ABSTRACT

A single i.p. injection of butylated hydroxytoluene (BHT) 6 h before a single urethan injection had varying effects on lung tumorigenesis in mice of different strains and ages. Strains exhibiting both high (A/J, SWR/J) and low (BALB/cByJ, 129/J, C57BL/6J) susceptibility to urethan tumorigenesis were tested in this study. BHT treatment decreased tumor multiplicity by an average of 32% in adult A/J mice but acted as a co-carcinogen by increasing tumor number 48% in adult SWR/J mice, 240% in adult C57BL/6J mice, 65% in adult 129/J mice, and 38% in 14-day-old A/J mice. The numbers of both alveolar type 2 cell-derived and bronchiolar Clara cell-derived lung adenomas were similarly affected by these BHT treatments. Such BHT pre-treatment had no effect on adenoma multiplicity in either young or adult BALB/cByJ mice. Multiplicity in young BALB/cByJ mice was unaffected by chronic BHT administration following urethan, even though multiplicities increased several-fold with such treatment in adult mice of this strain. Since the mice showing co-carcinogenesis by BHT include strains which are both highly susceptible and relatively resistant to urethan induction of lung tumors, our results support a distinction between genes regulating susceptibility to urethan carcinogenesis and to tumor modulation by BHT.

INTRODUCTION

BHT\(^1\) is a widely used food additive which modulates the actions of several genotoxic agents, either by enhancing or inhibiting the magnitude of their toxicity (1-3). Among the targets so affected by BHT are urethan-induced lung adenomas in mice. BHT administration to mice in the absence of treatment with carcinogens does not result in the induction of lung tumors (4). Chronic administration of BHT following a single urethan injection can increase lung tumor multiplicity up to 4 times the number induced by urethan alone (5, 6). Alternatively, a single BHT injection administered either a few hours prior to (7) or simultaneously with (8) urethan inhibits tumor formation by about 50% in some inbred strains.

Host factors such as genetic composition and age greatly influence an animal's response to the biological effects of BHT. Inbred strains vary in the degree to which chronic post-urethan treatment with BHT increases tumor multiplicity (6-8). A study using recombinant inbred strains of mice indicates that the genes regulating this sensitivity to BHT are probably not identical to those which determine the susceptibility to urethan induction (7). The host genome also regulates the cellular derivation of lung tumors. Lung adenomas can arise from either alveolar type 2 cells or from bronchiolar Clara cells, and tumors of these different cellular origins have distinctive morphologies (9, 10). Both tumor types can appear in the lungs of a single mouse, and the proportion of the two types varies with strain (11). Chronic BHT treatment after urethan can increase the number of tumors arising from both of these cell types (11).

BHT reversibly damages the lungs of adult mice of all strains tested but has no such effect on pre-weanlings (12). Agents which perturb the activities of enzymes involved in drug metabolism also abolish BHT-induced lung damage (12-14). Metabolites of BHT, rather than BHT itself, may be responsible for the lung-toxic actions of BHT. Immature mice may not synthesize the metabolite(s) of BHT responsible for lung toxicity or their lungs may not be sensitive to it (them), either of which would protect them against lung damage.

As an approach to understanding these phenomena we extended our examination of the effects of strain and age on BHT modulation of lung tumors and have determined the degree to which pre-urethan BHT treatment selectively affects either tumor type. Part of this work was mentioned in a preliminary report (15).

MATERIALS AND METHODS

The mice used for these studies included strains which are highly susceptible to urethan induction of lung tumors (A/J, SWR/J) and strains which are more resistant (BALB/cByJ, 129/J, and C57BL/6J), as described previously (6). Adult SWR, strain 129 mice, and B6 mice, 6-10 weeks old, were purchased from The Jackson Laboratory (Bar Harbor, ME) and injected 2 weeks following arrival. Five-day, 14-day, and adult A/J and cBy mice were bred in our facility, the original stock having been obtained from The Jackson Laboratory. Mice were maintained on aspen chip bedding, fed Wayne Lab Blox, and permitted tap water ad libitum and were kept on a 12-hour light-dark cycle.

The following injection procedures were used: urethan only, a single i.p. injection of 1 mg urethan (Sigma Chemical Co., St. Louis, MO) in 0.9% NaCl/g body weight; BHT/urethan, a single i.p. injection of 200 mg BHT (Sigma) dissolved in corn oil/kg body weight, followed 6 h later by a single urethan injection as above; cedrene/BHT/urethan, a single i.p. injection of 400 μg cedrene (Pfaltz and Bauer, Stanford, CT) diluted in corn oil/g body weight preceded the BHT injection by 2 h; urethan/BHT x 6, a single urethan injection was followed by six weekly BHT injections each at the above dose; urethan/(cedrene/BHT) x 6, each of the BHT injections was preceded 2 h earlier by a cedrene injection at the above dose. The earliest injection in any series was given shortly after the beginning of the light cycle. These procedures have been described previously in detail (6, 7).

Tumors were collected 14-16 weeks after urethan injection and prepared for histology as described previously (11). All tumors were assigned to either type 2 or Clara cell classes on the basis of the morphological criteria of Kaufman et al. (9). Alveolar tumors (type 2-derived) show a compact arrangement of cells with little compression of adjacent tissue; the edges of these tumors often extend into neighboring alveoli. Clara-derived tumors have well-delineated borders, and the cells are arranged as papillary structures which usually compress the surrounding normal lung tissue.

Total numbers of tumors/mouse in each histological classification were compared between treatment groups using a one-way analysis of variance. A value of $P < 0.05$ was the minimum probability level chosen to establish statistically significant differences between groups.

RESULTS

Strain Variation in the Effects of BHT Pretreatment on Urethan-induced Lung Tumorigenesis in Adult Mice. When adult A/J mice were injected with BHT before urethan treatment,
tumor multiplicity decreased (Table 1). This confirms our previous report (7). BHT pretreatment did not influence multiplicity in cBy mice relative to that with urethan alone to a statistically significant extent. It is noteworthy that cBy mice are especially sensitive to the lethal effects of a BHT/urethan protocol, just as this strain was to a urethan/BHT × 6 protocol (7). In this same experimental series BHT increased tumor multiplicity in SWR, B6, and 129 mice by 48, 240, and 655%, respectively. The percentage of mice that developed tumors increased in B6 mice from 65 to 93%, and in strain 129 mice it increased from 63 to 100%; the incidence in SWR mice was already 100% with urethan alone.

The relationship between the concentration of administered BHT and the magnitude of the co-carcinogenic effect was examined in strain 129 mice (Table 2). Augmentation by BHT of lung tumor multiplicity is dose-dependent, with a maximal effect observed at a dose of 50 mg/kg body weight. The dependence of prophylaxis in A/J mice on the concentration of BHT was noted previously (7). This dose-dependent increase in tumor multiplicity is an example of co-carcinogenesis, the stimulation of tumorigenesis by the prior or simultaneous application of a non-carcinogen along with a carcinogen (16), e.g., BHT/urethan. This is operationally and probably mechanistically different from tumor promotion, wherein the chronic application of a non-carcinogen following carcinogen administration increases tumor growth (16), e.g., urethan/BHT × 6.

Cedrene, the sesquiterpene which is a major component of cedarwood oil, modifies the in vivo activities of several drug-metabolizing enzymes (17, 18). Cedrene injection a few hours prior to BHT treatment abolished all pulmonary effects of BHT heretofore tested, including pneumotoxicity (12), tumor enhancement by the urethan/BHT × 6 protocol (7), and tumor prophylaxis in A/J mice by the BHT/urethan schedule (7). Cedrene may prevent the formation of those BHT metabolites responsible for each of these various pulmonary effects of BHT. We therefore tested whether cedrene would also block the co-carcinogenic action of BHT. Cedrene treatment prior to BHT injection abolished co-carcinogenesis in SWR mice (Table 1); the extent of the co-carcinogenic effect of BHT on 129 mice was decreased by cedrene.

Since urethan induces the formation of both type 2 and Clara-derived adenomas in A/J, SWR, and 129 mice (11), we asked whether the prophylactic effect of BHT in A/J mice or the co-carcinogenic action of BHT in SWR and 129 mice selectively affected either tumor type. BHT reduced the number of both tumor types in A/J mice and increased both types in SWR and strain 129 mice (Table 3). In 129 mice, the degree of stimulation of alveolar tumor growth was greater than that for papillary tumors. The effect of BHT co-carcinogenesis on tumor histogenesis was also tested in B6 mice. The relative proportions of alveolar and papillary tumors in B6 mice have not been described previously. Both tumor types were present, and, like the strain 129 mice, the number of alveolar tumors was most strongly increased by BHT treatment.

Effect of Age on the Modulatory Effects of BHT on Urethan-Induced Lung Tumorigenesis. Young mice differ from adults in two respects that are relevant to these studies, the rate of lung growth and the insensitivity of young mice to the pneumotoxic effects of BHT (12). Mice of two different ages were chosen for study because the proliferative rates of their lung epithelial cells differ during the postnatal period. Five days after birth corresponds to the beginning of the peak of the [3H]thymidine labeling index in randomly bred Swiss-Webster mice, while 14 days is the beginning of the decline in this index (19). In 14-day-old A/J mice a BHT/urethan regimen increased tumor number in each of three independent studies (Table 4). This is in contrast with the prophylactic effect of this same protocol in adult A/J mice (Table 1). Co-carcinogenesis in 14-day A/J mice was abolished if the BHT injection was preceded by cedrene. The relative proportion of alveolar and papillary tumors in 14-

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**Table 1** Effects of BHT treatment prior to urethan on lung tumorigenesis in different strains of adult mice

Mice were given injections i.p. of 200 mg BHT/kg body weight or with the corn oil vehicle 6 h before a single 1-mg urethan/g body weight i.p. injection. In some experiments mice received a 400 µg cedrene/g body weight i.p. injection 2 h before BHT administration. These results represent the combined data from 2 independent experiments, for a total of 16-22 treated mice per group.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Urethan</th>
<th>BHT/urethan</th>
<th>Cedrene/BHT/urethan</th>
<th>% of change (BHT/urethan) × 100°</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/J</td>
<td>0.8 ± 0.3* (4/6)*</td>
<td>2.4 ± 0.7 (5/5)*</td>
<td>5.6 ± 1.6 (5/5)*</td>
<td>3.9 ± 1.0*</td>
</tr>
<tr>
<td>cBy</td>
<td>0.8 ± 0.3* (4/6)*</td>
<td>2.4 ± 0.7 (5/5)*</td>
<td>5.6 ± 1.6 (5/5)*</td>
<td>3.9 ± 1.0*</td>
</tr>
<tr>
<td>SWR</td>
<td>21.9 ± 1.6 (0/16)*</td>
<td>21.9 ± 1.6 (0/16)*</td>
<td>21.9 ± 1.6 (0/16)*</td>
<td>-32</td>
</tr>
<tr>
<td>B6</td>
<td>2.4 ± 0.7 (5/5)*</td>
<td>2.4 ± 0.7 (5/5)*</td>
<td>2.4 ± 0.7 (5/5)*</td>
<td>+38</td>
</tr>
<tr>
<td>129</td>
<td>2.4 ± 0.7 (5/5)*</td>
<td>2.4 ± 0.7 (5/5)*</td>
<td>2.4 ± 0.7 (5/5)*</td>
<td>+38</td>
</tr>
</tbody>
</table>

* Percentage of tumor multiplicity induced by the BHT/urethan regimen as compared to the urethan only regimen.
* Mean ± SE.
* P < 0.005, BHT/urethan-treated mice compared to urethan-treated mice.
* P < 0.001, BHT/urethan-treated mice compared to urethan-treated mice.
* P < 0.05, cedrene/BHT/urethan compared to urethan-treated mice.
* P < 0.005, cedrene/BHT/urethan compared to urethan-treated mice.

**Table 2** Effect of dosage of BHT on co-carcinogenesis of strain 129/J mice

Mice were given injections of corn oil or of different amounts of BHT 6 h before a single 1-mg urethan/g body weight i.p. injection.

<table>
<thead>
<tr>
<th>Dosage of BHT (mg/kg body wt.)</th>
<th>Tumor multiplicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.8 ± 0.3* (4/6)*</td>
</tr>
<tr>
<td>10</td>
<td>2.4 ± 0.7 (5/5)*</td>
</tr>
<tr>
<td>50</td>
<td>5.6 ± 1.6 (5/5)*</td>
</tr>
<tr>
<td>100</td>
<td>1.5 ± 0.5 (4/6)*</td>
</tr>
<tr>
<td>200</td>
<td>4.8 ± 0.8 (4/4)*</td>
</tr>
</tbody>
</table>

* Mean ± SE.
* P < 0.005, compared to urethan-treated mice.
* P < 0.001, compared to urethan-treated mice.

**Table 3** Lung tumor morphology in mice treated with urethan or with BHT prior to urethan

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>Total no. of tumors</th>
<th>No. of tumors/mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/J</td>
<td>Urethan</td>
<td>247 (72)*</td>
<td>18.2 ± 1.3*</td>
</tr>
<tr>
<td></td>
<td>BHT/urethan</td>
<td>202 (78)</td>
<td>14.7 ± 1.2*</td>
</tr>
<tr>
<td>SWR</td>
<td>Urethan</td>
<td>166 (75)*</td>
<td>13.3 ± 1.2*</td>
</tr>
<tr>
<td></td>
<td>BHT/urethan</td>
<td>190 (69)</td>
<td>17.4 ± 1.4*</td>
</tr>
<tr>
<td>B6</td>
<td>Urethan</td>
<td>16 (76)</td>
<td>1.2 ± 0.2*</td>
</tr>
<tr>
<td></td>
<td>BHT/urethan</td>
<td>30 (86)</td>
<td>2.3 ± 0.4*</td>
</tr>
<tr>
<td>129</td>
<td>Urethan</td>
<td>9 (56)</td>
<td>0.6 ± 0.2*</td>
</tr>
<tr>
<td></td>
<td>BHT/urethan</td>
<td>57 (76)</td>
<td>4.8 ± 1.0*</td>
</tr>
</tbody>
</table>

* n, number of animals tested.
* Numbers in parentheses, percentage of tumors which are of a specific histological type.
* Mean ± SE.
* P < 0.05 between urethan and BHT/urethan groups.
* P < 0.001 between urethan and BHT/urethan groups.

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day A/J mice was similar to that observed in adults, in agreement with previous results comparing neonatal and adult cBy mice (11), and this proportion was unaffected by co-carcinogenesis (data not shown). BHT/urethan did not significantly change the tumor multiplicities in 5-day A/J, 5-day cBy, or 14-day cBy mice relative to administration of urethan alone (Table 4). Urethan/BHT × 6 did not affect multiplicity in two independent studies with 5-day cBy mice or 5-day A/J mice. cBy mice were used because such chronic BHT treatment affects adult cBy mice to a greater extent than any other strain thus far tested (6, 7).

**DISCUSSION**

A single administration protocol involving a carcinogen and a tumor modulatory agent has contrasting effects in mice of different strains and ages. BHT treatment before urethan injection is prophylactic for lung tumors in adult A/J mice but is co-carcinogenic in adult SWR, B6, and 129 mice and in 14 day A/J mice (Tables 1 and 4). These strain and age specificities are reminiscent of the organ specificity of tumor modulation by BHT in rats (20), where continuous BHT treatment before urethan injection inhibited hepatoma growth and adrenocortical nodule formation but enhanced the formation of bladder tumors. Tumor promotion probably involves selective clonal expansion of initiated cells, and minimal duration of exposure to the promoting agent is required following carcinogen treatment. For example, 4 weekly BHT injections were sufficient for lung tumor promotion in mice, while 2 injections were not (8). In contrast, prophylaxis and co-carcinogenesis can ensue following a single administration at approximately the same time as carcinogen application and probably perturb the initiation step itself (16). With regard to BHT and urethan, prophylaxis and co-carcinogenesis could result from a modification of urethan metabolism, the interaction of urethan with DNA, or the availability of target cells. Only a few compounds, including phorbol esters, have been reported to have both tumor promoting and co-carcinogenic activities (21, 22).

It is interesting that both type 2- and Clara cell-derived tumors were affected by the BHT/urethan protocol (Table 2). We found previously that the urethan/BHT × 6 regimen also affected both tumor types (11). There is some evidence that only Clara cell-derived tumors may progress toward cancer (10). These results may therefore differ from those in which phorbol ester application to mouse skin increased the number of benign papillomas only and did not affect the number of malignant skin tumors (23).

Two of the possible reasons for the strain and age-dependency of lung tumor modulation by BHT are variations in BHT metabolism and the action of Pk-C. Several metabolites of BHT have been described (24, 25). Strain and age-specific metabolic patterns may give rise to BHT metabolites in adult A/J mice which tend to detoxify urethan, yet other metabolites formed in young A/J mice and adult SWR, B6, and 129 mice augment initiation by urethan. 2-Tert-butyl-4-methylphenol, a structural analogue of BHT, causes lung toxicity and is prophylactic in adult A/J mice but does not promote lung tumors (7). Different structural features of the BHT molecule are thus apparently required for prophylaxis, promotion, and toxicity. Of the mice tested thus far, adult A/J mice are unique in their prophylactic response to BHT (Table 1) and in having a lower specific activity of pulmonary Pk-C than other strains (26). This is relevant because Pk-C may mediate the effects of other tumor modulatory agents, such as phorbol esters (27). BHT treatment of mice lowers pulmonary Pk-C activity and reduces the Pk-C catalyzed phosphorylation of an endogenous M, 36,000 lung protein in both A/J and cBy mice (28).

A significant tumor modulatory effect of BHT among immature animals was demonstrable only in 14-day A/J mice, which may be related to the fact that this strain also has a reduced susceptibility to urethan at 14 days (compare Tables 1 and 4). Investigators have recently used neonatal mice (treated within 24 h after birth) to test the putative carcinogenicity of polycyclic aromatic hydrocarbons by the ability of these compounds to induce lung tumors (29). This assay yielded a positive result for fluoranthene, even when the more conventionally used mouse skin tumor bioassay was negative (30). The greater susceptibility to co-carcinogenesis of 14-day A/J mice compared with 5-day mice suggests that 14-day A/J mice would be sensitive tools for bioassays of putative co-carcinogens. The effects of age on the tumor modulatory actions of BHT are complex. Young A/J mice exhibit deleterious effects of BHT via an administration protocol that is beneficial to adult mice. BHT is without effect in any protocol involving young cBy mice, while it promotes lung tumor formation in adult cBy mice to a greater degree than in any other strain studied (7).

These age dependencies may have potential toxicological consequences for humans, since 6–11-month-old infants are estimated to have the highest daily BHT dietary intake (31). It should also be noted that a few 5-day-old A/J and cBy mice given injections of BHT alone developed lung tumors, while no tumors were found in any 14-day or adult mouse treated only with BHT (data not shown). It would be of great interest to extend these findings to large-scale studies on BHT induction of lung tumors in 5-day-old animals of each strain.

**ACKNOWLEDGMENTS**

We thank Deborah S. Beer, James Dunlap, Cynthia A. Fernandez, and Barbara Macintyre for their excellent technical assistance and
PROPHYLAXIS AND CO-CARCINOGENESIS BY BHT

David G. Beer, Martin S. Butley, and John A. Thompson for their helpful comments on this manuscript.

Note Added in Proof

We have subsequently tested the effect of a BHT/urethan protocol on tumor multiplicity in 14-day SWR and strain 129 mice. BHT was co-carcinogenic in these pre-weanlings to the same extent that it was in adults of these strains. Thus, the qualitative difference between pre-weanling and adult A/J mice treated with a BHT/urethan protocol did not occur in the 129 or SWR strains.

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Effects of Strain and Age on Prophylaxis and Co-Carcinogenesis of Urethan-induced Mouse Lung Adenomas by Butylated Hydroxytoluene

Alvin M. Malkinson and Larry G. Thaete


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