

Effect of Methylene Chloride Inhalation on Replicative DNA Synthesis in the Lungs of Female B6C3F₁ Mice

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In the National Toxicology Program 2-year inhalation study of dichloromethane (DCM), there was a significant increase in pulmonary neoplasms in female B6C3F₁ mice exposed to 2000 ppm (overall rates of 30/48 versus 5/50 in control). Replicative DNA synthesis was examined to evaluate the potential role of treatment-induced lung cell proliferation on pulmonary carcinogenicity. Tritiated thymidine incorporation was assessed in methacrylate plastic sections after 1, 2, 3, or 4 weeks of inhalation exposure to 2000 ppm or 8000 ppm DCM. Similar measurements of labeling indexes were made after 13 and 26 weeks of exposure to 2000 ppm DCM using bromodeoxyuridine as the labeling agent. In all cases the labeling agent was delivered over a 6-day period using osmotic minipumps. The labeling index (LI) of bronchiolar epithelium (two branches proximal to the terminal bronchiole) of mice exposed to 2000 ppm DCM for 2-26 weeks decreased to 40-60% of the control. Terminal bronchioles showed a similar decrease in LI. Mice exposed to 8000 ppm DCM had a less dramatic decrease in LI. No pathological change was found in the exposed lungs. It is concluded that inhalation exposure to DCM for up to 26 weeks reduces cell turnover of bronchiolar cells in female B6C3F₁ mice.

Introduction

Dichloromethane (DCM; synonym, methylene chloride), an industrially and commercially important chemical, was carcinogenic in mouse liver and lung in 2-year inhalation studies (1). Although DCM was mutagenic for bacteria in the presence or absence of liver microsomes (2), previous assays for genetic damage in mammalian cells have provided conflicting results (3). Initial reports of short-term *in vivo* assays included failure to induce micronuclei in mouse bone marrow, absence of covalent binding to mouse liver DNA, and negative results in the *in vivo/in vitro* unscheduled DNA synthesis assay (4). More recently, inhalation of 4000 or 8000 ppm DCM for 10 days by

B6C3F₁ mice was reported to increase the frequency of sister chromatid exchanges in lung cells and lymphocytes and the frequency of micronuclei in erythrocytes (3). After 3 months of inhalation exposure to 2000 ppm DCM, B6C3F₁ mice had increases in lung cell sister chromatid exchanges and erythrocyte micronuclei (3). These recent findings support the contention that DCM may induce lung tumors by a genotoxic mechanism. This possibility is further supported by the observation that some lung tumors in DCM-exposed mice have point mutations in activated *K-ras* oncogenes (5). In contrast, liver tumors induced by exposure to DCM contained mutations in *H-ras* genes that were typical of what was observed in spontaneous liver tumors (5). Cell proliferation in the liver is reported elsewhere (6).

Cell proliferation is known to play an important role in the induction of neoplasia by both genotoxic and nongenotoxic carcinogens (7). To determine whether inhalation exposure to DCM was associated with alterations in cell proliferation in the lung, osmotic minipumps were used to label cells undergoing replicative DNA synthesis over a 6-day period. Treatment-related changes in cell turnover in lung were assessed by determining the labeling index for bronchiolar and alveolar cells.

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Materials and Methods

The chemical source of DCM and vapor generation methodology for inhalation exposure are described in detail elsewhere (8). A 30 mg/mL solution of bromodeoxyuridine (BrdU, Sigma Company, St. Louis, MO) was prepared in phosphate-buffered saline containing 0.1 N NaOH. Tritiated thymidine was purchased from DuPont (Boston, MA).

Two studies were conducted in series in which female B6C3F₁ mice were exposed to 2000 ppm or 8000 ppm of DCM (Fig. 1). In experiment A, 4- to 6-week-old mice were exposed to air containing DCM at concentrations of 0, 2000, or 8000 ppm, 6 hr per day, 5 days per week for up to 4 weeks. Interim sacrifices were done after 1, 2, 3, and 4 weeks of DCM exposure (Fig. 1). Each group consisted of five animals per exposure concentration. Six days before sacrifice, osmotic minipumps (Model 2001, Alzet Corporation, Palo Alto, CA) containing 200 μ L of tritiated thymidine (1.0 mCi/mL) were implanted subcutaneously (9). The radioisotope was delivered at a rate of 1 μ L/hr. All mice were killed by carbon dioxide asphyxiation, weighed, and necropsied. Lungs were inflated with 10% buffered formalin through the trachea and then immersed in 10% buffered formalin. Left, right apical, and right diaphragmatic lobes were sectioned into halves along the lobar bronchi, and each half was separately embedded in methacrylate plastic; 2- μ m sections were cut for autoradiography and hematoxylin and eosin staining.

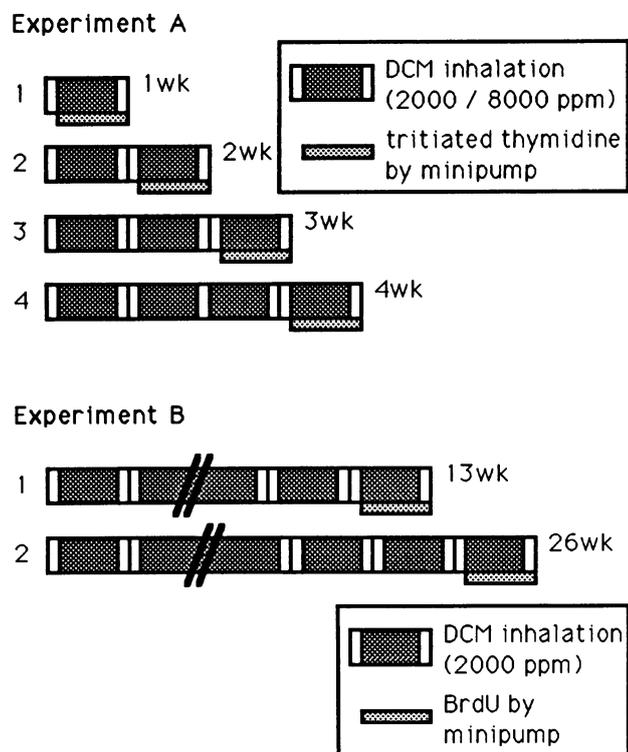


FIGURE 1. Experimental design of dichloromethane studies.

Selected blocks were sectioned and stained using the alcian blue-periodic acid Schiff (AB-PAS) reaction for detection of mucous metaplasia in the epithelium.

Standard autoradiographic labeling techniques were used to detect cells that incorporated tritiated thymidine. The slides were dipped in NBT2 emulsion (Eastman Kodak, Rochester, NY), exposed for 12 weeks, developed, and counterstained with hematoxylin and eosin. Cells with 10 or more silver grains over the nucleus were counted as labeled under high power (400 \times) microscopic fields of view.

In experiment B, 4- to 6-week-old mice were exposed to air containing DCM at concentrations of 0 and 2000 ppm, 6 hr per day, 5 days per week for up to 78 weeks. Interim sacrifices were done after 13, 26, 52, or 78 weeks of DCM exposure. Each group consisted of 10 animals per exposure concentration. Materials from the 13-week and 26-week sacrifices were used for this study. Six days before sacrifice, osmotic minipumps (Model 2001, Alzet Corporation, Palo Alto, CA) containing 200 μ L of bromodeoxyuridine (BrdU) were implanted subcutaneously (9). The labeling agent was delivered at a rate of 1 μ L/hr.

Methacrylate plastic sections of the lungs were prepared as described for experiment A. Two-micrometer sections were cut, and BrdU incorporation was identified by a modified immunohistochemical method using anti-BrdU antibody and AEC (aminoethyl carbazole) chromogen (10,11); tissues were counterstained with hematoxylin. Selected blocks were sectioned and stained using the AB-PAS reaction.

Epithelium of bronchioles that were two branches proximal to the terminal bronchiole was counted to generate labeling index (LI) data for bronchiolar epithelium. About 100 epithelial cells per mouse were counted. In addition, on the average, 200 epithelial cells from terminal bronchioles were counted per mouse for LI. Relative LI to control was calculated as the ratio of LI to the concurrent control value. Alveolar duct cells and cells associated with a possible bronchiolization of alveoli were not included as terminal bronchiolar cells. Ciliated and nonciliated cells were distinguished in sections from experiment A. Ciliated and non-ciliated cells were not distinguished in terminal bronchioles in either experiment.

Statistical comparisons between treated and control groups were performed using Student's *t*-test.

Results

Study results from experiments A and B are combined and presented according to two anatomical sites in the lungs.

Bronchioles

Labeling indexes of the bronchiolar epithelium (two branches proximal to the terminal bronchiole) in mice exposed to 2000 ppm DCM from week 2 to week 26

were significantly decreased compared to concurrent controls. Mice exposed to 8000 ppm DCM had a slight decrease in LIs from week 2 to 4 (Fig. 2). No treatment-related cytomorphological changes in either ciliated or non-ciliated bronchiolar cells were observed. AB-PAS staining showed no treatment-related increase or decrease in mucus-containing cells in the bronchioles. There were no significant changes in the ratio of ciliated to non-ciliated bronchial cells during the 4 weeks of treatment.

Terminal Bronchioles

Labeling indexes of the terminal bronchiolar epithelium in mice exposed to 2000 ppm DCM were significantly decreased compared to concurrent controls from week 3 of exposure up to week 26. Mice exposed to 8000 ppm DCM had a marked decrease in LI in week 2

and slight decrease in weeks 3 and 4 (Fig. 3). There were no treatment-related morphological changes in the terminal bronchioles. No AB-PAS positive bronchiolar cells were observed in treated and control animals.

Discussion

A 6-day continuous labeling protocol with osmotic mini-pumps was used in this study to detect alterations in cell proliferation in the lung (9). Under these experimental conditions we observed 30 to 50% LI in bronchiolar epithelium of control mice during the first 4 weeks of the study, and 20% LI in bronchiolar epithelium of control mice during weeks 13–26. These LIs are compatible with those of young growing and nongrowing adult mice (12,13). On the other hand, a 40%–60% decrease in LI of the bronchiolar epithelium of DCM-exposed mice was observed for 2–26 weeks.

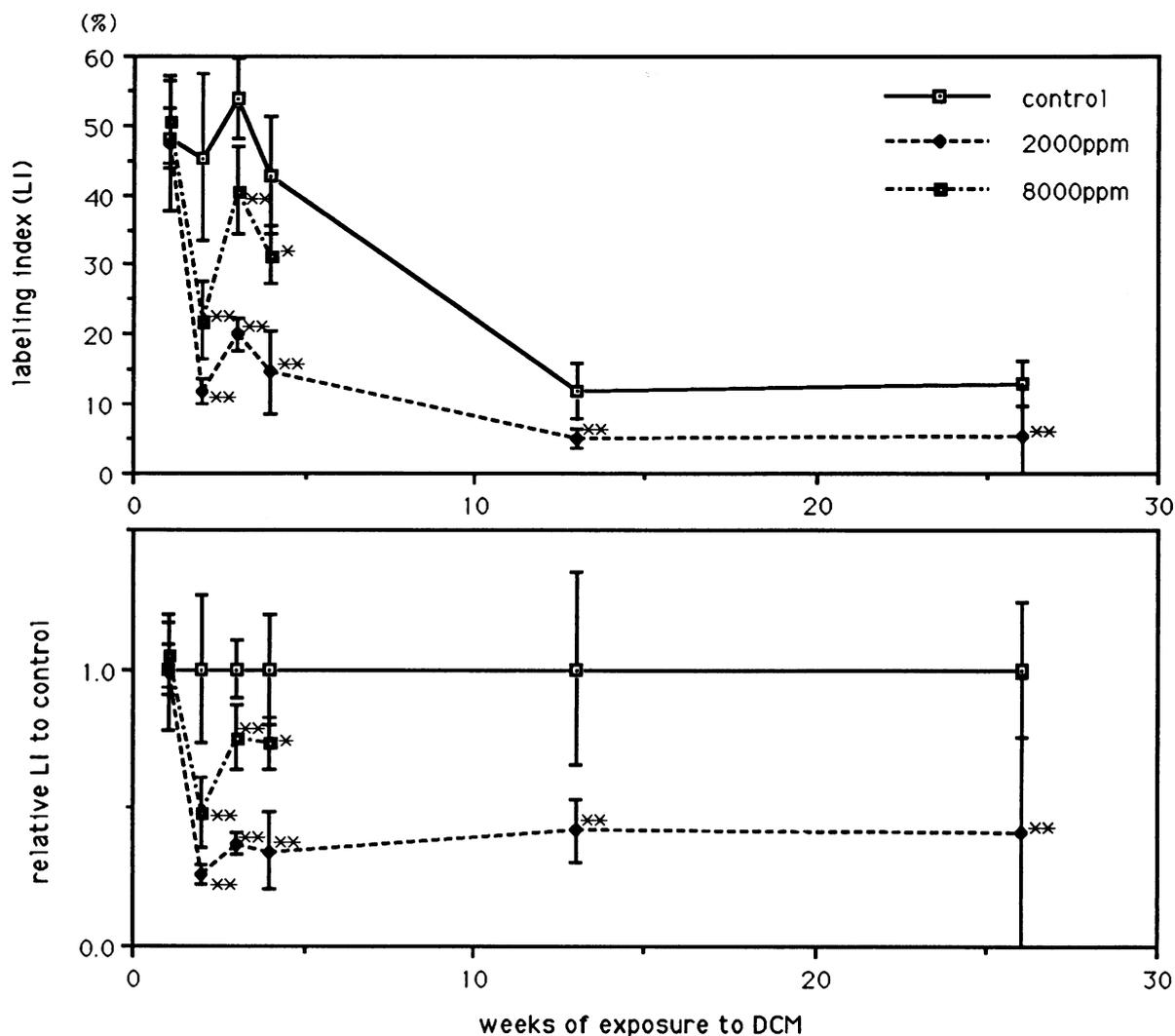


FIGURE 2. Labeling index (LI) of epithelium of bronchioles two branches proximal to the terminal bronchiole obtained by 6-day continuous labeling by osmotic minipump (top; mean \pm sd) and the relative LI to control (bottom). (**) $p < 0.01$, (*) $p < 0.05$ by t -test compared to control.

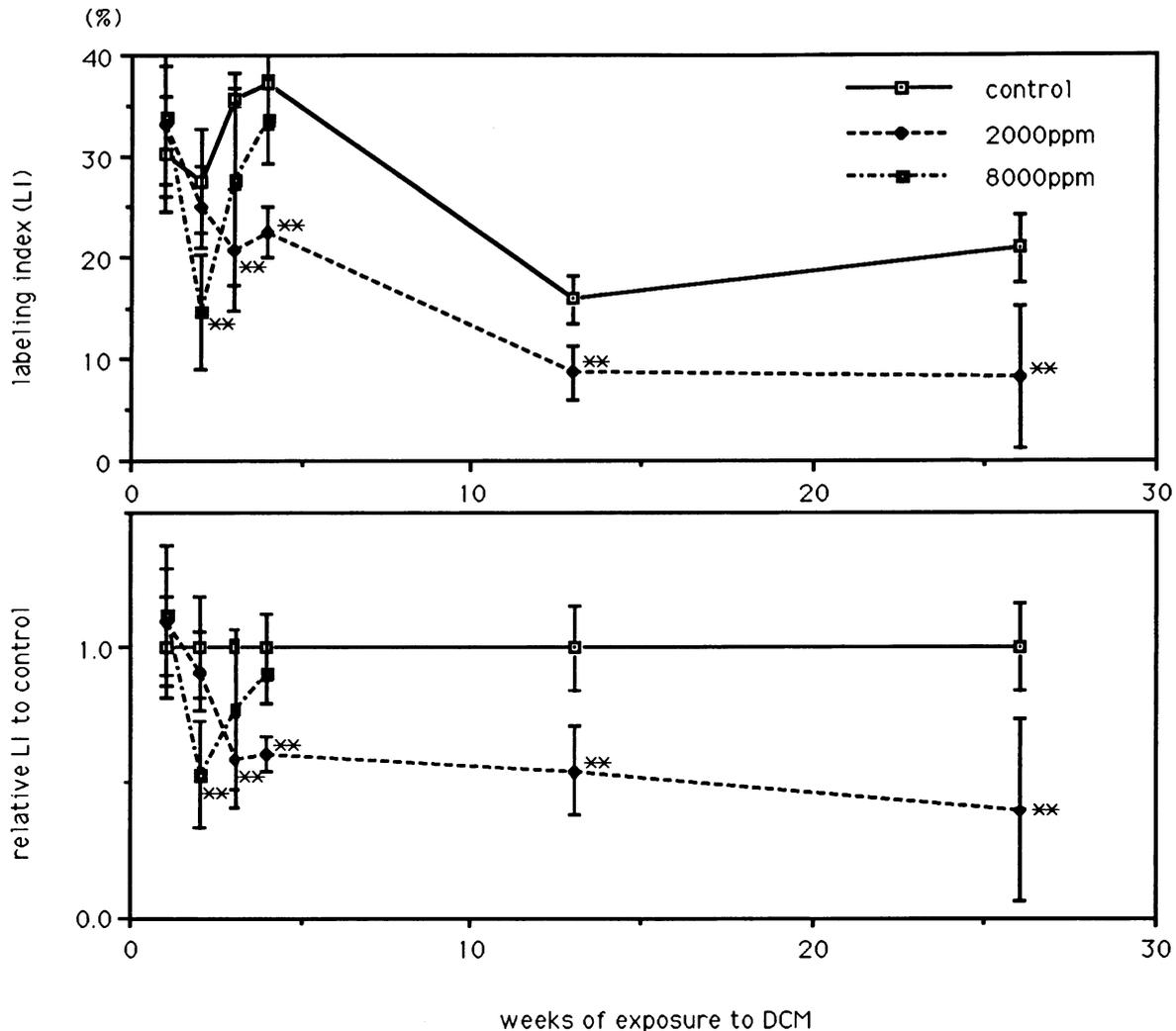


FIGURE 3. Labeling index (LI) of epithelium of terminal bronchioles by 6-day continuous labeling by osmotic minipump (top; mean \pm SD) and the relative LI to control (bottom). (**) $p < 0.01$ by t -test compared to control.

The LI data obtained by a 6-day continuous labeling technique are a function of at least two factors: the S-phase fraction and the rate of exfoliation of bronchiolar cells. Thus, the observed decreased LI can be interpreted in at least three different ways: *a*) DCM inhalation suppressed replicative DNA synthesis of bronchiolar epithelium for up to 26 weeks, *b*) DCM exposure prolonged generation time (length of cell cycle) of bronchiolar epithelium, or *c*) DCM induced selective cell death (exfoliation) in the growth fraction so the number of labeled cells in the epithelial layer decreased from that of control animals.

Mathematical simulations on bronchiolar cell turnover for the conditions *a*) and *c*) above are possible under assumptions that *a*) the epithelial cell number per unit length of basement membrane is constant and the length of the cell cycle is constant in either control or treated mice, *b*) the cell turnover rate of labeled and nonlabeled cells is same, and *c*) only aged cells (non-

labeled cells in this situation) are exfoliating in the control mice. A simulation for DCM suppressing replicative DNA synthesis shows that the 40–60% reduction in LI over 6 days can be attributed directly to the 40–60% reduction in the size of the S-phase fraction, if exfoliation in DCM-treated mice also occurs only in nonlabeled cell population. A simulation for DCM inducing selective cell death shows that up to a 50% reduction in LI over 6 days can be attributed to the selective killing effect of DCM without any changes in the size of S-phase fraction. Although additional studies on cell-cycle dynamics are needed for definitive interpretation of 6-day pump data, the reality seems to fall somewhere between these two extreme situations: DCM probably suppressed replicative DNA synthesis of the bronchiolar epithelium to a slight or moderate degree.

By light microscopy, there was no obvious treatment-related morphological change such as hyperpla-

sia, atrophy, necrosis, or metaplasia in bronchiolar regions up to 26 weeks. We also failed to detect significant changes in LI and morphology of alveolar type-II cells in the first 4 weeks of exposure to DCM (preliminary observation).

Lungs of mice exposed to 2000 ppm DCM up to 52 and 78 weeks had various lesions such as focal type-II cell hyperplasia, alveolar/bronchiolar (A/B) tumors, slight to moderate chronic inflammatory cell infiltration of the bronchi and bronchioles, and mucous metaplasia of the bronchial and bronchiolar epithelium (preliminary observation).

Alveolar/bronchiolar tumors were observed in B6C3F₁ mice inhaling 2000 and 4000 ppm DCM for up to 2 years (1,14). Because the lung contains a heterogeneous cell population, there has been considerable debate and speculation on the cell of origin of mouse A/B neoplasia (15-18). Morphological data implicate the type II cell as the probable cell of origin of mouse A/B neoplasms, especially when early time points are examined (19). On the other hand, biochemical data, especially that relating to metabolic and detoxification pathways, implicate the Clara cell as a main metabolic site for inhaled DCM in mouse lungs (2,20).

It was hoped that measurement of cell proliferation would help resolve the mechanism of lung tumor induction by DCM and give some indication of the cell of origin. Potent mouse pulmonary carcinogens have been reported to induce initial suppression in DNA synthesis in alveolar and bronchiolar epithelial cell population, followed by usually more than a 100% increase in cell proliferation (hyperplasia) within 4 weeks (21-24). Our results on replicative DNA synthesis in lung cells of DCM-exposed mice show a lack of such significant enhancement in cell proliferation in the latent period, suggesting that the mechanism of tumor induction by DCM is not associated with significant enhancement of cell proliferation. Further studies are necessary to elucidate the cell of origin and mechanism of induction of DCM-induced lung tumors in B6C3F₁ mice.

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