Alteration in Cell Proliferation in Mouse Lung Following Urethane Exposure

II. Effects of Chronic Exposure on Terminal Bronchiolar Epithelium

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Cell proliferation in bronchiolar epithelium of normal mouse lung and of lung chronically exposed to urethane was compared. Animals treated for 10 weeks with drinking water containing 0.1% urethane and their controls were injected with ^{3}H-thymidine; autoradiographs were made of lung tissue and mean grain counts of labeled nuclei in terminal bronchiolar epithelium compared in the control and urethane-treated groups. In controls, the decline in mean grain counts of labeled daughter cells between 24 hours and 4 days after ^{3}H-thymidine injection was compatible with a mean cell cycle time of about 60 hours. In contrast, the hyperplastic bronchiolar epithelium of the urethane-treated animals showed an elevated labeling index and deficient production of labeled cells over the 6-day period as judged by decline in mean grain counts. These findings were interpreted as showing that the hyperplasia and high labeling index did not represent faster turnover or shortened cycle but reflected an increase in cell cycle time or of population renewal time. (Amer J Path 64:531–540, 1971)

A SINGLE EXPOSURE of mice to urethane has been shown to give rise to lung adenomas [1] consisting of type 2 alveolar epithelial cells.[2] Although continuity between terminal bronchiolar epithelium and the peripheral adenomas was described occasionally,[3,4] serial sections of many tumorous lungs showed only a rare connection between them,[5] supporting the view that adenomas arose from type 2 epithelium alone, and not from extensions of the hyperplastic bronchiolar epithelium.[4,6]

A close functional relationship between the nonciliated cells of the terminal airways, Clara cells and the type 2 epithelium is suggested by the reports that either the Clara cells [7] rather than the type 2 cells produce surfactant [8] or that together [9,10] they form a unit for surfactant production.

In the present studies on the effects of urethane on cell proliferation in the lung, attention is directed toward the hyperplasia of bronchiolar

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epithelium; studies of the type 2 cell will be the subject of a later report. Principally, an attempt is made to outline the cycle time of dividing cells in bronchiolar epithelium of normal mice and compare this with the cycle time in hyperplastic epithelium of mice chronically exposed to urethane. For this purpose, animals were injected with ^{3}H-thymidine and autoradiographic grain counts of bronchiolar epithelial nuclei were made between the first and second division cycle after exposure to ^{3}H-thymidine.

Materials and Methods

Thirty-six Swiss-Webster mice (O'Grady, Bronx, NY) were divided into control and experimental groups. The test group received 0.1% urethane (ethyl carbamate) (Fisher Scientific, Springfield, NJ) in their drinking water; control animals received tap water. All animals were injected with 1 μCi ^{3}H-thymidine/g body weight (3 Ci/mmole; New England Nuclear, Boston, Mass) in the tenth week and subsequently killed in groups of 3 control and 3 test animals at each of the following times after ^{3}H-thymidine injections: 30 minutes, 24 hours, 1, 2, 3, 4, 5 and 6 days. Small sections of lung were fixed in osmium tetroxide and embedded in Epon. The remainder of the lung was fixed in acetic–alcohol for 1 hour, then transferred to 10% formalin–saline for 24 hours. Specimens of these lungs were vacuum-embedded in paraffin, cut at 3 μ and the histologic sections mounted on albumin-coated slides and dipped in NBT2 emulsion (Eastman Kodak) and exposed for 21 days at 4 C. Epon-embedded sections were coated with NBT2 emulsion and exposed for 30 days at 4 C. After development with Dektol, paraffin sections were stained with methyl green–thionin, and Epon sections, with toluidine blue.

A minimum of 1500 cells were counted from sections of terminal and respiratory bronchioles from each animal. In order to obtain a more homogeneous population and to avoid small bronchi, only those cells were counted that appeared in longitudinal sections of the terminal bronchiole as it opened into the respiratory bronchiole. Observations included the total number of nuclei and counts of grains over labeled nuclei. A nucleus covered with three or more grains was considered labeled. The labeling index (LI) was the number of nuclei labeled divided by the total number of nuclei, expressed as a percent.

In calculating the standard deviation of the labeling indices, each animal was considered one observation. Mean grain counts were determined from pooled counts of 50–100 labeled cells from 3 animals of each group. Autoradiographs of Epon-embedded material were used to determine the frequency of cell types in bronchiolar epithelium.

Observations

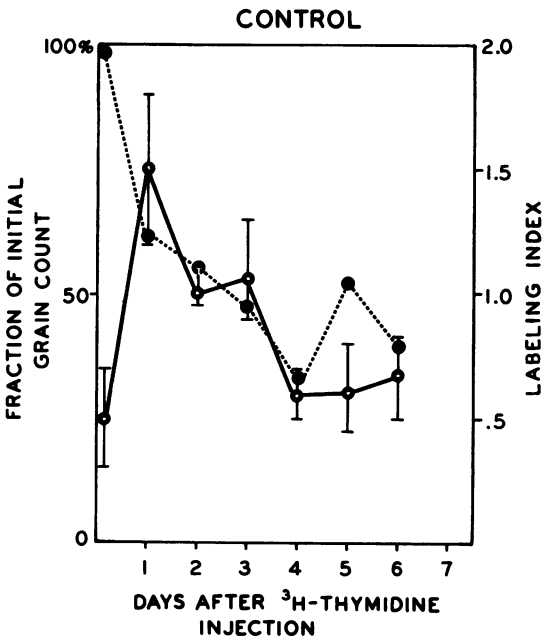
None of the lungs contained grossly recognizable tumors; microscopically, however, all of the experimental animals had small tumors (Fig 1). A striking difference between lungs of control and experimental animals, in addition to the tumors, was the hyperplasia of bronchiolar epithelium in the latter, where the cells often extended into the respiratory bronchioles (Fig 2). Comparison of the relative frequency of the cell types in the 2 groups showed that, in both,

the frequency of ciliated and nonciliated epithelial cells was approximately the same (Fig 3). Cells with labeled nuclei were frequently seen adjacent to the basement membrane of the bronchioles. Occasionally, migrating labeled cells could be recognized; these were believed to come from the collections of mononuclear cells adjacent to the bronchioles, which have a high labeling index.[11]

Labeling Indices and Mean Grain Counts in Controls

The mean labeling index (Fig 4) 30 minutes after injection of ^{3}H-thymidine was 0.52% ± 0.24%. It rose to 1.53% ± 0.39% at 24 hours and then declined to 1.00% ± 0.02% at 48 hours. A plateau of the labeling index at approximately 1.0% was maintained on the second and third days and then it declined between days 3 and 4 and stayed in the range of 0.6% for the remainder of the experiment.

Counts of the number of grains per labeled nucleus (Table 1 and Text-fig 1) showed a mean of 29.34 grains per labeled nucleus in animals killed 30 minutes after injection of ^{3}H-thymidine. Twenty-four hours later, the mean grain count was 18.24; it declined to 16.05 on the second day, 13.59 on the third day and 10.09 on the fourth day. The mean grain count then increased to 15.60 on the fifth day and was 12.29 on the sixth day.



TEXT-FIG 1—Comparison of the mean grain count and labeling index in terminal bronchioles at intervals after injecting ^{3}H-thymidine into control animals. Abscissa: days after injection with 1 μCi/g body weight. Ordinate: *dotted line,* fraction of initial mean grain count; *solid line,* percent of labeled nuclei.

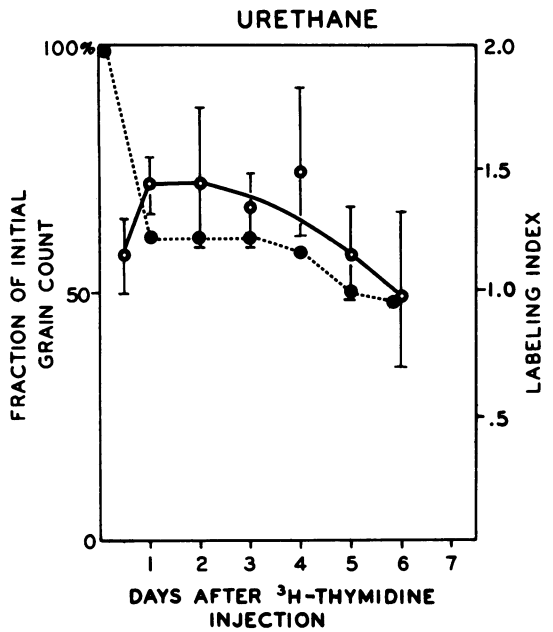
Table 1—Mean Grain Counts

Time after ^{3}H-thymidine injection	Mean grain count ± SD	
	Control	Urethane
30 minutes	29.34 ± 13.67	28.51 ± 13.60
24 hours	18.24 ± 12.75	17.22 ± 11.33
2 days	16.05 ± 8.92	17.33 ± 9.71
3 days	13.57 ± 9.24	17.44 ± 9.87
4 days	10.09 ± 7.43	16.40 ± 8.56
5 days	15.60 ± 15.64	14.66 ± 8.94
6 days	12.29 ± 7.55	14.09 ± 8.15

Labeling Indices and Mean Grain Counts in Animals Exposed to Urethane

The labeling index (Text-fig 2) was 1.16% ± 0.17% at 30 minutes after ^{3}H-thymidine injection and rose to 1.44% ± 0.08% at 24 hours. After remaining essentially unchanged for 4 days, it decreased somewhat between the fifth and sixth days.

The mean grain count 30 minutes after injection of ^{3}H-thymidine was 28.51 grains per labeled cell. It declined to 17.22 by 24 hours and remained in this range for 3 days; then, it began a slow decline, reaching 14.09 on the sixth day.



TEXT-FIG 2—Comparison of mean grain count and labeling index in terminal bronchioles at intervals after injecting ^{3}H-thymidine into animals drinking 0.1% urethane for 10 weeks. Abscissa: days after injection with 1 μCi/g body weight. Ordinate: *dotted line,* fraction of initial mean grain count; *solid line,* percent of labeled nuclei.

Proportion of Labeled Paired Cells

Thirty minutes after injection of ^{3}H-thymidine, no labeled nuclei were found adjacent to one another; however, 24 hours later, 36.6% of labeled bronchiolar epithelial cells in control animals, and 31.7% in test animals were paired. In control animals, the proportion of pairs increased to 52.3% on the second day and then declined. In animals exposed to urethane, the proportion of pairs increased to 37.9% on the second day, and remained at 32.9% for the third and fourth days.

Discussion

Autoradiographic studies of respiratory epithelium are difficult to interpret because of several factors: only a small proportion of cells are labeled by ^{3}H-thymidine; the various cell types that divide (granular, Clara and basal cells) may have different cycle times; and the differentiated, ciliated cells have undertermined life spans. Finally, labeled DNA is reutilized on the fourth day after injection of ^{3}H-thymidine [12] and possibly later.[13]

Reutilization of Thymidine-Labeled DNA

Baserga [12] demonstrated a sharp increase in the amount of tritium per gram of lung tissue on the fourth day after injection of ^{3}H-thymidine; he believed this increase could be due to the reuse of labeled tritiated DNA released from dead cells. Steel and Lamerton,[13] using liquid scintillation counting of lung tissue containing ^{3}H-thymidine, found a plateau of activity for 3 days after injection, followed by a steep decline of 3 days' duration. No further loss of tritium could be detected as long as 8 weeks later. In addition to reutilization of label, the migration of labeled cells was also suggested as an explanation for this phenomenon. In the present experiments, the decline in labeling index and mean grain count in controls lasted for 4 days. On the fifth and sixth day, the mean grain count increased and the labeling index leveled off. While the increased grain count might be explained by reutilization of DNA labeled with ^{3}H-thymidine, labeled cells were occasionally seen entering the respiratory epithelium from peribronchiolar cell collections, so it seems that migration of labeled cells through the epithelium could also account for the observed changes.

Cell Cycle in Normal Terminal Bronchioles

In adult respiratory epithelium, a steady state [14] is assumed, in that for each cell that divides, one cell will leave the dividing population, thus maintaining constancy of cell numbers. The expectation is that all cells incorporating ^{3}H-thymidine into DNA will divide within 24 hours;

the labeling index will double and the mean grain count will decrease by half. Upon completion of the first division after labeling, only half of the labeled daughter cells would subsequently divide and half would differentiate, the latter maintaining their grain count as it was at 24 hours. Division of this second generation, then, would decrease the mean grain count to two-thirds of its value at 24 hours.

The decline of almost 50% in the mean grain count over the 24-hour period after injection of ^{3}H-thymidine suggests that cells that did incorporate ^{3}H-thymidine divided over this period. Taking the mean grain count at 24 hours as a reference point for the end of the first division, the mean grain count would be expected to reach two-thirds of the 24-hour count (12.16 grain/labeled cell) upon completion of division of half of the labeled daughter cells. Reference to Table 1 shows that this occurred between the third and fourth days. Thus, the maximum cell cycle, beginning at 24 hours and ending between days 3 and 4, could be approximated as 60 hours. Assuming a 7-hour S period[15,16] and 2.5 hours for G_2 in bronchial epithelium,[16] and 1.5 hours for mitosis, the maximum G_1 period was 49 hours.

Bronchiolar Epithelium of Animals Exposed to Urethane

Labeling Index

The number of cells in the DNA synthetic period 30 minutes after ^{3}H-thymidine injection was twice the control value. This might have reflected in increase in length of the S period or an increase in the number of cells in the S period. Additionally, incorporation of ^{3}H-thymidine may have represented DNA repair or metabolic DNA.[17] The latter explanation was considered because urethane is known to damage chromosomes [18] and DNA repair might be anticipated. However, the similarity of the proportion of paired labeled cells and mean grain counts at 24 hours in urethane and control groups supports the interpretation that ^{3}H-thymidine incorporation into DNA represented premitotic synthesis.

Prolongation of the S period

A comparison of the number of labeled cells 24 hours after ^{3}H-thymidine injection favors the alternative that the S period was prolonged in epithelium of mice drinking urethane. For, if there were twice as many cells in the S period at 30 minutes, one would expect a proportionately high labeling index at 24 hours. Conversely, if the time spent in the S period was longer but the number of cells was the same, this would explain the similar labeling index and mean grain counts, and proportion of paired labeled cells in control and test groups at 24 hours.

Postmitotic Interphase

After completion of the first division cycle, the labeling index and mean grain counts showed little change from 24 hours to 4 days. Evidently, these cells were either delayed in the G_1 period or remained in the population as nondividing, labeled cells. Evidence from other experiments [19] suggests a real prolongation of the G_1 as well as the S period. Possibly, the slight elevation of labeling index and the beginning of the decline of mean grain count between the fifth and the sixth days may have indicated the completion of G_1, S, G_2 and mitosis in some of the bronchiolar cells of animals drinking urethane. The proportion of these cells was too small to decrease the mean grain count to less than 50% of its initial value.

Comparison of Response to Acute and Chronic Urethane Exposure

This study has brought out certain differences between the response of lung cells to acute and to chronic urethane exposure. After a single urethane injection, the labeling index of bronchiolar cells [20] as well as of parenchymal cells [11,20,21] was decreased and this was followed, after 3 days, by a dramatic increase in the labeling index and mitotic index that was interpreted as a compensatory period. Whether the cell cycle time was actually shortened or more cells were in cycle, the findings indicated a shortening of the population renewal time.[11] This is in contrast to the findings in the present experiments with chronic urethane exposure, where the population renewal time increased either due to lengthening of cycle time or due to failure of a large proportion of cells to divide at all. The anatomic result of this urethane-induced disturbance in the steady state was hyperplasia of bronchiolar epithelium, many cells of which were presumably damaged.

It is not possible, at the present time, to relate the effects of urethane on the cell cycle to its role in carcinogenesis. Shimken and co-workers [20] have made a study of the acute effects of urethane and other carcinogens on DNA synthesis, and concluded that the presumed inhibition of DNA synthesis may be an unrelated toxic effect of urethane. In chronic exposure, prolongation of the life-span of damaged cells may be of importance in view of the finding [22] that urethane is extensively bound to nuclear DNA and cytoplasmic RNA, and remains bound for long periods of time.

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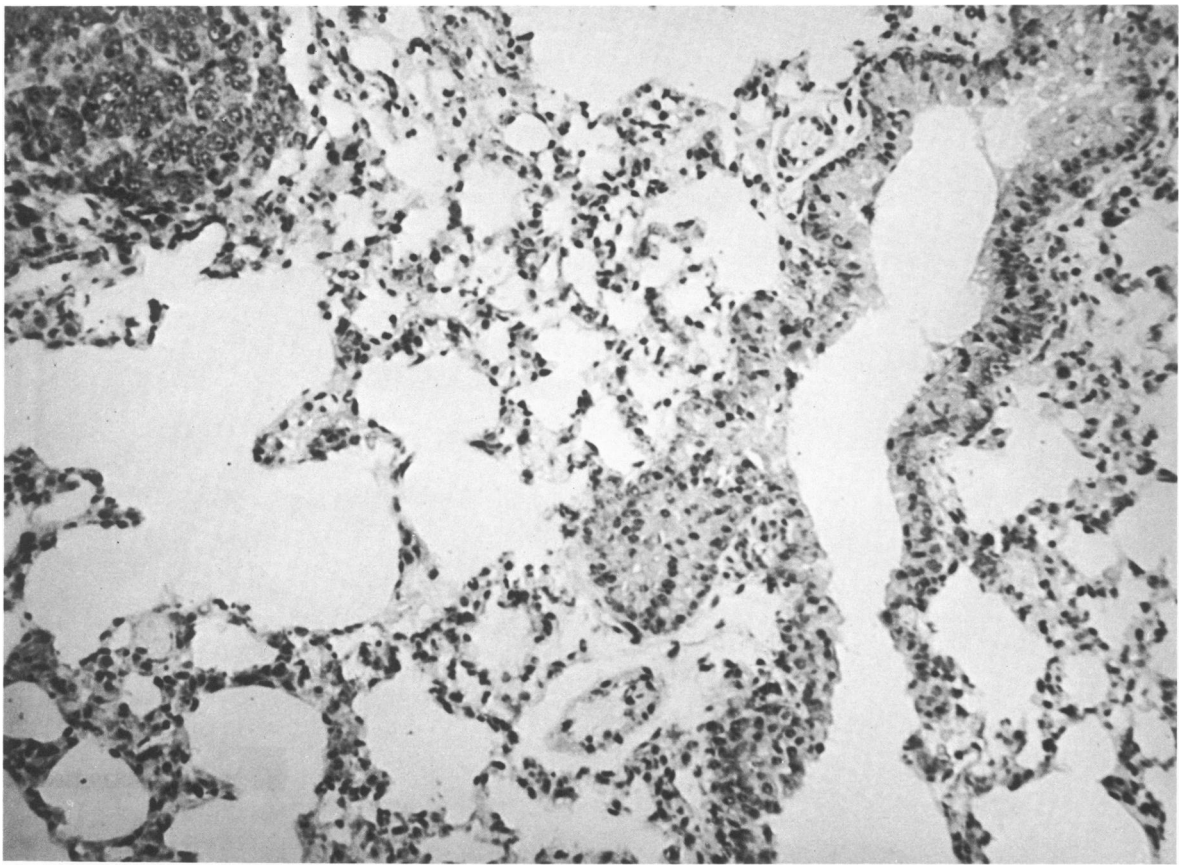

Fig 1—Lung of animal who drank 0.1% urethane solution for 10 weeks. A small adenoma of type 2 alveolar epithelial cells is shown in the left upper corner, adjacent to but not connected with the moderately hyperplastic epithelium of a terminal bronchiole (*right*) (methyl green–thionine, × 110).

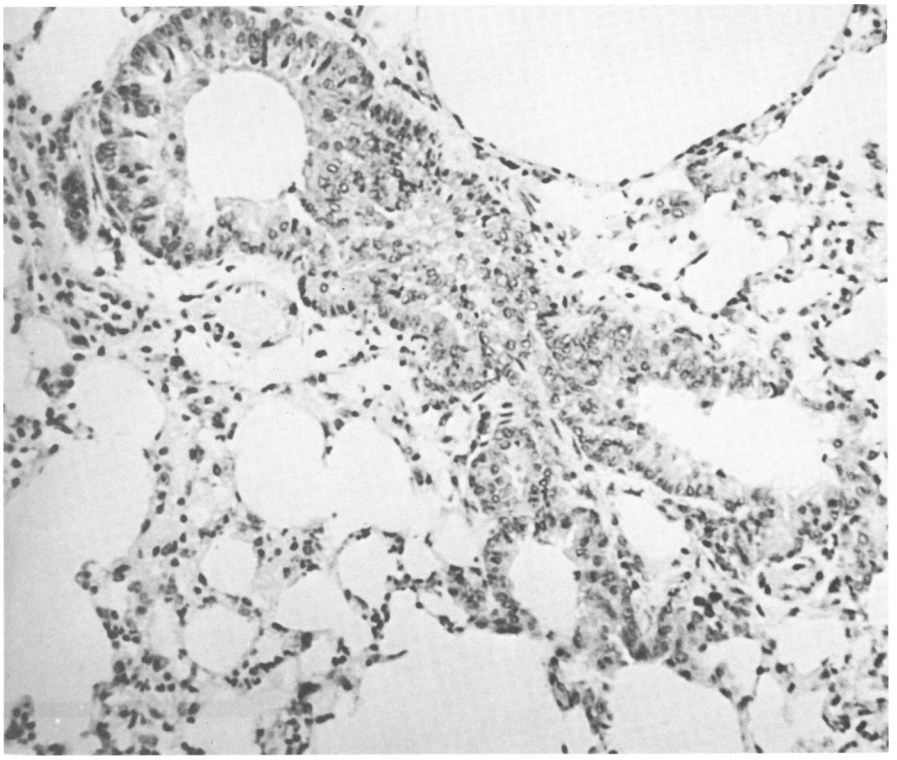

Fig 2—Typical appearance of hyperplastic terminal bronchioles of animals exposed to urethane for 10 weeks. Bronchiolar epithelium extends more distally, toward the alveoli, than normally (methyl green–thionine, × 110).

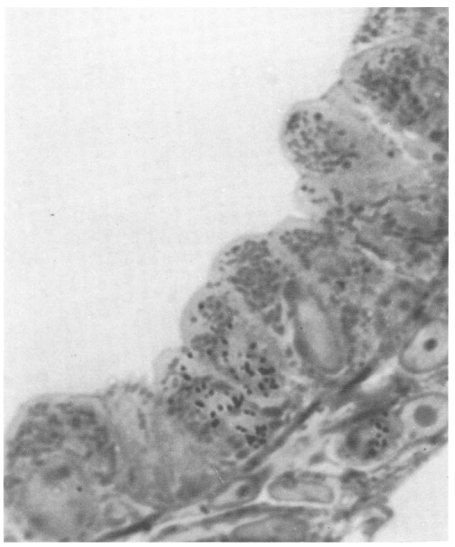

Fig 3—Autoradiograph of terminal bronchiole of animal exposed to urethane for 73 days; killed 3 days after ^{3}H-thymidine injection. A pair of nonciliated cells containing many mitochondria and few secretory granules (Clara cells) are labeled (toluidine blue, × 1000).