mood (25) using a 3-fold contingency table (24).

Calculation of the number of foci per liver, rather than the commonly used presentation of the mean number of focus transections per unit area, was used in this study since preliminary computations showed that the number of focus transections per unit area does not accurately reflect the number of foci present unless all of the foci are essentially the same size. This follows because the number of observed transections per unit area is a direct function of the number and an inverse function of the size of the foci in the liver. In computer experiments, simulated livers were designed to provide sections that contained focus transections with mean radii of 0.09 or 0.27 mm and a mean number of either 4 or 10 focus transections per sq cm. It was found that the numbers of foci per cu cm in the simulated livers with the larger mean focus transections were only 30% of those for the simulated livers which gave sections with the same number of focus transections but with only one-third the mean radius (10).

RESULTS

A similar overlapping progression of hepatic changes was
observed for the DEN-treated mice in each of the experiments. Definitive diagnoses of G-6-Pase-deficient or hyperbasophilic foci were not possible in liver sections from 4-week-old mice. On the other hand, many liver sections from 8-week-old mice contained readily discernible G-6-Pase-deficient focus transections, which were only slightly, if at all, hyperbasophilic (Figs. 1 to 3). At 8 weeks, most of the transections were about 100 µm in diameter; transections of this size contained about 25 cells so that, if the transection was through the focus center, the focus would contain about 95 cells. The number of G-6-Pase-deficient foci generally increased with time, and the range of sizes of the focus transections was much broader for the older mice (Fig. 4). By 16 weeks, most of the G-6-Pase-deficient focus transections that were at least 100 µm in diameter were also hyperbasophilic in serial sections stained with H & E.

The occurrence of some G-6-Pase-deficient focus transections of diameters ≥1 mm coincided (at about 16 weeks in male mice and 32 weeks in female mice) with the appearance of grossly visible gray-white nodules of 1- to 2-mm diameter on the surface and in the interior of the liver lobes. These gross nodules, which were diagnosed as type A hepatomas (21), increased in size and number with time (Figs. 5 and 6). At later times, occasional red-brown nodules, of the same color as the surrounding nonnodular liver, were evident in some of the DEN-treated livers in addition to the gray-white nodules. These red-brown nodules were also diagnosed primarily as type A hepatomas, although a few were diagnosed as type B hepatomas, and some were mixed type A-type B hepatomas (21). Despite the distinctive morphologies of type A and type B mouse hepatomas (21), their transplantability, as demonstrated earlier by Andervont and Dunn (1) and more recently by Williams et al. (49), indicates that both types of lesions may be classified as hepatocellular carcinomas. No consistent difference between the gray-white and red-brown type A hepatomas was observed from examination of H & E-stained sections. The type A and type B hepatomas, which are distinguished primarily by the thickness of the plates of hepatocytes, are illustrated in a previous paper from our laboratory (11). Except for some compression of the adjacent parenchyma by the foci of hyperbasophilic cells, the remainder of the liver showed no pathological changes throughout the period of observation. No gross metastatic tumors were noted.

The rate of progression of the above changes and the number and size of the microscopic foci and gross nodules depended on the strain and sex of the mice and on the dose of DEN, as described below. G-6-Pase-deficient focus transections were not observed up to 40 weeks in liver sections from any of the control mice that were either untreated or given injection only with the solvent.

**Development of G-6-Pase-deficient Foci in CD-1 Mice as a Function of Their Sex and the Dose of DEN.** In a preliminary experiment (Experiment 1), injection of 0.4 µmol of DEN per g body weight into male CD-1 mice within 24 hr after birth resulted in the development of G-6-Pase-deficient hepatic foci by 8 weeks. At that time, there was an average of 177 ± 42 G-6-Pase-deficient foci per liver with an average diameter of 113 µm; the largest transection diameter was 310 µm. The average number of foci per liver increased to 508 ± 150 (S.D.) and 887 ± 243 by 16 and 20 weeks, respectively. Gross gray-white nodules were first seen at 16 weeks, and by 28 weeks, the livers of the DEN-treated male mice contained an average of 30 gray-white hyperbasophilic G-6-Pase-deficient nodules. About 20% of these nodules were at least 0.5 cm in diameter. The greatly enlarged livers accounted for about 10% of the body weight at this time. By 40 weeks, some red-brown nodules were also evident. When the experiment was terminated at 48 weeks, the livers of the 15 surviving DEN-treated male mice all contained multiple confluent tumor masses, which were histologically confirmed as hepatomas (type A, type B, or mixed types A and B) (21). At 48 weeks, solitary grossly detectable liver tumors, diagnosed as type A hepatomas, were found in 3 of 28 male mice given injection only with the solvent, and one other mouse had 2 type A hepatomas. A microscopic granuloma was observed in the liver of one of 25 male mice from the control group not receiving injection.

In view of the above results, Experiment 2 was designed to compare the development of G-6-Pase-deficient foci in male and female mice given either 0.1 or 0.4 µmol DEN per g body weight within 24 hr after birth. In this experiment, the number of G-6-Pase-deficient foci per liver increased significantly with time from 8 to 24 weeks for male mice given either 0.4 (p < 0.001) or 0.1 (p < 0.001) µmol DEN per g (Chart 1). Furthermore, the number of observable G-6-Pase-deficient foci in the livers of these male mice suggested a dose-response effect (Chart 1). For the male mice given either dose, there were similar numbers of foci at 8 and 12 weeks (about 50 and 200, respectively). However, at 16, 20, and 24 weeks, the livers of...
the male mice that had received 0.4 μmol/g contained 2.8, 1.8, and 4.6 times, respectively, as many foci as did the livers of male mice that received the lower dose. Because of the broad standard deviations, only the latter difference was statistically significant (p = 0.03). To facilitate comparisons of our data with literature reports of hepatic focus transactions per sq cm, the data for the males given the higher dose of DEN have been tabulated in 3 ways, i.e., the number of transactions per sq cm, the number of foci per cu cm, and the number of foci per liver (Table 1).

In contrast to the results with male mice, the average number of observable G-6-Pase-deficient foci in the livers of female CD-1 mice given either dose of DEN did not exceed 100 per the 24-week period studied. Therefore, the sex difference for the number of G-6-Pase-deficient hepatic foci observable throughout the 8- to 24-week period was highly significant (p < 0.001) for either dose of DEN.

The mean transection diameters of the G-6-Pase-deficient foci did not differ significantly with either the dose of DEN or the sex of the CD-1 mice (Chart 2). However, the mean focus transection diameters in the livers of both male and female mice given either dose of DEN increased with time up to 16 weeks (p ≤ 0.001 for each group). The distributions of the focus transection diameters (Chart 2) indicated that the growth rates were not homogeneous. For each group, the majority of the transection diameters were smaller than the overall means. The observable number of enzyme-deficient foci per liver would be a function of both the number initiated and the time required for the clones of the altered hepatocytes to grow to a detectable size. Especially for the male mice, an increase in the mean focus transection diameter was reduced by the addition with time of many new small foci to those for which the transections had reached the minimum detectable size (about 0.05 mm in diameter) earlier. The focus transection diameters greater than that of the ninth decile were markedly larger for males than for females of the same age given the same dose of DEN (Chart 2). For this reason, and because the males had more hepatic foci per liver, the number of large G-6-Pase-deficient focus transactions was much greater in the livers of the males as compared to those of the females.

Development of G-6-Pase-deficient Foci and Hepatic Tumors in Male and Female B6C3F1 Mice. In view of the heterogeneous development of G-6-Pase-deficient foci in the livers of CD-1 mice, a similar experiment (Experiment 3) was carried out in which male and female B6C3F1 mice were given each a single i.p. dose of 0.2 μmol DEN per g body weight. As was observed for the CD-1 mice, a small number of G-6-Pase-deficient foci was detectable at 8 weeks in the livers of the DEN-treated inbred mice of both sexes. However, the mean number of foci per liver tended to increase with time up to 28 weeks (Table 2) for both sexes, but the difference was significant only for the males (p < 0.001). At each time point, except for 8 and 20 weeks, the mean number of observable foci per liver was smaller for female B6C3F1 mice than for the males. However, when the data for all the time points were considered, there was no statistically significant sex difference for the number of foci per liver (p = 0.6). Liver sections from trioctanoin-injected control mice or from those receiving no injection contained no detectable G-6-Pase-deficient focus transactions at any time.

A significant increase in the mean diameters of the G-6-Pase-deficient focus transactions with time was observed in the livers of both sexes (p < 0.001) (Chart 3). However, this rate of increase was significantly smaller (p < 0.001) for transactions from the livers of female B6C3F1 mice as compared to those of the males. As was observed for the CD-1 mice, the size of the 20% of the G-6-Pase-deficient focus transactions with the largest diameters, especially from the 16th to the 28th weeks, was markedly greater (p < 0.001) for the males (p < 0.001) as compared to the female mice (Chart 3).

The sequence of development of hepatic nodules in B6C3F1 mice was similar to that for male CD-1 mice in that the appearance of 1-mm gray-white nodules coincided with the detection of G-6-Pase-deficient, hyperbasophilic transactions of 1-mm diameter or larger at 16 weeks of age in males and at 32 weeks of age in the females. Like the enzyme-deficient foci, the gray-white nodules continued to grow with time in the absence of any experimentally imposed selecting environment. Although at 36 weeks the number of G-6-Pase-deficient foci per liver differed only by a factor of 2 for the DEN-treated
Hepatic G-6-Pase-deficient Foci

Data for Experiment 3, in which B6C3F, mice received 0.2 µmol of DEN per 0.01 ml of trioctanoin per g of body weight i.p. within 24 hr after birth. Control mice received either the same volume of trioctanoin alone or no injection. Five mice/sex/treatment were killed, except where noted, at 4-week intervals from 4 to 36 weeks of age.

Table 2

<table>
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<th>Treatment</th>
<th>Sex</th>
<th>4 wk</th>
<th>8 wk</th>
<th>12 wk</th>
<th>16 wk</th>
<th>20 wk</th>
<th>24 wk</th>
<th>28 wk</th>
<th>32 wk</th>
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<tr>
<td>DEN</td>
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<td>67 ± 31</td>
<td>79 ± 57</td>
<td>142 ± 60</td>
<td>147 ± 138</td>
<td>236 ± 105</td>
<td>326 ± 200</td>
<td>135 ± 31</td>
<td>264 ± 87</td>
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<tr>
<td>DEN</td>
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<td>0</td>
<td>98 ± 97</td>
<td>35 ± 34</td>
<td>78 ± 73</td>
<td>176 ± 68</td>
<td>101 ± 49</td>
<td>171 ± 69</td>
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* Livers of 10 mice/sex/treatment were analyzed.
* Livers of 20 mice/sex/treatment were analyzed.
* Mean ± S.D.

All of the gross nodule transections observed in liver sections (approximately 2 sq cm) from the male and the female mice killed at 36 weeks were diagnosed histologically as type A hepatomas. Furthermore, even though no gross nodules were seen in the livers from each of 8 DEN-treated female mice killed at 36 weeks, the sections from these livers contained one to 3 G-6-Pase-deficient, hyperbasophilic focus transections of about 1-mm diameter. These were the largest transections on the slides and were also diagnosed histologically as type A hepatoma.

Effect of Androgenization on the Number and Size of G-6-Pase-deficient Foci in the Livers of DEN-treated Female Mice. The effect of the hormonal environment on the development of hepatic G-6-Pase-deficient foci was further evident from examination of the livers of female mice that were ovariecctomized at 24 weeks and then given injections of testosterone propionate (Experiment 4). At 36 weeks of age, the number of detectable foci in the livers of these androgenized mice was twice that observed in the livers of unoperated or of sham-operated female controls not given testosterone (p < 0.001, Table 4). The mean number of G-6-Pase-deficient foci per liver for the androgenized females was nearly identical to that for male mice of the same age that were also treated as neonates with the same dose of DEN. The mean focus diameters for the livers of the 3 groups of female mice were similar, but these diameters were only one-half of that for the male mice. The androgenization thus apparently resulted in the growth to a detectable size of many additional hepatic foci during the 12-week period.

DISCUSSION

In either CD-1 or B6C3F, mice, a single neonatal treatment with DEN initiates the development of histochemically detectable foci of hepatocytes, grossly visible white nodules of hyperbasophilic parenchymal cells, and hepatomas, types A and B (11, 21). The deduction that these observed tissue changes are a continuum in the development of mouse hepatomas is supported by their sequential appearance in carcinogen-treated livers and by the observation that the growth of microscopic hepatic G-6-Pase-deficient focus transections to diameters of 1 mm and larger coincided with the appearance of grossly visible white nodules of at least 1-mm diameter. Quantitative comparisons of these lesions in carcinogen-treated male and female mice were consistent with this deduction. Thus, the detectable G-6-Pase-deficient foci were more...
The differences in the incidences of G-6-Pase-deficient foci and of hepatomas in male as compared to female mice do not appear to be related to a difference in the metabolism of DEN. Rao and Vesselinovitch (33) found no significant differences in the rate of dealkylation of DEN, apparently the essential step in its metabolic activation (12), by liver homogenates from neonatal male and female B6C3F1 mice. Furthermore, androgenization of females at 24 weeks of age caused an increase at 36 weeks in the number of detectable G-6-Pase-deficient foci per liver to a level observed for male mice of the same age that had also been treated with DEN as neonates. These data thus suggest that equal numbers of hepatocytes were initiated in mice of both sexes; however, in the hormonal environment of the female, most of these cells were apparently quiescent or very slowly replicating.

Although G-6-Pase-deficiency serves as a convenient marker for altered hepatocytes in mouse liver, there is no evidence that its deficiency or any other altered histochemical or phenotypic characteristic that has been reported (5, 14, 22, 49) has any functional meaning in the carcinogenic process. The phenotypic heterogeneity of histochemically distinguishable cells within a single hepatic focus or between foci within the same rat liver has been described (18, 28, 31). From the observations that a few G-6-Pase-deficient hyperbasophilic foci in the livers of intact female mice grew to gross size, while the majority did not, and that the ranges of the diameters of the focus transections were large, especially at later times, it is evident that these populations of hepatocytes are heterogeneous, at least with respect to growth rate (23, 32, 37).

The heterogeneity of growth potential among foci, even those within a single liver, probably contributed to the rather broad standard deviations obtained for some of the data. Another important factor was the smaller number of G-6-Pase-deficient focus transections observed at early time periods, especially for the females. It should be noted that calculated totals of 40 to 70 G-6-Pase-deficient foci per liver (characteristic of the early time periods, especially for the females) were based on actual observations of no more than 5 focus transections per 2-sq cm liver section. For more detailed analyses, larger section areas per liver and/or larger numbers of animals should be used when the number of foci may be small.

The administration of chemical carcinogens to mice prior to...
birth or as neonates and the subsequent quantitation of the carcinogen-induced histochemically detectable foci and hepatomas as a function of time constitute a useful model for the study of hepatocarcinogenesis in the mouse. Especially when administered prior to weaning, a single or limited number of doses of a variety of chemical carcinogens induces high incidences of multiple gross hepatic tumors in mice by about 1 year (2, 36, 45). This system now appears capable of dissection for analysis of initiation and promotion. The more rapid replication of hepatocytes in the very young mouse presumably facilitates the fixation of the carcinogen-induced initiating event (5). Among the carcinogen-altered hepatocytes, some apparently have a growth advantage which permits them to give rise to microscopically detectable groups, probably clones, of cells (2, 38). The nature of the ‘promotion’ stimulus that facilitates the development of these foci and later tumors in the adult mouse is not clear (2, 14, 35, 45). The greater susceptibility of mice treated prior to or just after birth suggests that some promotion may be associated with normal liver growth. Furthermore, the sex differences in the rates of appearance and growth of the microscopic foci and of the development of gross tumors, as well as the increased rate of growth of the foci in the livers of androgenized females, suggest that androgenic hormones may have a prominent role in the promotion phase of mouse hepatocarcinogenesis.

ACKNOWLEDGMENTS

We are especially appreciative to Dr. Harold Campbell of the McCord Laboratory for calling our attention to the papers by DeHoff (8), DeHoff and Rhines (9), and Fullman (16) after one of us (N. R. D.) had independently derived the same relationships for calculation of the number and average size of the foci (10). We would like to thank Carol Cooper and Randy Knibbs for excellent technical assistance and Jane Weeks, Mary Foltz, and Lana Barsness for preparation of the histological slides.

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Hepatic G-6-Pase-deficient Foci

Figs. 1 to 6. Sections were taken from the livers of male CD-1 mice given a single injection of DEN (0.4 μmol/g) within 24 hr after birth and killed at the stated times.

Figs. 1 and 2. Serial transections (diameters, 0.20 mm) stained with H & E (Fig. 1) and for G-6-Pase (Fig. 2) through the same hepatic focus from a mouse given DEN and killed at 8 weeks of age. Hepatocytes in the focus are small, hyperbasophilic, and closely packed (Fig. 1); and the distinct border and size of the focus can easily be distinguished as a deficiency of enzyme staining in Fig. 2. Frozen sections. x 740.

Fig. 3. Transection (diameter, 0.16 mm) of a hepatic focus from a mouse given DEN and killed at 8 weeks of age. Hepatocytes in the focus are generally smaller and more closely packed, have smaller nuclei, and are somewhat more basophilic than the surrounding normal hepatocytes. Paraffin section. H & E, x 1225.

Fig. 4. G-6-Pase staining of a section cut from a frozen tissue block composed of several liver lobes from a mouse given DEN and killed at 20 weeks of age. Multiple, randomly distributed transections of varying size of G-6-Pase-deficient foci of hepatocytes can be seen (arrows). Frozen section. x 6.5.

Fig. 5. Low-power magnification of a transection (diameter, 0.80 mm) of a hyperbasophilic, G-6-Pase-deficient focus present in the liver of a mouse given DEN and killed at 24 weeks of age. Hepatocytes in the focus are small but closely packed, exhibiting some compression of the surrounding parenchyma. The histological pattern is that seen in a mouse hepatoma, type A, with a few areas of cellular atypism. Paraffin section. H & E, x 310.

Fig. 6. High magnification of the border of a transection (diameter, 1.3 mm) of a large hyperbasophilic, G-6-Pase-deficient focus seen in the liver of a mouse given DEN and killed at 28 weeks of age. As seen in the lower right-hand portion of the micrograph, hepatocytes of the focus exhibit considerable cytoplasmic vacuolization and significant variation in cell size, nuclear size, and staining properties. The adjacent hepatic parenchyma is compressed. Paraffin section. H & E, x 625.

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