Genetic Variation in the Proliferation of Murine Pulmonary Type II Cells: Basal Rates and Alterations following Urethan Treatment

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ABSTRACT

Susceptibility to urethan-induced pulmonary tumorigenesis varies among inbred strains of mice. A genetic basis for this variation was sought using three strains with widely differing tumor multiplicities after urethan treatment. Twenty-one mice from each of strains A/J (high susceptibility), BALB/cByJ (intermediate susceptibility; hereafter called cBy), and C57BL/6J (low susceptibility; hereafter called B6) were treated i.p. with 1 mg urethan/g body weight, and sacrificed at 0 (no urethan), 12, 24, 36, 48, 65, and 80 days after treatment (three mice per strain per time point). Each mouse was given 1 μCi [ethyl-3H]thymidine/g body weight 45 min before sacrifice. Lungs were processed for autoradiography, and labeling indices were independently determined for non-tumor-associated type II cells and for tumor cells (most tumors arise from alveolar type II pneumocytes in A/J mice). Three categories of proliferative differences were found. First, statistically significant differences (P < 0.05) among all strains were found for type II cell labeling indices in untreated mice, and these differences persisted for 65 days after urethan treatment. Proliferative rates were highest in A/J mice and lowest in B6 mice, while cBy mice were intermediate. Secondly, the peak of type II cell labeling occurred 12 days following urethan in strains A/J and cBy, but at 24 days in B6 mice. This difference is consistent with the fact that tumors were observed earlier following urethan treatment in A/J and cBy mice (at 36 days) than in B6 mice (at 48 days). Finally, the labeling indices in A/J and B6 tumors were high at first (6 and 4%) and then declined to 1–1.5% by 80 days after urethan treatment, while cBy tumor labeling indices remained at about 1.5% throughout the experimental period. These results suggest that the variation in susceptibility to urethan-induced lung tumorigenesis among different strains of mice is related to the normal basal rates of lung mitoses in these strains. Mice may be particularly sensitive to urethan during cell division, making strains with a higher rate of mitosis more susceptible to tumorigenesis.

INTRODUCTION

The discovery by Nettleship and Henshaw (1) that urethan (ethyl carbamate) treatment stimulated pulmonary adenoma formation in mice has led to the use of this compound as a model inducing agent. Of the several cell types in the alveolar wall, the type II pneumocyte has been shown to be the target cell for this carcinogen (2). Type II cells secrete surfactant, but also function as stem cells for the replacement of type I cells which do not proliferate (3). Clara cells serve a similar stem cell function in the bronchioles (4), and are also a cell of origin of lung adenomas (5). Both cell types exhibit a low LI* in adult mice (2).

These cells proliferate selectively in response to urethan to form reproducible numbers of adenomas in a given strain of mice. Strain A/J is highly susceptible, with a tumor multiplicity of 20–25 tumors/mouse 14–16 weeks after a 1-mg/g body weight dose of urethan (6). BALB/cBy mice (hereafter called cBy) have an intermediate susceptibility (1–2 tumors/mouse) and strain C57BL/6J (hereafter called B6) exhibits a low susceptibility (<1 tumor/mouse) at this same urethan dose (7).

Both type II and Clara cell-derived tumors can develop within a single mouse, and strains vary in the relative proportions of these two tumor types (8). A/J and cBy mice have few Clara derived tumors and differ mainly in the number of alveolar type II-derived tumors (8); a single gene is apparently responsible for this difference (7). Multiple genes control susceptibility to carcinogens. By analysis of recombinant inbred strains of mice derived from A/J and B6 mice it was shown that three Pas (pulmonary adenoma susceptibility) genes are responsible for the different susceptibilities of the progenitor mice, with one gene, designated Pass-1, having a major effect (9).

Although genetic differences in lung tumorigenesis have been known for many years (10), the mechanisms of these genetic differences are not understood. One hypothesis is that there are inherent differences among these strains in the tendency of type II and Clara cells to proliferate. Or, the tumor cells themselves may display differing cell kinetics among these three strains. We examined these hypotheses.

MATERIALS AND METHODS

Sixty-three female, 8-week-old mice were used in this study. Twenty-one mice from each of strains A/J, cBy, and B6 were obtained from The Jackson Laboratory (Bar Harbor, ME), and housed in our animal facility according to NIH guidelines for the care and use of laboratory animals. All mice received Wayne Lab Blox and water ad libitum, and were exposed to a 12-h light, 12-h dark cycle throughout the experimental period.

Urethan (Sigma Chemical Co., St. Louis, MO) was dissolved in 0.9% NaCl and administered i.p. (1 mg/g body weight) to 18 mice from each strain. The remaining mice (controls) received only the NaCl vehicle (10 μl/g body weight). Forty-five min prior to sacrifice, each mouse was given an i.p. injection of 1 μCi [ethyl-3H]thymidine (20 Ci/mmol; New England Nuclear, Boston, MA)/g body weight. Three mice from each strain were sacrificed by cervical dislocation at 12, 24, 36, 48, 65, and 80 days after urethan treatment. Their lungs were removed and immersed in Bouin's fixative for 1 week, then dehydrated and embedded in paraffin; 5 μm sections (200 from each mouse) were mounted on glass slides, dipped into NTB-2 emulsion (Eastman Kodak Co., Rochester, NY), exposed for 10–14 days at ~70°C, developed in Dektol, and stained with hematoxylin and eosin.

Counting of labeled cells (3 or more photographic grains over a nucleus) was done on 2 fields of 100 cells, using a square eyepiece graticule at a magnification of ×400, on each of 15 sections from each lung. The sections represented 60–μm steps through the lung so that a significant portion of each lung was examined. Quantitation of labeled cells in both bronchioles and alveoli was attempted, but autoradiographic grains were found in sufficient numbers for meaningful measurement only in alveolar cells. A total of 3000 alveolar wall cells was counted for each mouse. Micropscopic fields containing bronchioles and large blood vessels were avoided while counting labeled alveolar cells. The alveolar wall cells examined included type I and type II pneumocytes, macrophages, sepal cells, and endothelial cells (Fig. 1). Relative proportions of labeled cell types were determined by classifying 300 labeled cells from each control or experimental group (100 cells/animal) into one of the following four categories: macrophages (large, irregular
nuclei and nonvacuolated cytoplasm); type II pneumocytes (rounded nucleus and vacuolated cytoplasm); type I pneumocytes, endothelial cells, and septal cells; and labeled cells which were free in the alveolus. Differential counts were done on control groups and on experimental groups at times surrounding the peak LI (see "Results"). The number of labeled cells/3000 cells counted represented the LI for non-tumor-associated alveolar cells in all groups. All 100-cell fields were used, including those with no labeled cells, in the determination of the LI. These data were then multiplied by the relative proportions of type II cells in each group (based on the differential counts), resulting in the type II cell LI. This LI was determined for each animal, then the results from the three animals in a group were combined for determination of group means and for statistical comparisons among groups.

An additional six animals, two from each strain, were used to determine the relative numbers of macrophages which could be obtained by lavage. Animals were sacrificed and two lavages with phosphate-buffered saline were done to remove unattached lung cells. Cells were counted using a hemocytometer, and the total number of lavaged macrophages was determined for each strain.

All microscopic sections were examined for the presence of tumors. The slide containing the largest cross-sectional profile of each tumor (the section nearest the center of the tumor) was used to determine the LI for tumor cells. The number of labeled cells/100 tumor cells represented the LI for lung adenomas in each group. Mean values were compared among strains and among times after urethan administration for each group using a one-way analysis of variance, with \( P < 0.05 \) as the minimum acceptable level for the establishment of statistically significant differences.

### RESULTS

Microscopic tumors, defined here as concentrated cell masses (10–15 or more cell diameters across) with clearly recognizable borders, exhibited labeled cells in a random distribution within each tumor. No lung areas containing high concentrations of labeled cells were observed in this study. There was no indication of detectable mitotic foci (11) or radiographic "hot spots." This was true for both non-tumor-associated alveoli and for tumors.

Type II pneumocytes constituted the largest group of labeled cells in animals of all strains. Fig. 2 summarizes the relative proportions of the various labeled cell types in these autoradiographic samples. It is readily apparent that there are no differences in the relative distribution of cell types in the labeled cell populations among experimental groups. Type II cells represented approximately 50% of the labeled cells at all times following urethan administration in each strain. There were also no differences among strains in the number of macrophages recovered by lavage. The range of macrophage counts was 5.2–7.5 \( \times 10^4 \) cells/animal with no significant differences between animals or among strains.

Table 1 is a summary of the LI for type II pneumocytes. The control animals, which received no urethan, exhibited significant differences in LI among the three strains \( (P < 0.05) \), the relative order being A/J > cBy > B6. These differences were maintained between strains A/J and cBy through day 65 posturethan. The data for strain B6 were more variable, as shown by the higher SE, than for the other two strains. Peak labeling in
Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Control</th>
<th>12</th>
<th>24</th>
<th>36</th>
<th>48</th>
<th>65</th>
<th>80</th>
</tr>
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<tr>
<td>A/J</td>
<td>15.2 ± 1.3&lt;sup&gt;a&lt;/sup&gt; (13-18)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31.4 ± 8.7&lt;sup&gt;c&lt;/sup&gt; (17-47)</td>
<td>12.7 ± 2.2&lt;sup&gt;d&lt;/sup&gt; (8-15)</td>
<td>7.8 ± 2.8 (3-13)</td>
<td>7.7 ± 0.3&lt;sup&gt;e&lt;/sup&gt; (7-8)</td>
<td>7.1 ± 0.1&lt;sup&gt;f&lt;/sup&gt; (7-7)</td>
<td>5.3 ± 0.7&lt;sup&gt;j&lt;/sup&gt; (4-6)</td>
</tr>
<tr>
<td>cBy</td>
<td>9.4 ± 1.8 (8-11)</td>
<td>17.1 ± 1.4&lt;sup&gt;c&lt;/sup&gt; (14-19)</td>
<td>5.9 ± 1.3 (4-9)</td>
<td>4.5 ± 0.7 (3-6)</td>
<td>4.7 ± 1.1 (3-7)</td>
<td>3.5 ± 0.9 (2-5)</td>
<td>7.7 ± 1.9 (4-11)</td>
</tr>
<tr>
<td>B6</td>
<td>5.4 ± 1.8 (4-7)</td>
<td>4.9 ± 2.0 (1-7)</td>
<td>17.6 ± 4.1&lt;sup&gt;j&lt;/sup&gt; (13-26)</td>
<td>5.3 ± 3.0 (2-13)</td>
<td>14.1 ± 5.2 (6-24)</td>
<td>4.6 ± 1.0 (3-6)</td>
<td>4.5 ± 1.7 (2-8)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± SE.
<sup>b</sup> A/J, cBy, and B6 differ significantly from each other at these times (P < 0.05).
<sup>c</sup> Peak indices differ significantly from other time points within each strain (P < 0.05).
<sup>d</sup> A/J and cBy differ significantly (P < 0.05).
<sup>e</sup> Significantly lower than control (P < 0.01).
<sup>f</sup> Numbers in parentheses, range.

non-tumor-associated alveoli from B6 lungs was 12 days later than for A/J and cBy. Both cBy and B6 LIIs returned to their control values by the end of the experimental period; cBy mice showed a decrease between days 24 and 65 and a return to normal by day 80 posturethan. The LI for A/J mice remained lower from day 36 through the end of the experimental period than that found at day zero.

The LIS within tumors are summarized in Table 2. All tumors observed in this study had a similar morphological appearance of uniform and compact cellularity and exhibited no central necrosis. Morphological differences which distinguish type II-derived from Clara-derived tumors 2 to 16 months after chemical induction (5, 8) were not evident in any of these early tumor specimens. Corresponding with previously reported susceptibilities of these strains to urethan, many more tumors were found in A/J lungs than in cBy, and very few were observed in lungs from B6 mice. The first appearance of tumors in the mice came 24 days after the peak LI for each strain. Thus, tumors were first observed in A/J and cBy mice at 36 days posturethan, and in B6 mice at 48 days.

Percentage of labeling of tumor cells was consistently higher than for nontumor-associated type II cells in all three strains. Labeling in tumors was 7 to 25 times that of the non-tumor-associated type II cells in each strain. cBy tumors showed a constant LI which was lower than that for A/J or B6 tumors at all times observed. In spite of the differences in tumor multiplicity, A/J- and B6-derived tumors exhibited parallel and sharply decreasing LIs which were almost identical. The decrease in LI for A/J tumors between days 36 and 48 and between days 48 and 65 is highly significant in each case (P < 0.001).

Sufficient numbers of tumors were not available in B6 mice to establish statistically significant differences between time points.

DISCUSSION

Our results have shown a significant difference in type II cell LI among three strains of mice which differ in their susceptibility to urethan-induced tumorigenesis. These LIs were determined in non-tumor-associated alveolar areas, and the tumor cells were analyzed separately. This proliferative difference among strains exists in both control mice and in those treated with urethan. Type II pneumocytes, theputative cell of origin of many murine lungadenomas, represented 50% of the labeled cell population in all groups. Type II cells have been shown to have an LI of 0.3% (rats; Ref. 12) and 0.36% (mice; Ref. 13).

The LIs reported for these cells for a study of another susceptible strain (randomly bred Swiss-Webster mice). The control LI was nearly identical with those in our study. The timing of the peak LI in Kauffman's study was delayed by 3 to 4 weeks and reached a higher level, possibly due to the extended time over which the carcinogen was administered (in drinking water) compared with a more concentrated bolus injection in our study. In another study of alveolar cell mitotic rates, Kauffman (15) measured both the LI with [H]thymidine and the mitotic index after colchicine in Swiss-Webster mice treated with a single dose of urethan. Elevated values for these two indices were found at 7–14 days, corresponding with the peak LI found for A/J and cBy mice in our study. Type II cells, then, respond similarly to urethan under these different treatment regimens in strains which are susceptible to its tumorigenic effects.

Our study is the first to report a significant difference in LI in normal type II cells in vivo in strains of mice differing in their susceptibility to urethan. Kolesnichenko and Gorkova (16) reported a significantly higher LI in strain A than in C57BL (strains not specified) in an organ culture study of lung explants. Simnett and Heppleston (17) determined lung cell kinetics in mice of strains A/Grs<sup>a</sup> and A/J (the latter two substrains not specified), and found no statistically significant differences in the LIs among these three strains, although the mitotic index was significantly higher for A than for C57BL mice. The LIs they reported are higher than those found in our study, but they investigated total alveolar wall cells and did not determine the relative proportions of various labeled cell types.

Percentages of different cell types in the labeled population can vary greatly. For example, Kauffman (15) found 12% of labeled alveolar cells to be type II pneumocytes while our study showed...
50% of labeled cells to be type II, yet the type II LIs were similar in both studies. Shimkin et al. (18) studied LIs in mice of three strains with varying susceptibility to urethan. Their results do not reveal a significant difference in normal alveolar cell LI among strains A/He, C3H, and C57BL (substrains not specified), but they do show some difference in cell kinetics which correlated with tumor susceptibility. Peak LIs were reached at the same times for the A/He and C57BL strains as we found with A/J and B6 strains. Dyson and Heppleston (19) studied the LI of both tumor and alveolar cells in A2G mice. Their alveolar LI varied from 2.0% (peak at 2 weeks) to 0.7%, which parallel our results with A/J mice very closely. The LI for tumor cells in their study decreased from 6.0% (peak at 4 weeks) to 1.5%, again corresponding very closely with our results in A/J mice. No strain comparison was made in that study.

Simnett and Heppleston (17) found a reduced LI with increasing age in all strains tested. This may explain our finding that A/J mice (and the other strains to a more limited extent) did not return to control levels but were actually lower after the 3-month experimental period.

DeMunter et al. (20) measured LI in lung and liver from GRS/A mice (highly susceptible to lung tumorigenesis but not to liver tumor induction) and C3Hf/A mice (opposite susceptibilities from GRS/A mice) after treatment with dimethylnitrosamine. The LI for lung was greatly increased in GRS/A but not in C3Hf/A mice after treatment with the carcinogen, while liver cells had the opposite labeling pattern. Our study shows not only that the specific target tissue (lung) has an increased LI after treatment with a carcinogen (corresponding with results by DeMunter et al., cited above), but also that the degree of increase is related to the relative susceptibility of the specific strain of mouse to tumorigenesis in this tissue. Yet another system which shows a correspondence between LI and susceptibility to carcinogenesis is the colon. Deschner et al. (21) have demonstrated that tumor multiplicities in the colon, after dimethylnitrosamine treatment, correspond with the normal proliferative rates of colonic cells among inbred mouse strains.

The growth rate of tumors, as revealed by tumor cell LI, did not completely parallel the relative susceptibilities of the strains to tumorigenesis in our study. Both A/J and B6 mice show a high initial tumor cell LI, but these strains have opposite susceptibilities. By mice, with an intermediate responsiveness to urethan, exhibited a constant LI in tumor cells throughout the experimental period. These data indicate that while susceptibility to urethan-induced tumorigenesis is related to the normal LI in these three strains, the growth of the tumors after induction is not. Mitosis in tumor cells is apparently regulated by a more complex mechanism than a simple increase in the normal alveolar cell mitotic rate. Complexity of the control of neoplastic growth in the lung is suggested by previous work in this laboratory (9) which revealed that at least three genetic loci influence tumor formation in A/J and B6 mice. The control of the normal proliferative rate of alveolar type II pneumocytes is but one of the mechanisms underlying strain differences in urethan carcinogenesis.

ACKNOWLEDGMENTS

We thank R. Dean Pecotte and James Dunlap for their excellent technical assistance, Dr. Emil Skamene for pointing out related studies on colon carcinogenesis, and Dr. Shirley L. Kauffman for a valuable critique of the manuscript.

REFERENCES

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