Tumorigenesis and Genotoxicity of Ethyl Carbamate and Vinyl Carbamate in Rodent Cells


ABSTRACT

Vinyl carbamate (VC) is a suspect metabolic intermediate in ethyl carbamate (EC) carcinogenesis. In the present studies, EC and VC were evaluated for their relative abilities to induce adenomas and sister chromatid exchanges (SCEs) in lung cells of A/J, C3HeB/FeJ, and C57BL/6J strain mice. For both end points, animals were administered a single i.p. injection of the test chemical. Percentage of mice with adenomas and number of adenomas per mouse were compared among the three strains 24 weeks following exposure to EC or VC. Although the relative order of strain sensitivity was the same for both chemicals: A/J > C57BL/6J > C3HeB/FeJ, VC was much more potent than EC. For SCE analysis of primary lung cells cultured from treated animals, EC and VC showed potency differences similar to those observed for tumorigenesis. All three mouse strains revealed significant dose-dependent increases in SCE frequency. However, there was no strain specificity for this effect. SCE persistence over time was also compared in treated A/J and C57BL/6J mice. Although EC- and VC-induced SCE frequencies declined over a 2-week observation period, again, there was no strain specificity for this effect. VC was also tested for enhancement of SA7 virus transformation of Syrian hamster embryo cells. Significant concentration-dependent increases in cell transformation frequency were observed.

INTRODUCTION

EC, or urethane, has long been studied for its potential to induce mutations and cancer (1; for reviews see Refs. 2-4). Although this substance is tumorigenic in many different species and tissues (2, 3, 5), it exerts a high level of mouse strain specificity for lung adenoma induction (6, 7). Strains which are normally predisposed to lung adenoma development respond to EC exposure with an increased incidence of adenomas after a relatively short latent period. Strains showing few spontaneous lung adenomas are largely resistant to the formation of this type of tumor after EC exposure. A view widely held (8, 9), although with some exception (10), is that EC acts to accelerate the development of predetermined tumors rather than to initiate them. This possibility seems unlikely since untreated A-strain mice 1-2 years of age rarely develop more than one or two spontaneous tumors per lung, whereas EC-treated mice develop multiple tumors (depending upon dose) in 6 months or less (11, 12). Retention of original strain specificities for EC tumorigenesis following lung tissue transplantation between differentially sensitive strains suggests that the important physiological determinants are intrinsic to the lung (7).

Cytogenetic studies have been conducted in order to determine if mouse strains with different sensitivities to EC tumorigenesis exhibit corresponding differences in susceptibility to, or expression of, EC-induced chromosome damage. EC and a metabolic product, N-hydroxyurethane, appear relatively more clastogenic for embryonic lung cultures when cells are derived from tumor-sensitive strains than when they are from tumor-resistant strains (13). EC also induces more chromosome aberrations in hematopoietic tissues from mice prone to EC leukemogenesis than in those from leukemia-resistant mice (14). In vitro studies with VC, a suspect metabolite of EC (15), have also shown it to be more efficiently processed to SCE-inducing and mutagenic metabolites when added enzyme fractions are from adenoma-susceptible mice (strain A/J) than when they are from adenoma-resistant mice (strain C57BL) (16). However, analyses of SCE induction in bone marrow cells after in vivo exposure to EC or to VC (16, 17) have not revealed any strain-specific effects that can be related to patterns of EC tumor induction. Thus, evidence of strain specificity for EC chromosome effects is equivocal.

VC is suspected to be the most important proximate carcinogenic metabolite of EC. Dahl et al. (15, 18) showed that VC causes, with much greater potency, the same types of tumors produced by EC in rodents. Also, after in vivo exposure of rodents to EC or VC, SCE induction levels are highest in tumor-prone tissues, and they reflect potency differences for the two chemicals that roughly parallel those observed for tumorigenesis (19-22). Further demonstration that EC-induced SCE lesions are highly persistent has led to speculation that this property may be related to tumorigenesis (23). When tested for oncogenic cell transformation in the C3H10T1/2CL8 mouse fibroblast system, neither EC nor VC was positive (21). However, genotoxicity studies in bacterial and mammalian cells have indicated that both EC and VC require metabolic activation to express maximal activity and, in cell transformation systems with greater metabolic potential, EC proved positive (24, 25).

The present studies were designed to address three questions regarding EC and VC activities. First, using several well-characterized mouse strains, does VC show strain-specific tumorigenesis patterns similar to those of EC? Second, is there a strain specificity for SCE induction and/or persistence in lung cells by EC and VC which corresponds to strain sensitivities for lung-adenoma induction? Third, does VC remain negative for cell transformation when tested in a primary cell system with high metabolizing capabilities? Tumors and SCEs were analyzed in lung cells from i.p. exposed A/J, C3H, and C57BL...
strains of mice. Transformation was tested by enhancement of SA7 virus effects in primary Syrian hamster embryo cells.

MATERIALS AND METHODS

Chemicals. EC (99% pure) and BrdUrd were purchased from Sigma Chemical Co., St. Louis, MO. VC (99% pure) was synthesized by Midwest Research Institute, Kansas City, MO. BrdUrd was dissolved in water; EC and VC were dissolved in 0.85% NaCl solution.

Mice. Male and female mice used in tumorigenesis and SCE induction studies were 3–5 months old and were derived from the same shipment/colony group. C3HeB/FeJ and C57BL/6J mice were obtained from Jackson Laboratory, Bar Harbor, ME. Strain A/J mice were from the breeding colony (originally derived from A/J strain, Jackson Laboratory) of Dr. Gary Stoner, Medical College of Ohio, Toledo, OH. The mice used for tumorigenesis studies were quarantined for a minimum of 14 days in high efficiency particulate air-filtered rooms with air (15 cycles/h) at 50-60% relative humidity. Room temperatures were maintained at 68–72°F with a 12-h light-dark cycle. The animals were maintained in cages with corn-cob bedding and fed lab chow (Purina) and water ad libitum. and VC (10–60 mg/kg) were usually administered to 3 mice (for each chemical dose). BrdUrd was dissolved in water, EC and VC were dissolved in 0.85% NaCl solution. Mice were acclimatized for a minimum of 10 days in laminar-flow rooms with air (15 cycles/h) at 50–60% relative humidity. Room temperatures were maintained at 68–72°F with a 12-h light-dark cycle. The animals were fed lab chow (Purina) and water ad libitum.

Lung Tumorigenesis. Lung tumorigenesis studies were carried out by administering a single i.p. injection (0.1 ml) of either EC or VC to mouse strains A/J, C57BL, and C57BL EC doses ranging from 30 to 1000 mg/kg and VC doses ranging from 1 to 60 mg/kg were evaluated. In most instances, 32 mice (16 males and 16 females) were used per treatment group for each strain. All animals were killed 24 weeks following injection. The lungs were removed and fixed in Tellyesniczkys fluid. After fixation, the tumors were counted under a dissecting microscope. Lung tumor responses (percentage of mice with tumors and number of tumors per mouse) were compared among the three strains and their appropriate control groups. Lung tumors were evaluated only in animals that survived the full 24 weeks.

SCE Induction. For SCE induction studies, EC (300 or 1000 mg/kg) and VC (10–60 mg/kg) were usually administered to 3 mice (for each of strains A/J, C3H, and C57BL) as a single i.p. injection (0.2–0.3 ml). Detailed procedures for harvesting and culturing lung cells for cytogenetic analysis have been reported elsewhere (26). Briefly, for dose-response studies, lung cells were collected 16 h following carbamate injection by digesting the lung tissue with a solution of trypsin, EDTA, and collagenase infused via the trachea. The isolated cells, showing viabilities of 77–93%, were added at a final density of 1.6 x 10^5 cells/ml. BrdUrd was carried out for two cell cycles (48 h). Cell hypotonic (0.075 M KCl) and fixation (methanol:acetic acid, 3:1) treatments, followed by slide preparation, differential staining of chromosomes (fluorescence-plus-Giemsa), and SCE analyses were in accordance with standard cytogenetic procedures (27). For SCE persistence studies, A/J and C57BL mice were given injections of EC (1000 mg/kg) or VC (60 mg/kg), and lung cells were harvested at times ranging from 4–320 h later. The lung cells were processed as described above for cytogenetic analyses (except that total culture times were 56 h). A minimum of 25 cells with clear sister chromatid differentiation per culture was evaluated. As a measure of toxicity, 100 metaphases from each culture were also analyzed for cell cycle kinetics and replicative indices calculated (28).

Cell Transformation. Procedures used for cell transformation studies have been reported elsewhere (29). Briefly, primary Syrian hamster embryo cells were treated with VC (concentrations from 31 to 1000 μM BrdUrd) for 20 h. The chemical was removed, and SA7 virus was allowed to adsorb for 3 h. The cells were then subcultured and dishes seeded for survival (666 cells/60-mm dish) and transformation (2 x 10^4 cells/60-mm dish) assays. Eight to 10 days posttreatment, the survival assay dishes were fixed and stained and plating efficiencies of treated versus control dishes were calculated. Twenty-eight days after treatment, transformation assay dishes were fixed and stained and the number of virus foci quantitated. Transformation frequencies per 10^6 surviving cells were calculated.

Statistical Analysis. For each chemical, tumor multiplicity was analyzed using a three-way AOV model in which all interactive, as well as main, effects of strain, sex, and chemical dose were tested. Tumor counts were square root or rank transformed to make AOV assumptions more tenable. SCE data for each chemical were examined in a 2-way AOV with strain and chemical dose as factors. Rather than analyzing individual cell counts, mean SCEs per cell per mouse were weighted by the inverse of the variance of cell frequency within each animal. Cell transformation data were analyzed using Dunnett's (30) test to determine which transformation frequencies significantly differed from medium control.

RESULTS

Lung tumor incidence following exposure to the chemicals VC and EC was analyzed using two approaches: the percentage of mice with tumors and the average number of tumors per mouse. At the time of analysis, when animals were approximately 10 months of age, 25–28% of control A/J mice revealed spontaneous adenomas with a frequency of approximately 0.4/mouse. Only 3–6% (1–2 mice) of C3H and C57BL strains revealed spontaneous adenomas with frequencies of ≤0.1/mouse.

EC induced a dose-related increase in the percentage of mice with lung tumors in both male and female A/J, C3H, and C57BL mice (Table 1; Fig. 1). The A/J strain showed the greatest overall increase in percentage of mice with tumors, C3H showed the smallest, and C57BL showed an intermediate response. In the A/J strain, the females exhibited the maximal response (100% mice with tumors) at a lower exposure (100 mg/kg) than the males (300 mg/kg).

There was also a strain-specific response in the average number of tumors per mouse induced by EC (Table 1; Fig. 2). A/J mice of both sexes revealed dramatic increases in the number of tumors, which C3H and C57BL mice showed comparatively little or no increase over control levels. These effects were also statistically evident as AOV showed a strong strain-by-dose interaction (P < 0.001) but no significant effects involving sex.

VC exposure induced a dose-related increase in the percentage of mice with tumors (Table 1) for both males and females of all three strains. The A/J strain showed the most dramatic increase, followed by the C57BL and C3H strains, respectively (Fig. 1). Whereas 70% of C57BL mice had tumors at the 1000 mg/kg dose of EC, this strain (both sexes) showed a maximal (100% mice with tumors) response to VC at the much lower dose of 60 mg/kg.

The number of lung tumors per mouse increased to the greatest extent in the A/J strain (Fig. 2). The response was dose related in the 1–30-mg/kg range and appeared to plateau. The apparent decrease in tumors at the 60-mg/kg dose may have been due to toxicity for the lung, which can interfere with carcinogenicity in A/J mice (9). The C57BL strain showed a small increase, and the C3H strain remained at control levels (Fig. 2). Analysis of variance showed evidence (P = 0.01) of an interaction involving VC dose, strain, and sex. While slightly greater numbers of tumors at high doses were seen in A/J males compared to females, this interaction was probably a statistical artifact. After rank transforming the data and reanalyzing with the same AOV model, this effect could not be detected (P = 0.20). The strong strain by dose interaction (P < 0.001), regardless of sex, can be seen in Fig. 2.

In the AOV of SCE per cell, there was no evidence of a
Table 1 Lung tumor response of A/J, C3H, and C57BL mice to ethyl carbamate or vinyl carbamate

<table>
<thead>
<tr>
<th>Strain</th>
<th>Chemical</th>
<th>% of mice with lung tumors</th>
<th>Lung tumors/mouse</th>
<th>% of mice with lung tumors</th>
<th>Lung tumors/mouse</th>
<th>% of mice with lung tumors</th>
<th>Lung tumors/mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>n=28</td>
<td>25</td>
<td>0.3 ± 0.54*</td>
<td></td>
<td>n=29</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>0.87% NaCl</td>
<td>n=32</td>
<td>28</td>
<td>0.4 ± 0.71</td>
<td></td>
<td>n=32</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>EC (mg/kg)</td>
<td>n=31</td>
<td>31</td>
<td>0.9 ± 0.75</td>
<td></td>
<td>n=31</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>n=100</td>
<td>94</td>
<td>1.7 ± 0.96</td>
<td></td>
<td>n=14</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>n=100</td>
<td>7.3 ± 2.86</td>
<td></td>
<td></td>
<td>n=10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>n=100</td>
<td>29.3 ± 7.67</td>
<td></td>
<td></td>
<td>n=14</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>VC (mg/kg)</td>
<td>n=30</td>
<td>30</td>
<td>0.4 ± 0.68</td>
<td></td>
<td>n=30</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>n=32</td>
<td>33</td>
<td>1.4 ± 1.08</td>
<td></td>
<td>n=31</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>n=32</td>
<td>81</td>
<td>7.2 ± 4.16</td>
<td></td>
<td>n=20</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>n=31</td>
<td>100</td>
<td>43.0 ± 12.33</td>
<td></td>
<td>n=32</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>n=26</td>
<td>100</td>
<td>40.2 ± 14.07</td>
<td></td>
<td>n=16*</td>
<td>43</td>
</tr>
</tbody>
</table>

*Mean ± SD.

These exposure groups had 16 animals (8 male, 8 female) at the beginning of the experiment.

Strain-by-EC dose interaction (P = 0.89) or of an effect due to strain (P = 0.79) alone. The common dose-related response (P < 0.001) exhibited a baseline (Fig. 3) of approximately 13 SCEs/cell and a maximum increase to approximately 19 SCEs/cell at the 1000 mg/kg dose. A time course study also showed no difference between A/J and C57BL mice in the persistence of SCEs following a single 1000-mg/kg EC dose. The SCE frequency for control animals of both strains was approximately 10 SCEs per cell, while treated animals showed nearly identical increases: an initial rise to 20 SCEs per cell followed by a gradual decrease to 17 SCEs per cell at 320 h.

The same pattern of SCE induction occurred after VC exposure, although this chemical was much more potent. An increase in SCEs to approximately 50 SCEs per cell (P < 0.001) with no apparent difference in response due to mouse strain (P = 0.80) (Fig. 3) was observed. As with EC, SCE persistence following VC exposure (60 mg/kg) was similar for both strains: an initial increase to 50 SCEs per cell followed by a decline to 30 SCEs per cell at 320 h.

Replicative indices were calculated in both the SCE induction and SCE persistence experiments. These values did not reveal any consistent evidence of significant toxic effects upon cell cycling as a function either of EC or VC dose, or of postexposure times at which cultures were initiated (data not shown).

The cell-transforming activity of VC was determined using a bioassay that measures the chemical enhancement of a primate adenovirus (SA7) transformation in primary Syrian hamster...
EC AND VC TUMORIGENESIS AND GENOTOXICITY

Table 2 Survival and transformation of Syrian hamster embryo cells treated with vinyl carbamate and SA7 virus

<table>
<thead>
<tr>
<th>Concentration, μg/ml</th>
<th>% of survival relative to control</th>
<th>Total SA7 foci/10 dishes</th>
<th>Transformation frequency/106 surviving cells</th>
<th>Enhancement ratio: TF treated/TF control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>0 ± 0</td>
<td>0.70 ± 0.94</td>
<td>0 ± 0</td>
<td>0</td>
</tr>
<tr>
<td>500</td>
<td>25.7 ± 8.6</td>
<td>33.7 ± 13.3</td>
<td>6.48 ± 2.87b</td>
<td>6.4</td>
</tr>
<tr>
<td>250</td>
<td>50.3 ± 11.7</td>
<td>34.3 ± 13.4</td>
<td>3.11 ± 0.92b</td>
<td>3.2</td>
</tr>
<tr>
<td>125</td>
<td>80.0 ± 5.4</td>
<td>33.7 ± 16.1</td>
<td>1.89 ± 0.56b</td>
<td>1.9</td>
</tr>
<tr>
<td>62</td>
<td>91.7 ± 6.0</td>
<td>24.7 ± 4.7</td>
<td>1.28 ± 0.51b</td>
<td>1.4</td>
</tr>
<tr>
<td>31</td>
<td>88.0 ± 7.5</td>
<td>23.3 ± 6.6</td>
<td>1.30 ± 0.70b</td>
<td>1.4</td>
</tr>
<tr>
<td>Medium control</td>
<td>100 ± 0.0</td>
<td>21.3 ± 4.8</td>
<td>0.97 ± 0.21</td>
<td>1.0</td>
</tr>
<tr>
<td>0.50 B(a)P</td>
<td>13.0 ± 3.0</td>
<td>11.5 ± 2.5</td>
<td>4.83 ± 0.93b</td>
<td>5.6</td>
</tr>
<tr>
<td>0.25 B(a)P</td>
<td>64.7 ± 25.8</td>
<td>37.7 ± 12.3</td>
<td>2.95 ± 1.51b</td>
<td>3.2</td>
</tr>
<tr>
<td>0.5% Ethanol control</td>
<td>100 ± 0.0</td>
<td>20.3 ± 5.4</td>
<td>0.93 ± 0.15</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Values are means ± SD for three replicate determinations except for the 0.5-μg/ml concentration B(a)P (two determinations). Cells were treated for 20 h, the chemical removed, and virus absorbed for 3 h. Cells were then subcultured and seeded for cytotoxicity and transformation. VC was dissolved in medium while B(a)P was dissolved in 0.5% ethanol. The absolute plating efficiency ranged from 9-12%.

DISCUSSION

In the present study, mouse strain sensitivities for spontaneous and EC-induced lung adenomas followed the expected (31, 32) order: A/J > C57BL and C3H. Mice bearing spontaneous lung tumors were roughly 8-10 times more frequent in the A/J strain than in the C3H and C57BL strains. A/J mice showed a 3-4-fold higher multiplicity of tumors per mouse, as well. In other studies (33), C3H mice have yielded "intermediate" frequencies of spontaneous and EC-induced lung tumors, clearly higher than those of C57BL mice. Our observation that the C3H strain is at least, if not more, tumor resistant than the C57BL strain may reflect study differences in either the mouse substrains or in the experimental conditions used. This relative order of sensitivity, C3H ≤ C57BL, and the tumorigenic potencies of EC at the doses used for all the strains, are very close to those recently reported in another study (32) using experimental conditions similar to ours. Gender differences in mice have, in general, been considered to be unimportant in pulmonary tumor induction (9, 11, 12). We observed only minimal and inconsistent gender differences in response to EC and VC.

Dahl et al. (15) first reported that VC was more active than EC for inducing similar types of tumors in rats and various strains of mice. They also reported that A/J mice were more sensitive than CD-1 mice to EC- and VC-induced lung adenomas. Our data indicate that the dose of VC that causes 100% of A/J mice to develop tumors is less than or equal to one-tenth the dose of EC required for this effect. At the comparable dose of 30 mg/kg, the number of tumors per mouse was approximately 50 times greater for VC than for EC exposure. VC was also much more active than EC in the less tumor-prone C57BL and C3H strains. However, the differences in potency between EC and VC were not as great as that seen in the A/J strain. Clearly, VC could completely overcome the inherent resistance of C57BL mice to tumor formation. VC (60 mg/kg) caused 100% of mice in this strain to develop tumors, although tumor incidence per mouse never reached the very high levels (>40/mouse) observed in A/J mice.

The C3H strain response to VC exposure appeared to level off with approximately 50% of the mice showing tumors. In addition, increases in the number of tumors per animal were minimal. The possibility that higher doses might produce greater responses in this strain cannot be ruled out. However, the similarity of response between the two highest doses, 30 and 60 mg/kg, argues against this. Despite the differences in potency for lung tumor induction by EC and VC, the order of strain sensitivities to these two chemicals was the same: A/J > C57BL > C3H.

EC and VC differences in potency for inducing SCEs in marrow, liver, and lung macrophages have been reported to approximate their potency difference for lung tumorigenesis (21, 22). This relationship was maintained in lung cells from the present study, i.e., the 1000-mg/kg dose of EC caused less than a 2-fold increase for all strains while the 60-mg/kg dose of VC caused an increase of 4-5-fold. However, the lung cell SCE responses to both chemicals were considerably weaker than those observed for the other tissues, i.e., marrow cells undergo 6-fold increases after a 400-mg/kg dose of EC and nearly 8-fold increases after a 25-mg/kg dose of VC (16, 21). Since EC is reported to be distributed fairly evenly to mouse lung and marrow cells (34), pharmacokinetic differences would seem an unlikely explanation for the observed tissue-specific SCE levels. Rather, the noncycling status of the lung cells at the time of treatment and other differences from marrow, such as posttreatment BrdUrd substitution, longer cycle times, cell selection factors, and possibly increased lesion repair capabilities, may have contributed to the reduced SCE frequencies. Noncycling peripheral blood lymphocytes have also shown lower in vivo EC-induced SCE-induction levels as compared with bone marrow and alveolar macrophage cells (35).

Despite some evidence suggesting that A/J mice may metabolize VC more proficiently than do C57BL mice (16), SCE levels in these two strains (and in the C3H strain) were indistinguishable. Neither marrow cells (16) nor lung cells (present study) have revealed any A/J versus C57BL strain-related differences in SCE induction or persistence after in vivo exposure to EC or VC. SCE formation may be unrelated to tumorigenesis processes and may reflect a more generalized genetic damage than that leading to tumor formation. Alternatively, the proliferating lung cells in which SCEs were measured may, like bone marrow, be unrepresentative of the type II cells and Clara cells from which the adenomas are thought to be derived (9, 36, 37).

In the present study, there was no effort to enrich for a particular cell type; the composition of the proliferating cell population was unknown.

It has been previously reported that EC and VC do not transform C3H10T1/2/CL18 mouse embryo fibroblasts (21). Although these cells have some of the cytochrome P-450 mixed-function oxidases required for the metabolic activation of procarcinogens, their specificity for activation is limited to polycyclic aromatic hydrocarbons and related chemical classes. Therefore, it is not surprising that they are not able to be transformed by EC and VC. Primary hamster embryo cells contain high metabolic activation capability and are transformed by a wide variety of classes of chemical carcinogens. Although EC did not transform these cells (data not shown), VC was active, although only at relatively high concentrations. If VC is indeed a metabolic intermediate of EC, these results suggest that primary Syrian hamster embryo cells may be incapable of converting EC to VC but appear to be capable of
converting VC to forms which enhance SA7 virus transformability of the resistance characterizing C57BL/Fa mice in comparison with susceptible A/Fa mice (38). However, a long-standing view has been that no strain is without some potential to undergo cell transformation demonstrated in the present study is consistent with the latter. Regardless, the three strains analyzed here clearly differ in some inherent potential for induced tumor development, and the nature of the susceptibility differences may vary among the strains. Even though tumorigenesis and SCE induction properties by these carbamates correlate in terms of potency, our data are not supportive of any direct involvement of SCE formation in the strain-specific tendencies to develop lung tumors.

REFERENCES


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James W. Allen, Gary D. Stoner, Michael A. Pereira, et al.