A Mouse Lung Tumor Model of Tobacco Smoke Carcinogenesis

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We examined the possibility of developing an animal model of tobacco smoke carcinogenesis. Male Balb/c and SWR mice were exposed for 5 months to tobacco smoke (6 h/day, 5 days/week; average concentration, 122 mg/m³ of total suspended particulates [TSP]) followed by a recovery period of 4 months in air. In both strains there was an increase in lung tumor multiplicities and incidence, although statistical significance was only observed with lung tumor multiplicity in the SWR mice. An analysis of 11 previous and independently conducted assays with strain A/J mice that followed the same protocol was performed. In each experiment, lung tumor multiplicities were significantly higher in tobacco smoke-exposed mice compared with air-exposed controls, and a good correlation between exposure (average tobacco smoke concentrations multiplied by length of exposure in months) and lung tumor multiplicities was found. In 7 experiments involving tobacco smoke concentrations greater than 100 mg/m³ of TSP, lung tumor incidences were 5 times higher than in control mice. Tobacco smoke-exposed mice had a smaller percentage of adenomas with carcinomatous foci or adenocarcinomas than air-exposed controls, and no differences between the two groups were found in an analysis of Ki-ras mutations. After 6 h of exposure to tobacco smoke, plasma cotinine levels in mice were comparable to those found in active human smokers. The lung tumor model might be suitable for future evaluation of chemopreventive agents or modified tobacco products.

Key Words: tobacco smoke; lung tumors; strain A/J mice; Balb/c mice; SWR mice; plasma cotinine; K-ras mutations; dose-response relationship; tobacco smoke inhalation

Smoking of cigarettes became affordable with the invention of machines that could produce cigarettes in large quantities. Consumption of cigarettes increased dramatically during and after World War I. Clinicians began to suspect a link between cigarette smoking and the hitherto "unusual" disease of lung cancer in the late 1920s. Epidemiological studies from Germany, Great Britain, and the United States firmly established smoking as the most important risk factor for the development of lung cancer (Witschi, 2001). Today, lung cancer causes more deaths annually than breast, prostate, and colon cancers combined (Szabo, 2001).

Attempts to duplicate the human experience in experimental animals have, for the most part, been unsuccessful. Mohr and Reznik (1978, p. 347), in their comprehensive review of the literature, concluded that, despite an enormous amount of work, "no researcher has succeeded as yet in producing a significant incidence of pulmonary tumors." In 1986 the International Agency for Research on Cancer (IARC) summarized the then-available evidence and found that of 4 rat studies judged to be adequate for critical analysis only one yielded unequivocal evidence for the carcinogenicity of tobacco smoke. Tumor incidence in the exposed group was below 10%. Hamsters developed laryngeal tumors but no tumors in the lower respiratory tract. Several mouse studies discussed in the IARC document failed to provide clear evidence for carcinogenicity by tobacco smoke, as did another large study conducted in a single laboratory (Henry and Kouri, 1986). Coggins, in a 1998 review of 14 chronic animal inhalation studies with mainstream cigarette smoke, indicated that "significant increases in the numbers of malignant tumors were not produced in the respiratory tract of rats or mice exposed chronically to inhalation of cigarette smoke" (Coggins, 1998, p. 313).

It is acknowledged that there probably will always be a certain population of smokers who are unable or unwilling to quit. Efforts are underway to find new ways to achieve tobacco harm reduction. Along these lines, the tobacco industry has made major efforts in product improvement through the development of a less harmful cigarette (Wagner et al., 2000). For several reasons, it is desirable to have an animal model of tobacco smoke carcinogenesis. A committee of the Institute of Medicine recommended that animal models be used to test the potential adverse health effects of tobacco smoke or any proposed additives (Stratton et al., 2001). Animal models of tobacco smoke carcinogenesis might also be useful in preclinical testing of chemopreventive agents. For example, animal studies with tobacco smoke showed that it is not always possible to predict effective chemoprevention if putative chemopreventive agents are only evaluated in lung tumor models.

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induced by constituents of tobacco smoke (Witschi, 2000). Animal studies showed that β-carotene (Obermueller-Jevic et al., in press) and N-acetylcysteine (Witschi et al., 1998) are ineffective against tobacco smoke, an outcome that would have predicted the disappointing results of two major clinical trials (Ommen, 1998; van Zandwijk et al., 2000). Finally, it might be possible to develop risk assessments for environmental tobacco smoke exposure by complementing data from epidemiological studies with dose-response information derived from animal experiments. A recent attempt showed a surprisingly good correlation between tumorigenic potency of environmental tobacco smoke in animals and increased risk derived from human case-control studies (Bogen and Witschi, 2002).

During the last few years, we have developed a murine model of tobacco smoke-induced lung carcinogenesis. The model uses strain A/J mice exposed to a mixture of cigarette sidestream and mainstream smoke. A key element in increasing the development of lung tumors after exposure to tobacco smoke was the design of a nonconventional protocol. Instead of being exposed for their entire lifespan to the smoke, the mice are exposed to tobacco smoke for 5 months only. After cessation of smoke exposure, the mice are allowed to recover in air for another 4 months before being evaluated for tumor development (Witschi et al., 1997a, 1997b). The results of these studies have been confirmed in a different laboratory (D’Agostini et al., 2001a). In the current study, we add evidence that a similar protocol might be successfully used in other mouse strains, and we present an overall analysis of 11 previous and independently conducted experiments using the strain A/J mouse model.

**MATERIALS AND METHODS**

**Animals**

Male strain A/J mice, 6 to 8 weeks old, were purchased from Jackson Laboratories (Bar Harbor, ME) and male Balb/c and SWR mice from B&K Universal (Fremont, CA). A few mice were randomly selected for a standard rodent health surveillance screen (Comparative Pathology Laboratory, University of California, Davis). No evidence of infectious disease (pathogenic agents) or the presence of parasites or ova in pelage and cecum was found. Serology was negative for mouse hepatitis virus, Sendai virus, Reovirus type 3, pneumonia virus, parvovirus, ectromelia, and mycoplasma pulmonis. The mice were housed in groups of 4 in polypropylene cages with tight-fitting wire screen lids on conventional bedding material. At all times, including the smoke exposure period, water and diets were provided ad libitum. The mice were monitored daily and weighed weekly. All experimental protocols had been approved by the Animal Welfare Committee, University of California, Davis, and were in agreement with current National Institutes of Health guidelines.

**Experimental Design for Carcinogenesis Studies**

In most experiments, the original protocol for evaluation of tobacco smoke carcinogenesis was followed (Witschi et al., 1997a). Mice were exposed for 5 months (6 h/day, 5 days/week) to the tobacco smoke and then allowed to recover for another 4 months in air. In two additional experiments, the mice were kept for 9 months in tobacco smoke; concomitant controls were kept in air throughout. At the end of the experiment, they were killed by pentobarbital overdose. For analysis of tumor incidence and multiplicity, the lungs were manually expanded to inspiratory volume by intratracheal instillation of Tel-elysinczyk’s fluid and fixed for at least 24 h in the same fixative before being transferred into 70% ethanol. After counting the tumors, a few lungs were selected and embedded in paraffin. Sections 5 μm thick were stained with hematoxylin-eosin (H&E) and examined under light microscope.

**Exposure System**

The tobacco smoke exposure system was identical to that used in our previous studies (Witschi et al., 1997a, 1997b, 1998, 1999, 2000). Briefly, mice were exposed to a mixture of 89% sidestream and 11% mainstream smoke generated from burning Kentucky 1R4F reference cigarettes (purchased from the Tobacco Research Institute, University of Kentucky, Lexington, KY). Chamber atmospheres were monitored for nicotine, CO, and total suspended particulates (TSP). Within the exposure chambers, all cages were periodically rotated so that each cage occupied at least once all possible locations within the exposure chambers. After an initial acclimatization period of 5 weeks involving increasing concentrations of tobacco smoke within the chamber, tobacco smoke concentrations used for the individual experiments ranged from 50 and 150 mg/m³ of TSP. The TSP concentrations for the individual experiments are listed in Tables 1 and 2.

**Analysis for Ki-ras Mutations in Lung Tumors**

**Extraction and amplification of DNA from paraffin-embedded tissue.** Twenty slides containing lung tumors, 10 from tobacco smoke-exposed and 10 from control air-exposed mice, were analyzed for mutations in the Ki-ras gene. Polymerase chain reaction (PCR) products were obtained from 9 of the 10 tissue samples in each group. Five-micrometer sections were cut from prepared paraffin-embedded blocks and placed on glass slides. Using stained H&E slides as a guide, the area containing the tumors was cut from the unstained slides with a fresh razor blade and placed in irradiated microcentrifuge tubes. The samples were deparaffinized by 2 rounds of incubation with 1 ml of mixed xylenes for 30 min on a rotary shaker followed by 2 rinses with 0.5 ml of 100% ethanol as described previously (Wessner et al., 1996, Wright and Manos, 1990). The tubes were air dried and incubated overnight at 37°C with 100 μl of digestion buffer (50 mmol Tris HCl, pH 8.0, 0.1 mmol molten acid, 0.5% Tween 20) containing 200 μg/ml of proteinase K. The proteinase K was then inactivated by incubation at 95°C for 8 min, and the samples were stored at −20°C until use.

A 2:1 aliquot of the lysate was used for the PCR. All of the reactions were carried out in a 100-μl reaction volume that consisted of reaction buffer (10 mmol Tris HCl, pH 8.0, 2.5 mmol MgCl₂, 50 mmol KCl), 200 μM dNTPs, and two units of AmpliTaq Gold (Perkin Elmer Life Sciences, Boston, MA). Amplifiers for exons 1 or 2 of the Ki-ras gene were added at a final concentration of 0.2 μM. The samples were overlaid with 100 μl of mineral oil and amplified by the standard procedure of Saiki et al. (1988). After denatur-

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Weeks 1–2</th>
<th>Weeks 3–5</th>
<th>Weeks 6–20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative humidity (%)</td>
<td>54 ± 7 (9)</td>
<td>68 ± 8 (24)</td>
<td>64 ± 11 (54)</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>22 ± 1 (9)</td>
<td>22 ± 1 (24)</td>
<td>22 ± 1 (151)</td>
</tr>
<tr>
<td>CO (ppm)</td>
<td>273 ± 30 (9)</td>
<td>331 ± 14 (24)</td>
<td>340 ± 28 (151)</td>
</tr>
<tr>
<td>TSP (mg/m³)</td>
<td>83 ± 8 (9)</td>
<td>100 ± 12 (24)</td>
<td>122 ± 25 (151)</td>
</tr>
<tr>
<td>Nicotine (mg/m³)</td>
<td>ND</td>
<td>ND</td>
<td>13.2 ± 5.1 (27)</td>
</tr>
</tbody>
</table>

*Note. All measurements were made within the inhalation chambers. Data are given as means ± SD; number of determinations are in parentheses. ND, no data; CO, carbon monoxide; TSP, total suspended particulates.*

**TABLE 1**

Exposure Data for Carcinogenesis Assay with Balb/c and SWR Mice
at 72 °C for 1 min at 94 °C, for 2 min at 94 °C, and the samples were amplified by 40 cycles of denaturation for 2 min at 94°C, annealing for 2 min at 55°C, and extension for 2 min at 72°C, followed by a final extension step for 7 min at 72°C. Primers for exons 1 and 2 of Ki-ras were synthesized by Integrated DNA Technologies (Cor-
liville, IA). Primer sequences for exon 1 were forward 5'-ATGACTGAG-
TATAAATGTGT, and reverse 5'-TCGTACTCATCCAAAGATG, which
produced a 98-bp fragment; primer sequences for exon 2 were forward 5'-
TACAGGAAACAAGTAGTAATTGATGGAGAA, and reverse 5'-ATAAT-
GATGATATCTTCATCAAATGATTTAGT, which produced a 171-bp fragment.

Each reaction included deparaffinization procedure controls, which lacked
tumor tissue but were mock extracted and taken through the entire protocol,
and negative buffer controls for the PCR amplification reactions. All samples
were amplified in a BioRad iQCyclus thermocycler. The sizes of the PCR
products were confirmed using 2% agarose gel.

**Allele-specific oligonucleotide hybridization (ASO).** Thirty microliters of
PCR products, diluted in 170 μl of sterile water, were heat denatured and
blotted directly onto a Nitram membrane filter (Schleicher & Schuell, Keene,
NH) using a Schleicher & Schuell minifold II slot blot apparatus. The ampli-
fied DNA products were fixed to the membrane by ultraviolet crosslinking, and
the filters were prehybridized in 5 × SSC, pH 7.0, 50 mmol sodium phosphate,
pH 7.05 × Denhardt’s solution, and 0.5% SDS/100 μg/ml of salmon sperm
DNA at 37°C for 1.5 h. The filters were hybridized overnight in the same
buffer containing 5 × 10^{-6} cpn/pmol of a 20-bp oligonucleotide to mouse K-ras
codons 12, 13, or 61 (Clontech, Palo Alto, CA). The oligomers were 5'-end-
labeled with phosphorus 32 to a specific activity of more than 10^{7} cpn/pmol
using T4 polynucleotide kinase. After hybridization, the filters were washed
under stringent conditions (3°C below the T_{m}) that allowed only fully matched
probes to remain bound to DNA (Mattes and Miller, 2000; Miller et al., 1994,
2000). The blots were visualized on a Molecular Dynamics PhosphorImager
445Si (Sunnyvale, CA). Results are based on two independent amplifications
from the lysates to ensure that none of the observed mutations were the result
of Taq-induced errors.

The Ki-ras gene was sequenced directly from the initial PCR products by
the Wake Forest University School of Medicine DNA Sequencing and Gene
Analysis Facility. Direct PCR sequencing was performed using the ABI
PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer
Life Sciences) according to the manufacturer’s instructions. The DNA se-
quences was analyzed using DNASIS software (Hitachi Software Engineering
America, Ltd., San Bruno, CA).

**Plasma Cotinine Levels.**

A/J mice were exposed for 6 h to three different tobacco smoke concentra-
tions (80, 120, and 160 mg/m^{3} of TSP). Immediately after tobacco exposure,
half of the mice were deeply anesthetized with pentobarbital for blood collec-
tion. The other half were allowed to survive for another 18 h before being
killed for blood collection. Plasma cotinine levels were measured using a
commercially available enzyme-linked immunosorbent assay kit (Cotinine
Serum Micro-Plate EIA, STC Technologies, Inc., Bethlehem, PA).

**Statistical Analysis.**

For tumor analysis, the number of nodules visible on the lung surface were
counted and the results expressed as tumor incidence (i.e., percentage of mice
with one or several lung tumors) and tumor multiplicity (the average number
of tumors per lung, including non-tumor-bearing animals). All numerical data
were calculated as mean ± SD or SE. Comparisons of tumor multiplicity
between tobacco smoke-exposed mice and air-exposed controls were made by
Welch’s alternate test or, in the case of multiple comparisons, by analysis
of variance followed by the Tukey-Kramer test. Tumor incidences were
compared using Fisher’s exact test. A p value of 0.05 or less was considered
significant.

**RESULTS.**

**Lung Tumor Development in Balb/c and SWR Mice.**

We examined the possibility of increasing lung tumor incidences and
multiplicities in Balb/c and SWR mice, strains that are somewhat less suscepti-
bile to carcinogen-induced lung tumor formation than are strain A/J mice. Male mice of both
strains were exposed for 5 months to an average tobacco smoke
concentration of 122 mg/m^{3} of TSP (see Table 1). Smoke exposure was followed by a 4-month recovery period in air.
The mice tolerated the tobacco smoke exposure well, and no exposure-related deaths were observed. In Balb/c mice, tobacco smoke initially produced a sharp reduction in weight gain. On removal from the smoke, the mice rapidly gained weight and, by the end of the experiment, had reached a similar body weight as the corresponding controls, kept in air throughout the 9 months. On the other hand, tobacco smoke exposure had no influence on body weight in SWR mice; tobacco smoke-exposed mice and air-exposed mice gained weight at identical rates (Fig. 1).

Tumor data are shown in Table 3. In both strains, exposure to tobacco smoke produced an increase in lung tumor multiplicities and lung tumor incidences. In Balb/c mice, lung tumor multiplicities more than doubled, but the difference compared with controls was not statistically significant. In the SWR mice, lung tumor multiplicities were increased by a factor of almost 10, and the difference compared with controls was statistically significant \( p < 0.05 \). Although tumor incidences were higher in both strains when exposed to tobacco smoke, they did not reach statistical significance compared with controls.

### Dose-response Relationships and Overall Tumor Incidences in Strain A/J Mice

During the last 6 years, we conducted 11 independent studies in which we always used the same protocol: exposure of male strain A/J mice to tobacco smoke from 3.5 to 5 months (6 h/day 5 days/week) followed by a recovery period in air. In each experiment, the mice were killed exactly 9 months after initiation of exposure. Because all mice entered the experiment at the same age (8–10 weeks) and were killed at the same age, absolute number of tumor multiplicities and incidences can be compared directly from one experiment to another. As exposure parameters, we calculated concentrations-months (i.e., the average tobacco smoke concentration in a given experiment, expressed as milligrams per meter cubed of TSP, multiplied by the duration, in months, of exposure).

The results of this analysis are shown in Figure 2 and Table 2. It can be seen that whenever we used the protocol in which mice were exposed to tobacco smoke first and then given a recovery period in air (split protocol), amounting to exposure-months ranging from 133 to 735, a good dose-response relationship was observed between exposure-months and lung tumor multiplicity (Fig. 2). In every single one of the 11 experiments, the difference between the controls and the tobacco smoke-exposed mice was statistically significant \( p < 0.05 \). Linear regression indicated a statistically significant increase in tumor multiplicity with dose \( p < 0.01 \). However, in mice that were kept for the full 9 months in tobacco smoke (exposure-months of 1250 and 1350) and not allowed to re-

### Table 3

**Lung Tumor Data for Balb/c and SWR Exposed to Tobacco Smoke**

<table>
<thead>
<tr>
<th>Strain and treatment</th>
<th>Lung tumor multiplicity ( a )</th>
<th>Lung tumor incidence ( b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balb/c, tobacco smoke</td>
<td>0.44 ( \pm ) 0.13 (27)</td>
<td>9/27 (33%)</td>
</tr>
<tr>
<td>Balb/c, air</td>
<td>0.20 ( \pm ) 0.07 (29)</td>
<td>6/30 (20%)</td>
</tr>
<tr>
<td>SWR, tobacco smoke</td>
<td>0.35 ( \pm ) 0.14 (31)*</td>
<td>6/31 (19%)</td>
</tr>
<tr>
<td>SWR, air</td>
<td>0.04 ( \pm ) 0.04 (27)</td>
<td>1/26 (4%)</td>
</tr>
</tbody>
</table>

*Note.* Data are given as means \( \pm \) SE. The number of mice is given in parentheses.

\( a \)Average number of tumors per lung; sample includes non-tumor-bearing mice.

\( b \)Number of tumor-bearing mice per total number of mice at risk.

*Significantly different \( p < 0.05 \) compared with SWR mice kept in air.
cover in air, lung tumor multiplicities were not higher than in mice subjected to the split protocol, although their “dose” was almost twice as high. Lung tumor incidences for the experiments involving exposure to tobacco smoke followed by a recovery period in air are listed in Table 2. In all experiments, lung tumor incidence was higher in the tobacco smoke-exposed mice (range, 58–100%) than in the corresponding controls (range, 42–80%). In the mice exposed to tobacco smoke concentrations below 100 mg/m$^3$ of TSP, differences were not statistically significant, whereas in those exposed to higher concentrations (>100 mg/m$^3$ of TSP), differences were statistically significant in 5 of 7 experiments.

**Terminal Body Weight Versus Lung Tumor Multiplicity**

 Dietary restriction is known to inhibit lung tumor development in strain A/J mice. Because exposure to tobacco smoke adversely affects weight loss, the correlation between final body weight of mice and lung tumor multiplicity was examined. Figure 3 shows that there was no correlation between final body weights in mice exposed to tobacco smoke and lung tumor multiplicity. In control mice, there even seemed to be a tendency to associate fewer lung tumors with increased body weight.

**Tumor Types and Ki-ras Mutations**

In all experiments with strain A/J mice, selected tumors were examined under light microscope. Proliferative pulmonary lesions were morphologically similar between control mice and those exposed to tobacco smoke and were typical of pulmonary lesions commonly observed in strain A mice. Lesions were categorized as focal alveolar epithelial hyperplasia, alveolobronchiolar adenomas, and alveolobronchiolar adenocarcinomas (Witschi et al., 1997a). There was usually no difference between the two groups in the relative distribution of adenocarcinomas and adenomas, although not infrequently the tobacco smoke-exposed mice seemed to have fewer carcinomas than the control mice. We subsequently tabulated all available histopathological diagnoses from our experiments and divided the tumors into two groups: adenomas and adenomas with carcinomatous foci or fully developed adenocarcinomas. The results of this analysis are shown in Table 4. In mice exposed to tobacco smoke, the percentage of malignant tumors was significantly lower than for mice exposed to air.

**Ki-ras mutations** have frequently been described in murine lung tumors, and, in comparing smoke-exposed and control mice, we have found evidence of a shift in mutations in codon 61 (Witschi et al., 1995). This observation was obtained by examining tumors from mice exposed to a low concentration (4 mg/m$^3$ of TSP). We speculated that higher doses might accentuate this lesion. Tumor tissue from a small number of air-exposed and and tobacco smoke-exposed mice (average concentration, 130 mg/m$^3$ of TSP) was harvested by microdissection as described in the Materials and Methods section and subjected to rigorous analysis for K-ras mutations. Codons 12, 13, and 61 were screened by ASO with 6 or 7 different mutant 20-bp oligonucleotide probes differing from the wild-type sequence by a single base pair to detect mutations in the Ki-ras gene. Within the control group of air-exposed mice, 7 of the 9 tumors examined contained the Glu$^{61} \rightarrow$ Arg$^{61}$ mutation within exon 2, whereas the two remaining tumors exhibited wild-type sequence. Similarly, 6 of the 9 tumors from tobacco smoke-exposed mice also contained the Glu$^{61} \rightarrow$ Arg$^{61}$ mutation; the remaining three tumors exhibited the wild-type sequence at this exon. None of the tumors from either the air- or tobacco smoke-exposed mice demonstrated mutations at exon 1. No significant difference was seen in the frequency or mutational spectrum of the codon 61 mutations when comparing the tobacco smoke- and air-exposed groups. All of the PCR products for exon 2 were sequenced in both the forward and reverse directions to confirm the results of the ASO analysis. Five PCR samples for exon 1 were also sequenced in both directions to confirm the lack of mutations observed in these samples. None of the procedure or negative control samples produced a signal in the ASO.

**TABLE 4**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Adenomas</th>
<th>Adenomas +</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobacco smoke</td>
<td>301</td>
<td>280 (93%)</td>
<td>21 (7%)*</td>
</tr>
<tr>
<td>Filtered air</td>
<td>144</td>
<td>118 (82%)</td>
<td>26 (18%)</td>
</tr>
</tbody>
</table>

*Percentage of adenomas with carcinomatous foci or carcinomas in the tobacco smoke-exposed mice is significantly lower than in mice kept in air ($p < 0.02$ with Fisher’s exact test).
Plasma Cotinine Levels in Strain A/J Mice Exposed to Tobacco Smoke

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Cotinine levels (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>160 mg/m³ TSP</td>
<td>133 ± 1 (3) 94 ± 8 (3)</td>
</tr>
<tr>
<td>120 mg/m³ TSP</td>
<td>126 ± 1 (3) 82 ± 1 (3)</td>
</tr>
<tr>
<td>80 mg/m³ TSP</td>
<td>112 ± 2 (3) 91 ± 3 (3)</td>
</tr>
<tr>
<td>Filtered air</td>
<td>0.3 ± 0.2 (6) ND</td>
</tr>
</tbody>
</table>

**Note.** Male strain A mice were exposed for 6 h to three different concentrations of tobacco smoke. Mice were killed and plasma was collected for cotinine determination immediately after smoke exposure and 18 h after the initiation of smoke exposure. Cotinine values are given as mean ± SE; number of determinations are in parenthesis. Concentration, tobacco smoke concentration in chamber; ND, no data; TSP, total suspended particulates.

**Plasma Cotinine Concentrations**

Plasma cotinine concentrations were measured in mice exposed for 6 h to tobacco smoke and then again in mice that had been removed from the smoke atmosphere for 18 h. The data are given in Table 5. Plasma cotinine levels were dose-dependent and fell once the mice were removed from the smoke atmosphere.

**DISCUSSION**

We have shown, in a series of repeated and independent experiments, that it is possible to produce increases in lung tumor multiplicity in mice exposed to a mixture of cigarette mainstream and sidestream smoke. The effect is most pronounced in strain A/J mice, a strain that is inherently susceptible to a multitude of carcinogens (Shimkin and Stoner, 1975). In SWR and Balb/c mice, the response to tobacco smoke in terms of absolute numbers of tumors per lung is lower, although the relative increase over controls is comparable to A/J mice in the Balb/c strain and much higher in the SWR mice. The difference compared with controls is significant in the SWR mice but not in the Balb/c mice. Inhalation of tobacco smoke also increases lung tumor incidence. In the strain A/J mice, a significant increase in lung tumor incidence was observed when mice were exposed to chamber concentrations of tobacco smoke greater than 100 mg/m³ of TSP. In Balb/c and SWR mice, the increase in lung tumor incidence was not significant. It should be noted, however, that a tumor incidence of 19%, as was seen in tobacco smoke-exposed SWR mice, versus 4% in the controls would be significant with a group size of 50 mice, the number usually used in more conventional cancer bioassays. In addition, SWR mice do not show decreased weight gain while exposed to tobacco smoke. This is in stark contrast to the other strains, in which weight gain in smoke-exposed mice often is 15–20% lower than in controls. It is thus possible to obtain a significant tumor response in SWR mice without exceeding the maximum tolerated dose.

Murine lung tumors, on the basis of site of origin and histology, do not resemble human bronchial squamous cell carcinoma. Mouse lung tumors develop in the peripheral lung first as areas of hyperplasia and then grow into adenomas, eventually progressing to adenocarcinomas. At 1 year of age, approximately 80% of the tumors are adenomas and 20% are adenomas containing carcinomatous areas or are fully developed adenocarcinomas with solid, papillary, or mixed growth pattern (Witschi et al., 1997a). Invasion of adjacent tissues, blood vessels, or lymphatics by malignant cells is seen on occasion. If the mice were allowed to live out their lifespan, the tumors eventually would fill entire lobes and kill them. At this stage, all tumors are carcinomas (Foley et al., 1991). The tumors thus resemble human lung adenocarcinoma, which has increased in incidence and by now is the most prevalent histological type of lung tumor observed clinically in the United States (Thun et al., 1997). Existing mechanistic information on the multiple similarities between human and murine lung tumors in biochemistry, signal transduction pathways, and alterations in oncogenes reinforces the postulate that mouse lung tumors are a good model for adenocarcinoma of the human lung (Malkinson, 1998). Lung tumors in strain A mice have become the most widely used animal model for the evaluation of chemopreventive agents (Stoner, 1998).

A key element in the successful model development was a split protocol in which the mice were exposed to tobacco smoke first and then given a recovery period in air. This approach can be justified by the following observations. In mice exposed to tobacco smoke from 3.5 to 5 months and then given a recovery time in air, there is a very good dose response to the product of smoke concentration and length of exposure (see Figure 2 and Table 2). Mice exposed to tobacco smoke for the full 9 months and not allowed to recover in air have a response that is similar to, but not higher than, that in mice subjected to the split protocol. This could indicate that inhalation of tobacco smoke not only initiates the tumorigenic process but also interferes with tumor progression. The data in Table 4, presenting distribution of tumor types in several hundred specimens, show that tobacco smoke delays tumor progression: Significantly fewer carcinomas have been diagnosed in mice exposed to tobacco smoke compared with those kept in air. Interestingly, a similar effect on tumor progression could be achieved with chemopreventive agents (Conaway et al., 1998). Several observations might explain the basically counterintuitive finding of tobacco smoke interfering with tumor development (D’Agostini et al., 2001a). One possibility is that smoke-induced stress imposed on the mice, coupled with caloric restriction, interferes with lung tumor development in strain A mice (Droms and Malkinson, 1991; Pashko and Schwartz, 1996). However, Figure 3 indicates that body weight, at least as determined at the end of an experiment, is not correlated with lung tumor multiplicity. Tobacco smoke is
capable of inducing apoptosis in the epithelial cells of mice and rats exposed to tobacco smoke (D’Agostini et al., 2001b) as well as in human pulmonary cells (Vayssier et al., 1998). Increased apoptosis could slow down tumor development by eliminating cells containing mutagenic and potentially procarcinogenic lesions, as might cytotoxicity caused by selected tobacco smoke constituents (e.g., acrolein). It remains speculative how this would apply to the natural history of lung cancer in smokers. It is interesting to note that, immediately after smoking cessation, former smokers are, for a few years, actually at higher risk for lung tumors than current or never smokers, although eventually the risk decreases (Wynder and Stellman, 1977; Postmus, 1998). In former smokers, the initial removal of the cytotoxic or apoptosis-enhancing elements present in tobacco smoke might allow tumors to proliferate more rapidly than they would in the presence of tobacco smoke. This should, of course, not be construed to imply that to quit smoking is not beneficial; it is. However, it might indicate that attention to thorough diagnostics and perhaps chemopreventive measures should particularly be directed toward former smokers.

Several studies have shown that mutations in K-ras are a frequent and early event in both murine and human lung adenocarcinomas (You et al., 1989). Studies using transgenic mice have clearly shown the importance of mutated K-ras genes for the initiation and maintenance of lung cancer (Fisher et al., 2001). In addition, mutation in the K-ras gene shows a strong association with tobacco smoke in patients with adenocarcinoma of the lung (Ahrendt et al., 2001). We have previously found that exposure to tobacco smoke at comparatively low concentrations (4 mg/m³ of TSP), which did not produce an increased tumor response, seemed to target mutations of the Ki-ras gene to exon 2 for mutations (Witschi et al., 1995). We repeated this analysis in selected tumors in mice that had been exposed to a much higher, tumorigenic concentration of tobacco smoke (130 mg/m³ of TSP), which did not produce an increased tumor response, seemed to target mutations of the Ki-ras gene to exon 2 for mutations (Witschi et al., 2001). In former smokers, the initial removal of the cytotoxic or apoptosis-enhancing elements present in tobacco smoke might allow tumors to proliferate more rapidly than they would in the presence of tobacco smoke. This should, of course, not be construed to imply that to quit smoking is not beneficial; it is. However, it might indicate that attention to thorough diagnostics and perhaps chemopreventive measures should particularly be directed toward former smokers.

We were surprised to find no difference in the mutation spectrum between air-exposed and smoke-exposed mice. Interestingly, we found no mutations in exon 1 in the air-exposed mice in this small study, thus accounting for the apparent lack of shift in the mutational spectrum observed previously (Witschi et al., 1995). Several studies have shown that different carcinogens induce specific mutations at the Ki-ras gene locus (You et al., 1989). Thus, although our result can be explained by the fact that the number of tumors examined in the current study was too small to detect a significant difference between the two treatment groups (9 tumors each in the control and smoke-exposed mice), the notion must also be entertained that Ki-ras mutations are not as dominant an event in tobacco smoke carcinogenesis in mouse lung tumors as is often assumed. In addition, because we selected the largest tumors for molecular analysis, we may have biased our study by specifically examining those tumors most likely to harbor the Arg61 mutant allele. Alternatively, it is also possible that tobacco smoke may promote the outgrowth of spontaneously mutated lesions in the A/J lung already predisposed to develop Ki-ras mutations. In the latter case, one would not expect the mutational spectrum between the air- and smoke-exposed mouse to differ. Studies using the transplacental administration of the polycyclic aromatic hydrocarbon, 3-methylcholanthrene, to crosses between C57BL/6 and DBA mice (Leone-Kabler et al., 1997; Miller et al., 2000) or to Balb/c mice (Gressani et al., 1999; Miller et al., 2000) have documented differences in the Ki-ras mutational spectrum as a function of the histological stage of the tumors, organ site, and mouse strain. Further studies with larger numbers of tumors from different strains of mice are required to determine whether smoke exposure does have an effect on the mutational spectrum of the Ki-ras gene or merely promotes the proliferation of cells with preexisting mutations at this locus.

The mouse lung tumor assay could help in the evaluation of the eventual full carcinogenic potential of new or modified tobacco products. To date, such information is usually developed by using mutagenesis assays and skin-painting studies (Wagner et al., 2000). Mouse skin tumors resemble lung tumors inasmuch as they develop in particularly sensitive mouse strains (e.g., SENCAR mice) and progress from benign papillomas to malignant neoplasms. Whereas in inhalation studies the full complex mixture of tobacco smoke can be evaluated, consisting of both gas phase and tar phase, skin-painting studies allow only the examination of smoke condensates. Information currently available suggests, however, that the gas phase is as carcinogenic as full tobacco smoke (Hausmann et al., 2001; Leuchtenberger and Leuchtenberger, 1974; Witschi et al., 1997b). The positive tumor responses found in mice exposed to the gas phase alone also allows the conclusion that, even under conditions of whole body exposure, the carcinogenic response in the lung is due to inhaled carcinogens rather than particulate matter, which is deposited on the skin of mice and then ingested after grooming.

In conclusion, it is possible to produce lung tumors in different mouse strains by inhalation of tobacco smoke. A key element in the protocol is that mice are allowed to recover a few months in air after smoke exposure. Oxidative stress,
possibly resulting in increased apoptosis or cytotoxicity, may interfere with tumor growth and progression as long as the mice are kept in the smoke atmosphere. Mice that are exposed almost twice as long to tobacco smoke and are not allowed to recover in air do not show an increased tumor response despite having been exposed to almost twice the tobacco smoke dose. Although it is not necessary to document, using animal studies, tobacco smoke as a lung carcinogen—this fact is widely recognized through the human experience alone—the mouse lung tumor system might be further explored for future studies on tobacco smoke toxicity and mechanisms of carcinogenic action as well as for preclinical evaluation of putative chemopreventive agents.

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