Growth Kinetics of Microscopic Hepatocellular Neoplasms in Carcinogen-resistant and Carcinogen-responsive Strains of Mice

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ABSTRACT

The initiation and growth of microscopic hepatocellular neoplasms in C57BL/6 mice, considered relatively resistant to hepatocarcinogenesis, was compared with that of the more responsive C3H and B6C3F1 (C57BL/6 × C3H) strains. Tumors were induced by giving male mice injections of diethylnitrosamine when they were 15 days old. During the first 18 weeks postinjection, the growth rates of neoplasms in the three strains were almost identical (doubling time of 2.1 to 2.5 weeks). However, after that time, only the growth rates of the C57BL/6 neoplasms slowed; between 30 and 42 weeks the doubling time had increased to 13 weeks. In addition, at all sacrifice times the number of neoplasms in the C3H strain was at least 2.5 times higher than in the C57BL/6 and B6C3F1, strains. These results suggest that the genetic determinant(s) for inhibited tumor growth (expressed only in C57BL/6 mice) are recessive to those for unimpeded tumor growth (expressed in C3H and B6C3F1 mice), while the determinant(s) for large numbers of tumors (expressed only in C3H mice) are recessive to those for small numbers of tumors (expressed in C57BL/6 and B6C3F1 mice).

In addition to the interstrain differences in tumor growth, two other types of tumor growth heterogeneity were identified. First, in each of the three strains, the largest tumors were found to grow faster than the smaller tumors. This suggests that the very broad range in tumor size that is seen in this model results from the long-term differences in the growth rates of individual neoplasms. Second, we found that in microscopic hepatic neoplasms in B6C3F1 mice, the thymidine labeling indices were 2.3 times greater in the outer 50-μm layer (2 cells thick) than in the next deeper 50-μm layer of cells. This suggests that even in these minute neoplasms, gradients in blood-borne oxygen, nutrients, or growth factors are responsible for heterogeneous growth.

INTRODUCTION

Strain-dependent variation in the response of mice to hepatocarcinogens usually parallels the spontaneous incidence of liver tumors and has been known for many years (reviewed in Refs. 1 and 2). For example, after exposure to carcinogens, the responsive C3H strain shows a high incidence of liver tumors and short tumor latency (time to tumor); in contrast, the C57BL/6, A/J, and SWR strains are much more resistant to carcinogens. Recently there has been increased interest in characterizing this genetic variation (3–6), especially since the hybrid B6C3F1 mouse, widely used to assess the carcinogenicity of drugs and toxins (7), is a cross between the responsive C3H and resistant C57BL/6 strains. The genetic analyses have been facilitated, in part, by the development of stereological methods to quantify the early microscopic neoplasms (8, 9) and more importantly by the refinement of a neonatal mouse liver tumor model (10), in which single doses of carcinogens induce many hundreds of microscopic hepatocellular neoplasms (8, 11). The model has the advantage of providing a long interval (9 to 10 months) for gauging the impact of postinitiation modifiers and yielding a large data base for analyzing a process with stochastic features.

Recently, we documented (12), and others confirmed (13), a distinctive morphological change in the hepatic microneoplasms (neoplasms measuring less than 1 mm in diameter) of C57BL/6 mice. This consisted of focal, randomly distributed clusters of cells that contain cytoplasmic inclusions of secretory protein. Interestingly, in the human disease α1-antitrypsin deficiency, almost identical-appearing inclusions are associated with the development of liver cell injury and cirrhosis (14). This observation has led to a theory that, in the human disease, the accumulation of excess secretory protein is responsible for the liver cell injury (15). Strong support for this “accumulation theory” has recently come from studies in transgenic mice, where insertion of the mutant human (Z) allele resulted in accumulations of the inclusions and in liver cell injury (16). Accordingly, we assessed the relationship between the presence of inclusions and cell replication; we discovered that tritiated thymidine labeling indices in the inclusion-rich cells were only one-third those of the inclusion-free cells (12). This led us to hypothesize that the impairment would slow the overall growth rates of hepatocellular neoplasms in the C57BL/6 mice and that it might be revealed by comparison with the tumor growth rates in the C3H and B6C3F1 strains.

MATERIALS AND METHODS

Animal Treatment

Male C57BL/6J × C3HeB/FeJ (B6C3F1) and C57BL/6J (C57BL/6) mice used in this study were bred from male C3HeB/FeJ mice and C57BL/6J mice of both sexes supplied by the Jackson Laboratory (Bar Harbor, ME). C3He/HeNHsd BR (C3H) mice were bred from parental stocks from Sprague Dawley, Inc. (Madison, WI). Studies involving the C57BL/6 and C3H mice were carried out, in their entirety, at the University of Wisconsin Medical School. Breeding, maintenance, and sacrifice of the B6C3F1 mice and fixation of liver tissues were carried out at the University of Chicago. Animal maintenance, diets (Teklad 4% chow, ad libitum), and light-dark cycles were as previously described (11) and similar at the two institutions. Animals received injections of DENA (5 mg/kg body weight) when they were 15 days old and were weaned at 28 days of age. The B6C3F1 mice (5 to 8/group) were killed at 10, 20, and 28 weeks after carcinogen; the C57BL/6 mice (7 or 8/group) were killed at 12, 18, 30, and 42 weeks; and the C3H mice (5 or 6/group) were killed at 12, 18, and 30 weeks. In preparation for autoradiographic studies, the mice received injections of tritiated thymidine, in six divided doses, over a 36-hour period just prior to sacrifice (12). For the B6C3F1 and C3H mice, controls (3–8/group) received injections of saline and killed at the same times as the experiments, while for the C57BL/6 strain 12 controls were killed at 38 weeks after injection.

Classification of Neoplasms

Proliferating hepatocellular lesions were classified as trabecular carcinomas when arranged in plates that were 3 or more cells thick or if...
they showed severe anaplasia; they were considered non trabecular hepato cellular neoplasms when these features were absent (12). Lesions measuring less than 1 mm in diameter were considered microneoplasms; larger ones were referred to as macroneoplasms.

Quantitative Stereology

Areas of Tumor Profiles and Number and Volumes of Tumors. To ensure adequate sampling of tumor profiles (a profile is a 2-dimensional tracing or slice through a 3-dimensional object), they were identified in 3- or 5-μm-thick step serial sections through 1-mm-thick blocks of liver (between 2.6 and 5.2% of the total liver volume) taken from the left, median, right, and caudate lobes; at a minimum, every fifth section was available for study. However, in B6C3F1 mice killed at 36 weeks and in C3H mice killed at 30 weeks, where many profiles were readily available on single “face” sections from tissue blocks, it was not necessary to carry out the serial sectioning procedure.

In order to estimate the total number of neoplasms and their size distributions at each sacrifice time in the C57BL/6 and the C3H strains, a stereological method (5, 18) was applied to pooled profile data. In these two strains the lower size limit for reliable recognition of profiles was established as 122 μm in diameter; the comparable truncation point for the reconstructed B6C3F1 microneoplasms was 77 μm. In the latter strain, 3-dimensional reconstruction of all the neoplasms at 10, 20, and 28 weeks was as previously described (5).

Tumor Burdens. In the C3H and C57BL/6 strains, estimates of the tumor burden (in grams) were made by assuming that the specific gravity of the liver was one and multiplying the fractional cross-section area of liver occupied by neoplasms by the liver weights (17). In the B6C3F1 mice the tumor burden was determined by summing the volumes of the individual neoplasms at each time.

Tumor Cohorts (Size Determined). The mean size of B6C3F1 tumors (at 10 and 20 weeks) in a cohort of the largest neoplasms and in a cohort of small neoplasms was determined by respectively averaging the sizes of the 1st to 15th and the 101st to 115th largest tumors from the pooled data at each sacrifice time. However, since the “weighted” fraction of sampled livers (mean percentage of liver sampled multiplied by the number of animals) was 67% greater at 28 weeks than at 10 or 20 weeks, the means of the largest and small tumor cohorts at 28 weeks were derived from the 1st to 25th and the 101st to 125th largest tumors, respectively. An adjustment was not deemed necessary for comparable studies in the C3H and C57BL/6 mice, since the “weighted” fractions differed by less than 20% from one sacrifice time to the next in each of these strains.

The average size of the 15 largest C57BL/6 (12, 18, 30, and 42 weeks) and C3H neoplasms (12 and 18 weeks) was determined from magnified tracings of tissue generated from step-serial sections. To do this, all of the identified profiles belonging to the 15 largest tumors in each group of sacrificed mice were first identified and measured with computer-assisted planimetry (5); the size of each neoplasm was then estimated by applying a modification (see Equation A, below) of a stereological method to at least four sections through each tumor.

Improved Method for Estimating Sizes of Individual Neoplasms. Whereas the earlier method for estimating the sizes of individual neoplasms was constrained by the assumption that the shapes of the neoplasms approximated spheres (19), the new technique permits more variation, by allowing the shapes of neoplasms to be ellipsoids. The volume of each ellipsoid is estimated by integrating the area under the curve described by the equation

\[ y = -ax^2 + bx + c \]  \hspace{1cm} (A)

The parabolic curve represents the best fit to a second-order regression that is generated from a series of points in which each y is the measured cross-sectional area through the neoplasm, and each x value represents the distance of the section from a predetermined section in the series; the generated values for a, b, and c represent parameters that reflect the shape, size, and position of the lesions. In a study of 50 microneoplasms from the livers of C57BL/6 mice, killed at 42 weeks (data not shown), the volumes that were estimated by the parabolic method corresponded almost exactly to their actual volumes.

Statistical Analyses

For each strain, differences in tumor burdens between successive sacrifice times were evaluated by the two-sample Wilcoxon rank test using the Minitab Statistics Package (20).

RESULTS

The neoplasms of all three strains had orderly non trabecular patterns. None of the livers from any of the control animals contained neoplasms.

Tumor Growth Kinetics in B6C3F1 Mice. For comparing the three strains, the estimated number of neoplasms larger than 122 μm/liver and their aggregate volumes, at the various sacrifice times, are shown in Figs. 1 and 2. Using a lower truncation point (75 μm), the estimates of B6C3F1 tumors were 313 at 10 weeks, 922 at 20 weeks, and 1021 at 28 weeks (Fig. 3A). Only at the earliest sacrifice time did the neoplasms in the smallest size classes have a considerable effect on the aggregate tumor burden. In contrast, at 28 weeks, the 15 largest neoplasms, only 4% of the total sample, accounted for fully two-thirds of the total tumor burden.

The growth of tumor burden and of the tumor subsets was close to exponential over the entire 26 weeks of data collection; the doubling time of the aggregate tumor burden was 2.2 weeks (Fig. 4A). During the first 10 weeks of this period the mean of the largest tumor cohort had increased 37-fold in size (5.2 doublings), while the mean of the small tumor cohort increased only 11-fold (3.7 doublings). Accordingly, the growth rate of the largest tumor cohort was about 50% greater. Not surpris-

Fig. 1. Comparison of the estimated total number of neoplasms per liver, larger than 122 μm in diameter, in mice from the three strains, killed at various intervals after injection with DENA. The numbers were obtained by totaling the number of lesions estimated to be present in each of the bins (exclusive of bin number 1) in Fig. 3.

Fig. 2. Comparison of total tumor burdens, per liver, estimated to be contributed by the sum of all lesions for the three mouse strains.
Fig. 3. Histogram of the size distributions of the estimated number of neoplasms per liver in the three strains (A, B6C3F; B, C57BL/6; C, C3H), each at 3 or 4 sacrifice times. Each bin includes all lesions having 4 times the volume of the previous bin. Bin 1 contains all lesions with diameters between 0.077 and 0.122 mm. The largest bin (bin number 8) contained neoplasms with diameters between 1.91 and 3.13 mm. •, earliest sacrifice times (10 weeks for B6C3F mice and 12 weeks for C57BL/6 and C3H mice); □, second sacrifice times (20 weeks for B6C3F mice and 18 weeks for C57BL/6 and C3H mice); △, third sacrifice times (28 weeks for the B6C3F mice and 30 weeks for the C57BL/6 and C3H mice); ◇, fourth sacrifice time (42 weeks in the C57BL/6 strain).

Fig. 4. Comparison of the change in mean tumor volume for the largest tumor cohorts (○), with the aggregate tumor volumes (△) for the three strains (as in Fig. 3). For the B6C3F strain, data for the small tumor cohort (*) are also included. For purposes of comparison, the volumes at the earliest sacrifice time were normalized by assigning them the value of 1 (10^0). The actual tumor volumes at the earliest sacrifice times were: for B6C3F mice, 1.47 x 10^-6 g for the largest tumor cohort; 1.03 x 10^-7 g for the small tumor cohort; and 2.34 x 10^-5 g for the aggregate burden. For C57BL/6 mice, the aggregate burden of neoplasms was 3.8 x 10^-4 g, and the mean size of the largest tumor cohort was 3 x 10^-4 g. For C3H mice, the aggregate size of the neoplasms was 4.1 x 10^-3 g/liver, and the mean size of the largest tumor cohort was 1.0 x 10^-2 g. Bars, SD.

Table 1 Tritiated thymidine labeling indices in serially sectioned neoplasms from B6C3F mice, killed at 28 weeks after DENA

<table>
<thead>
<tr>
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<th>Outer 50 µm</th>
<th>Deeper 50 µm</th>
<th>Core</th>
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<tr>
<td>Mean ± SD</td>
<td>7.57 ± 3.40*</td>
<td>3.31 ± 1.69*</td>
<td>2.85 ± 1.61*</td>
</tr>
<tr>
<td>Ratio (average)</td>
<td>2.66</td>
<td>1.16</td>
<td>1.00</td>
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* P < 0.0001.

Fig. 4. Comparison of the change in mean tumor volume for the largest tumor cohorts (○), with the aggregate tumor volumes (△) for the three strains (as in Fig. 3). For the B6C3F strain, data for the small tumor cohort (*) are also included. For purposes of comparison, the volumes at the earliest sacrifice time were normalized by assigning them the value of 1 (10^0). The actual tumor volumes at the earliest sacrifice times were: for B6C3F mice, 1.47 x 10^-6 g for the largest tumor cohort; 1.03 x 10^-7 g for the small tumor cohort; and 2.34 x 10^-5 g for the aggregate burden. For C57BL/6 mice, the aggregate burden of neoplasms was 3.8 x 10^-4 g, and the mean size of the largest tumor cohort was 3 x 10^-4 g. For C3H mice, the aggregate size of the neoplasms was 4.1 x 10^-3 g/liver, and the mean size of the largest tumor cohort was 1.0 x 10^-2 g. Bars, SD.

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The volumes of the lesions were determined by reconstruction; they included 19 microneoplasms and 2 macroneoplasms. In the 9 lesions measuring less than 500 µm in diameter, the cores included all cells other than those in the outermost 50-µm "shells." The distribution of tritiated thymidine-labeled cells was determined by autoradiography on a group of 21 randomly selected neoplasms from two mice killed at 28 weeks after DENA (Table 1). A steep gradient was apparent with the mean labeling in the outer 50-µm-thick (2-cell-thick) shells more than twice that in the next deeper "shells" or in the "cores" of the neoplasms.

Tumor Growth Kinetics in C57BL/6 Mice. In this strain the estimated numbers of tumors (larger than 122 µm), 100 at 12 weeks, 256 at 18 weeks, 483 at 30 weeks, and 537 at 42 weeks (Figs. 1 and 3B), were rather similar to those for the B6C3F mice but considerably less than those for the C3H mice. Between 12 to 18 weeks, the doubling time of the aggregate tumor burden, 2.1 weeks (Figs. 2 and 4B), was similar to that of the two other strains. However, tumor growth began to slow after 18 weeks, and between 30 and 42 weeks the doubling time had increased to 13 weeks. Again, as in the case of the B6C3F mice, the shape of the growth curve of the largest tumor cohort approximated, but was slightly higher than, that of the fractional tumor volume. The tumor burdens at 30 to 42 weeks in C57BL/6 mice were the only two sequential estimates, among all three strains, whose values did not differ significantly from...
change of the tumor burden from the 12th to the 18th weeks, were highest in this strain (Figs. 1 and 3C). The rate of change of the tumor burden from the 12th to the 18th weeks (Figs. 2 and 4C; doubling time of 2.45 weeks) was very similar to that in the B6C3F1 mice and did not decline between 18 and 30 weeks; again, as in the two other strains, the cohort of largest tumors increased at a greater rate (between 12 and 18 weeks) than the aggregate tumor burden.

DISCUSSION

Results of this study highlight the factors that contribute to the perception of "carcinogen resistance" in C57BL/6 mice. The single most important finding was an impairment in the growth of hepatocellular neoplasms that was unique to this strain. Significantly, the growth rates of the tumors in the C57BL/6, the C3H, and the B6C3F1 strains were all similar during the earliest interval (12 to 18 weeks after DENA). However, beginning between 18 and 30 weeks, and becoming most prominent between 30 and 42 weeks, there was a progressive slowing in the growth of the C57BL/6 neoplasms; during these successive intervals, the growth rates of the neoplasms declined by more than 80%. Since we found that the number of growth-retarded cells containing secretory protein inclusions also increased during these periods (12), it appears reasonable to suggest that the impaired growth of neoplasms in C57BL/6 mice is related to the cellular defect in protein secretion.

In a recent report, Hanigan et al. (9) also observed that microneoplasms in C57BL/6 mice grew more slowly than in C3H mice. However, they did not observe the progressive nature of the slowing. While differences in diet could explain part of this discrepancy (the diet that they used contained more fat and less protein), differences in analytical methods are probably more important. For example, the authors expressed their results as first-order regressions, based on pooling the data from two different experiments; this method of analysis would not reflect changes in growth rates even if they did occur. Again, Hanigan et al. completed their study of C57BL/6 mice after 32 weeks, while we assessed C57BL/6 tumor growth for 42 weeks. This 10-week difference could be especially important, since we observed maximum slowing during this interval. Finally, the more rapid early growth of the C57BL/6 tumors that we observed could have been obscured by the manner in which the authors plotted the rate of change in the mean size of neoplasms. The mean size was calculated by dividing the estimated total tumor burden by the estimated number of tumors (itself derived from random profile data). The emergence, with time, of more and more identifiable (but very small) tumors would artifactually lower the estimated average sizes of the neoplasms needed for comparison. In their study, this effect was greatest during the earliest interval (12 to 18 weeks), when the emergence of quantifiable tumors was most rapid. In fact, in the C57BL/6 strain, they found that the number of neoplasms larger than the truncation point (135 \( \mu \)m in diameter) increased 8-16-fold during this time. In contrast, in the present study, by following the change in size of a cohort of the largest tumors, the growth kinetics of the most life-threatening subset of tumors was evaluated.

While tumors grew more slowly in the C57BL/6 than in the C3H and B6C3F1 mice, they were more numerous in the C3H mice than in the two other strains. This additional difference was apparent at all of the times when growth rates in the strains were similar. Thus, there was no need to consider the possibility that the inequality might reflect an artifact resulting from the retarded outgrowth of neoplasms in the C57BL/6 mice (9).

Taken together, the findings are consistent with a polygenic model for mouse hepatocarcinogenesis (6) in which the determinants for two traits, rate of tumor growth and number of initiated tumor cells, are responsible for the perception of strain-dependent variability in carcinogen responsiveness. Thus, the data indicate that the growth impairment of hepatocellular neoplasms in C57BL/6 mice, hypothesized to result from the early appearance of secretory inclusions, is a recessive trait that is not expressed in the hybrid mice. Similarly, regardless of whether one or more genes are responsible for the large number of tumors in C3H mice, it appears that they are unexpressed in the B6C3F1 mice and are therefore recessive to those responsible for inducing smaller numbers of tumors.

Other results of this study have more general relevance to neoplastic growth kinetics. For example, one of the unexplained features of the microneoplasms in the infant mouse model, already commented on, is the great variation in tumor size; within 6 months of injecting DENA the tumors ranged in volume through more than 3 orders of magnitude (5). Two simple growth kinetic models could explain this diversity (21). The first, referred to as the "random rate" model, assumes that all neoplasms start to grow at the same time but with different growth rates. The second, the "random delay" model, assumes that all tumors have the same growth rate, but the tumors commence their growth at different times.

Our results support the random rate rather than the random delay model. The evidence was strongest in the B6C3F1 mice, where all of the sampled tumors were measured directly from serial section reconstructions. Here, the cohort of the largest tumors showed a 50% higher rate of growth than a comparable cohort of small tumors. Additionally, in the two other strains the largest tumor cohort grew at a rate exceeding that of the total tumor burden. Since the latter parameter is "diluted" by the smaller, more slowly growing tumors, it is not surprising that aggregate tumor growth was slower. Of course, in order to carry out these analyses, it was assumed that, among a very large number of neoplasms, arranged according to size, cohorts of 15 to 25 tumors would, over time, maintain their mean rank order. The assumption seems reasonable even though it is known that the growth of neoplasms may fluctuate somewhat, apparently in response to changes in the hosts' nutritional or hormonal status (22, 23).

A second type of growth heterogeneity, also with potential relevance for other types of tumors, was demonstrated within the individual mouse hepatic neoplasms. Rather surprisingly, a very sharp gradient in thymidine labeling indices was demonstrated in B6C3F1 microneoplasms; the concentration of labeled cells in the outer 50-\( \mu \)m shells was more than double that in the next, deeper layer of cells. It is noteworthy that many transplantable neoplasms often grow more slowly as they enlarge (24). This phenomenon, mathematically characterized as Gompertz kinetics (25), is primarily a result of ischemia, which leads to growth retardation (26) and ultimately necrosis of cells that are deep within neoplasms. While analogous gradients of growth, reflected by the pattern of thymidine labeling, have been observed in artificially aggregated microscopic tumor...
spheroids (27), the present report is the first documentation of replicative gradients in autochthonous microscopic neoplasms. Of course, as indicated earlier, variation in thymidine labeling within individual tumors was also observed in microneoplasms of the C57BL/6 mice (12). However, in that case the randomly distributed clusters of growth-retarded cells would probably have obscured the zonal gradients.

The impressive variation in tumor size in the neonatal mouse liver tumor model could have important implications for the use of the B6C3F1 mouse in standard carcinogenic bioassays (7). Earlier, we reported that a 10-fold increase in DENA, from 5 to 50 mg/kg, resulted in a 3.7-fold increase in the number of tumors, but the distribution of tumor sizes, reflecting their different growth rates, was not changed by the higher dose (28). However, to our knowledge, there is no comparable information on how this distribution might be altered by different classes of carcinogens. Since the duration of tumor latency is an important factor in assessing carcinogenic bioassays (29), which is dependent on the absolute number of induced neoplasms and the distribution of their growth rates, future experiments should also evaluate this latter parameter when attempting to develop meaningful human risk assessments (30).

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