The carcinogenicity of environmental tobacco smoke

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Male strain A/J mice were exposed for 6 h a day, 5 days a week to environmental tobacco smoke (ETS) generated from Kentucky 1R4F reference cigarettes. Chamber concentrations were 87 mg/m^3 of total suspended particulate matter (TSP), 246 p.p.m. of CO and 16 mg/m^3 of nicotine. After 5 months, 33% of the ETS exposed and 11% of the control animals had one or several lung tumors; the difference was statistically not significant. A second group of animals exposed for 5 months to ETS was allowed to recover for another 4 months in filtered air. When they were killed, 85% of the ETS animals had lung tumors (average number per lung: 1.4 ± 0.2), whereas in the control group 38% had lung tumors (average number of lung tumors in all animals 0.5 ± 0.2). The differences in tumor incidence and multiplicity were statistically significant. More than 80% of all tumors were adenomas, the rest adenocarcinomas. When animals were pretreated with a carcinogen, lung tumor multiplicity was lower in the ETS exposed animals after 5 months compared with controls injected with a carcinogen and kept in air. However, after an additional 4 month recovery period in air, lung tumor multiplicities were the same in ETS plus carcinogen exposed mice as in carcinogen-treated air-exposed controls. Histopathologic and morphometric analysis of the lung tissue failed to reveal any differences between ETS exposed and control animals. However, immediately after ETS exposure, immunohistochemistry revealed increased staining for CYP1A1 in airway epithelia and lung parenchyma; following recovery in air, the staining disappeared again. Analysis of cell kinetics showed an initial burst of increased DNA synthesis in the epithelial cells of the airways and a smaller early positive response in the parenchyma. Feeding of butylated hydroxytoluene during ETS exposure did not modulate lung tumor development. It was concluded that ETS is a pulmonary carcinogen in strain A/J mice.

Introduction

In 1964, the Surgeon General warned in his first report on ‘Smoking and Health’ that active smoking causes disease and death (1). Today, the health risks of active smoking are well known. It also has become obvious that exposure to environmental tobacco smoke (ETS*), i.e., ‘passive’ or involuntary smoking in the workplace, public places or in the home is more than a simple nuisance to non-smokers. Of particular concern is the question whether ETS increases the risk of non-smokers to develop lung cancer. In 1992, the US Environmental Protection Agency analysed the available evidence on adverse respiratory health effects, including lung cancer, in non-smoking spouses of smokers and concluded that ETS is a human carcinogen (2). From available exposure data and anticipated size of the US population exposed to ETS, it was calculated that ETS might be responsible for ~3000 lung cancers per year in non-smokers aged 35 and over. Another analysis of epidemiological findings concluded that, taking all studies together, there was a statistically significant 20–30% increased risk for non-smokers married to smokers to develop lung cancer. ETS was judged to be ‘of carcinogenic importance’ (3). Involuntary exposure to ETS must be added to voluntary smoking as a risk factor. The implications for public health worldwide are considerable.

ETS is a mixture of cigarette sidestream smoke (SS) and cigarette mainstream smoke (MS). It contains 15% of MS, the smoke first inhaled by an active smoker and then exhaled; while briefly retained in the lung, the smoke is scrubbed of some of its constituents, most notably nicotine, CO and much of the particulate matter. Approximately 85% of ETS is SS, the smoke curling off the end of a lit cigarette between puffs. SS is generated at lower burning temperatures than is MS, has a different chemical composition and ages rapidly. Most notably, carcinogens in cigarette smoke are found in higher concentrations in SS than in MS (2). On the other hand, non-smokers are exposed to ETS diluted by several orders of magnitude compared to the MS inhaled by active smokers.

The toxicity of ETS is usually studied by exposing small laboratory rodents to pure SS. Acute (a few days) or subchronic (up to 6 months) exposure elicits minimal histopathological changes in the lower respiratory tract of adult rats and hamsters (4,5). In animals exposed perinatally, SS modifies post-natal lung development (6), increases the number of pulmonary neuroendocrine cells (7) and alters airway reactivity (8,9). Additional systemic toxic effects include enhanced development of arteriosclerotic lesions in the vessels of rats and rabbits (10–12), increased myocardial infarct size in rats (13), reduced birthweight in rats (14) and increased activity of drug metabolizing enzymes in the liver after few days exposure (15). On the other hand, exposure to ETS for 15 weeks down-regulates hepatic cytochrome P450 in ferrets (16). Thus, with the exception of carcinogenesis, exposure of animals to ETS has mimicked several conditions believed to occur in humans exposed to ETS. The carcinogenic potential and potency of SS needs to be examined, particularly in view of the observation that, in skin painting studies, SS condensates are much more tumorigenic than are MS condensates (17).

We recently conducted a carcinogenesis bioassay with SS (18) by using the strain A/J mouse lung tumor model (19).
This particular mouse strain had been used previously to assess the potential carcinogenic effect of full cigarette smoke. In 1952, Essenberg found a 91% lung tumor incidence in strain A/J mice exposed for 1 year to cigarette smoke, compared to a 59% incidence in controls; the difference was statistically significant (20). A later study by the same author was confirmatory, whereas another was not (21,22). Unfortunately, no data on chamber concentrations of smoke constituents are available from these studies. In our own experiment, we exposed male strain A/J mice to a SS concentration of 4 mg/m³ of total suspended particulate matter (TSP). After 6 months, the number of animals bearing lung tumors and the average number of tumors per lung (multiplicity) were identical to that of the controls. Analysis of the tumors for Ki-ras mutations indicated that SS had produced a shift in the mutation spectrum of lung tumors. This suggested that SS might have some effects on lung tumorigenesis in strain A/J mice (18).

Cigarette smoke is a comparatively weak carcinogen in experimental animals (23,24). It is possible that in our first attempt to produce lung tumours by SS the smoke concentration used had been too low. We therefore repeated the experiment by exposing A/J mice to a much higher SS concentration that was reinforced with MS in order to more closely duplicate ETS. In addition, we examined whether ETS might be a promoting agent. We found that ETS alone produced a significant increase in lung tumor multiplicity and incidence and at the same time, rather than being a promoting agent, delayed the formation of lung tumors induced by a chemical carcinogen.

Materials and methods

Animals

Male strain A/J mice, 6 to 8 weeks old, were purchased from Jackson Laboratories, Bar Harbor, ME. Selection of males only was dictated by available chamber space. Randomly chosen animals were sent to the Comparative Pathology Laboratory, UC Davis, for a standard health screen; no evidence for infectious or parasitic diseases were found with the exception of *P. mirabilis* in the cecum of the mice from one shipment. The animals were housed, 4 to a cage, in polypropylene boxes with tightly fitting wire screen lids on conventional bedding material. At all times during the experiment the animals had free access to conventional laboratory chow and to water *ad libitum*, including during smoke exposure.

Materials

Reference cigarettes Kentucky 1R4F were obtained from the Tobacco Research Institute, University of Kentucky, Lexington KY and Alitex osmotic minipumps (model 2001, nominal pumping rate 1 μl/h) from Alza Corporation, Palo Alto, CA. Urethan (ethyl carbamate), 3-methylcholanthrene and 5-bromo-2′-deoxyuridine (BrdU) were from Sigma Chemical Co., St Louis, MO and anti-BrdU antibody from Boehringer-Mannheim, Indianapolis, IN. The Peroxidase Vectastain Elite ABC Kit, mouse IgG, was from Vector Laboratories, Burlingame, CA and Immuno-Bed™ from Polyclinics, Inc., Warrington, PA. BHT-containing diets (TD 80139 and TD 81142) were obtained from Harlan-Teklad, Madison, WI. The control diet contained 20% casein, 0.3% methionine, 15% corn starch, 55.7% sucrose, 5% corn oil, vitamins and mineral mix AIN-76 (3.5%). Butylated hydroxytoluene (BHT) was added at a concentration of 0.5% with an adjustment in sucrose.

Experimental design

An overview of all experiments is given in Table I.

### Carcinogenesis study and tumor modulation experiment

For the carcinogenesis study, a total of 96 mice, 12 weeks old, were randomly divided into two groups and placed within their plastic cages into glass and stainless steel Hinners-type exposure chambers (volume: 0.44 m³). The experimental group (*n* = 48) was exposed for 6 h a day, 5 days per week (Monday through Friday) to ETS produced from burning Kentucky 1R4F reference cigarettes. The control group (*n* = 48) was kept in filtered air. A second experiment was designed to examine whether ETS would ‘promote’ chemically induced lung tumors. Another 96 mice were randomly divided into two groups. One group received one single intraperitoneal (i.p.) injection of urethan (500 mg/kg) and the other one single i.p. injection of 3-methylcholanthrene (20 mg/kg). These doses may be expected to give within 4 months a 100% tumor incidence and an average of 7 to 10 tumors per lung (25,26). Three days later, each group was randomly divided into two groups and placed within their plastic cages into glass and stainless steel chambers (volume: 0.44 m³). The air controls for both groups were ventilated with filtered air. Half of the animals after 5 months, the other half after another 4 months of recovery in air; tumor count, pathology, morphometry and immunocytochemistry in animals killed after 5 months and in animals killed 9 months after beginning of the experiment.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment groups</th>
<th>Exposure conditions</th>
<th>Sacrifice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinogenesis</td>
<td>ETS exposed (<em>n</em> = 48) and filtered air in controls (<em>n</em> = 48)</td>
<td>87.3 mg/m³ of TSP</td>
<td>5 months</td>
</tr>
<tr>
<td>Tumor modulation</td>
<td>Urethan and ETS (<em>n</em> = 24) or air (<em>n</em> = 24); methylcholanthrene and ETS (<em>n</em> = 24) or air (<em>n</em> = 24)</td>
<td>87.3 mg/m³ of TSP</td>
<td>5 months</td>
</tr>
<tr>
<td>BHT and ETS co-carcinogenesis</td>
<td>BHT diet and ETS (<em>n</em> = 24) or air (<em>n</em> = 24); control diet and ETS (<em>n</em> = 24) or air (<em>n</em> = 24)</td>
<td>52.6 mg/m³ of TSP</td>
<td>2.5 months</td>
</tr>
<tr>
<td>Cell kinetics</td>
<td>ETS exposed and filtered air controls (<em>n</em> = 5 per group)</td>
<td>83.5 mg/m³ of TSP</td>
<td>10 weeks</td>
</tr>
</tbody>
</table>

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The average exposure concentration in the inhalation chambers was 87.3 mg/m³ of TSP (Table II). In the chambers, the cages were placed on a top and a bottom rack, six cages on each rack, three in a front and three in a back row. Every week, the position of all cages was rotated in a systematic way so that during the entire experiment every cage at least twice occupied each of the 12 possible positions within the chamber. The air controls for each group were kept in glass and stainless steel chambers that were ventilated with filtered air. The animals were observed daily and weighed weekly. After 5 months, half of the animals in each group were killed with a pentobarbital overdose and the lungs were processed for histopathology and tumor count. The remaining animals were removed from the inhalation chambers and kept for another 4 months within their cages in a humidity and temperature controlled environment (20–21°C and 40–70% relative humidity). Nine months after the beginning of the experiment, the animals were killed and the lungs analysed for tumor presence and histopathology.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Carcinogenesis studies</th>
<th>Cell kinetic studies</th>
<th>BHT co-carcinogenesis studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative humidity (%)d</td>
<td>38 ± 10 (n = 145)</td>
<td>41 ± 8 (n = 48)</td>
<td>41 ± 8 (n = 48)</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>22.4 ± 1.7 (n = 145)</td>
<td>22.6 ± 0.9 (n = 48)</td>
<td>22.7 ± 0.9 (n = 48)</td>
</tr>
<tr>
<td>Carbon monoxide (p.p.m.)</td>
<td>16.1 ± 3.9 (n = 175)</td>
<td>18.9 ± 3.9 (n = 41)</td>
<td>17.0 ± 5.1 (n = 43)</td>
</tr>
<tr>
<td>Nicotine (mg/m³)</td>
<td>87.3 ± 21.0 (n = 191)</td>
<td>83.5 ± 7.9 (n = 45)</td>
<td>52.6 ± 8.3 (n = 46)</td>
</tr>
<tr>
<td>Mass median diameter (MMD, µn)</td>
<td>0.41 ± 0.02 (n = 3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Geometric standard deviation</td>
<td>1.87 ± 0.12 (n = 3)</td>
<td>6.3 ± 3.9 (n = 5)</td>
<td></td>
</tr>
</tbody>
</table>

Tissue preparation

For analysis of tumor incidence and multiplicity, the lungs were manually expanded with Telleyesniczky's fluid and fixed for at least 24 h. The number of tumor nodules visible on the lung surface was counted by two different observers. The procedure has been shown to accurately give the total number of all macroscopically discernible tumors in a mouse lung (28). The results were expressed as tumor incidence, i.e., percentage of animals with one or several lung tumors and as tumor multiplicity, the average number of tumors per lung. Tumor multiplicity was calculated as average number of tumors per lung of all, including non-tumor bearing animals (19) and for tumor-bearing animals only. Histopathology of lung tumors was done on hematoxylin and eosin (H&E) stained paraffin sections.

For determination of labeling indices, the lungs were inflated through the trachea with 1% paraformaldehyde/0.1% glutaraldehyde solution and 1 h later were transferred into 70% ethanol. Slides were cut and stained for visualization of BrdU with the procedures described in detail before (29). Labeling indices were determined on coded slides for the alveolar zone, the terminal bronchiolae and the large intrapulmonary airways. In the alveolar zone, 500 to 1000 cells per lung were counted in randomly selected fields and in the conducting airways a minimum of 500 cells per lung was counted. Terminal bronchiolae were defined by their opening into alveolar ducts and intrapulmonary large airways by their diameter (0.5 to 1.5 mm). The labeling index was calculated as the percentage of labeled epithelial cells per total number of epithelial cells counted (29).

Preparation for immunocytochemistry

The lungs from air control mice and mice exposed to ETS in the carcinogenesis experiment and killed after 5 months smoke exposure (Group 1) and 4 months recovery (Group 2) were fixed in situ via intratracheal instillation of 1% paraformaldehyde for 1 h and embedded in paraffin. Tissue slides were prepared as described previously (30). A polyclonal antibody against purified rat P450IA1 (Gentest Corp, Woburn, MA) produced in goat was used to detect P450IA1. A polyclonal antibody against purified rat P4502B1 (Gentest Corp., Woburn, MA) produced in goat was used to detect P4502B1. A polyclonal antibody against rat P4502E1 produced in goat (31) was used to detect P4502E1 in the lungs (Gentest Corp., Woburn, MA). Purified liver microsomes demonstrate a single band for CYP2B1 and CYP2E1 by Western blot. The polyclonal antibody for CYP1A1 shows a double band with slight cross-reactivity to CYP1A2, as a minor band, and a second band with strong staining to 1A1. Each of these antibodies (1A1, 2B1 and 2E1) have been shown to be cross-reactive in the mouse. A detailed description of the immunocytochemical procedures is provided in a previous publication (30).

Preparation for morphometric analysis

For morphometry, animals from the carcinogenesis study were killed after 5 months of ETS exposure (Group 1) and after a 4 month recovery period (Group 2). They were deeply anesthetized by sodium pentobarbital and the trachea was cannulated. The abdomen was opened, the lungs collapsed and instilled with 0.6% glutaraldehyde, 0.9% paraformaldehyde, pH 7.4 and 0.2% glutaraldehyde. The lungs were then inflated with 1% paraformaldehyde/0.1% glutaraldehyde and stored in methanol.

Butylated hydroxytoluene (BHT) co-carcinogenesis studies

In this experiment we investigated whether TS-induced lung tumor yield could be modified by concomitant feeding of BHT. A total of 96 mice were randomly divided into four groups: BHT diet and exposed to ETS (n = 24), control diet and exposed to ETS (n = 24), BHT diet and kept in filtered air (n = 24) and control diet and kept in filtered air (n = 24). ETS exposure and feeding of the BHT diet were begun simultaneously. Originally it was intended to expose the animals to the same ETS concentration as in the other experiments; however, during the first weeks several animals in the ETS and BHT group died, making it necessary to reduce the ETS concentration to 52.6 mg/m³ of TSP (Table II). After 2.5 months in ETS, the animals were moved to clean air and, while continuously being fed the BHT or control diets, were allowed to recover until 9 months after the beginning of the experiment. At this time they were killed and the lungs were analysed for presence of lung tumors.

Cell kinetic studies

For determination of cell kinetics, animals were exposed to ETS at a chamber concentration of 83.5 mg/m³ of TSP (Table II). Control animals were kept in filtered air. The cumulative labeling indices were determined during weeks 1, 2, 3, 4, 6 and 10 of ETS exposure. At the end of week 10, the animals were removed from the exposure chamber and cell proliferation was measured during the recovery period in weeks 11 (first week of recovery), 12, 14 and 20 (second, fourth and tenth week of recovery, respectively). In order to measure labeling indices, five animals exposed to ETS and five animals kept in filtered air were lightly anesthetized with methoxyflurane at the beginning of each week indicated above. An osmotic minipump filled with BrdU solution (20 mg/ml) was implanted under the skin of the back. One week later, the animals were killed by pentobarbital overdose and the lungs processed for immunohistochemistry.

Exposure system

The ETS exposure system was identical to the one described by Teague et al. (27) and previously described in detail (18). Briefly, SS was generated by burning conditioned (48 h in 60% humidity) Kentucky 1R4F reference cigarettes in a smoking machine with standardized 35 ml puffs of 2 s duration, once every min, for a total of eight puffs per cigarette. After dilution and aging in a conditioning chamber (2 min), the SS produced between puffs was drawn into the exposure chambers. In order to reach the desired target concentrations of smoke in the exposure chamber, the SS atmosphere was reinforced every 58 s with a 2 s puff of MS (representing 11% of the total smoke mixture). The chamber atmosphere was monitored for CO, nicotine and total suspended particulate matter (TSP). Total suspended particulate matter was measured by weighing material collected on filters and carbon monoxide was monitored with a model 880 non-dispersive-infrared (NDIR) analyser (Beckmann Industries, La Habra, CA). Nicotine concentrations were measured by drawing air samples through sorbent tubes, extracting the nicotine and analysis with gas chromatography. All procedures have been described in detail (18).

Environmental tobacco smoke
Results

Carcinogenesis study

In the carcinogenesis study, male A/J mice were exposed for 5 months to ETS at an average TSP concentration of 87.3 mg/m³. All animals survived in the smoke atmosphere. While being exposed to ETS, they lost almost 15% of their initial body weight within the first month of exposure (Table III). The animals failed to gain weight until removed from the smoke inhalation chambers. Within 2 weeks following transfer into clean air, they had regained all lost weight and body weights stayed comparable to controls until the end of the experiment.

Lung tumor incidence and multiplicities were determined in animals killed immediately after the 5 month smoke exposure and also in animals given an additional 4 month recovery period in air. The data are shown in Table IV. After 5 months of ETS exposure, both tumor incidence and tumor multiplicity were somewhat higher in the ETS exposed animals than in the corresponding controls; however, the differences were not statistically significant. It only became obvious after the 4 month recovery period that ETS had a tumorigenic effect. Lung tumor incidence (83%) was more than twice that found in controls (38%). According to Fisher’s exact test, this difference is considered to be very significant. Lung tumor multiplicity (calculated for all animals, including non-tumor bearing ones) was higher than 1.0 and almost three times more than in controls. According to the criteria developed by Shimkin and Stoner (19), the finding constitutes an unequivocal positive response in the strain A/J mouse lung tumor model. It can be concluded that ETS produces lung tumors in mice.

Proliferative pulmonary lesions were morphologically

### Table III. Body weights during exposure to ETS (87 mg/m³ of TSP)\(^a\)

<table>
<thead>
<tr>
<th>Month of exposure</th>
<th>Filtered air exposed</th>
<th>ETS exposed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Begin</td>
<td>27.2 ± 2.0</td>
<td>27.0 ± 1.9</td>
</tr>
<tr>
<td>1</td>
<td>27.2 ± 1.9</td>
<td>23.7 ± 1.5</td>
</tr>
<tr>
<td>2</td>
<td>27.5 ± 1.6</td>
<td>23.4 ± 1.4</td>
</tr>
<tr>
<td>3</td>
<td>27.4 ± 1.8</td>
<td>22.6 ± 1.4</td>
</tr>
<tr>
<td>4</td>
<td>28.2 ± 1.7</td>
<td>23.9 ± 1.4</td>
</tr>
<tr>
<td>5</td>
<td>28.4 ± 2.1</td>
<td>24.2 ± 1.4</td>
</tr>
<tr>
<td>6</td>
<td>28.1 ± 1.5</td>
<td>27.9 ± 1.9</td>
</tr>
<tr>
<td>7</td>
<td>29.0 ± 1.5</td>
<td>28.4 ± 2.1</td>
</tr>
<tr>
<td>8</td>
<td>29.3 ± 1.6</td>
<td>28.6 ± 2.1</td>
</tr>
<tr>
<td>9</td>
<td>30.0 ± 1.7</td>
<td>29.5 ± 2.3</td>
</tr>
</tbody>
</table>

\(^a\) All data (g body weight) given as means ± SE. At the beginning of the experiment, the number of animals in each group was \( n = 48 \). After 5 months of exposure, 24 animals in each group were killed and all the other animals were allowed to recover in filtered air until 9 months. The animals in the ETS exposed group returned to control weights within 2 weeks following cessation of smoke exposure.

### Table IV. Lung tumors in strain A/J mice exposed to ETS\(^a\)

<table>
<thead>
<tr>
<th>Exposure conditions</th>
<th>Parameter</th>
<th>Air controls(^b)</th>
<th>ETS exposed(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 months in ETS</td>
<td>Tumor incidence(^c)</td>
<td>2/24 (8%)</td>
<td>6/24 (25%)</td>
</tr>
<tr>
<td>(87 mg TSP/m³)</td>
<td>Tumor multiplicity, all animals(^d)</td>
<td>0.1 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>(( n = 24))</td>
<td>(( n = 24))</td>
<td>(( n = 24))</td>
</tr>
<tr>
<td>and 4 months air</td>
<td>Tumor multiplicity, tumor bearing animals only(^e)</td>
<td>1.0 ± 0.0</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>(( n = 2))</td>
<td>(( n = 6))</td>
<td>(( n = 6))</td>
</tr>
<tr>
<td>2.5 months in ETS</td>
<td>Tumor incidence</td>
<td>9/24 (38%)</td>
<td>20/24 (83%)</td>
</tr>
<tr>
<td>(53 mg TSP/m³)</td>
<td>Tumor multiplicity, all animals</td>
<td>0.5 ± 0.2</td>
<td>1.4 ± 0.2(^f)</td>
</tr>
<tr>
<td></td>
<td>(( n = 24))</td>
<td>(( n = 24))</td>
<td>(( n = 24))</td>
</tr>
<tr>
<td>and 6.5 months air</td>
<td>Tumor multiplicity, tumor bearing animals only</td>
<td>1.3 ± 0.2</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>(( n = 9))</td>
<td>(( n = 20))</td>
<td>(( n = 20))</td>
</tr>
</tbody>
</table>

\(^a\) A/J mice were exposed to ETS at concentrations and times indicated, 6 h/day, 5 days/week.

\(^b\) Where appropriate, data are given as mean ± SE with the number of animals in parentheses.

\(^c\) Number of tumor bearing animals/total number of animals (%).

\(^d\) Total number of lung tumors divided by total number of animals, inc. non-tumor bearing animals.

\(^e\) Average number of lung tumors per tumor bearing animal.

\(^f\) Total number of tumor bearing animals only.

\(^p\) Compared with controls by Fisher’s exact test.

\(^p\) Compared with controls with Welch’s alternate \( t \)-test.

\(^\ast\) Pooled data from Table VI.
similar between control mice and those exposed to ETS and were typical of pulmonary lesions commonly observed in strain A mice (33). Lesions were categorized as focal alveolar epithelial hyperplasia, alveolar/bronchiolar adenomas, and alveolar/bronchiolar adenocarcinomas based upon previously published diagnostic criteria (34). The majority of adenomas had a solid growth pattern with contiguous neoplastic cells filling alveolar spaces. There was no difference in the relative distribution of adenocarcinomas and of adenomas between the two groups. In the ETS exposed animals, 17% of all tumors had a pattern consistent with the diagnosis of adenocarcinoma and in the control group 20% did so. Of the four carcinomas observed, two had a solid growth pattern, one had a mixed obviously, ETS does not enhance the development of chemically-initiated lung tumors. As an alternative, we examined whether the development of ETS-induced lung tumors could be modulated. For this purpose we treated the animals with the antioxidant BHT, an agent known to enhance lung tumor development in mice (35). Originally it was planned to expose animals to a high ETS concentration (87 mg of TSP/m³) as in the carcinogenesis study while feeding them a diet containing 0.5% BHT for 2.5 months, a time sufficient to have a substantial promoting effect (36). During the first 2 weeks of ETS exposure, it was found that several animals kept on a BHT diet and exposed to ETS died. This made it necessary to reduce the mixture of 89% SS and 11% MS to an average ETS concentration of 52.6 mg/m³ of TSP. At this reduced concentration, no more animals died directly because of ETS exposure; a few deaths were caused by fighting and other unrelated causes. After 2.5 months, the animals were removed from ETS and, while still being fed the BHT diet, were allowed to recover in air for 6.5 months. All animals were killed 9 months after the beginning of the experiment. We found that tumor incidence and tumor multiplicity in animals exposed to ETS remained significantly lower than in the controls. This inhibitory action of ETS was only of a transitory nature. Nine months after the beginning of the experiment, both groups had the same number of tumors per lung, indicating that removal of the animals from ETS and recovery in air allowed tumor multiplicity to increase at a faster rate than in control animals. Essentially the same observation was made in the animals treated with a single injection of 3-methylcholanthrene. After 5 months, the number of tumors was significantly lower in the ETS exposed animals, but then approached the number of tumors found in control lungs after an additional 4 month recovery period in air.

**Table V. Tumor modulating study**

<table>
<thead>
<tr>
<th>Time of sacrifice</th>
<th>Animals treated with urethan</th>
<th>Animals treated with 3-methylcholanthrene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Air exposed (n)</td>
<td>ETS exposed (n)</td>
</tr>
<tr>
<td>After 5 months in ETS</td>
<td>11.3 ± 1.2 (12)</td>
<td>5.3 ± 0.9 (12)</td>
</tr>
<tr>
<td>After 5 months in ETS and 4 months in air</td>
<td>16.7 ± 0.9 (11)</td>
<td>16.3 ± 1.4 (12)</td>
</tr>
</tbody>
</table>

*Table VI. Lung tumor data in animals fed BHT*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ETS and BHT diet</th>
<th>ETS and control diet</th>
<th>Filtered air and BHT diet</th>
<th>Filtered air and control diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor incidence</td>
<td>13/17 (76%)</td>
<td>15/21 (71%)</td>
<td>10/18 (56%)</td>
<td>13/23 (57%)</td>
</tr>
<tr>
<td>(all animals)</td>
<td>(n = 17)</td>
<td>(n = 21)</td>
<td>(n = 18)</td>
<td>(n = 23)</td>
</tr>
<tr>
<td>Tumor multiplicity a</td>
<td>1.5 ± 0.3</td>
<td>1.1 ± 0.2</td>
<td>0.8 ± 0.2</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>(tumor bearing only)</td>
<td>(n = 13)</td>
<td>(n = 15)</td>
<td>(n = 10)</td>
<td>(n = 13)</td>
</tr>
</tbody>
</table>

*Animals were exposed for 5 months to ETS (87 mg/m³ of TSP, 6 h/day, 5 days/week). After 5 months, half the animals were killed and the remaining animals allowed to survive in air for another 4 months. All data are given as mean ± SE. Lung tumor incidence was 100% in all groups.*

*500 mg/kg of urethan i.p.*

*20 mg/kg of 3-methylcholanthrene i.p.*

*p < 0.05 compared with air-exposed controls.*
ETS and fed a BHT diet was slightly higher than in animals exposed to ETS and fed the control diet; however, the difference was statistically not significant (Table VI). BHT had no tumor-modulating effect in animals exposed to filtered air. The data from all the animals exposed to ETS, whether fed BHT or not, were then pooled and so were the control data. Table IV shows the results of this analysis. It was found that lung tumor multiplicity was again significantly higher than it was in controls, although in this experiment animals had been exposed to a much lower ETS concentration for only 2.5 months. Lung tumor incidence in the ETS exposed animals was comparable to the incidence found in the first experiment, but a somewhat higher lung tumor incidence in the control group made the difference not significant ($P > 0.1$).

**Histomorphometry**

The lungs of animals exposed to the high concentration of ETS (87 mg/m$^3$ of TSP) were examined 5 months after ETS (Group 1) and after an additional 4 month recovery period in air (Group 2). Under the light microscope, no obvious histopathological lesions were identified in the ETS exposed animals. A detailed morphometric analysis of the lung parenchyma was conducted (Table VII). There was no measurable effect of ETS on alveolar air or tissue volumes or the number of alveolar type II cells or alveolar macrophages. In control animals, all parameters evaluated were essentially the same in 7 and 11 month old animals. The only significant changes in morphology, as well as signs of continually increased cell proliferation were found in the nasal septum. These observations will be reported elsewhere.

**Immunocytochemistry**

Immunocytochemical staining for the cytochrome P450 isozymes were examined in two manners. The first was to examine the general staining of airways and lung parenchyma following exposure to ETS for 5 months (Group 1) and again following 4 months after recovery in filtered air (Group 2). The second approach was to examine regions within the lung containing them. The distribution of staining for each isozyme was determined.

Staining with cytochrome P4501A1 in control lungs was not evident. In contrast, for those animals that had been exposed to ETS for 5 months, immunocytochemistry showed significant staining of airway epithelium and the lung parenchyma (Figure 1A and B). Punctate staining of the non-ciliated bronchiolar epithelium was noted throughout all airways, but not for every non-ciliated bronchiolar cell. Staining of the lung parenchyma for alveolar septal cells was diffuse, beginning at the bronchiole-alveolar duct junction and extending throughout the parenchyma to the subpleural tissues. Specific cell types staining within the parenchyma were endothelial cells, best identified in large blood vessels. Alveolar septal cells were less clearly identified, but were suggestive of endothelial and epithelial cells, although definitive identification was not possible. Four months following the end of ETS exposure, no staining for CYP1A1 could be detected in the airway epithelium or the lung parenchyma (Figure 2C).

Cytochrome P450 2B1 and 2E1 were present in the lungs and strongly stained the epithelial cells of the airways. No differences were noted between control and ETS-exposed animals. No staining of the lung parenchyma was noted for CYP2E1, however, discrete staining of alveolar septal cells was noted for CYP2B1. Although specific cell types for 2B1 could not be identified, no endothelial cells stained. Those cells staining were cuboidal in shape and appeared to be epithelial type II cells. The staining pattern noted for both CYP2B1 and 2E1 was similar 4 months following the end of smoke exposure with no differences noted between animals exposed only to filtered air or allowed to recover for 4 months after a 5-month exposure to ETS.

The presence of cytochrome isozymes within lung tumors compared with the lung parenchyma was similar. No enhanced staining was noted for CYP 1A1, 2B1 or 2E1. The majority of tumors examined were in the lungs of animals at 4 months after the end of ETS exposure. Like the rest of the lung parenchyma, no enhanced staining for CYP1A1 was found within any tumor examined. CYP2B1 staining was most evident within the lung tumors examined, but no greater than that observed throughout the lung parenchyma. CYP2B1 staining within the tumor mass appeared to be in epithelial cells lining thin slits of airspace found throughout the tumor.

**Cell kinetics**

Cumulative 1-week labeling indices were measured in the alveolar zone and in the airways during weeks 1, 2, 4, 6 and 10 of ETS exposure and during weeks 1, 2, 4 and 10 of a recovery period in air. Concentrations of ETS (83 mg/m$^3$) were comparable to the ones used in the carcinogenesis study. The data are presented in Table VIII and Figures 2 and 3. In the alveolar zone, the labeling indices were significantly increased during the first 2 weeks of exposure. For the remainder of the experiment, they stayed slightly higher than labeling indices in controls, although the difference, with one exception at 14 weeks was no longer statistically significant. In the airways, both in the large intrapulmonary airways

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**Table VII. Morphometry of lung parenchyma**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>5 months ETS exposure (Group 1)</th>
<th>4 months recovery (Group 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Air controls</td>
<td>ETS exposed</td>
</tr>
<tr>
<td>Fixed lung volume (cm$^3$)</td>
<td>1.51 ± 0.08</td>
<td>1.48 ± 0.12</td>
</tr>
<tr>
<td>Alveolar air volume (cm$^3$)</td>
<td>0.95 ± 0.06</td>
<td>0.91 ± 0.09</td>
</tr>
<tr>
<td>Tissue volume (cm$^3$)</td>
<td>0.16 ± 0.02</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>Capillary volume (cm$^3$)</td>
<td>0.09 ± 0.01</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>Macrophage volume (cm$^3$)</td>
<td>0.015 ± 0.007</td>
<td>0.018 ± 0.007</td>
</tr>
<tr>
<td>Alveolar surface area both lungs (cm$^2$)</td>
<td>1511 ± 192</td>
<td>1479 ± 130</td>
</tr>
<tr>
<td>No. of alveolar, Type II cells</td>
<td>8.6 ± 0.9×10$^6$</td>
<td>7.1 ± 0.7×10$^6$</td>
</tr>
<tr>
<td>No. of alveolar, macrophages</td>
<td>2.1 ± 0.7×10$^6$</td>
<td>2.1 ± 0.6×10$^6$</td>
</tr>
</tbody>
</table>

*Animals were exposed for 5 months to 87 mg/m$^3$ of TSP and then allowed to recover for another 4 months in filtered air. All data are given as mean ± SD; the number of animals in each group was $n = 6$. 

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Environmental tobacco smoke

Fig. 1. Cytochrome P4501A1 immunostaining following exposure to ETS (87.3 mg/m³ of TSP, 6 h/day, 5 days/week). Numerous non-ciliated bronchiolar epithelial cells of the lung airways intensely stained for CYP1A1 as well as many alveolar septal cells throughout the lung parenchyma following 5 months of exposure to sidestream cigarette smoke (panels A and B). Stained cells included endothelial cells lining the capillaries and large vessels and cuboidal cells probably representing alveolar epithelial cells, although the identification of these cells as epithelial could not be definitively determined. The lungs from control animals exposed to filtered air only failed to demonstrate any detectable staining for CYP1A1 for the airways or the parenchyma. Four months recovery from ETS was associated with a complete loss of staining for CYP1A1 in the airways and the lung parenchyma (panel C).

Discussion

In the present study we have shown that exposure of strain A/J mice to ETS results in a significantly increased lung tumor incidence and multiplicity. In animals exposed for 5 months to an average concentration of 87 mg/m³ of TSP, the tumor incidence was twice as high and tumor multiplicity (average number of tumors in all animals, including non-tumor bearing mice) essentially three times as high as in a corresponding control group. In a second experiment, in which animals were exposed for half the length of time to a lower concentration of ETS, lung tumor multiplicity was still twice as high as in controls. These observations agree in principle with the findings of Essenberg (20) who found a significantly higher lung tumor incidence (91%) in A/J mice exposed for 14 months to cigarette smoke than in controls (59%). In later studies, exposing animals to smoke produced from cigarettes containing less nicotine and tar resulted in a weaker response (21,37). The lack of proper exposure data, e.g. concentration of smoke constituents within the inhalation chamber, preclude

(Figure 2) and in the terminal bronchioles (Figure 3), we found an initial burst of cell proliferation during the first 2 weeks. This was followed by a sustained increase in the number of BrdU positive cells for another 4 weeks. At 10 weeks, labeling indices were practically identical in ETS exposed and control animals and remained so for the entire recovery period.

Fig. 2. Cumulative labeling indices (percentage of labeled cells) in large intrapulmonary airways. Experimental animals (filled circles) were exposed for 6 h/day, 5 days/week to ETS produced by burning Kentucky 1R4F reference cigarettes (chamber concentration 83.5 mg/m³ of TSP). Control animals (open circles) were kept in filtered air. Cumulative labeling indices were determined during the weeks indicated on the abscissa. All data are plotted as mean ± SE; where there is no SE plotted, it was smaller than the symbol. The number of animals per group was n = 5 throughout. Values labeled with an asterisk were significantly different (P < 0.05) from the corresponding control group.

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Table VIII. Labeling indices in alveolar zone*  

Week | Filtered air exposed | Sidestream smoke exposed  
------- | --------------------- | ------------------------  
1 | 6.9 ± 0.6 | 10.4 ± 1.0b  
2 | 8.2 ± 0.5 | 9.8 ± 0.4b  
4 | 6.5 ± 1.0 | 8.7 ± 1.5  
6 | 7.4 ± 0.9 | 8.5 ± 0.8  
10 | 5.1 ± 0.6 | 6.0 ± 0.2  
11 (1 of recovery period) | 3.7 ± 0.5 | 6.8 ± 1.4  
12 (2 of recovery period) | 3.0 ± 0.4 | 3.2 ± 0.6  
14 (4 of recovery period) | 3.7 ± 0.7 | 6.7 ± 0.9b  
20 (10 of recovery period) | 3.2 ± 0.6 | 5.4 ± 2.1  

*Animals were exposed to 83.5 mg/m³ of TSP for 10 weeks and then removed into air. All data are given as means ± SE. The number of animals was n = 5 throughout.  
bP < 0.05 compared to filtered air controls.

for a total of 26 weeks), but smoke exposure concentrations were much higher (248 mg/m³ of TSP). The animals were given only a 5-week recovery period and no lung tumor effect was observed (45). It is equally interesting to note that rats exposed to another complex carcinogenic mixture, diesel exhaust, had a higher lung tumor incidence after exposure for 12 months and allowed a 28 month recovery period than did animals who had been exposed for 24 months, but allowed only a 6 month recovery period (48).

There are several reasons why a recovery period might be important. In both our experiment and the one reported by Finch et al. (45), animals exposed to cigarette smoke initially lost weight and then failed to gain weight at the same rate as did the controls. At the end of the exposure period, weights of the exposed animals were only 85% of control values. ETS may have resulted in appetite depression and reduced food intake, which became immediately and fully reversible upon return of the animals to clean air (Table III). It is well established that caloric restriction suppresses tumorigenesis (49,50), and also in strain A/J mice treated with a polycyclic aromatic hydrocarbon (51). An alternative explanation might be that the metabolic rate in ETS-exposed mice was greater than in controls; this might enhance detoxification pathways. A third possibility might be that ETS suppresses or slows the progression of initiated cells into tumors. In this paper we have clearly demonstrated that the growth of urethan- or methylcholanthrene-induced lung tumors in A/J mice can be significantly suppressed as long as the animals are kept in ETS. However, as soon as the animals are removed from the ETS atmosphere, tumors grow and, after 4 months recovery, the number of tumors per lung is similar to that of the controls (Table VI). Since lung tumors were induced with only a single administration of the chemical given 3 days before ETS exposure, it may be concluded that ETS did not affect initiating events, but rather subsequent progression growth of initiated cells to visible tumors. It has been demonstrated by others that tobacco smoke may prevent the development of chemically-
induced lung tumors in mice (52), although the effect depended upon the experimental protocol. Other agents such as oxygen (53) or ozone (54) have a similar effect. The precise mechanism for the tumor-inhibiting action of ETS or other oxidants is not known, but might involve increased susceptibility of newly transformed cells to oxidant damage. This may prevent rapid progression to tumors while still conserving their eventual tumorigenic potential.

Tobacco smoke is usually not thought of as an agent that may prevent lung cancer development, although at least two epidemiologic studies in humans also concluded that tobacco smoke may interfere with pulmonary carcinogenesis induced by other agents. Continuous cigarette smoking partially inhibited lung cancer development in workers exposed to chloromethyl ethers (55). In metal ore miners, non-smokers developed more lung tumors than did smokers; on the other hand, smoking shortened the tumor latency period and was called a promoting stimulus (56). In considering possible explanations, it was speculated that a thickened mucus layer in the airways of smokers might have been responsible for this unexpected finding. A thickened layer might favor hydrolysis of chloromethyl ethers to non-carcinogens or increase the distance between deposited alpha-radiation emitting particles and cellular targets. However, the overwhelming majority of epidemiological studies show unequivocally that in man, smoking is a risk to develop lung cancer, but by a substantial decrease in risk (57). The observations of Weiss (55) and of Axelson and Sundell (56) may represent special situations and do not apply to smoking in general.

A rather surprising finding was the absence of any obvious non-cancer related histopathological lesions in the lungs of the mice exposed to ETS. Histological examination of the tissues under the light microscope did not reveal any abnormalities and the detailed morphometric analysis of the lung tissue failed to show any significant differences between ETS-exposed animals and controls. Perhaps the most unexpected observation was the absence of any response of the alveolar macrophage population to ETS. Contrary to what was expected, the number or volume of alveolar macrophages did not increase, nor did they show any signs of cytoplasmic accumulation of ETS particles. Although the morphometric parameters examined in the lungs of ETS-exposed animals were consistently reduced by a factor of 5–10% (e.g. alveolar air volume), the reductions were small and not statistically significant. Previous studies with SS resulted in no or only minimal histopathological lesions in the respiratory tract of experimental animals. Rats and hamsters exposed to considerably lower SS concentrations, 10 mg/m³ of TSP, had only hyperplasia, metaplasia and increased cell proliferation in the epithelium of the nasal septum. No changes in the lung parenchyma were reported (4,5). This agrees fully with our findings: the epithelium lining the nasal turbinates was the only site to show signs of ETS induced lesions and had a greatly increased epithelial cell proliferative rate (unpublished observations). The nasal epithelium may thus be in obligatory nose breathers the main target for ETS. Ferrets exposed to 38 mg/m³ of ETS, 2 h/day for 15 weeks, had a two- to threefold increase in collagen deposits in the submucosa of the respiratory bronchioles (58).

Immunocytochemistry of the lungs of the animals exposed to ETS showed significant immunostaining for cytochrome P450 isoenzyme 1A1. CYP1A1 induction was observed through-out the lungs, particularly in the non-ciliated bronchiolar epithelial cells and at all levels of the pulmonary parenchyma, including in endothelial cells and cells of the alveolar septa. The findings essentially confirm the observations by Forkert et al., who described increased levels of protein in endothelial and alveolar septal cells of strain A/J mice treated with 3-methylcholanthrene (59). This was in striking contrast to a previous study, in which A/J mice exposed for 6 months to a concentration of 4 mg/m³ of TSP had CYP1A1 immunostaining only in the vicinity of the bronchiolo–alveolar junction and limited to endothelial cells of the pulmonary parenchyma, but not in the bronchiolar non-ciliated epithelial cells (30). The most probable explanation is the increase in smoke concentration. Enzyme induction appeared to be only a transitory phenomenon and all signs of a presence of increased protein had disappeared after the recovery period in air. Two other isoforms, 2B1 and 2E1 were not found to be affected by ETS exposure at any time.

Sufficient tumor material to conduct immunohistochemical studies was only available from animals that had been allowed to recover in air. No evidence of increased levels of isozyme activities were detected. It could not be ascertained whether tumors induced by ETS, as do other tumors in A/J or SWR mouse lungs (59,60), respond less to an inducing stimulus than does the surrounding parenchyma, since the only tumors examined were from animals in which CYP1A1 activities had already returned to control levels. Analysis of cell proliferation was performed by measuring the cumulative 1-week labeling index over selected time periods during and following ETS exposure. Initially, there was a marked rise in labeling indices in the large intrapulmonary airways and in the terminal bronchioles, consistent with the idea that tumorigenesis may be linked to increased cell proliferation. In the fourth exposure week, airway labeling indices dropped, and eventually approached those found in controls and at the end of the exposure time, were identical to controls. Removal of the animals from ETS did not result in any rebound phenomenon. In the alveolar zone, where tumors can also be expected to form, there was a weaker response, significant only during the first 2 weeks. Basically the observations agree with previous studies. In rats exposed to 10 mg/m³ of TSP, an increase in labeling indices was only seen in the terminal airways after 5 days but not after 28, 90 or 180 days (61). In A/J mice exposed to 4 mg/m³ of TSP, airway labeling indices were briefly above control values in weeks 1–3 (18). However, no increase in the alveolar zone was seen. Hamsters appear to be more resistant, since no increased cell proliferation was seen in the airways in animals while they were exposed to cigarette smoke (62) and a slight increase was seen only in animals allowed to recover in air (63). It appears that, similar to what is found with other inhaled oxidants such as ozone (64), the cells lining the airways may become ‘adapted’ to continuous oxidative stress and no longer respond with increased proliferation. Cells in the epithelium lining the nasal cavity seem to make an exception, since signs of increased BrdU incorporation can be observed practically as long as the animals are exposed to ETS (18,61,63).

Lung tumors in strain A/J mice are thought to originate either from the type II alveolar cells or from the non-ciliated bronchiolar epithelial (Clara) cells. Increased rates of cell proliferation have usually been associated with tumor development, although mouse lung seems to make on occasion an exception from this general rule. Lung tumor formation can
be depressed even in the presence of sustained cell proliferation (65) and, vice versa, tumor development may be enhanced although there are no measurable signs of increased target cell proliferation (66). In the present experiment, cells in the two putative sites of tumorigenesis, the bronchiolar and the alveolar epithelia, showed signs of increased DNA synthesis only early and in a transitory fashion. It is possible that the dose of ETS, and the eventual tumor yield were too small to elicit any detectable sustained signs of cell proliferation following the initial burst and eventual ‘adaptation’. It may also be remembered that other experiments have shown that only some carcinogens, such as urethan, produce a measurable proliferation of the type II cell population, whereas other compounds, such as the equally potent lung tumorigen 3-methylcholanthrene, fail to do so (67).

The usefulness of our animal model for the study of human tobacco-smoke induced lung cancer remains to be established. The ETS concentrations required to produce lung tumors in A/J mice are considerably higher than are ETS concentrations found in ‘the real world’ where they rarely if ever seem to exceed TSP concentrations of more than 1 mg/m³ (68). However, the A/J mouse model is considered to be an excellent model for the study of human lung adenocarcinoma (69). The occurrence of this tumor type seems to be on the increase and a change in nicotine and tar content of newer cigarette brands is considered to be responsible (70). The mouse lung tumor model might become useful for the screening and development of chemopreventive agents. As long as cigarettes are legal in the US, and since nicotine is highly addictive, there will be smokers to protect. Even more important, there is now evidence that non-smokers, particularly children are at risk from ETS exposure (2,71). Smoking may thus affect a much larger population than just smokers. Furthermore, smoking cannot be regulated in the home of smokers where there is the highest exposure of non-smokers. Chemoprevention might thus be a viable, even vital option for those (spouses, children) who cannot be protected otherwise from the harmful effects of cigarette smoke. Unfortunately, the field was recently severely set back when it became obvious that beta carotene, one of the most extensively studied chemopreventive agents, had to be withdrawn from a large NCI sponsored clinical trial because it was inefficient, or possibly even carcinogenic (72,73). However, there are many other important and actively investigated chemopreventive agents, such as the isothiocyanates present in vegetables or polyphenols found in green and black teas. They have been shown to prevent the development of lung tumors in mice exposed to polycyclic aromatic hydrocarbons (74) or to tobacco smoke specific nitrosamines particularly NNK (75–77). The strain A/J mouse lung tumor model has been a most useful test system with which to examine the efficiency of these compounds (78). It will be interesting to evaluate the effects of these agents in A/J mice exposed to the full complex mixture of ETS rather than to just one model compound.

Acknowledgements

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