Lung Tumorigenicity in A/J and rasH2 Transgenic Mice Following Mainstream Tobacco Smoke Inhalation

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Hypothesizing that their respective genetic backgrounds would confer an increased sensitivity to lung tumorigenesis, the plausibility of selected rodent models for the inhalation testing of mainstream tobacco smoke (MTS) was evaluated. Strain A/J and rasH2 transgenic (Tg) mice were exposed to MTS from Kentucky 1R4F research cigarettes using either a whole-body or nose-only exposure regimen. The whole-body regimen consisted of a 20-week exposure period [0.200 mg wet total particulate matter/liter (WTPM/l), 6 h/day, 5 days/week]; nose-only dosing proceeded for 28 weeks [0.040, 0.125, or 0.400 mg WTPM/l, 3 h/day, 5 days/week]. Both regimens included a 16-week recovery period. Gross and microscopic examinations of the lungs were used to evaluate tumor formation, with experimental results supporting the following conclusions:

1. Evaluation of MTS-induced tumorigenicity based on gross evaluation versus microscopic confirmation provides strikingly disparate results, indicating that serial sectioning is necessary for a definitive assessment of lung tumors.
2. While the dosing regimens employed do not allow for a definitive comparison, whole-body exposure appeared to be more effective for inducing statistical changes in tumor multiplicity and incidence compared to nose-only exposure.
3. Exposure-related stress, evidenced as reductions in both body weight gain and background tumor formation, represents a potential confounder during inhalation testing of MTS tumorigenicity, with additional investigation warranted to validate the specificity of exposure-related responses.
4. Comparative findings between A/J and rasH2 Tg mice suggest that the former may be overly sensitive to exposure-related stress, potentially influencing tumorigenic responses.

Key Words: mainstream tobacco smoke; mouse lung tumorigenicity; strain A/J; rasH2 transgenic.

Subchronic exposure of A/J mice to a mixture of 89% sidestream/11% mainstream tobacco smoke has been reported to yield statistically significant increases in lung tumor formation compared to air-exposed controls (Witschi et al., 1997a,b, 1998, 1999, 2000). Collectively, mice were exposed whole-body to inhalation chamber concentrations ranging from 78.5 to 137 mg total suspended particulates (TSP)/m3 (unfiltered smoke, 6 h/day, 5 days/week), with ad libitum access to food and water maintained during exposures. Demonstration of increased tumor formation required that the standardized 20-week exposure period be followed by a 16-week recovery period, during which mice were provided filtered air. Bogen and Witschi (2002) provided justification for the recovery period; more specifically, it was suggested that tobacco smoke exposure suppresses the growth of premalignant foci, and that smoke-induced lung tumor risk occurs predominantly via a genotoxic mechanism. Consequently, the recovery period allows tobacco smoke–induced genetic damage to progress to tumors.

Employing a similar mixture of sidestream and mainstream smoke, D’Agostini et al. (2001a) confirmed that A/J mice were capable of exhibiting smoke-induced increases in lung tumor formation; however, subsequent experiments using the original testing protocol failed to replicate these findings (De Flora et al., 2003a). Interestingly, the latter study also employed a transgenic mouse (UL53-3×A/J) possessing a dominant-negative p53 mutation, purportedly to examine the impact of apoptotic signaling during tobacco smoke tumorigenesis. While sensitivity of the mutant mice to tobacco smoke–induced lung tumorigenicity was not striking, it was significantly enhanced relative to (wild-type) A/J mice, providing statistically significant increases in lung tumor multiplicity and incidence according to both the standardized 20-week exposure/18-week recovery protocol and in response to continuous 38-week inhalation exposure.

Impacting the ability of A/J mice to exhibit statistically significant increases in tobacco smoke–induced lung tumorigenesis is an inherently high level of spontaneous tumor formation. Recent studies by Witschi et al. (2002) examined the plausibility of additional rodent models for assessing tobacco smoke–induced lung tumor formation. While Balb/c mice exhibited only modest increases in tumor multiplicity and incidence in response to a mixture of sidestream and mainstream tobacco smoke, Swiss albino mice demonstrated approximately 9- and 5-fold increases for these indices, respectively. The Swiss mouse model benefits from a low incidence of spontaneous lung tumors and a higher tolerance for smoke-induced toxicity, as

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reflected by minimal body weight losses during the exposure period. Supporting the contention that these mice are similar to A/J mice in terms of sensitivity to tobacco smoke, De Flora et al. (2003b) reported that tumor multiplicity and incidence were increased more than 4-fold in smoke-exposed Swiss mice compared to air-exposed controls.

Demonstration of tobacco smoke–induced lung tumor formation using Swiss mice is encouraging when considering that interpretation of the A/J mouse data may be complicated by significant reductions in body weight (and subsequent gains during recovery) for smoke-exposed mice compared to sham-exposed controls and assessments of lung tumorigenicity based on gross examination (D’Agostini et al., 2001a; De Flora et al., 2003a; Witschi et al., 1997a,b, 1998, 1999, 2000). Significant modulation of body weight during both the exposure and recovery periods suggests a potential for altered animal homeostasis associated with test article dosing (reviewed by Hart et al., 1999) and the possibility that dissimilar promotion stimuli (i.e., associated with weight gain) were provided to air- and smoke-exposed mice during recovery. Regarding gross determination of tumors, sole reliance on this approach specific to tobacco smoke–induced lung tumor formation has yet to be appropriately validated.

Finally, concerning the genetic backgrounds of rodent models selected for the current study, the increased sensitivity exhibited by A/J mice to lung carcinogens has been linked to genes desigselected for the current study, the increased sensitivity exhibited formation has yet to be appropriately validated.

within its own promoter region (Saitoh et al., 1990). Proposed as a model for rapid carcinogenicity testing, the rasH2 Tg mouse possesses an increased capacity for detecting various types of mutagenic and nonmutagenic carcinogens, with no significant tumor induction observed with either mutagenic or nonmutagenic noncarcinogens (Yamamoto et al., 1998). Incidences of spontaneous tumors are generally low in the rasH2 Tg mouse during 6-month carcinogenicity studies, with reasonable dose-response relationships observed for carcinogen exposures (Mitsumori et al., 1998).

**MATERIALS AND METHODS**

**Animals.** Male strain A/J mice (ages 6–8 weeks) were purchased from Jackson Laboratories (Bar Harbor, ME) in sufficient quantities for six experimental groups (44 mice/group), a sentinel group (60 mice), and prestudy health screening. Male transgenic (hemizygous) and wild-type rasH2 mice (from the same founder population, ages 6–10 weeks) were acquired from Taconic (Germantown, NY), with the six experimental groups (44 mice/group) comprised of transgenic mice; wild-type mice were used for sentinels and health screening. Within 48 h of delivery, sera from health screen animals were processed for routine measurement of antibodies to disease; serology was negative for the battery of assays employed. Also, lungs from health screen animals were examined microscopically for evidence of infectious disease, with no adverse findings reported.

Mice were housed and cared for in accordance with the Institute of Laboratory Animal Resources (ILAR), Commission of Life Sciences, National Research Council document entitled, Guide for the Care and Use of Laboratory Animals (1996). After completion of the quarantine period, mice were housed in a vivarium with controlled lighting (12 h of darkness, from 6:00 P.M. EST ± 30 min), temperature (71.2 ± 0.7°F), relative humidity (55 ± 4.5% RH), and air flow (greater than 120 partial air changes/h). Mice were individually housed in stainless steel, wire-bottomed cages (3x × 9 × 5 in.) suspended on stainless steel racks.

A/J and rasH2 mice were provided ad libitum access to AIN-76A Diet (Dyets, Bethlehem, PA) and NIH 31 M Rodent Diet (Taconic), respectively. Use of the AIN-76A diet was consistent with published reports on tobacco smoke–induced lung tumorigenicity in A/J mice (Witschi et al., 1997b, 1999, 2000), while the NIH 31 M diet was employed for rasH2 Tg mice based on animal vendor recommendations and the desire to maintain conditions similar to those used during colony generation. Water was provided ad libitum through an automatic system, with water originating from the municipal supply and subsequently filtered through activated carbon and 5-μm particulate filters.

**Experimental design.** Mice were assigned to experimental and control groups using PATHFOX software (version 4.2.2; Xybion Medical Systems, Cedar Knolls, NJ). Group mean body weights were compared by ANOVA and least significant difference criteria and demonstrated not to be significantly different at a 5% two-sided risk level. Mice assigned to the whole-body MTS exposure regimen (6 h/day, 5 days/week, for 28 weeks) received 0.200 ± 0.002 mg wet total particulate matter/liter (WTPM/l); mice assigned to the nose-only exposure regimen (3 h/day, 5 days/week, for 28 weeks) received 0.040 ± 0.001, 0.125 ± 0.003, or 0.399 ± 0.012 mg WTPM/l. Sham-exposed mice received filtered and humidified air. Nose-only exposure proceeded using conventional mouse exposure tubes and whole-body exposure was accomplished using oversized (rat, nose-only) tubes as described by Dorman et al. (1996). Animal feed and water were not present during animal dosing. Each exposure regimen was followed by a 16-week recovery period, with necropsies scheduled to coincide with completion of the exposure (n = 11–15 mice) and recovery (n = 25–30 mice) phases.

Individual body weights were collected prior to the first sham or smoke exposure of the corresponding week. Each mouse was weighed according to a biweekly schedule (i.e., odd- and even-numbered animals weighed on alternating weeks) to accommodate pre-exposure weighing and the whole-body dosing regimen (6 h/day) selected for investigation.

**Exposure system.** Mainstream tobacco smoke was generated using 1R4F research cigarettes purchased from the University of Kentucky and smoke machines initially described by Baumgartner and Coggins (1980). Smoke machines were operated under standard Federal Trade Commission conditions, resulting in the generation of a 35-ml puff, 2 s in duration, taken once per minute. Several modifications have been incorporated into the basic design of the smoking machine (Ayres et al., 1990), allowing for computer-controlled loading and unloading and puff counting for cigarettes.

Use of an exposure controller system allowed the smoke concentrations delivered to the mice to be maintained at the target concentration for each exposure. Smoke-exposure concentrations were confirmed (prior to animal presentation) via analysis of inhalation chamber breathing zones. Dilution air was passed through a Del-Monox compressed air purification system (Delttech, Ocota, FL) and humidified. Exposure atmospheres possessed a relative humidity of 45 ± 5.2% and temperature of 73.4 ± 1.0°F.

Separate chambers were used for sham (filtered and humidified air) and MTS exposures, with each exposure device consisting of four 24-port circular tiers. Total flow rates for the supply of incoming air and diluted smoke streams were adjusted to ensure adequate exposure deliveries of ~500 ml/min/port.

**Evaluation of lung tumors.** Individual lung lobes from all experimental and control animals were examined grossly by a board-certified veterinary pathologist at the time of necropsy. After gross examination, lungs were fixed...
in 10% neutral-buffered formalin (using gravity filling, ~25 cm water pressure) for subsequent histopathology analysis. Lung lobes were then separated from the respiratory tree and oriented within a single paraffin block to achieve a longitudinal cut (across the lobes). Paraffin-embedded lungs were serially sectioned at 400 μm, and a veterinary pathologist performed the microscopic evaluation of tumors. Maps of individual sections were prepared to document microscopic evaluation of each lobe, thereby minimizing the possibility that larger tumors would be counted more than once. A review of tumors sizes (i.e., based on greatest linear dimension) from subgroups representative of both sham- and MTS-exposed A/J and rasH2 Tg mice (i.e., ~15% of all tumors generated) indicated that less than 5% of microscopically confirmed tumors were smaller than 400 μm in diameter.

Statistical analysis. Statistical evaluations of body weight data were made using tests provided by the PATH/TOX software, i.e., one-way analysis of variance (ANOVA) followed by Bartlett’s test for homogeneity of variance and the Dunnett’s test. Tumor multiplicity and incidence data were evaluated using the unpaired t-test and Fisher’s exact test, respectively, with statistical significance assigned according to a two-sided p value < 0.05.

RESULTS

Body Weight Changes Induced by MTS Exposures

Experimental and control animals were weighed prior to the initial exposure of the corresponding week, according to an alternating schedule; for example, all odd-numbered mice were weighed prior to the first exposure during each odd-numbered week. Group mean body weight data for whole-body-exposed A/J and rasH2 Tg mice are presented in Figure 1 and data for nose-only-exposed mice are presented in Figure 2.

Whole-body exposure of A/J mice to MTS (0.200 mg WTPM/l, 6 h/day, 5 days/week) resulted in body weight reductions of about 5–10% compared to air-exposed controls, with MTS-exposed mice maintaining modest weight gains during the 20-week exposure period. For the majority of the exposure phase, group mean body weights for MTS-exposed mice were statistically lower than those of sham controls. Moreover, MTS-exposed mice demonstrated ~2-fold increases in weight gain compared to air-exposed controls during the ensuing 16-week recovery period, with most of the increase occurring within the initial 4–5 weeks.

For rasH2 Tg mice exposed whole-body to MTS (0.200 mg WTPM/l, 6 h/day, 5 days/week), weight reductions (compared to air-exposed controls) were more modest (i.e., <5%) and occurred during the initial 6 weeks of exposure. Statistically significant differences observed beyond this initial exposure period were spurious and most likely a function of the alternate animal weighing schedule. During the 16-week recovery period, weight profiles for the sham- and MTS-exposed mice were similar.

Nose-only exposure of A/J mice to MTS (0.200 mg WTPM/l, 6 h/day, 5 days/week) resulted in body weight reductions of about 5–15% compared to air-exposed controls. Reductions appeared to be dose-dependent, with the 0.040–mg WTPM/l exposure resulting in minimal suppression (compared to sham) and exhibiting statistically significant differences in a spurious manner, likely associated with the alternate weighing schedule. Mice provided the intermediate dose of 0.125 mg WTPM/l continued to gain weight during MTS exposure, but at a level that was statistically reduced compared to sham-exposed controls for the duration of the exposure period. Finally, the 0.400–mg WTPM/l exposure completely suppressed body weight gain during MTS dosing. During the 16-week recovery period, MTS-exposed mice exhibited disproportionate, exposure-related increases in body weight gain. Mice exposed nose-only to 0.040, 0.125, or 0.400 mg WTPM/l MTS demonstrated weight gains of about 11, 16, or 29%, respectively, while air-exposed controls gained ~12% body weight.

Similar to the results from the whole-body dosing regimen, nose-only exposure of rasH2 Tg mice had less of an effect on body weight compared to A/J mice. Generally, reductions failed to exhibit a dose-related effect, with body weights for sham- and MTS-exposed mice remaining fairly stable during dosing. While statistically significant differences were observed for MTS-exposed groups compared to sham-exposed controls, reductions were somewhat spurious and possibly related to the alternate
weighing schedule. After completion of the exposure period, both sham- and MTS-exposed mice gained weight in a similar manner. Hence, for nose-only-exposed rasH2 Tg mice there were no apparent exposure-related decreases or (postexposure) increases in body weight gain.

Grossly Observed Lung Tumors

Gross evaluation of lung tumors was conducted for all mice surviving until scheduled necropsy. The impact of excluding early-death animals was deemed minimal, considering that the overall survival indices for the A/J and rasH2 Tg mice were 93.6 and 90.9%, respectively.

Whole-body exposure of A/J mice to MTS (0.200 mg WTPM/l, 6 h/day, 5 days/week) resulted in ~50% reductions of both tumor multiplicity (tumors/animal) and incidence (tumor-bearing animals, TBA) compared to background levels evident in air-exposed controls (Table 1). Multiplicity and incidence were reduced from 0.50 ± 0.67 to 0.23 ± 0.44 tumors/animal and from 42 to 23% TBA. After completion of the recovery phase, both tumor multiplicity and incidence for MTS-exposed animals were elevated compared to sham-exposed controls. Multiplicity increased from 0.35 ± 0.56 to 0.62 ± 0.9 tumors/animal, and incidence increased from 31 to 38% TBA; these differences were not statistically significant.

In contrast, whole-body exposure of rasH2 Tg mice did not yield reductions in either tumor multiplicity or incidence (Table 1). Multiplicity for MTS-exposed mice was increased (relative to shams) from 0.23 ± 0.44 to 0.36 ± 0.63 tumors/animal, and incidence was increased from 23 to 29% TBA; neither of these changes was statistically significant. As a consequence of the 16-week recovery period, multiplicity and incidence for MTS-exposed mice were elevated relative to air-exposed controls. Multiplicity was statistically increased ($p < 0.05$) from 0.36 ± 0.49 to 0.79 ± 0.9 tumors/animal, and incidence increased from 36 to 59% TBA.

A trend similar to that observed for whole-body MTS exposure of A/J mice was evident with nose-only dosing (Table 2). Specifically, tumor multiplicity and incidence for MTS-exposed A/J mice were reduced ~40–70% compared to air-exposed controls during the 28-week exposure period; none of the reductions were statistically significant. Multiplicity and incidence demonstrated nonstatistical yet dose-related increases as a consequence of the ensuing 16-week recovery period. Groups previously exposed to MTS at concentrations of 0.040, 0.125, or 0.400 mg WTPM/l yielded $0.34 \pm 0.55$, $0.62 \pm 0.82$, or $0.69 \pm 0.66$ tumors/animal and 31, 45, or 59% TBA, respectively, compared to $0.36 \pm 0.56$ tumors/animal and 32% TBA for air-exposed controls.

Nose-only MTS exposure of rasH2 Tg mice yielded minimal changes for tumor multiplicity and incidence compared to air-exposed controls (Table 2), with only the 0.125–mg WTPM/l, MTS-exposed animals exhibiting reductions. After completion of the 16-week recovery period, groups previously exposed to MTS at concentrations of 0.040, 0.125, or 0.400 mg WTPM/l yielded $0.36 \pm 0.56$, $0.41 \pm 0.78$ tumors/animal and 42, 32, or 28% TBA, respectively, compared to $0.36 \pm 0.86$ tumors/animal and 24% TBA for air-exposed controls.

After gross examination at necropsy, lungs from representative subgroups of A/J and rasH2 Tg mice (i.e., 24 animals/strain, subjected to either sham or MTS exposure) were reevaluated postfixation, and then microscopically to assess the need to proceed with serial sectioning of all experimental animals (data not shown). Consequently, it was determined that gross observation of fixed tissues provided tumor counts that were not appreciably different from necropsy evaluations; but, appreciably different results were obtained during microscopic evaluation. This was especially true for mice that failed to demonstrate grossly discernible tumors at necropsy, whereby no additional tumors were observed with fixed tissues, while 13 previously undetected tumors ($n = 24$ animals at risk) were confirmed microscopically.

![FIG. 2. Group mean body weight data for A/J (top panel) and rasH2 Tg (bottom panel) mice during nose-only exposure to humidified and filtered air (boxes), 0.040 (triangles), 0.125 (diamonds), or 0.400 (circles) mg WTPM/l MTS. Group mean body weights are provided for both the 28-week exposure and 16 week-recovery periods, with statistically significant reductions in MTS-exposed mice compared to sham-exposed controls noted (solid triangles, 0.040; solid diamonds, 0.125; and solid circles, 0.400 mg WTPM/l; $p = 0.05$).](image-url)
Microscopically Confirmed Lung Tumors (Lung Lobes Serially Sectioned at 400 μm)

As was the case for gross determinations, microscopic evaluation of lung tumors was conducted for all mice surviving until scheduled necropsy. The exclusion of early-death animals ensured valid comparisons of MTS-induced lung tumor formation across different dosing concentrations, with the impact of these exclusions deemed minimal based on the overall survival indices for the A/J and rasH2 Tg mice. With regard to the histology of lung tumors generated during this study, A/J mice developed a total of 161 microscopically confirmed lung tumors.

### TABLE 1
Tumor Multiplicity and Incidence in Response to Whole-Body Mainstream Tobacco Smoke Exposure

<table>
<thead>
<tr>
<th>Gross examination treatment group</th>
<th>Exposure phase</th>
<th>Recovery phase (postexposure)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Tumor multiplicity</td>
<td>Tumor incidence</td>
</tr>
<tr>
<td>A/J, sham exposure</td>
<td>0.50 ± 0.67</td>
<td>5/12 (42%)</td>
</tr>
<tr>
<td>A/J, 0.200 mg WTPM/l</td>
<td>0.23 ± 0.44</td>
<td>3/13 (23%)</td>
</tr>
<tr>
<td>rasH2 Tg, sham exposure</td>
<td>0.23 ± 0.44</td>
<td>3/13 (23%)</td>
</tr>
<tr>
<td>rasH2 Tg, 0.200 mg WTPM/l</td>
<td>0.36 ± 0.63</td>
<td>4/14 (29%)</td>
</tr>
</tbody>
</table>

Note. Tumor multiplicity is given ± SD; mg, milligram(s); WTPM/l, wet total particulate matter per liter.

*Statistically significant difference compared to sham-exposed control (two-sided p value < 0.05).

<table>
<thead>
<tr>
<th>Microscopic examination treatment group</th>
<th>Exposure phase</th>
<th>Recovery phase (postexposure)</th>
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<tbody>
<tr>
<td></td>
<td>Tumor multiplicity</td>
<td>Tumor incidence</td>
</tr>
<tr>
<td>A/J, sham exposure</td>
<td>0.42 ± 0.51</td>
<td>5/12 (42%)</td>
</tr>
<tr>
<td>A/J, 0.200 mg WTPM/l</td>
<td>0.23 ± 0.44</td>
<td>3/13 (23%)</td>
</tr>
<tr>
<td>rasH2 Tg, sham exposure</td>
<td>0.23 ± 0.44</td>
<td>3/13 (23%)</td>
</tr>
<tr>
<td>rasH2 Tg, 0.200 mg WTPM/l</td>
<td>0.71 ± 0.83</td>
<td>8/14 (57%)</td>
</tr>
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### TABLE 2
Tumor Multiplicity and Incidence in Response to Nose-Only Mainstream Tobacco Smoke Exposure

<table>
<thead>
<tr>
<th>Gross examination treatment group</th>
<th>Exposure phase</th>
<th>Recovery phase (postexposure)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tumor multiplicity</td>
<td>Tumor incidence</td>
</tr>
<tr>
<td>A/J, sham exposure</td>
<td>0.25 ± 0.45</td>
<td>3/12 (25%)</td>
</tr>
<tr>
<td>A/J, 0.040 mg WTPM/l</td>
<td>0.14 ± 0.36</td>
<td>2/14 (14%)</td>
</tr>
<tr>
<td>A/J, 0.125 mg WTPM/l</td>
<td>0.08 ± 0.28</td>
<td>1/13 (8%)</td>
</tr>
<tr>
<td>A/J, 0.400 mg WTPM/l</td>
<td>0.15 ± 0.38</td>
<td>2/13 (15%)</td>
</tr>
<tr>
<td>rasH2 Tg, sham exposure</td>
<td>0.23 ± 0.83</td>
<td>1/13 (8%)</td>
</tr>
<tr>
<td>rasH2 Tg, 0.040 mg WTPM/l</td>
<td>0.27 ± 0.65</td>
<td>2/11 (18%)</td>
</tr>
<tr>
<td>rasH2 Tg, 0.125 mg WTPM/l</td>
<td>0.07 ± 0.27</td>
<td>1/14 (7%)</td>
</tr>
<tr>
<td>rasH2 Tg, 0.400 mg WTPM/l</td>
<td>0.31 ± 0.63</td>
<td>3/13 (23%)</td>
</tr>
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</table>

Microscopically confirmed lung tumors (lung lobes serially sectioned at 400 μm)
(109 bronchioalveolar adenomas and 52 carcinomas); approximately 55 and 72% of tumors present within sham- and MTS-exposed mice were adenomas, respectively. For rasH2 Tg mice, a total of 179 lung tumors were microscopically confirmed (133 bronchioalveolar adenomas and 46 carcinomas); ~74% of tumors present within both sham- and MTS-exposed mice were adenomas.

Microscopic examination of lungs collected from whole-body, MTS-exposed A/J mice confirmed the ~50% reductions in both tumor multiplicity and incidence (compared to background levels of shams) demonstrated by gross evaluation (Table 1). Specifically, multiplicity and incidence were reduced from 0.42 ± 0.51 to 0.23 ± 0.44 tumors/animal and from 42 to 23% TBA. After completion of the recovery phase, both tumor multiplicity and incidence for MTS-exposed animals were statistically elevated ($p < 0.05$) compared to sham-exposed controls. Multiplicity increased from 0.46 ± 0.58 to 1.17 ± 0.85 tumors/animal and incidence increased from 42 to 79% TBA. In contrast to gross determinations, microscopic examinations provided statistically significant increases for both indices after completion of the recovery period.

Contrasting the A/J mouse data, whole-body exposure of rasH2 Tg mice did not yield reductions in tumor multiplicity or incidence (Table 1). Instead, tumor multiplicity ($p = 0.07$) and incidence ($p = 0.12$) were increased from 0.23 ± 0.44 to 0.71 ± 0.83 tumors/animal and from 23 to 57% TBA, respectively, compared to air-exposed controls. Hence, microscopic examination of the lungs from rasH2 Tg mice following whole-body MTS exposure revealed increases in tumor multiplicity and incidence not readily apparent with gross examination. Moreover, microscopic examination of recovery tissues yielded statistically significant increases ($p < 0.05$) in multiplicity and incidence for MTS-exposed animals relative to air-exposed controls. Multiplicity was increased from 0.60 ± 0.76 to 1.38 ± 0.94 tumors/animal, and incidence increased from 44 to 83% TBA. Gross evaluation of the recovery tissues had resulted in a statistical change for multiplicity alone.

Tumor multiplicity and incidence for nose-only, MTS-exposed A/J mice were reduced as much as 57% compared to air-exposed controls during the 28-week exposure phase. Generally, reductions were less than indicated by gross evaluation, despite higher background levels (sham controls) for both indices. Multiplicity and incidence demonstrated nonstatistical yet dose-related increases during the ensuing 16-week recovery period. Although similar dose-related trends were observed for A/J mice after recovery, results from gross and microscopic evaluations were considerably different, with the latter identifying greater numbers of tumors (and TBA) in both sham- and MTS-exposed groups.

Nose-only MTS exposure of rasH2 Tg mice yielded nonstatistical reductions in tumor multiplicity and incidence compared to air-exposed controls; these reductions were not as significant as those exhibited for similarly exposed A/J mice. After completion of the 16-week recovery period, nonstatistical (dose-related) increases in both multiplicity and incidence were observed for MTS-exposed mice; this was in contrast to gross determinations that indicated a greater response following 0.040 mg WTPM/l exposure. Although no statistical significance was associated with the MTS-induced increases in tumor incidence and multiplicity, both A/J-strain and rasH2 Tg mice exhibited dose-related effects in response to increasing concentrations of MTS provided by nose-only exposure.

**DISCUSSION**

This report details efforts to evaluate the suitability of strain A/J and rasH2 Tg mice for subchronic inhalation testing of MTS tumorigenicity, with the hypothesis being that the respective genetic backgrounds would confer an increased sensitivity to tobacco smoke. Experimental results from studies employing both gross and microscopic examinations of lung tumors generated in response to either whole-body or nose-only MTS exposure support the following conclusions:

1. Strikingly disparate results provided by gross evaluation versus microscopic confirmation of lung tumors support the contention that serial sectioning is necessary to effectively evaluate MTS tumorigenicity.

2. While the exposure regimens employed did not allow for definitive comparison, whole-body exposure to MTS appeared to be more effective for inducing statistically significant changes in tumor multiplicity and incidence compared to nose-only exposure.

3. Exposure-induced stress, evidenced as reductions in body weight gain and background tumor formation, represents a potential confounder during inhalation testing of tobacco smoke tumorigenicity; moreover, dissimilar body weight gains for sham- versus MTS-exposed mice may provide a disproportionate promotion stimulus during recovery.

4. Comparative findings for A/J and rasH2 Tg mice suggest that the former may be overly sensitive to experimentally induced stress, potentially influencing the interpretation of tumorigenic responses.

Previous reports demonstrating A/J mouse sensitivity to tobacco smoke–induced lung tumorigenicity (D’Agostini et al., 2001a; De Flora et al., 2003a; Witschi et al., 1997a,b, 1998, 1999, 2000) relied solely on gross evaluation of tumors. Interestingly, none of these studies directly examined the effectiveness of gross versus microscopic examination, but instead relied on published reports employing test articles unrelated to tobacco smoke (Shimkin and Stoner, 1975; Witschi, 1981) or rodent models different from the A/J mouse (Balansky, 1995). Results from our experiments, with few exceptions, demonstrate that gross determination of lung tumors has the potential to significantly underestimate actual tumor burden as revealed through microscopic examination. In fact, nearly all instances of increased lung tumor formation by MTS exposure were
observed as a consequence of serially sectioning the lungs and evaluating masses microscopically. Intuitively, inhalation exposure to the particulate and vapor phase constituents present in tobacco smoke would impact the entire lung and not just the area beneath the pleura. Though more costly and time-consuming, definitive assessment of lung tumor formation would appear to require the stringent approach of microscopic examination.

Statistically significant increases in tumor multiplicity and incidence were observed for A/J and rasH2 Tg mice after recovery from 20-week, whole-body MTS exposure. Moreover, rasH2 Tg mice exhibited near statistical increases in multiplicity ($p = 0.07$) and incidence ($p = 0.12$) after completion of the exposure period. In contrast, no statistically significant increases in tumor multiplicity or incidence were observed in response to 28 weeks of nose-only exposure or after completion of the corresponding recovery period. While the exposure-related trends for nose-only MTS dosing are encouraging, a reasonable conclusion from our experiments would be that whole-body exposure may present a more effective approach for inducing statistically significant changes in lung tumor multiplicity and incidence. It should be noted that nose-only exposure possesses several advantages over whole-body exposure during tobacco smoke inhalation studies, including a more accurate and precise delivery of smoke and the minimization of a secondary route of exposure from a preening dose.

It is noteworthy that recent studies employing MTS, in contrast to a mixture of sidestream and mainstream smoke, have been unsuccessful in demonstrating increased tumor formation in A/J mice. Finch et al. (1996) exposed A/J mice to 248 mg TSP/m$^3$ (0.248 mg WTPM/l) MTS for 6 months (6 h/day, 5 days/week). After 5 weeks of recovery, MTS-exposed animals were devoid of grossly observable lung tumors, while air-exposed shams possessed 0.3 tumors/animal with an incidence of 26% TBA. Also, D’Agostini et al. (2001a) used a 20-week exposure and 16-week recovery regimen with A/J mice exposed to 1300 mg TSP/m$^3$ MTS (1 h/day, 5 days/week); neither tumor multiplicity nor incidence were increased relative to sham after completion of the recovery period. Hence, results from this study, demonstrating statistical increases for whole-body MTS exposure and dose-related increases for nose-only MTS exposure, represent unique findings.

Results from published studies likewise demonstrate that tobacco smoke exposure possesses the potential for inhibiting background tumor formation (Finch et al., 1996) and reducing urethane- and methylcholanthrene-induced tumorigenicity in A/J mice (Witschi et al., 1997b). Moreover, the pervasive body weight losses exhibited by A/J mice during tobacco smoke exposure (D’Agostini et al., 2001a; De Flora et al., 2003a; Witschi et al., 1997a,b, 1998, 1999, 2000) are indicative of impaired food intake and/or systemic toxicity (De Flora et al., 2003b). A significant amount of experimental evidence supports an inhibitory effect for reduced caloric intake on preneoplastic progression (reviewed by Hart et al., 1999). For example, Hikita et al. (1999) reported that short-term fasting of rats, previously initiated with diethylnitrosamine and promoted with phenobarbital, resulted in the loss of virtually all measurable altered hepatic foci (AHF). Loss of AHF was accompanied by a marked decrease in bromodeoxyuridine labeling of hepatocytes, concomitant with a significant increase in apoptosis. Upon refeeding, AHF growth was significantly accelerated, with the numbers and volume percentage of altered foci comparable to nonfasted controls within 2 weeks; AHF proliferative index was 10-fold higher than observed for surrounding, nonfocal hepatocytes. Administration of phenobarbital during the fasting period did not alter these results, although AHF reappeared more rapidly with promoter treatment. These results were consistent with longer-term studies employing both food withdrawal and caloric restriction (Grasl-Kraupp et al., 1994).

Regarding a possible explanation for these changes, Dunn et al. (1997) reported on the effects of feeding ad libitum, dietary restriction (DR), and DR concomitant with IGF-I supplementation on carcinogen-induced preneoplasia in mice. IGF-I is lowered during DR in humans and rodents and is a known modulator of cell proliferation, apoptosis, and tumorigenesis. Results from these studies demonstrated that serum IGF-I was reduced 24% by DR but was completely restored in the IGF-I/DR–treated mice, that tumor development was decreased in DR animals, and that restoration of IGF-I serum levels in DR-treated mice increased the stage of observed tumors. Consistent with these published reports, A/J mice subjected to 40% DR for 20 weeks (5 days/week) in the absence of MTS dosing exhibited an 80% reduction in lung tumor multiplicity compared to ad libitum controls (data not shown). Multiplicity was increased nearly 4-fold for DR animals fed ad libitum during the 16-week recovery period; control animals failed to exhibit any meaningful change. That no significant body weight reduction was observed for DR mice compared to controls effectively demonstrates the consequences of experimentally induced stress on lung tumor formation within the A/J mouse.

Our study attempted to account for potential confounders, in part by identifying exposure concentrations that would have minimal impact on animal weight gains yet retain the potential for tumorigenesis (data not shown) and through the use of whole-body exposure tubes (Dorman et al., 1996), which allowed for individual exposure of animals without tainting food or water. Body weight losses associated with whole-body exposure to MTS were notably reduced (i.e., ≤10% compared to air-exposed mice) compared to published reports demonstrating ~20–25% reductions after exposure to 89% sidestream/11% mainstream tobacco smoke (D’Agostini et al., 2001a; Witschi et al., 1997a,b, 1998, 1999, 2000). This is especially pertinent when considering that the tobacco smoke concentration employed was nearly 50% higher than that previously used to provide increased lung tumor formation in A/J mice (Bogen and Witschi, 2002). A disproportionate body weight gain was still noted for A/J mice during the initial weeks of recovery. Moreover, nose-only exposure of A/J mice to three concentrations of
tobacco smoke resulted in a dose-dependent reduction of body weight gain (i.e., ~5–15%), with notably dissimilar increases in body weight gain during recovery.

Finally, comparative findings for A/J and rasH2 Tg mice suggest that the former may be overly sensitive to experimentally induced stress, with a potential for influencing tobacco smoke–induced tumorigenic responses. Whole-body exposure of A/J mice reduced background tumor multiplicity and incidence at a concentration that appeared to have no negative impact on rasH2 Tg mice. Tobacco smoke exposure possesses the potential to depress lung tumor formation, as evidenced by recent findings that MTS exposure of rats for either 18 or 100 consecutive days produced a significant and time-dependent increase in the proportion of apoptotic cells within the bronchial and bronchiolar epithelium (D’Agostini et al., 2001b). Apoptosis induction by tobacco smoke may be especially pertinent to the A/J mouse, given that genetic susceptibility of this model to lung tumor formation has been linked to the deletion of the EcoR1-generated, 0.55-kb K-ras fragment (Malkinson, 1992) and that preneoplastic cells are acutely more sensitive to apoptotic signaling than the surrounding non-neoplastic cells (Dunn et al., 1997). Ongoing studies are evaluating the effects of tobacco smoke–inhalation exposure on lung cell proliferation and apoptosis and the impact of exposure-related losses and gains in body weight during tumor formation in the A/J mouse model.

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REFERENCES


