Chronic Inhalation Exposure to Mainstream Cigarette Smoke Increases Lung and Nasal Tumor Incidence in Rats

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An animal model of lung carcinogenicity induced by chronic inhalation of mainstream cigarette smoke would be useful for research on carcinogenic mechanisms, smoke composition-response relationships, co-carcinogenicity, and chemoprevention. A study was conducted to determine if chronic whole-body exposures of rats would significantly increase lung tumor incidence. Male and female F344 rats (n = 81 to 178/gender) were exposed whole-body 6 h/day, 5 days/week for up to 30 months to smoke from 1R3 research cigarettes diluted to 100 (LS) or 250 (HS) mg total particulate matter/m3, or sham-exposed to clean air (C). Gross respiratory tract lesions and standard lung and nasal sections were evaluated by light microscopy. A slight reduction of survival suggested that the HS level was at the maximum tolerated dose as commonly defined. Cigarette smoke exposure significantly increased the incidences of non-neoplastic and neoplastic proliferative lung lesions in females, while nonsignificant increases were observed in males. The combined incidence of bronchioloalveolar adenomas and carcinomas in females were: HS = 14%; LS = 6%; and C = 0%. These incidences represented minima because only standard lung sections and gross lesions were evaluated. Mutations in codon 12 of the K-ras gene occurred in 4 of 23 (17%) tumors. Three mutations were G to A transitions and one was a G to T transversion. The incidence of neoplasia of the nasal cavity was significantly increased at the HS, but not the LS level in both males and females (HS = 6%, LS = 0.3%, C = 0.4% for combined genders). These results demonstrate that chronic whole-body exposure of rats to cigarette smoke can induce lung cancer.

Key Words: lung cancer; nasal cancer; tumors; neoplasia; cigarette smoke; inhalation; rats.

Despite the decline in smoking in the United States, tobacco-related disorders contribute substantially to the burden of respiratory and nonrespiratory illness and disease. It is estimated that 46.2 million adults in the United States smoke cigarettes and that smoking causes more than 440 thousand deaths in the United States each year (DHHS, 2004). Lung cancer is a particularly intractable disease, and smoking is thought to cause 85–90% of lung cancer deaths (DHHS, 1989; NRC, 1998; Peto et al., 1994). Respiratory tract cancers constituted 14% of new cancer cases in 2003, but they accounted for 29% of the 556,500 cancer deaths (Jemal et al., 2003), a mortality rate twice the average for all cancers. Smoking is also known to act synergistically with other environmental and workplace carcinogens to cause lung cancer (e.g., radon [NRC, 1998] and asbestos [Erren et al., 1999]).

The robust epidemiological evidence precludes a need to demonstrate the carcinogenicity of cigarette smoke in animals for the purpose of identifying a cancer hazard or estimating risk factors for humans. However, a positive chronic inhalation cancer bioassay paralleling those used for testing the carcinogenicity of other inhaled agents would be useful for research on the mechanisms of tobacco smoke-related carcinogenicity, interactions between smoking and other exposures, the effects of changing smoke composition, and chemoprevention. Cigarette smoke contains thousands of compounds, including many known carcinogens, tumor initiators, and tumor promoters (DHHS, 1989). There is interest in reducing the toxicity of cigarette smoke by altering its composition, to complement harm-reduction strategies such as smoking prevention and cessation programs (IOM, 2001; Shields, 2002). Studies of the potential effects of changing cigarette ingredients or other differences in smoke composition have involved chemical analyses and acute and subchronic biological assays (e.g., Ayres et al., 2001; Haussmann et al., 1998; Rustemeier et al., 2002). A reduction of cancer hazard could be tested with greater confidence using a chronic inhalation bioassay similar to the protocols commonly used to evaluate other inhaled materials. Chemoprevention for high-risk individuals, including prevention of recurrence after resection of an initial tumor, is another active area of investigation (e.g., Belinsky et al., 2003) that could benefit from a positive bioassay model.

The difficulty in reproducing in animals the obvious lung carcinogenicity of smoking in humans is a perplexing, long-standing toxicological dilemma. Coggins (1998, 2001, 2002) reviewed several chronic inhalation studies using rodents, dogs, and nonhuman primates, and concluded that no study...
has produced a statistically significant increase in lung tumors. Dalbey et al. (1980) reported a significant (p < 0.05) increase in respiratory tract (nasal plus lung) neoplasms among female F344 rats exposed nose-only seven times daily 5 days/week for 2.5 years compared to a combined group of untreated and sham-exposed controls, but did not report the significance of the lung tumor response separately. Lesser responses have resulted from other attempts. The experience with mainstream cigarette smoke is doubly troubling because it calls into question the adequacy of chronic inhalation bioassays to detect true carcinogenic hazards of chemically- and physically-complex mixtures and particularly the products of combustion or pyrolysis.

Increases in the incidence of lung adenomas have been produced in A/J, Balb/c, and SWR mice by a few months of exposure to simulated environmental tobacco smoke followed by a nonexposure period (D’Agostini et al., 2001; Witschi et al., 2002). However, adenomas were not increased in this assay among mice exposed to mainstream smoke either 6 h/day (Finch et al., 1996) or 1 h/day (D’Agostini et al., 2001). The variability of results and lack of increase in malignant tumors in this short-term assay raises questions about its utility for evaluating the mechanisms of carcinogenesis induced in humans by chronic smoke exposure.

There are several possible reasons for the failure to demonstrate the lung carcinogenicity of inhaled cigarette smoke in animals. Two key factors may have been small group sizes and a failure to dose the lungs of animals sufficiently. Most previous studies used the standardized puffing profile (2 s, 35 ml puff once/min) established by the Federal Trade Commission (32 CFR 11,187, 1967) for testing cigarette yield (NCI, 1996), which impeded the generation of large volumes of smoke. Attempting to mimic human smoking patterns using “puff-by-puff” exposures of rodents in nose-only exposure tubes limits the length and number of daily exposures and incurs stresses from frequent handling and confinement. Logistical constraints limit the practical sizes of groups exposed in tubes. Larger species present even greater challenges. The groups of dogs exposed via tracheostomy and nonhuman primates addicted to voluntary oral smoking have been small, and the exposures have not encompassed a majority of life span.

It is doubtful that previous animal studies reproduced the high doses achieved by human heavy smokers who puff in a manner that maximizes deposition. One problem is a failure to use exposures having sufficient concentration \( \times \) time integrals to mimic daily dosing of humans. For example, rats in the Dalbey et al. (1980) study were placed in tubes and exposed on seven separate occasions/day, 5 days/week to eight 30-s “puffs” of nominally 10% smoke, a total of only 2.3 h/week. Another problem is that rodents alter their breathing pattern during intermittent “puffs” in a manner that reduces exposure (Kendrick et al., 1976). Masse et al. (1985) discussed this “underdosing” problem and elected to use whole-body exposures of rats to achieve greater time-integrated daily exposures in their studies of carcinogenic interactions between cigarette smoke and radon. Their intermittent whole-body exposures for only 4 days/week for 1 year failed to increase lung tumors in a smoke-only group; however, the response to radon was increased by co-exposure to smoke (Monchaux et al., 1994). Our preliminary calculations (Finch et al., 1998) suggested that 6 h/day, 5 days/week chronic whole-body exposures could provide weekly deposited lung doses of smoke total particulate matter (TPM) simulating heavy human smoking using smoke concentrations compatible with long-term survival.

The present study was conducted to determine whether chronic whole-body exposure of F344 rats to mainstream cigarette smoke could produce significant, dose-related increases in the incidence of lung neoplasia. The goal was to establish an experimental model for research on mechanisms of smoke-induced lung cancer, carcinogenic interactions among combined exposures to multiple agents, and chemoprevention. The criteria for the model’s success were: (1) a statistically significant exposure concentration-related increase in the incidence of lung neoplasms, and (2) an increase in malignant, as well as benign, lung neoplasms. Pending an increase in lung tumors, a further goal was to determine whether the tumors develop in part through mutation of the K-ras gene, which occurs in 20–30% of adenocarcinomas of human smokers (Pulling et al., 2003). Carcinogenesis in nonrespiratory tissues, smoke dosimetry, and smoke composition-response relationships were not addressed in this study. Parallel studies using the same exposure atmosphere addressing effects of short-term exposure on lung (March et al., 1999) and nasal (Hotchkiss et al., 1995) cell proliferation and mucus production, effects of short-term exposure on lung tumors in A/J mice (Finch et al., 1996), effects of short-term exposure on lung and nasal cytochrome P450 induction (Wardlaw et al., 1998), and effects of chronic exposure on lung retention of radiotracer particles (Finch et al., 1998) were reported previously.

**MATERIALS AND METHODS**

**Animals and maintenance.** Male and female CD\(^\text{F}(344)/\text{CrlBR}\) rats (Charles River Laboratories, Raleigh, NC) were received at 28 ± 3 days of age and conditioned for 2 weeks in m\(^3\) whole-body inhalation exposure chambers (H2000, Lab Products, Maywood, NJ) ventilated with clean air. After conditioning, rats were randomly assigned by weight to experimental groups and identified by unique alphanumeric tail tattoos. The 753 rats included in the present analysis made up the three experimental groups listed in Table 1. The experimental group sizes were weighted differently in view of the expected background lung tumor incidence and to enhance detection of increases in the low-exposure group. For logistical reasons, rats in all treatment groups were entered into the study in three blocks over an 8-month period.

Rats were housed throughout the study in individual cages in exposure chambers ventilated at 12 ± 2 air changes per h, and maintained at 20 to 24°C and 40 to 70% relative humidity. Lights were on 12-h cycle (on during 0600–1800). Chambers and cages were washed weekly. Bacteriostatic liners (Shepherd Specialty Papers, Kalamazoo, MI) in excreta trays were changed twice daily, and trays were washed daily. Rats were observed visually twice daily, and moribund animals were euthanized and necropsied. Rats were weighed before exposure and monthly thereafter using a computerized data acquisition system (PathTox, Xybian, Cedar Knolls, NJ). Sera from rats selected throughout
glass bulbs, cryofocused using liquid nitrogen, and analyzed by GC/MS using
deuterated internal standards. Analytical accuracy was confirmed using NIST
and laboratory standards. Certain components were also analyzed in the low
exposure level as a check on scaling with TPM concentration.

**Necropsy and histology.** Moribund and terminal sacrifice rats were euthan-
ized by overdose of intraperitoneal sodium pentobarbital and exsanguination;
these and rats found dead underwent complete necropsy. The thoracic and abdom-
nal cavities and major organs (at minimum: adrenal glands, femur, heart, kid-
neys, larynx, liver, lungs and associated lymph nodes, head/face, spleen, thyroid
gland, trachea and urinary bladder) were examined, and gross lesions were
recorded. Major organs and the entire respiratory tract were fixed in 4% para-
formaldehyde in saline (PFA). Lungs were weighed, perfused intratracheally with
PFA, and submerged in PFA for fixation. Nasal cavities were flushed gently with
10% neutral buffered formalin (NBF) through the nasopharyngeal orifice and
submerged in NBF. The fixed left lung and right cranial, middle, and caudal lung
lobes were trimmed and embedded, and a single section of each was made along
the major axial airway. Gross lung lesions were also sectioned. Heads were
decalciﬁed in 13% formic acid and sectioned transversely at speciﬁc anatomic
locations to provide four sections of the nasal cavity (Young, 1981). Tissues were
embedded in parafﬁn, sectioned at 5 μm, and stained with hematoxylin-eosin.
Respiratory tissues were ﬁrst stained with alcin blue to enhance visibility of acid
mucosubstances, and then stained with hematoxylin-eosin.

**Histopathologic evaluation.** Lung and nasal cavity sections were ﬁrst
examined and classiﬁed by a single board-certiﬁed veterinary pathologist
(APG). Proliferative lesions were then reviewed by two additional board-
certiﬁed veterinary pathologists (THM and FFH), and consensus diagnoses
were developed. Lesion classiﬁcation followed the most recent guidelines pub-
lished in Guides for Toxicologic Pathology (Renne et al., 2003; Schwartz et al.,
1994). Gross lesions in other tissues were examined histologically to rule out
pulmonary metastases from nonpulmonary tumors and to identify metastases
from nasal or lung tumors. Evaluations of lung sections focused on proliferative
lesions. Evaluations of nasal sections included all neoplasms, which were clas-
siﬁed by site of origin (nasal cavity, oral cavity, and other [e.g., nasolacrimal duct,
or uncertain]) and tissue of origin (epithelial, mesenchymal, or uncertain).

**Analysis of mutations in codon 12 of K-ras gene.** Tumors of sufﬁcient size
were analyzed for the presence of a mutation in codon 12 of the K-ras gene in
addition to histopathology. DNA was obtained from adenomas and adenocarcin-
omas ≥4 mm by microdissection. DNA was isolated by digestion of the tissue
with pronase (1%) followed by phenol-chloroform extraction and ethanol pre-
cipitation. The DNA was analyzed for mutations in codon 12 of the K-ras gene
using BstN1 mutant allele enrichment. With this method, exon 1 of the K-ras gene
was amplified, and the resulting PCR product was digested with the BstN1
restriction enzyme (New England BioLabs) to generate fragments of 27 and
89 base pairs for wild-type codon 12 (Kahn et al., 1991). Point mutations at
either of the ﬁrst two positions of codon 12 block digestion by the restriction
enzyme and the intact DNA were then reampliﬁed for direct sequencing to
identify the speciﬁc mutation within codon 12.

**Statistical procedures.** Statistical signiﬁcance was tested and reported at
multiple levels; however, the minimum criterion for statistical signiﬁcance was
set at \( p = 0.05 \) for all comparisons.

**Similarity of entry blocks.** The consistency of lesion incidence rates across
the three entry blocks was evaluated using the Cochran-Mantel-Haenszel general
association statistic (Cochran, 1954; Mantel and Haenszel, 1959) to pool evid-
cence of systematic block-related differences across experimental groups
(C, LS, HS).

**Survival.** The product-limit method of Kaplan and Meier (1958) was used to
obtain survival estimates as a function of weeks on study. Cox’s proportional
hazards model (1972) was used to test equality of survival rates between cigarette
smoke-exposed groups and sham controls. Tarone’s (1975) extension of the
proportional-hazards method was used to evaluate exposure-related trends.
Reported \( p \) values are two-sided.

**Body and lung weights.** Individual body weights were averaged over 6
month intervals and analyzed using one-way analysis of variance, followed

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### TABLE 1 Experimental Group Sizes

<table>
<thead>
<tr>
<th>Group</th>
<th>Gender</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>High smoke (HS)</td>
<td>Male</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>163</td>
</tr>
<tr>
<td>Low smoke (LS)</td>
<td>Male</td>
<td>178</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>175</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>353</td>
</tr>
<tr>
<td>Control (C)</td>
<td>Male</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>237</td>
</tr>
</tbody>
</table>

the study were determined to be negative for antibodies against common rodent
pathogens (Standard Level II Antibody Proﬁle, Microbiological Associates,
Rockville, MD). Tap water was available via automatic valves ad libitum,
and pelleted ration (Certified Wayne Lab Blox, Allied Mills, Chicago, IL)
was available outside exposure hours. The laboratory is fully accredited by
the Association for Assessment and Accreditation of Laboratory Animal Care
and the protocol was approved by the Institute’s Animal Care and Use
Committee.

**Cigarette smoke exposures.** Rats were exposed (whole-body) 6 h/day,
5 days/week to either mainstream cigarette smoke diluted to 250 (high smoke,
“HS”) or 100 (low smoke, “LS”) mg TPM/m³ as described previously (Chen
et al., 1992), or to HEPA-ﬁltered air as controls (C). Rats were exposed at half the
target concentrations for the ﬁrst week for acclimation. After 6 weeks of smoke
exposure, all groups were exposed to only HEPA-ﬁltered air for 1 week to parallel
the brief suspension of exposure accompanying groups not included in this report
(Finch et al., 1998). Smoke exposures then resumed and continued for 30 months.

Smoke was generated using a modiﬁed commercial machine (Type 1300,
AMESA, Geneva, SW) from high-yield (nominally 27.1 mg TPM) unﬁltered
1R3 research cigarettes (Tobacco Health Research Institute, Lexington, KY).
Cigarettes were stored in a freezer and conditioned overnight before use at 24ºC
and 50 to 70% relative humidity. Cigarettes were puffed twice per min using
a 70 ml, 2 s puff generated by a peristaltic pump, and smoke was diluted with
HEPA-ﬁltered air.

Exposure TPM concentrations were determined gravimetrically from 2-h
ﬁlter samples taken three times daily from the mid-point of each chamber
using 25 mm glass ﬁber ﬁlters (Type A/E, Gelman, Ann Arbor, MI) weighed
on an electronic microbalance (Model C-31, Cahn, Cerritos, CA). Particle size
distribution was measured periodically using a multi-jet Mercer cascade im-
parator (Chen et al., 1990). Exposures were also analyzed periodically for carbon
monoxide (CO) by infrared absorption (Model 865, Beckman, Fullerton, CA), for
oxides of nitrogen (NOx) by chemiluminescence (Model 14T, ThermoElectron,
Hopkinton, MA), and for total gaseous hydrocarbons (HC) by infrared absorption
for total HC (Model 820-1, Beckman, Fullerton, CA), and for total gaseous hydrocarbons (HC) by infrared absorption
using 25 mm glass ﬁber ﬁlters (Type A/E, Gelman, Ann Arbor, MI) weighed
on an electronic microbalance (Model C-31, Cahn, Cerritos, CA). Particle size
distribution was measured periodically using a multi-jet Mercer cascade im-
parator (Chen et al., 1990).
by Dunnett’s multiple comparison test (Dunnett, 1955, 1980). Both absolute lung weight and lung weight as a percentage of terminal body weight were analyzed using one-way analysis of variance, followed by Dunnett’s multiple comparison test (Dunnett, 1955, 1980).

Neoplastic and non-neoplastic lesions. All lesions were assumed to be incidental to death, and were analyzed using logistic regression (Dinse and Haseman, 1986; Dinse and Lagakos, 1983). Lesion incidence was modeled as a logistic function of exposure concentration and time. Linear and quadratic temporal terms were used, and the quadratic term was eliminated if it did not significantly enhance the fit of the model. Both pairwise comparisons of smoke-exposed groups to controls and an overall trend test were performed. When the logistic regression failed to converge mathematically, the continuity-corrected incidental tumor test (Peto et al., 1980) was applied. This approach is identical to that which the National Toxicology Program applies in its evaluations of incidental tumor and non-neoplastic lesion incidence. Reported p-values are one-sided, to facilitate testing the hypothesis that smoke exposure increases the incidence of lesions.

RESULTS

Exposures

Both the daily and the study average smoke TPM concentrations were maintained very close to target levels (HS = 244 ± 27 vs. 250 mg/m³, LS = 98 ± 11 vs. 100 mg/m³, mean ± SD of daily values). Concentrations of gases measured periodically scaled roughly with TPM concentrations (HS = 230 ppm CO, 5 ppm NOx, and 133 ppm HC; LS = 97 ppm CO, 2 ppm NOx, and 61 ppm HC). The mass median aerodynamic diameters and geometric standard deviations of particle size were: HS = 0.62 μm, 1.34 σg (n = 93); LS = 0.56 μm, 1.34 σg (n = 169).

The results of analyses of particulate and vapor-phase components of the high level exposure atmosphere are expressed in Table 2 per mg of TPM mass. Measurements of selected components at both exposure levels yielded very similar concentrations per unit of particle mass.

Similarity Among Entry Blocks

There was no statistical evidence that responses differed significantly among the entry blocks within experimental groups. The blocks were combined for all analyses.

Survival

Median (50%) survival times for the C, LS, and HS groups were 752, 779, and 749 days, respectively, for males and 807, 845, and 766 days, respectively, for females. Survival of the HS females was significantly shortened compared to C females (p = 0.02), but survival of HS males was not significantly shortened. In contrast, survival of both males and females at the LS level was significantly lengthened compared to C groups (p = 0.01 for males, p = 0.003 for females).

Body and Lung Weights

An exposure-related reduction of body weight gain is illustrated in Figure 1. Body weights of both smoke-exposed groups were significantly (p < 0.01) lower than C weights at all 6-month averaging intervals. Although no attempt was made to measure food consumption in a definitive manner, feeder trays supplying a multiple-animal cage unit at each exposure level were weighed daily during exposure weeks 2 and 61. During week 2, food consumption for a unit of 12 males and 12 females averaged 17, 15, and 11 g/day/rat for the C, LS, and HS groups respectively. During week 61, food consumption for a unit of 16 males was 17, 16, and 15 g/day/rat for the C, LS, and HS groups respectively. These limited results suggested that exposure caused a dose-related depression of food consumption.

Mean absolute lung weights of both male and female HS rats were significantly greater than those of C rats (Table 3), but lung weights of the LS group did not differ significantly from C lung weights. The same relationships pertained to lung weights as percentages of terminal body weight (data not shown).

Histopathology

Lungs from all rats listed in Table 1 were examined. Nasal sections were not available for one C female that died after 90 days of exposure. Only two of the four nasal sections were available for another C female rat dying at 880 days, but because

<table>
<thead>
<tr>
<th>Particle phase</th>
<th>Mean (μg/mg TPM)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>103.1</td>
<td>2.0</td>
</tr>
<tr>
<td>Nicotine</td>
<td>51.5</td>
<td>18.3</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>10.9</td>
<td>8.1</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>5.7</td>
<td>1.4</td>
</tr>
<tr>
<td>Catechol</td>
<td>4.8</td>
<td>0.8</td>
</tr>
<tr>
<td>p-Cresol</td>
<td>4.2</td>
<td>1.1</td>
</tr>
<tr>
<td>m-Cresol</td>
<td>2.3</td>
<td>0.8</td>
</tr>
<tr>
<td>o-Cresol</td>
<td>1.9</td>
<td>0.7</td>
</tr>
<tr>
<td>Polycyclic aromatic hydrocarbons</td>
<td>(ng/mg TPM)</td>
<td></td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>10.3</td>
<td>1.6</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>6.1</td>
<td>0.7</td>
</tr>
<tr>
<td>Pyrene</td>
<td>4.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Anthracene</td>
<td>4.6</td>
<td>2.5</td>
</tr>
<tr>
<td>Benzantracene/chrysene</td>
<td>3.9</td>
<td>0.8</td>
</tr>
<tr>
<td>Vapor phase</td>
<td>(μg/mg TPM)</td>
<td></td>
</tr>
<tr>
<td>Acetone</td>
<td>37.4</td>
<td>9.0</td>
</tr>
<tr>
<td>Toluene</td>
<td>8.7</td>
<td>1.7</td>
</tr>
<tr>
<td>Limonene</td>
<td>5.1</td>
<td>2.1</td>
</tr>
<tr>
<td>2-Methylfuran</td>
<td>3.2</td>
<td>1.4</td>
</tr>
<tr>
<td>Benzene</td>
<td>2.3</td>
<td>0.3</td>
</tr>
<tr>
<td>m,p-Xylene</td>
<td>2.3</td>
<td>0.6</td>
</tr>
<tr>
<td>o-Xylene</td>
<td>0.8</td>
<td>0.2</td>
</tr>
</tbody>
</table>

*Measurements were performed six times during the exposure. Measurements of most analytes were replicated at each time. Results are presented as means and standard deviations of the six mean values.

TABLE 2
Concentrations of Selected Components in the High Level Exposure Atmosphere (250 μg/m³)"
there were no gross nasal lesions and the available sections had no evidence of proliferative lesions, that rat was considered to have no proliferative nasal lesions.

**Nonproliferative lung lesions.** The primary lesion associated with both HS and LS exposure consisted of aggregates of macrophages laden with golden-brown pigment. Macrophages containing pigment occurred singly and in groups ranging from small clusters of discrete cells within alveoli up to densely packed, solid nodular aggregates. These aggregates were typically admixed with scattered neutrophils, and often had associated type II pneumocyte hyperplasia and mild septal fibrosis (Fig. 2B). Such foci were essentially randomly disseminated throughout the lung parenchyma and were larger and more common in HS than in LS rats. Ciliated cuboidal cell metaplasia was common in alveolar ducts of HS rats and occurred to a lesser degree in LS rats (Fig. 2C). Squamous metaplasia of alveolar ducts was observed rarely in HS rats (Fig. 2D) and not at all in LS rats. None of the above changes was present in C rats.

Only minor histological abnormalities were observed in larger airways of exposed rats. Mild bronchiolar epithelial cell hypertrophy was present, sometimes accompanied by a mild increase in goblet cells. It was uncommon to observe substantive inflammation surrounding larger airways, even in HS rats, although scattered pigment- and particle-containing macrophages were observed in the peribronchial lymphoid tissue.

**Hyperplastic responses** consisted primarily of focal alveolar epithelial hyperplasia. The hyperplastic foci retained alveolar architecture and were lined by cuboidal epithelium, usually in a single layer. Atypia was usually lacking, although some foci had areas of mild nuclear atypia. Intervening air spaces sometimes contained desquamated epithelial cells and/or macrophages, and varying, but usually mild, degrees of septal fibrosis were present (Fig. 3A). In addition, keratinizing squamous cysts were found in one male and two female HS rats.

**Proliferative lung lesions** occurred at greater incidence in exposed females than in males with the exception of hyperplasia in the LS males and malignant neoplasia in HS males. Trends with exposure for all lesion categories were highly significant for females. No trend with exposure was significant for males, although the trend for all types of neoplasia combined was nearly significant ($p = 0.055$). The incidence of all neoplasias was significantly increased above the C incidence in both LS and HS females. The increases in neither benign nor malignant neoplasias alone reached significance in LS females. The incidences of all, benign, and malignant neoplasms in the HS females were approximately 14%, 9%, and 5%, respectively, and no neoplasms were observed in C females. No difference from C was significant for any individual lesion category for either the LS or HS males. The incidences of all, benign, and malignant

### Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS</td>
<td>$2.90 \pm 0.10^b$</td>
<td>$2.41 \pm 0.08^c$</td>
</tr>
<tr>
<td>LS</td>
<td>$2.53 \pm 0.06$</td>
<td>$1.79 \pm 0.06$</td>
</tr>
<tr>
<td>C</td>
<td>$2.54 \pm 0.09$</td>
<td>$1.71 \pm 0.06$</td>
</tr>
</tbody>
</table>

$^a$Group sizes identical to Table 1.

$^b p < 0.01$ versus C.

$^c p < 0.001$ versus C.

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**FIG. 1.** Mean body weights of experimental groups during exposure. C = control, LS = low smoke exposure level, and HS = high smoke exposure level.
neoplasms in the HS males were approximately 9%, 2%, and 6%, respectively, and incidences in C males were approximately 3%, 1%, and 3%, respectively.

Benign neoplasms were all bronchioloalveolar adenomas characterized by an alveolar pattern and a loss of normal alveolar architecture, within a relatively well-circumscribed area of high epithelial cell density. The neoplastic epithelium was fairly uniform with little cellular atypia or pleomorphism, and there was occasionally compression, but not invasion, of adjacent tissues (Fig. 3B).

Malignant neoplasms consisted of bronchioloalveolar carcinomas. In these neoplasms, alveolar architecture was obliterated by an area of high epithelial cell density accompanied by one or more features of malignancy (Fig. 3C), including nuclear/cellular atypia and invasion of adjacent vessels or airways. The 17 carcinomas varied in pattern, with 9 having an alveolar pattern, 5 papillary, 2 tubular, and 1 characterized as adenosquamous. No metastases from the carcinomas were observed.

The time to first observation of hyperplastic lesions was shortened by exposure among females, but not in a dose-related manner. The time to first observation of hyperplastic lesions was not shortened by exposure among males. Overall, exposure shortened the time to observation of neoplasms. Both benign and malignant neoplasms were observed earlier in HS than in LS females (111 and 154 days earlier, respectively). Benign and malignant neoplasms were observed earlier in both LS and HS males than in C males, but the shortening was not dose-related.

Proliferative nasal lesions. A similar spectrum of proliferative lesions was found in nasal cavities of LS and HS rats (Fig. 4), and was more pronounced in the HS group. There was no consistent gender difference in the incidence of nasal lesions. Epithelial hyperplasia varying from minimal to marked was common. Squamous metaplasia of transitional and respiratory epithelium was typically pronounced in HS rats, but only occasionally present and less severe in LS rats. Mucous cell metaplasia and hyperplasia were also common at both exposure levels, but more severe in HS rats. Minimal to mild mixed inflammatory infiltrates (lymphocytes, plasma cells, neutrophils, and histiocytes) were often present in the lamina propria of exposed animals, but more common in the HS rats.

The incidence of neoplasms of the nasal cavity was significantly increased among both males and females at the HS level, but not at the LS level (Table 5). The exposure trend was
Significant for both genders, although one neoplasm was observed in both the C and LS male groups, and none was observed in either C or LS females. Both benign and malignant tumors contributed to the response. Most of the nasal cavity tumors were of epithelial origin. The benign epithelial tumors were adenomas, which were noninvasive, exophytic, and contained ciliated cells. Most had invaginated, acinus-like structures, while the fourth mutation was a GGT to GTT transversion.

Mutations in Codon 12 of the K-ras Gene

Twenty-three lung tumors from rats exposed to cigarette smoke, 8 adenomas and 15 carcinomas, were of sufficient size to accommodate both histopathology and analysis of K-ras mutations. These tumors were from 6 LS and 15 HS rats. Four of 23 (17%) tumors were positive for mutation within codon 12 of the K-ras gene. All positive tumors were carcinomas, two from one HS female, one from another HS female, and the fourth from a LS female. Three carcinomas from C rats were also analyzed for K-ras mutations. None of the tumors from C rats were positive for K-ras mutation. Direct sequencing identified mutations in three positive tumors as GGT to GAT transitions, while the fourth mutation was a GGT to GTT transversion.

DISCUSSION

This study demonstrated for the first time that chronic (near life span) whole-body inhalation exposure of a common test strain of rat (F344) to cigarette smoke can produce statistically significant exposure-related increases in benign and malignant lung and nasal neoplasms. Evaluation of standard lung sections yielded incidences meeting the criteria for success (significant increase in lung neoplasia, including malignant neoplasia) for both genders (not listed in Table 5).
FIG. 3. Histology of proliferative lung lesions. (A) Focal alveolar epithelial hyperplasia in a female LS rat. (B) Bronchioloalveolar adenoma in a male HS rat. (C) Bronchioloalveolar carcinoma in a female HS rat. Inset shows a similar carcinoma from a female LS rat demonstrating vascular invasion. Note islands of neoplastic cells (arrowheads) invading wall of a large pulmonary vein and protruding into the vascular lumen (V).

FIG. 4. Histology of nasal lesions. Standard sections of rostral nasal cavity at level just caudal to the incisors. (A, C) Low and higher power views of a normal female C rat. (B, D) Low and higher power views of male HS rat nasal section. Note abundant epithelial hyperplasia (arrows), mucous metaplasia (small arrows), squamous metaplasia (arrowheads) with foci of keratinization (K), and neutrophilic inflammatory exudates (E). S = nasal septum, T = turbinate.
The composition of smoke in the exposure chambers undoubtedly differed slightly from that of smoke inhaled directly from a cigarette, especially with regard to highly reactive, short-lived chemical species. Indeed, other than voluntary smoking by addicted nonhuman primates, it is likely that no animal study has exactly reproduced human exposures to cigarette smoke. As an aid to designing the present study, Chen et al. (1989, 1992) compared the composition of smoke exposure atmospheres among different puffing profiles and between nose-only and whole-body chambers. Concentrations of most smoke components in the whole-body chamber were similar to those in nose-only chambers. Perhaps due to a greater opportunity for volatilization caused by the longer residence time, concentrations of a few vapor species (e.g., o-xylene, limonene) were slightly higher, and some particle components (e.g., glycerol, nicotine) were slightly lower in the whole-body chamber. Overall, the exposure in the present study was considered a reasonable test of the carcinogenicity of the major components of mainstream cigarette smoke, especially in view of the diversity of human exposures deriving from different cigarette types and puffing patterns.

Direct comparison of the responses of rats in the present study to those of human smokers is fraught with difficulty; however, some parallels can be drawn. Although statistically significant, lung neoplasia occurred at low incidence in the rats (HS ≈ 14%, LS ≈ 6% for females, and HS ≈ 9% for males). Similarly in humans, although cigarette smoke is the predominant cause of lung cancer, it is not an extremely potent carcinogen in view of the huge doses received by heavy smokers. Smoking-induced lung cancer is most obvious in men, in which the lifetime cumulative risk is approximately 16% (Peto et al., 2000; Vineis et al., 2004). The risk for lung cancer among women smokers continues to grow as large populations of smoking women age and smoking patterns of women become more similar to those of men. The lifetime lung cancer risk among women was reported in 2000 to be approximately 10% (Peto et al., 2000).

The lung tumor incidences observed in the present study likely underestimate the actual incidences. Tumors were located by examining standard histological sections plus evaluating all gross nodules identified during necropsy. Some neoplasms identified on sections were not evident grossly; conversely, some gross nodules were not neoplastic. Although the tumor incidences would have likely been higher had the entire lungs been sectioned serially, the method used in this study provided a standardized sample, and the exposure-related increases are valid, albeit likely minimal, indicators of exposure-induced carcinogenicity.

The types of lung lesions induced by exposure of the rats differ somewhat from those in human smokers, but also have parallels. The rat tumors were bronchioloalveolar in origin, with approximately equal numbers of adenomas and adenocarcinomas; no squamous cell carcinomas were observed, and no tumors were found in central airways. Lung tumors in human smokers are predominately malignant. Although squamous cell carcinomas

| TABLE 5 |
| Nasal Section Neoplasia |
| Males | Females |
| Nasal cavity — all types |
| Incidence — malignant epithelial |
| Rate (%) | 0.0 | 0.0 |
| Difference vs. C (p =) | 0.00 | 0.00 |
| Exposure trend (p =) | 0.08 | 0.00 |
| First observation (days) | 753 | 720 |
| Nasal cavity — all epithelial |
| Incidence — malignant epithelial |
| Rate (%) | 0.0 | 0.0 |
| Difference vs. C (p =) | 0.00 | 0.00 |
| Exposure trend (p =) | 0.08 | 0.00 |
| First observation (days) | 753 | 720 |

a No. with lesion/No. examined.
in large airways predominated in early studies of human smokers, a shift toward adenocarcinomas and more peripheral locations became evident in the late 1970s, and adenocarcinoma is now the most common type (Travis et al., 1995, Wingo et al., 1999). The exposure-related scattered epithelial hyperplasia and metaplasia, macrophage accumulation, and mild septal fibrosis, in rats were reminiscent of those found in lungs of human smokers (IOM, 2001).

Although the present study did not examine genetic alterations in the lung tumors in detail, the finding of mutations in the K-ras gene in 17% of the tumors examined parallels the finding of K-ras mutations in 20–30% of lung tumors from human smokers (Pulling et al., 2003). The lack of K-ras mutations in lung tumors of rats exposed to diesel emissions and carbon black (Belinsky et al., 1995) suggests that the mutations in the present study were likely a response to the specific composition of the cigarette smoke exposure and not a general phenomenon. This premise is supported by the fact that the GGT to GAT transition mutation in codon 12 commonly associated with lung tumors induced in the A/J mouse lung by the tobacco-specific nitrosamine NNK (Belinsky et al., 1989) was also observed in three of four cigarette smoke-induced tumors positive for K-ras mutation.

The greater incidence of lung tumors among females than among males was consistent with results from chronic inhalation studies of diesel exhaust (Mauderly et al., 1987; Nikula et al., 1995) and some poorly soluble particles such as talc (Hobbs et al., 1994) and carbon black (Nikula et al., 1995). Although the difference in the control incidence between males (3.4%) and females (0%) undoubtedly contributed to the differences in statistical significance of the responses, the zero incidence among females was not solely responsible for the significance of their response. The incidences among the exposed groups were clearly higher in females than in males, and the control incidences for both genders were within the historic range (e.g., NTP chronic studies with F344 rats, see http://ntp-server.niehs.nih.gov/main_pages/spec_rpts.html).

At least four factors may have contributed to the gender difference among the rats. First, the slightly longer life span of the females provided a longer time for development of detectable tumors. The LS and HS females had median survivals 66 and 17 days, respectively, longer than males. Second, there might have been a gender difference in the deposited or retained dose per unit of target tissue. Although neither respiration nor the dose of smoke components were measured in this study, there are data demonstrating that female F344 rats have greater ventilation per unit body weight than males (Mauderly, 1986), and that the accumulated lung burdens of some exposure materials (diesel soot and carbon black) are greater per unit of lung size in chronically-exposed females than in males (Mauderly et al., 1994). Third, there may be gender differences in the metabolism, accumulation, and excretion of carcinogens or procarcinogens, the formation of DNA adducts, DNA repair, or immune surveillance against tumor cells. For example, the evidence suggesting that human females are more susceptible than males to cigarette smoke-induced carcinogenesis is accompanied by growing evidence that estrogen receptor pathways may be important in carcinogenesis and, thus, may contribute to a gender difference (Stabile and Siegfried, 2004). Fourth, tumor-promoting epithelial proliferative responses may have been greater in females than in males. Because the incidence of lung hyperplasia in this study was slightly higher in HS, but not in LS, females than in the corresponding males, it seems unlikely that this was a major factor. Although the present study was not designed to examine the influence of the above factors, the plausibility of a real gender difference is clearly supported by multiple mechanism-based possibilities.

It is uncertain whether the gender difference in the tumor response of the rats has a parallel among human smokers. Males have historically predominated lung cancer incidence among human smokers, in parallel with their higher smoking rates. Although growing evidence suggests that females may be more susceptible than males at equal exposure rates (Risch et al., 1993; Stabile and Siegfried, 2004; Zang and Wynder, 1996), it is not yet clear whether a true gender difference exists in human susceptibility and, if so, what the mechanism of the difference might be.

The significant exposure-related increase in the incidence of nasal tumors in the rats also paralleled increases in nasal cancer among human smokers. The incidence of neoplasms of the nasal cavity (HS ≈ 6%, combined genders) was lower than the incidence of lung tumors, but higher than observed among human smokers. Although tumors in respiratory tract locations above the larynx account for only 2% of respiratory tract cancer in humans (Jemal et al., 2003), epidemiology has repeatedly identified smoking as a risk factor for human nasal cancer (reviewed in Benninger, 1999). A greater nasal:lung neoplasia ratio in rats than in humans could result from the fact that rats are obligatory nasal breathers and exposure of humans is dominated by oral inhalation. As noted above for lung tumors, skin and gastrointestinal absorption may also have contributed to the nasal response of rats.

It seems likely that the carcinogenicity in the present study was a response to chemical carcinogens rather than a nonspecific response to “particle overloading,” such as that caused by exposures to diesel emissions and some non-mutagenic solid particles administered above the “maximum tolerated dose” (MTD) (Mauderly, 1996). MTD is generally defined as the highest dose (exposure) that does not cause life span shortening for causes other than carcinogenicity (Haseman and Lockhart, 1994). The HS level met this definition because it significantly, although only slightly, shortened survival among HS females, but not males, and the tumors were not considered to have caused death. For exposures to poorly soluble particles that accumulate in the lung, the NTP Toxicology Design Review Committee proposed the additional criterion of setting the highest level at or below the minimum causing retardation of particle clearance by “overloading” particle clearance mechanisms (Lewis et al., 1989). Neither the lung burden of retained material
nor the clearance of deposited material were measured in this study. However, identical exposures in a parallel study were found to retard clearance of inhaled poorly soluble plutonium-239 dioxide particles in HS and LS females and HS males (Finch et al., 1998).

Although the HS exposure might have exceeded the MTD (which may also be true for exposures of human heavy smokers), it is unlikely that the LS level reached the MTD. The increased neoplasia at the LS level suggests that the tumors were not solely a response to overwhelming exposures. In addition, only three keratinizing squamous cysts were observed among the 163 HS rats, a lesion found at much higher incidence in “overloading” exposures of rats to diesel emissions or carbon black (Nikula et al., 1995). Finally, the finding of mutations in the K-ras gene in tumors in the present study, a lesion not induced in tumors in rats exposed heavily to diesel emissions or carbon black (Belinsky et al., 1995), is further evidence that the lung carcinogenicity in the present study was not a nonspecific response to “particle overloading.”

In summary, this study demonstrated that chronic, whole-body inhalation exposure of rats to cigarette smoke at and below the MTD produces exposure-related increases in the incidences of lung and nasal tumors and other proliferative and nonproliferative lesions. This finding suggests that whole-body exposures, constitute a research platform that might be useful for studying carcinogenic mechanisms, chemoprevention, and the co-carcinogenicity of chronic cigarette smoking, and by implication, chronic exposures to other physically and chemically complex mixtures.

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