# Histological Alterations in Male A/J Mice Following Nose-Only Exposure to Tobacco Smoke

By: J. T. Hamm, S. Yee, N. Rajendran, R. L. Morrissey, S. J. Richter, and M. Misra.

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## Abstract:

The incidence and multiplicity of grossly observed and microscopic lesions of the respiratory tract of A/J mice exposed nose-only to mainstream smoke (50, 200, or 400 mg total particulate matter/$m^3$ from 2R4F cigarettes) was compared to those of filtered air controls. Animals were necropsied at the end of exposure (5 mo) or following 4 or 7 mo of recovery. Lungs were visually inspected for tumors at all necropsies and examined histopathologically at 9 and 12 mo. At 5 mo no tumors were recorded. No significant elevations in tumor incidence or multiplicity were recorded although at 9 mo multiplicity was elevated in the mid-exposure group (0.90 versus 0.55 tumors per animal for controls). At 12 mo, multiplicity was increased over the 9-mo necropsy at all exposures except 200 mg/$m^3$; however, there were no dose-related trends in multiplicity or incidence. Histopathological alterations included hyperplasia, metaplasia, and inflammation of the nose and larynx and proliferative lesions of the lungs. At 9 mo, the multiplicity of focal lung lesions was 1.4 per animal in controls but averaged 1.0 among smoke-exposed groups. There was an inverse relation ($p < .059$) between smoke concentration and the percentage of hyperplastic lesions at 9 mo. At 12 mo the high-exposure group had slightly increased multiplicity of 2.3 lesions compared with 1.6 among controls, while the percentage of hyperplasic lesions was similar between groups. Nose-only inhalation of mainstream tobacco smoke resulted in chronic inflammatory changes of the respiratory tract yet failed to produce statistically significant changes in tumor incidence or multiplicity.

**Keywords:** tobacco | respiratory | mice | hyperplastic lesions | tumor | inflammatory

## Article:

The International Agency for Research on Cancer (IARC, 2004) has classified cigarette smoke as a known human carcinogen. An appropriately sensitive, reproducible, and relevant animal model for smoke-induced lung tumorigenesis has not been clearly identified. Reviews on the use of rats

and mice (Coggins, 2000) as well as hamsters, dogs, and nonhuman primates (Coggins, 2001) report that studies of cigarette smoke exposure fail to demonstrate a robust increase in the incidence of respiratory tract tumors. A follow-up review by Coggins (2002) reiterated the lack of respiratory-tract tumors in experimental animals. However, Hutt et al. (2005) recently reported a modest increase in pulmonary carcinoma in B6C3F1 mice exposed whole body for the entire life span. Hecht (2005) provides an up-to-date review of cigarette smoke inhalation studies that highlights the positive results of Hutt et al. (2005) and provides brief discussion of results with the A/J mouse.

Studies employing the A/J mouse have reportedly shown cigarette smoke-induced tumorigenesis. Witschi et al. (1997) exposed male A/J mice to a 89% sidestream/11% mainstream smoke (MS) mixture and necropsied animals at the end of the 5-mo exposure or after an additional 4 mo of recovery in air. After a recovery period, smoke exposure was associated with increased multiplicity of lung tumors. Witschi (2005a) reviewed 18 carcinogenesis studies using the A/J mouse exposed to tobacco smoke and reported tobacco smoke exposure was associated with increased tumor multiplicity and incidence in 83% and 56% of the studies, respectively. Witschi performed an additional review of the A/J mouse as a model system for tobacco smoke tumorigenesis in which multiple single-dose exposures studies were pieced together to generate a dose-response analysis of tumor multiplicity (Witschi, 2005b). While there is a relationship between smoke dose and lung tumor multiplicity, the slope of the curve is shallow (Witschi, 2005b). Based on the shallowness of the dose-response and the generally low lung tumor multiplicity following smoke exposure, Witschi (2005a, 2005b) concluded that cigarette smoke is a weak animal carcinogen. However, despite the weak response, studies in multiple laboratories (D'Agostini, 2001; Curtin et al., 2004; Stinn et al., 2005) have been able to reproduce similar increases in tumor incidence and multiplicity in the A/J mouse.

The objective of this study was to assess histological lesions in A/J mice exposed nose-only to mainstream smoke (MS) generated from 2R4F Kentucky reference cigarettes at concentrations of 50, 200, or 400 mg total particulate matter (TPM)/m3.A negative control of filtered air (Sham control group) with 0 mg TPM/m3 was compared with the MS exposure groups. Also, a 50 ppm sulfur dioxide (SO2) group was included as a positive control for the possible effects of chronic irritation on proliferative processes in the lung. However, complete histopathology was not conducted on this study arm therefore the data is not shown. As part of the evaluation, lungs were fixed and evaluated histologically for the presence of additional tumors and preneoplastic, proliferative lesions.

## Methods

### Test Article

The test article was the MS from 2R4F Kentucky reference cigarettes purchased from the University of Kentucky, Lexington, KY. All cigarettes were stored at room temperature in a secure location in their original cartons until use. All cigarettes were conditioned prior to use by storage in an enclosure for at least 48 h at 70 ± 2◦F and 60 ± 3% humidity.

### Animals and Animal Maintenance

*Receipt, Housing, Group Assignment, and Environment*

Three hundred seventy-five male A/J mice 6 to 8 wk old were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals received water and certified rodent diet 5002 (PMI Nutrition International, Inc., Brentwood, MO) ad libitum, except during exposure periods. Blood was collected from six animals prior to the initiation of exposures and three surviving animals at the time of the terminal necropsies and screened for a variety of common respiratory infectious agents. All serological findings were negative.

During nonexposure hours the animals were housed in 2-m3 model H-2000 exposure chambers (Hazelton Systems, Inc., Aberdeen, MD). The animals were housed individually in suspended stainless-steel wire mesh cages equipped with an automatic drinking water supply system and food trays. Following quarantine, suitable animals (total 260) were randomly assigned to control or treatment groups using a body weight stratification procedure. Three housing chambers were used: one for the air control group and two for smoke-exposed groups. Temperature, relative humidity (RH), and airflow were monitored continuously; temperature was maintained between 20 and 24◦C, the relative humidity (RH) between 50 and 70%, and there were 15 ± 3 air changes/h.

*Experimental Design*

Groups of 65 male mice were assigned to 1 of 4 exposure groups and exposed to diluted MS generated from 2R4F cigarettes or filtered air (sham control). Cigarette smoke exposures were conducted at TPM target concentrations of 50, 200, or 400 mg/m3 (low, mid, and high exposure groups, respectively) of diluted MS. Exposures were conducted for 3 h/day, 5 days/wk for 5 mo. The experimental design is summarized in Table 1. Briefly, each group of animals was divided into five subgroups: interim, terminal, first recovery, second recovery, and surveillance. The interim group consisted of 5 animals per exposure group and was necropsied at the end of 1 wk of exposures (5 consecutive days). Tissues, bone marrow, and blood were collected for weighing or future analysis. Following the interim necropsy, five additional animals were placed in each group and assigned to the second recovery group. The terminal group consisted of 15 animals per exposure group and was necropsied at the end of a 5-mo exposure period. The first and second recovery groups consisted of 20 animals each per exposure group and were maintained following exposure termination and necropsied at either 9 mo (first recovery group) or 12 mo (second recovery group). Finally, the surveillance animals consisted of five sentinel mice per exposure group and through the measurement of carboxyhemoglobin, nicotine, and cotine were used to verify and generally quantify at periodic intervals the exposure regimen. During the course of the study, tissues were also collected for gene expression studies (data not presented).

**Inhalation Exposure Laboratory Conditions**

Cigarette smoke exposures were conducted in a laboratory equipped with 64-port nose-only inhalation exposure chambers (Lab Products Inc., Seaford, DE) contained in acrylic enclosures. During the inhalation exposures the mice were held in nose-only exposure animal holding tubes (CH Technologies (USA), Inc., Westwood, NJ). Animal tube loading and unloading and tube insertion and removal from the exposure chamber manifold were performed according to

standard procedures designed to minimize stress to mice. The mice were acclimated to the holding tubes for 3 days prior to exposure. Following exposure, the holders were removed from the chamber. The mice were removed from the holders and returned to their cages.

TABLE 1
Experimental study design

| Group number | Test article | Target concentration | Number of mice | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Total | Toxicology[a] | | | | |
| | | | | Interim | Terminal | Recovery[b] | | Sentinel[c] |
| | | | | | | A[d] | B[d] | |
| 1 | Filtered air–sham control, mg TPM/m$^3$ | 0 | 65 | 5 | 15 | 20 | 20 | 5 |
| 2 | Cigarette smoke–low, mg TPM/m$^3$ | 50 | 65 | 5 | 15 | 20 | 20 | 5 |
| 3 | Cigarette smoke–mid, mg TPM/m$^3$ | 200 | 65 | 5 | 15 | 20 | 20 | 5 |
| 4 | Cigarette smoke–high, mg TPM/m$^3$ | 400 | 65 | 5 | 15 | 20 | 20 | 5 |
| | | Totals: | 260 | 20 | 60 | 80 | 80 | 20 |

## Test Atmosphere Generation

The cigarette smoke test atmospheres were generated using 30-port Condor smoking machines (Borgwaldt-KC, Richmond, VA). Each exposure chamber had a dedicated smoking machine. Conditioned cigarettes were smoked in basic conformity with ISO Standards 3308 and 4387. The smoking machines were supplied with pressure-regulated, filtered, conditioned air. Vacuum exhaust for the smoke machines was provided by a dedicated regenerative ring blower and exhausted outside the building. The mainstream smoke was diluted in two steps with breathable quality air: first a 1:60 dilution immediately at the exit of the smoke pump and with a second dilution set to the levels required to attain the target smoke concentration.

In this study, the puffs per cigarette ranged from 9.2 to 9.4 for all cigarettes used in this study with a 35 ± 0.5 mm butt length and a puff volume of 35 ml over a two-second puffing period taken once each minute. Sham control animals were exposed to filtered air in an AMESA (AMESA Electronics, Geneva, Switzerland) air control chamber.

## Test Atmosphere Monitoring

During each inhalation exposure period, the TPM concentration for smoke-exposed groups was determined at least three times per exposure in each smoke chamber (once from filtered air) using a gravimetric filter-collection method. Samples were collected from exposure ports with sampling parameters chosen to collect approximately 4 mg TPM per filter. In addition, real-time monitors (RAM sensor) were used to monitor aerosol concentration.

CO levels were monitored continuously in the smoke exposure and sham control chambers with an infrared gas analyzer (Model ZRH, California Analytical Instruments, Inc., Orange, CA). The instrument was calibrated during the test atmosphere development, and the calibration was checked prior to exposure with a zero air standard and an appropriate CO standard.

Aerosol particle size distribution was determined monthly in each chamber (excluding sham control) using a quartz crystal microbalance (QCM) based 10-stage cascade impactor (California Measurements, Inc., Sierra Madre, CA) at a sampling rate of 0.24 L/min. The mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were calculated from the mass accumulated on each stage of the QCM.

**Exposure Biomarkers**

During exposure wk 3, 11, 19, and recovery wk 1, 5 mice per group designated as sentinels were bled for determination of carboxyhemoglobin (COHb) levels in blood. As soon as possible (within minutes) after being removed from the exposure chamber, the animals were anesthetized with 70% CO2/30% O2, bled from the retro-orbital sinus using a tube with ethylenediamine tetraacetic acid (EDTA), and the level of COHb was determined using an IL-482 CO-oximeter (Instrumentation Laboratory, Inc., Lexington, MA). During exposure wk 7, 15 and recovery wk 4, sentinel mice were bled for nicotine and cotinine blood level determinations by radioimmunoassay (Van Vunakis et al., 1987).

**Toxicology Endpoints**

*Body Weights and Clinical Observations*

Animals were observed twice daily for mortality or moribundity. Body weights were measured on study day 8 and at weekly intervals thereafter, including during the recovery period.

*Hematology and Clinical Chemistry*

Hematology and clinical chemistry studies were performed on those mice designated for terminal necropsy at exposure termination. Blood was obtained from the retro-orbital sinus of anesthetized (70% CO2/30% O2) animals in a predesignated order stratifying exposure groups over bleed time. Analysis of hematology and clinical chemistry samples was performed as detailed in Heck et al. (2002) with the exception that hematological parameters were evaluated using the ADVIA 120 hematology system (Bayer Corp., Tarrytown, NY).

**Mortem and Postmortem Procedures**

*Euthanization and Necropsy*

At necropsy, mice were euthanized by intraperitoneal injection of an overdose of sodium pentobarbital. Moribund animals were euthanized by intraperitoneal injection of an overdose of sodium pentobarbital or carbon dioxide asphyxiation. Complete gross postmortem examinations were performed on all animals. The surface of the lungs of each animal was examined for visible lesions. The weights of the brain, heart, kidneys, liver, lungs, spleen, testes, and thymus were recorded.

*Tissue Preservation*

The following tissues were collected and preserved in 10% neutral buffered formalin: bone marrow (femur), brain (medulla/pons, cerebrum, and cerebellum), heart, kidneys, larynx, liver, lungs, nose (nasal turbinates), trachea, and gross lesions. In addition, nasal passages were flushed with formalin before being immersed in fixative and the lungs were inflated with formalin via the trachea.

*Histopathological Evaluations*

Histopathology evaluations were performed on the following tissues collected at the 5-, 9-, and 12-mo necropsies: larynx, lungs (with mainstem bronchi), nose (nasal turbinates), trachea, and gross lesions. These tissues were embedded in paraffin, processed by routine histological methods, stained with hematoxylin and eosin, and evaluated microscopically by a board-certified veterinary pathologist. Additional histopathology was performed on the lungs from animals at 9- and 12-mo time points and consisted of sections taken every 200 $\mu$m and stained with hematoxylin and eosin for evaluation.

*Respiratory-Tract Tissue Sectioning*

The lungs were sectioned according to Dungworth et al. (1976). Laryngeal sections were taken in the narrowly delineated base of the epiglottis, overlying glands in the ventral epiglottal area (Coggins et al., 1980). If subepithelial glands were absent from the section, the section was considered to be in the wrong area for evaluation. A block containing a longitudinal section of distal trachea (distal to larynx) was prepared to include the bronchial bifurcation. Using the technique of Young (1981) the respiratory epithelium of the nasoturbinate, maxilloturbinate, and walls of the nasal cavity were examined.

**Statistical Procedures**

Clinical observations were tabulated, but not statistically analyzed. All other toxicology data were analyzed using Tukey's HSD procedure. Tukey's HSD procedure is a pairwise comparison procedure, wherein all treatment groups are compared to all other groups, which controls the Type I error rate for the family of all pairwise comparisons. While all groups were compared, for purposes of this study, the comparisons of interest consisted of each cigarette group with the sham control group. Data were analyzed with SYSTAT (Systat Software, Inc., Point Richmond, CA, version 10.2). A significance level of $\alpha = .05$ was used for all comparisons.

*Proliferation Data*

The ratio was defined as

$$\text{Ratio} = \frac{\#\text{hyperplasias}}{(\#\text{adenomas} + \#\text{carcinomas})}$$

Animals with at least one hyperplasia but no adenomas or carcinomas were all given the same very large (999) ratio. The value chosen is inconsequential to the analysis (described later), as

long as it is larger than all other observed ratios. Animals judged not to have any of the three histopathologic conditions were omitted from the analysis. The Jonckheere–Terpstra (JT) test (Richter & Higgins, 2006) was used to assess the null hypothesis of no difference in Ratio distribution across the groups against the alternative hypothesis that the Ratio tends to decrease across groups (i.e., as concentration increases).

*Incidence of Histological Alterations*

Logistic regression was used to assess change in incidence of lesions as a function of group. Since sample sizes were small and there often occurred perfect discrimination among groups (all 0% or 100% incidence rates); exact inference using conditional maximum likelihood estimation was used to improve results (Agresti, 1996). Reported are odds ratio estimates and *p* values for testing the hypothesis that the odds ratio is equal to 1 (equal odds). The explanatory variable, Group, was treated as an ordinal variable assuming equally spaced categories. Thus, an odds ratio is interpreted as the factor by which the odds of a lesion are higher when comparing a Group category to the previous category.

The family-wise confidence level for Tables 4 to 6 was 95%, which means that there is simultaneous 95% confidence that all intervals in the table contain the true population odds ratio. In order to achieve this, individual confidence intervals were computed using a conservative $(1 - 0.05/25)*100\% = 99.8\%$ confidence level for Table 4 and $(1 - 0.05/17)*100\% = 99.7\%$ level for Tables 5 and 6. Any intervals with a lower bound greater than 1 indicate statistically significant evidence of increased incidence of lesions at the family-wise 5% level (equivalently, statistical significance can be concluded if the *p* value is less than .002 for Table 4 or less than .003 for Tables 5 and 6).

For example, for the first variable (Hyperplasia, respiratory epithelium) in Table 4, the odds of lesion for a given Group are estimated to be 59.8 times the odds for the Group with the next lowest concentration. With 99.8% confidence, this factor is at least 3.19. Since this lower bound is greater than 1, this result is statistically significant at the 5% (familywise) level. (Equivalently, the *p* value less than .002 also indicates a statistically significant result.)

*Note*: In some cases, due to the pattern of responses, only a lower bound on the confidence interval can be computed, and the upper bound is denoted as "∞" (infinity).

## RESULTS

### Smoke Exposure

The overall mean TPM concentrations with standard deviations for the target levels set at 50, 200, and 400 mg TPM/m3 for 2R4F cigarette smoke were 51 ± 3.5, 205 ± 12.8 and 402 ± 19.3 mg TPM/m3, respectively. The overall mean nicotine concentrations with standard deviations were 3.3 ± 0.8, 16.6 ± 2.6, and 31.3 ± 3.5 mg/m3 at each of the test atmosphere target concentrations, respectively. The particle size distribution of the smoke generated from each cigarette type was well within the respirable range. The mass median aerodynamic diameters (MMADs) ranged from 0.30 to 0.33 $\mu$m and the geometric standard deviations (GSDs) ranged

from 1.86 to 1.95. The overall mean CO concentrations with standard deviations were 67 ± 5.0, 226 ± 14.4, and 453 ± 28.4 ppm at each of the test atmosphere target concentrations, respectively.

**Mortality and Gross Observations**

Forty-one deaths occurred during the study, including 19 in the low exposure group; however, due to the lack of correlation with smoke concentration, mortality was generally not considered attributable to smoke exposure. Clinical signs generally observed in all exposure groups during the exposure period included alopecia, lesions, salivation, and wet inguinal fur, while increased clinical observations of tremors and coldness to touch were seen in only smoke exposure groups, particularly in the 200 and 400 mg TPM/m3 smoke-exposure groups. The incidences of these clinical signs were notably diminished or absent during the recovery period.

The mean body weight increased in all groups throughout the course of the study, even though the weight of those mice exposed to cigarette smoke was statistically significantly decreased a majority of the time during the exposure period (see Figure 1). The body weight for the 50 mg TPM/m3 smoke-exposure group was significantly depressed for the second month of the study but at no other time. Following 1 wk of exposure, the mean body weight of animals in the 200 and 400 mg TPM/m3 smoke-exposure groups was significantly depressed. The 200 mg TPM/m3 group remained depressed throughout exposure; however, by study day 183, approximately 4 wk postexposure, mean body weights for animals in this group were similar to those of the filtered air (sham) group. In contrast, the 400 TPM/m3 group remained significantly depressed through study day 246. All affected mean body weight and mean weight gains were similar to those of the sham exposure group by the end of the study.

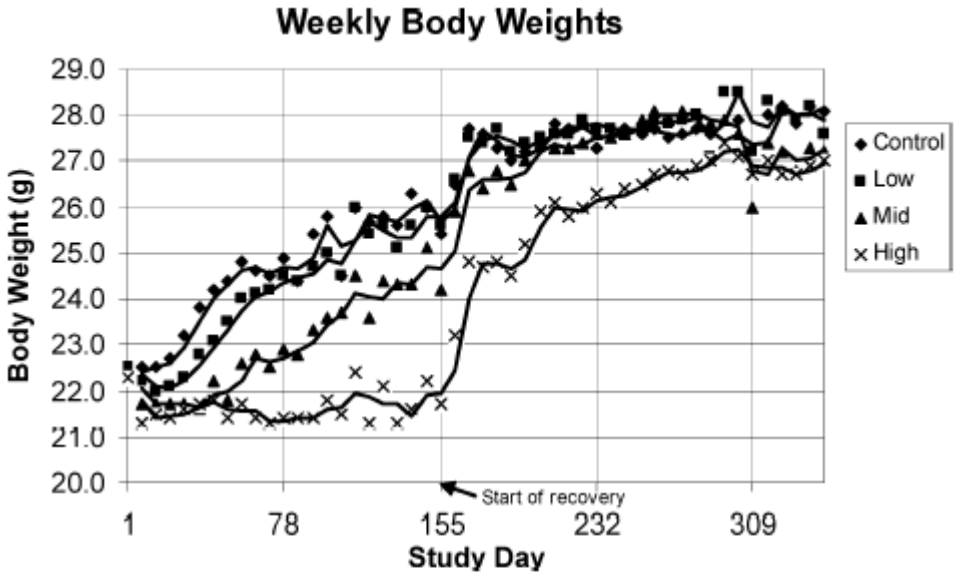


FIG. 1. Body weights were recorded on a weekly basis throughout the 5-mo exposure and 7-mo recovery period. Smoke exposure resulted in significant decreases in body weight gain in all smoke exposure groups. Body weight was depressed in the low-exposure group during mo 2 of exposure, whereas the mid- and high-exposure groups displayed decreased body weights beginning in wk 2 of exposure and continuing into the recovery period.

**Clinical Chemistry**

The following hematology and clinical chemistry parameters were found to be statistically significantly affected: hematocrit, total platelet count, percent neutrophil, percent lymphocyte, percent eosinophil, absolute neutrophil count, absolute lymphocyte count, absolute eosinophil count, absolute large unstained cells, and alkaline phosphatase. In the high exposure group, the numbers and percentage of neutrophils were significantly increased whereas the numbers of lymphocytes, eosinophils, and large unstained cells were all decreased. The mid concentration exposure group also had significant decreases in the numbers of lymphocytes. In addition, the percentage of neutrophils was increased while the percentage of lymphocytes and eosinophils were both decreased.

Blood oximetry data showed no biologically significant effects on total hemoglobin levels in any of the comparisons during the course of the study. Carboxyhemoglobin levels were statistically significantly increased in all smoke- exposed groups compared to the filtered air (sham) group in a concentration-dependent manner during the exposure period (Table 2). Mean serum nicotine and serum cotinine levels showed increases in a concentration-dependent manner in all smoke-

exposed groups compared to the filtered air (sham) group during the exposure period (Table 3); increases were statistically significant for the mid and high exposure groups.

TABLE 2
Serum carboxyhemoglobin levels

| Exposure group | wk 3 | wk 11 | wk 19 | Recovery wk 1 |
|---|---|---|---|---|
| 0 | $0.7 \pm 0.29$ | $0.6 \pm 0.58$ | $0.7 \pm 0.26$ ($n = 3$) | $0.9 \pm 0.10$ ($n = 3$) |
| 50 | $7.1 \pm 0.63$* | $7.5 \pm 0.76$* | $5.7 \pm 1.55$ ($n = 3$) | $1.0 \pm 0.36$ |
| 200 | $25.2 \pm 0.98$* | $27.8 \pm 2.05$* | $24.1 \pm 3.14$* | $0.9 \pm 0.45$ ($n = 4$) |
| 400 | $35.8 \pm 3.11$* | $37.1 \pm 1.63$* | $33.9 \pm 1.94$* | $0.9 \pm 0.36$ |

*Note*. Values represent mean percent carboxyhemoglobin level ± standard deviation; $n = 5$, except where noted in parentheses. Asterisk indicates significant difference from sham control ($p < .05$).

## Organ Weights

Absolute organ weights affected included: *Terminal Necropsy*: liver and testes (400 mg TPM/m3). Relative organ weights affected included: *Terminal Necropsy*: brain (400 mg TPM/m3), and final body weight (400 mg TPM/m3); *First Recovery Necropsy*: final body weight (400 mg TPM/m3). All gross lesions observed at necropsy were considered incidental and unrelated to smoke exposure.

## General Histopathology

Normal epithelium of the nasal passages was one to two cell layers thick, the ventral epiglottal floor was two to three cell layers thick, and the trachea possessed a single cell layer. Hyperplasia of the epithelium was a prominent finding throughout the respiratory tract, and severity was determined by the degree to which the nucleated cell layer was thickened. Within the nasal passages, minimal hyperplasia was characterized by loss of the eosinophilic nuclear-free layer at the mucosal surface. Increasing severity was scored based upon additional thickness of the nucleated cell layer. In addition, cilia that were present in the less severe cases of hyperplasia were absent in more severe instances. Metaplasia was diagnosed by the presence of cells with polarity altered such that they were oriented horizontal to the basal membrane. In severe metaplastic alterations, keratinization was noted. Submucosal inflammation was diagnosed in nasal passages and larynx when the submucosa was slightly thickened and diffusely infiltrated with neutrophils and mononuclear inflammatory cells.

Histopathological alterations are detailed in Tables 4 through 6. At the terminal necropsy, hyperplasia of the respiratory epithelium was a prominent feature noted in the nasal passages, larynx and trachea. In general, severity of hyperplasia decreased from the anterior to posterior nasal regions, was highest in the larynx and lowest in the trachea. At the lowest smoke exposure (50 mg TPM/m3), hyperplasia of the epithelium of the nose and the larynx were noted. In addition to changes observed at the low smoke concentration, exposure to 200 mg TPM/m3 produced severe hyperplasia and metaplastic alterations in nasal level 1. In addition, 200 mg TPM/m3 produced lumen exudate within the nasal passages with nasopharyngeal duct epithelia hyperplasia, keratinization and chronic inflammation of the larynx, epithelial hyperplasia of the trachea, and infiltration and focal accumulation of pigmented macrophages in the lung. At the

high smoke level (400 mg TPM/m3) there was also subacute submucosal inflammation and epithelial atrophy of the nasal passages.

Significant repair was noted in the histopathological lesions observed from the first recovery necropsy (detailed in Table 5), but residual lesions were present. By the first recovery necropsy, no metaplastic changes of the nasal epithelium were noted and keratinization of the larynx/epiglottis was no longer observed. The severity of hyperplasia was greatly reduced; for example severity of hyperplasia in nose level 1 had dropped from 3.9 to 0.8. However, respiratory epithelium hyperplasia in nose and epithelial cell hyperplasia/metaplasia in larynx with associated chronic inflammation were still present in animals recovering from exposure to cigarette smoke at 50, 200, or 400 mg TPM/m3. Olfactory epithelium atrophy (often associated with subacute submucosal inflammation and lumen exudate) in nose and focal pigmented macrophage accumulation in lung were still present at the end of the initial recovery period in animals exposed to cigarette smoke at 200 or 400 mg TPM/m3. Further recovery was noted in the histopathology lesions observed from the second recovery necropsy (detailed in Table 6), but residual lesions (atrophy of olfactory epithelium that often included sub-acute submucosal inflammation and lumen exudate in nose and focal pigmented macrophage accumulation in lung) were still present at 200 or 400 mg TPM/m3. Only atrophy of the olfactory epithelium and focal pigmented macrophages in the lungs remained significantly altered within animals from the 400 mg TPM/m3exposure group.

TABLE 3
Serum nicotine and cotinine levels

| Exposure group | Serum nicotine levels | | | Serum Cotinine levels | | |
|---|---|---|---|---|---|---|
| | wk 7 | wk 15 | Recovery wk 4 | wk 7 | wk 15 | Recovery wk 4 |
| 0 | $0.1 \pm 0.13$ | $1.9 \pm 0.64$ ($n = 2$) | $0.6 \pm 1.01$ ($n = 3$) | $0.2$ ($n = 1$) | ND | $0.3 \pm 0.58$ ($n = 3$) |
| 50 | $95.8 \pm 50.51$ | $85.3 \pm 22.63$ | $2.8 \pm 2.94$ | $285.2 \pm 64.82$ | $188.9 \pm 50.29$ | $0.1 \pm 0.12$ |
| 200 | $275.0 \pm 119.91$* | $216.9 \pm 65.16$* | $8.9 \pm 13.8$ | $627.8 \pm 157.51$* | $443.9 \pm 138.00$* | $1.5 \pm 2.26$ |
| 400 | $332.3 \pm 42.74$* | $409.2 \pm 133.4$* | $9.9 \pm 15.66$ | $851.2 \pm 117.70$* | $743.2 \pm 105.88$* | $0.7 \pm 0.59$ |

*Note.* Values represent means ± standard deviation; $n = 5$, except where noted in parentheses. Asterisk indicates significant difference from sham control ($p < .05$). ND, not determined due to insufficient serum sample.

TABLE 4
Histopathological alterations at the 5-mo terminal necropsy

| Lesion | Group/target concentration (mg TPM/m³) | | | | 98.7% Confidence intervals for odds ratios | | |
|---|---|---|---|---|---|---|---|
| | I / 0 | II / 50 | III / 200 | IV / 400 | 10 unit change | 100 unit change | *p* Value |
| Nose, Level 1 | | | | | | | |
| Hyperplasia, respiratory epithelium | 0/10 | 5/5 (1.60)* | 8/8 (3.00) | 10/10 (3.90) | 1.15, ∞ | 3.96, ∞ | <.0001* |
| Squamous metaplasia | 0/10 | 0/5 | 3/8 (0.38) | 10/10 (2.40) | 1.06, ∞ | 1.71, ∞ | <.0001* |
| Inflammation, subacute, submucosal | 0/10 | 0/5 | 0/8 | 10/10 (2.10) | 1.06, ∞ | 1.73, ∞ | <.0001* |
| Exudate, lumen | 0/10 | 0/5 | 0/8 | 9/10 (1.90) | 1.06, ∞ | 1.73, ∞ | <.0001* |
| Nose, Level 2 | | | | | | | |
| Hyperplasia, respiratory epithelium | 0/10 | 0/5 | 8/9 (0.89) | 10/10 (3.00) | 1.08, ∞ | 2.19, ∞ | <.0001* |
| Squamous metaplasia | 0/10 | 0/5 | 0/9 | 0/10 | — | — | — |
| Atrophy, olfactory epithelium | 0/10 | 0/5 | 0/9 | 10/10 (2.40) | 1.08, ∞ | 2.17, ∞ | <.0001* |
| Inflammation, subacute, submucosal | 0/10 | 0/5 | 0/9 | 9/10 (1.70) | 1.06, ∞ | 1.77, ∞ | <.0001* |
| Exudate, lumen | 0/10 | 0/5 | 0/9 | 7/10 (1.20) | 1.03, ∞ | 1.33, ∞ | <.0001* |
| Nose, Level 3 | | | | | | | |
| Atrophy, olfactory epithelium | 0/10 | 0/5 | 0/9 | 10/10 (2.60) | 1.08, ∞ | 2.17, ∞ | <.0001* |
| Inflammation, subacute, submucosal | 0/10 | 0/5 | 0/9 | 10/10 (2.00) | 1.08, ∞ | 2.17, ∞ | <.0001* |
| Exudate, lumen | 0/10 | 0/5 | 1/9 (0.22) | 9/10 (2.00) | 1.05, 1.77 | 1.58, 300 | <.0001* |
| Nose, Level 4 | | | | | | | |
| Atrophy, olfactory epithelium | 0/10 | 0/5 | 0/9 | 6/10 (1.40) | 1.02, ∞ | 1.16, ∞ | 0.0003* |
| Inflammation, subacute, submucosal | 0/10 | 0/5 | 0/9 | 4/10 (0.70) | 0.98, ∞ | 0.86, ∞ | 0.0091 |
| Exudate, lumen | 0/10 | 0/5 | 0/9 | 4/10 (1.10) | 0.98, ∞ | 0.86, ∞ | 0.0091 |
| Hyperplasia, epithelium, nasopharyngeal duct | 0/10 | 0/5 | 7/9 (0.78) | 9/10 (1.60) | 1.04, 1.64 | 1.49, 139 | <.0001* |
| Larynx, epiglottis | | | | | | | |
| Epithelial cell hyperplasia/metaplasia | 0/8 | 5/5 (4.00) | 8/8 (4.00) | 10/10 (4.00) | 1.12, ∞ | 3.03, ∞ | <.0001* |
| Keratinization | 0/8 | 0/5 | 8/8 (2.38) | 10/10 (2.10) | 1.09, ∞ | 2.38, ∞ | <.0001* |
| Inflammation, chronic | 1/8 (0.12) | 0/5 | 8/8 (1.00) | 10/10 (1.20) | 1.07, 4.37 | 1.92, ∞ | <.0001 |
| Trachea | | | | | | | |
| Hyperplasia, respiratory epithelium | 0/6 | 0/1 | 2/3 (1.33) | 8/8 (1.75) | 1.02, ∞ | 1.23, ∞ | 0.0001* |
| Lung, left | | | | | | | |
| Infiltration, pigmented macrophage, diffuse | 0/10 | 0/5 | 9/9 (1.00) | 10/10 (1.10) | 1.10, ∞ | 2.68, ∞ | <.0001* |
| Accumulation, pigmented macrophage, focal | 0/10 | 0/5 | 5/9 (0.89) | 7/10 (2.40) | 1.01, ∞ | 1.12, 10.58 | 0.0003* |
| Lung, right | | | | | | | |
| Infiltration, pigmented macrophage, diffuse | 0/10 | 1/5 (0.20) | 9/9 (1.00) | 10/10 (1.10) | 1.09, ∞ | 2.37, ∞ | <.0001* |
| Accumulation, pigmented macrophage, focal | 0/10 | 0/5 | 5/9 (0.78) | 9/10 (2.40) | 1.04, ∞ | 1.44, 129.2 | <.0001* |

*Incidence (mean group severity score).

TABLE 5
Histopathological alterations at the 9-mo recovery necropsy

| Organ–lesion | Group/target concentration (mg TPM/m$^3$) | | | | 98.7% Confidence intervals for odds ratios | | |
|---|---|---|---|---|---|---|---|
| | I / 0 | II / 50 | III / 200 | IV / 400 | 10 unit change | 100 unit change | *p* Value |
| Nose, Level 1 | | | | | | | |
| Hyperplasia, respiratory epithelium | 0/9 | 2/9 (0.22)* | 4/10 (0.40) | 8/10 (0.80) | 1.02, 1.23 | 1.18, 7.99 | .0002* |
| Inflammation, subacute, submucosal | 0/9 | 0/9 | 7/10 (0.80) | 5/10 (0.90) | 1.00, 1.18 | 0.99, 5.11 | .0034 |
| Exudate, lumen | 0/9 | 0/9 | 0/10 | 1/10 (0.30) | 0.86, ∞ | 0.21, ∞ | .526 |
| Nose, Level 2 | | | | | | | |
| Hyperplasia, respiratory epithelium | 0/9 | 0/9 | 0/10 | 0/10 | — | — | — |
| Atrophy, olfactory epithelium | 1/9 (0.22) | 0/9 | 2/10 (0.50) | 9/10 (2.00) | 1.03, 1.36 | 1.38, 21.07 | <.0001* |
| Inflammation, subacute, submucosal | 0/9 | 0/9 | 0/10 | 7/10 (1.10) | 1.04, ∞ | 1.46, ∞ | <.0001* |
| Exudate, lumen | 1/9 (0.11) | 0/9 | 0/10 | 3/10 (0.50) | 0.95, 1.26 | 0.60, 9.96 | .152 |
| Nose, Level 3 | | | | | | | |
| Atrophy, olfactory epithelium | 1/9 (0.33) | 0/9 | 0/10 | 7/10 (1.80) | 1.01, 1.35 | 1.14, 19.70 | .0005* |
| Inflammation, subacute, submucosal | 0/9 | 0/9 | 0/10 | 6/10 (0.90) | 1.03, ∞ | 1.28, ∞ | .0002* |
| Exudate, lumen | 1/9 (0.22) | 2/9 (0.33) | 2/10 (0.20) | 1/10 (0.30) | 0.89, 1.08 | 0.32, 2.18 | .863 |
| Nose, Level 4 | | | | | | | |
| Atrophy, olfactory epithelium | 0/9 | 0/9 | 0/10 | 2/10 (0.50) | 0.94, ∞ | 0.53, ∞ | .128 |
| Inflammation, subacute, submucosal | 0/9 | 0/9 | 0/10 | 2/10 (0.40) | 0.94, ∞ | 0.53, ∞ | .128 |
| Exudate, lumen | 0/9 | 0/9 | 0/10 | 1/10 (0.30) | 0.86, ∞ | 0.21, ∞ | .526* |
| Larynx, epiglottis | | | | | | | |
| Epithelial cell hyperplasia/metaplasia | 0/10 | 5/9 (0.67) | 8/8 (2.50) | 10/10 (2.80) | 1.10, ∞ | 2.51, ∞ | <.0001* |
| Inflammation, chronic | 0/10 | 1/9 (0.11) | 7/8 (0.88) | 8/10 (0.90) | 1.03, 1.30 | 1.33, 13.36 | <.0001* |
| Lung, left | | | | | | | |
| Accumulation, pigmented macrophage, focal | 0/10 | 0/10 | 7/10 (0.80) | 10/10 (2.10) | 1.08, ∞ | 2.24, ∞ | <.0001* |
| Lung, right | | | | | | | |
| Accumulation, pigmented macrophage, focal | 0/10 | 1/10 (0.10) | 8/10 (0.90) | 10/10 (2.20) | 1.08, 2.00 | 2.14, 986 | <.0001* |

*Incidence (mean group severity score).

## Lung Tumorigenesis

The incidence and multiplicity of lesions on the surface of the lung are summarized in Table 7. At the 9-mo recovery necropsy, exposure to cigarette smoke at 200 mg TPM/m3 produced increased multiplicity of masses observed on the surface of the lungs, however this elevation was not statistically significant ( $p = .065$). In Table 8, the lesions on the surface of the lungs of animals that underwent histological examination are reported. In all groups, the animals that were randomly assigned to the histology group had lower incidence and multiplicity of lung lesions.

Histological examination of the lungs, revealed focal proliferative lesions not detected by visual inspection at the time of necropsy (Table 9). These lesions included focal hyperplasia, adenoma and adenocarcinoma; lesions are depicted in Figure 2A–D.

In the current study, smoke exposure was associated with increased progression of focal hyperplasia to adenoma at 9 mo (Table 10) such that the predominant lesion in air-exposed animals was focal hyperplasia and with increasing concentrations of smoke exposure there is a trend toward increasing tumor prevalence. However, this trend was analyzed for statistical significance using the Jonckheere–Terpstra test and analysis ( $p$ value is .0592) indicated moderate but not statistically convincing evidence that the ratio of hyperplasias to adenomas and carcinomas tends to decrease as smoke concentration increases. The 12-mo data were also analyzed for trend in the conversion of hyperplasia to

adenoma. The *p* value is .477, which fails to provide convincing statistical evidence that the ratio tends to decrease as group number increases.

## DISCUSSION

This study was performed to evaluate the response of A/J mice exposed nose-only to cigarette mainstream smoke. The evaluation included thorough histological evaluation of the respiratory tract and step-sectioning of the lungs. Nose-only inhalation produced histopathological alterations consistent with prior rodent inhalation studies (Coggins, 1998; Heck et al., 2002). Alterations included epithelial hyperplasia and inflammation of the nose and larynx as well as accumulation of macrophages in the lungs. Examination during recovery demonstrated that a number of the alterations decreased in severity but persisted at study termination. Following 4 mo of recovery, animals continued to display atrophy of the olfactory epithelium, hyperplasia of the laryngeal epithelium and accumulations of pigmented macrophages in the lung. This persistence underscores the severity of the exposure.

The tumorigenicity of tobacco smoke was difficult to assess in the current study due to the small number of animals, high incidence of spontaneous tumors and low tumor multiplicity over exposure groups. At the 9-mo necropsy, multiplicity of visually identified lung lesions was elevated in the mid-exposure group, although this was not a statistically significant finding. Curtin et al. (2004) compared nose-only to whole-body exposure of A/J mice. While whole-body exposure produced statistically significant increases in lung tumor incidence and multiplicity, nose-only exposure showed only an increasing trend. In addition, most investigations of tobacco smoke tumorigenicity in A/J mice have utilized a mixture composed primarily of sidestream smoke, and it is conceivable that the response to a sidestream mixture is different than it would be to mainstream smoke only. Perhaps the current findings are a reflection of lowered responsiveness of nose-only inhalation or mainstream smoke combined with the relatively weak carcinogenicity of tobacco smoke in A/J mice (Witschi et al., 2004; Witschi, 2005a).

Most cigarette smoke tumorigenicity studies employing A/J mice have relied on tumor counts on the lung surface (Witschi, 2005a; D'Agostini et al., 2001). Curtin et al. (2004), however, demonstrated that histological examination of the lung identifies tumors not observed through visual inspection of the lungs' surface. In addition, Curtin et al. demonstrated that microscopic examination produced statistically significant elevations in tumor incidence and multiplicity of mainstream smoke-exposed A/J mice that were likely to escape detection by gross examination only. Similarly, the current study identified numerous additional proliferative lesions using microscopic examination, and this emphasizes the need for histological analysis of the lung in order to optimize the data yielded.

In the current study, tumors were predominantly adenomas with a small percentage containing carcinomatous foci. This is consistent with recent reports that 80% of tumors occurring in cigarette smoke exposed A/J mice are adenomas, with additional tumors being adenoma with carcinomatous foci or carcinomas (Witschi, 2005a). Similarly, Dixon et al. (1991) performed a survey of lung tumors in the A/J mouse and reported that 67% of spontaneous tumors were adenomas and the remainder carcinomas. It should be noted that the predominance of adenomas represents a possible limitation of murine models (Malkinson, 1998; Hutt et al., 2005). While

there has been an apparent shift in histologic type of human lung cancer favoring adenocarcinoma (Wingo et al., 1999), human lung cancers are more histologically diverse than murine and human patients are generally diagnosed with adenocarcinoma rather than adenoma. In addition to tumors, histological evaluation of the lung identified a relatively high incidence of hyperplastic foci. Witschi et al. (1997) reported that proliferative lesions within the lungs of smoke- exposed A/J mice included hyperplasia, adenoma, and adenocarcinoma. While the incidence of adenomas and adenocarcinomas was reportedly similar between control and exposed groups, no mention is made of the relative prevalence of hyperplastic lesions. In addition, Stinn et al. (2005) reported the predominant tumor type in A/J mice is bronchioloalveolar adenoma and that approximately 20% of mice had hyperplastic lesions, although no breakdown by exposure group was provided. The presence of hyperplastic foci generally appears incompletely classified.

Auerbach et al. (1957) reported a histological study of the respiratory epithelium using step sections of the tracheobronchial tree of 150 male patients characterized by smoking habits. The authors concluded that epithelial-cell hyperplasia, stratification, and metaplasia had the lowest incidence in nonsmokers and increased in frequency in proportion to the amount of smoking. As the study progressed the authors published additional findings that characterized sections of epithelium for hyperplasia, atypical cell morphology, the absence of cilia, and lesions containing multiple alterations (Auerbach et al., 1961). Of interest to the current study is the observation that smoking appeared to influence epithelial hyperplasia. Even low levels of smoking (<1/2 package per day consumption) resulted in over 50% of sections with hyperplasia and above 1 pack per day the percentage of sections with hyperplasia decreased in proportion to the average daily consumption of cigarettes. The decreasing proportion of sections from human patients containing hyperplasia is similar to findings in the current study. However, in contrast to the current study in A/J mice, sections from nonsmoking human patients contained a very small incidence of epithelial hyperplasia (2.4%), whereas epithelial hyperplasia was the most frequent lesion observed in the air-exposed mice.

TABLE 6
Histopathological alterations at the 12 mo recovery necropsy

| ORGAN–lesion | Group / target concentration (mg TPM/m3 ) | | | | 98.7% confidence intervals for odds ratios | | |
|---|---|---|---|---|---|---|---|
| | I / 0 | II / 50 | III / 200 | IV / 400 | 10 unit change | 100 unit change | p-value |
| NOSE, LEVEL 1 | | | | | | | |
| Hyperplasia, respiratory epithelium | 0/7 | 0/1 | 0/10 | 0/10 | — | — | — |
| Inflammation, subacute, submucosal | 0/7 | 0/1 | 0/10 | 0/10 | — | — | — |
| Exudate, lumen | 0/7 | 0/1 | 1/10 (0.20) | 4/10 (0.70) | 0.97, 1.54 | 0.74, 74.5 | 0.048 |
| NOSE, LEVEL 2 | | | | | | | |
| Hyperplasia, respiratory epithelium | 0/7 | 0/1 | 0/10 | 0/10 | — | — | — |
| Atrophy, olfactory epithelium | 0/7 | 0/1 | 3/10 (0.50) | 9/10 (2.50) | 1.03, 1.64 | 1.29, 137 | <.0001* |
| Inflammation, subacute, submucosal | 0/7 | 0/1 | 0/10 | 0/10 | — | — | — |
| Exudate, lumen | 0/7 | 0/1 | 3/10 (0.40) | 4/10 (0.60) | 0.96, 1.22 | 0.70, 7.56 | 0.102 |
| NOSE, LEVEL 3 | | | | | | | |
| Atrophy, olfactory epithelium | 0/7 | 0/1 | 2/10 (0.60) | 9/10 (2.70) | 1.03, 1.68 | 1.36, 174 | <.0001* |
| Inflammation, subacute, submucosal | 0/7 | 0/1 | 0/10 | 2/10 (0.40) | 0.92, ∞ | 0.45, ∞ | 0.238 |
| Exudate, lumen | 1/7 (0.28) | 0/1 | 2/10 (0.40) | 4/10 (0.70) | 0.95, 1.17 | 0.58, 4.82 | 0.348 |
| NOSE, LEVEL 4 | | | | | | | |
| Atrophy, olfactory epithelium | 0/7 | 0/1 | 1/10 (0.20) | 3/10 (0.40) | 0.95, 1.51 | 0.61, 61.7 | 0.138 |
| Inflammation, subacute, submucosal | 0/7 | 0/1 | 0/0 | 0/10 | — | — | — |
| Exudate, lumen | 0/7 | 0/1 | 0/0 | 1/10 (0.10) | 0.844, ∞ | 0.18, ∞ | 1.000 |
| LARYNX, EPIGLOTTIS | | | | | | | |
| Epithelial cell hyperplasia/metaplasia | 0/7 | 0/1 | 7/10 (1.40) | 10/10 (1.90) | 1.04, ∞ | 1.50, ∞ | <.0001* |
| Inflammation, chronic | 0/7 | 0/1 | 7/10 (0.80) | 3/10 (0.30) | 0.95, 1.13 | 0.60, 3.29 | 0.388 |
| LUNG, LEFT | | | | | | | |
| Accumulation, pigmented macrophage, focal | 0/7 | 0/1 | 7/10 (0.70) | 10/10 (2.80) | 1.04, ∞ | 1.50, ∞ | <0.0001* |
| LUNG, RIGHT | | | | | | | |
| Accumulation, pigmented macrophage, focal | 0/7 | 0/1 | 8/10 (1.00) | 10/10 (2.80) | 1.05, ∞ | 1.57, ∞ | <0.0001* |

TABLE 7
Incidence and multiplicity of lesions observed on the surface of the lungs from all study animals at necropsy

| | Air-exposed | 50 mg/m$^3$ | 200 mg/m$^3$ | 400 mg/m$^3$ |
|---|---|---|---|---|
| 5 mo | 0/14 (0) | 0/10 (0) | 0/14 (0) | 0/14 (0) |
| 9 mo | 8/20 (0.55) | 9/20 (0.55) | 11/20 (0.9) | 8/20 (0.55) |
| 12 mo | 7/12 (0.83) | 5/6 (1.17) | 6/15 (0.8) | 8/15 (0.8) |

*Note*. Incidence is expressed as the number of tumor-bearing animals observed divided by the total animals necropsied. Multiplicity is in parentheses.

TABLE 8
Incidence and multiplicity of lesions observed on the surface of the lungs from histology animals at necropsy

| | Air-exposed | 50 mg/m$^3$ | 200 mg/m$^3$ | 400 mg/m$^3$ |
|---|---|---|---|---|
| 9 mo | 3/10[1] | 3/10 | 3/10 | 2/10 |
| | (0.30)[2] | (0.40) | (0.50) | (0.20) |
| 12 mo | 2/7 | 2/2 | 3/10 | 3/10 |
| | (0.43) | (1.0) | (0.50) | (0.40) |

*Note*. Incidence is expressed as the number of tumor bearing animals observed divided by the total animals necropsied. Multiplicity is in parentheses.

TABLE 9
Multiplicity of histologically identified focal lung lesions

| Group | Focal lung lesions/ animal at 9 mo | Focal lung lesions/ animal at 12 mo |
|---|---|---|
| Air-exposed | 1.4 (10) | 1.6 (7) |
| 50 mg/m$^3$ | 0.8 (10) | 1.5 (2) |
| 200 mg/m$^3$ | 1.1 (10) | 1.6 (10) |
| 400 mg/m$^3$ | 1.2 (10) | 2.3 (10) |

*Note*. Number of animals examined is in parentheses. No statistically significant ($p \leq .05$) differences in multiplicity were recorded.

A potential mechanism of cigarette smoke-induced lung tumorigenesis in A/J mice may involve inflammation acting to promote the prevalent spontaneous hyperplastic foci. Promotion has been suggested to be a dominant mechanism of lung cancer mortality in human smokers (Hazelton et al., 2005), and an association between lung cancer and pulmonary inflammation has been suggested (Smith et al., 2006). In addition, cigarette smoke has been shown to be inflammatory in murine models (D'hulst et al., 2005; Seagrave et al., 2004). In the current study, cigarette smoke exposure was associated with inflammatory changes of the upper respiratory tract and changes in peripheral white cell counts indicative of a systemic inflammatory response. Inflammation could promote spontaneous hyperplastic lesions in A/J mice. The influence of inflammation on tumorigenicity of mice has been demonstrated using strains that differ in their sensitivity to tumor promotion by butylated hydroytoluene (BHT). Bauer et al. (2001) demonstrated that a sensitive mouse strain displays inflammation following BHT administration whereas a tumor- resistant model does not develop an inflammatory response to BHT.

The anti-inflammatory agents aspirin and sulindac (Duperron & Castonguay, 1997) and indomethacin (Moody et al., 2001) have been shown to decrease tumorigenicity in the A/J mouse. Witschi et al. (2005) demonstrated that the synthetic glucocorticoid dexamethasone decreases A/J mouse lung tumor multiplicities. Dexamethasone decreased tumors in smoke-exposed animals by 35%, and while this was not statistically significant, significant decreases

occurred in tumor multiplicity of air-exposed mice. Similarly, Estensen et al. (2004) report that the synthetic glucocorticoid, budesonide, delays hyperplasia and adenoma formation following benzo[a]pyrene (BaP) exposure.

TABLE 10
Association of smoke exposure with tumor progression

| | 9 mo | | 12 mo | |
|---|---|---|---|---|
| Group | Percent hyperplasia | Percent tumor[a] | Percent hyperplasia | Percent tumor[a] |
| Air-exposed | 71.4 | 28.6 | 36.4 | 63.6 |
| 50 mg/m$^3$ | 62.5 | 37.5 | 0 | 100 |
| 200 mg/m$^3$ | 36.4 | 63.6 | 31.2 | 68.8 |
| 400 mg/m$^3$ | 16.7 | 83.3 | 47.8 | 52.2 |

*Note*. Analysis of the ratio of hyperplasia to tumors using the Jonckheere–Terpstra test failed to confirm a statistically significant trend at 9 mo ($p$ value is .0592) or at 12 mo ($p$ value is .477).
[a]Percentage of focal lung lesions diagnosed as either adenoma or adenocarcinoma.

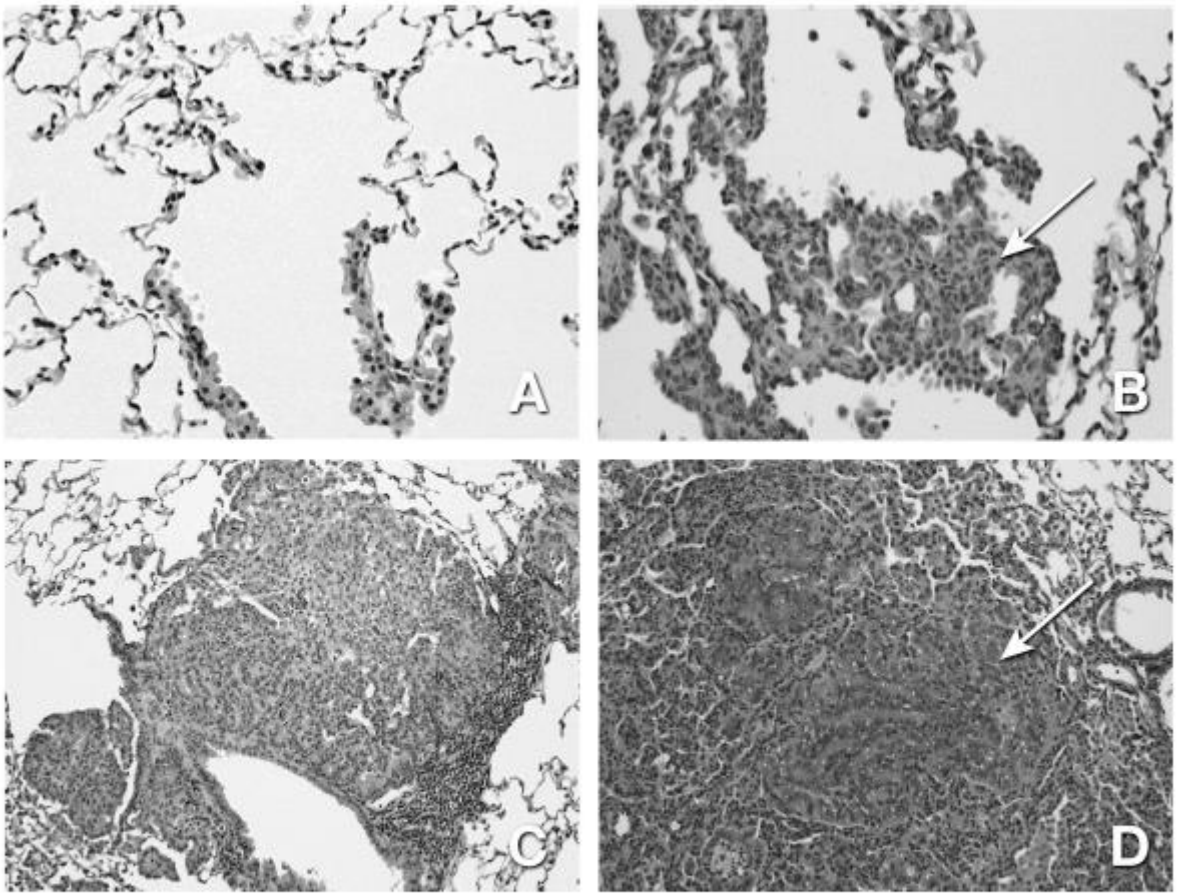


FIG. 2. Histological sections of A/J mouse lung. (A) Normal lung alveolar epithelium. (B) Focal hyperplasia; note proliferation of alveolar epithelial cells (arrow) without significant compression of adjacent lung tissue. (C) Adenoma; focal proliferation of alveolar epithelial cells with compression of adjacent lung tissue but no significant cellular atypia or focal alterations of cell morphology. (D) Adenocarcinoma; characterized by the presence of foci of larger, more basophilic cells (arrow) within what would otherwise be called an adenoma.

Studying the mechanism of tobacco smoke-associated lung tumorigenesis, Stinn et al. (2005) demonstrated that smoke exposure was associated with significant elevations in corticosterone. Corticosterone is a glucocorticoid and exerts an anti-inflammatory effect. Further evidence for the importance of inflammation, and specifically glucocorticoids, in lung tumorienesis was provided by Pashko and Schwartz (1996), who reported that adrenalectomized mice develop greater numbers of tumors.

The current study demonstrates persistent histological evidence of inflammation within the respiratory tract of exposed animals. However, inconsistent with the present results, Stinn et al. (2005) report that corticosterone levels rapidly return to control levels during the postexposure period. Therefore, with the anti-inflammatory influence of elevated corticosterone dissipating with exposure termination, a residual inflammatory stimulus may predominate in the postexposure period. A recovery period has been shown to be critical for the development of tumors in cigarette smoke-exposed A/J mice (Witschi et al., 1997; D'Agostini et al., 2001), whereas 9 mo of continuous cigarette smoke exposure has failed to produce increases in lung tumor incidence or multiplicity (Witschi, 2000; D'Agostini et al., 2001). Similarly, Finch et al. (1996), using a shortened recovery period, reported an inhibition of tumor formation in smoke-exposed A/J mice. Interestingly, administration of dexamethasone and myoinositol in the diet of smoke-exposed A/J mice after exposure termination prevented the development of tumors seen in animals maintained on a control diet following exposure (Witschi et al., 2000).

Our study is the first to report a thorough histopathological examination of the respiratory tract from smoke-exposed A/J mice. Results demonstrate a chronic inflammatory response that is not resolved after 7 mo of recovery. In addition, there was some indication that smoke exposure might influence the progression of hyperplastic lesions. However, greater numbers of animals need to be evaluated in order to properly determine the influence of smoke exposure on the progression of spontaneous hyperplastic lesions. Regardless, results further demonstrate the need for histological examination of the respiratory tract for accurate assessment of lung tumorigenicity in A/J mice.

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