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An Updated Review of Inhalation Studies with Cigarette Smoke in Laboratory Animals

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Until recently, the published literature on inhalation studies with laboratory animals and cigarette smoke consisted entirely of negative findings, as far as neoplastic disease is concerned. This paper brings readers up to date, with analyses of recent studies that do indeed appear to report success after so many years of failure. The paper consists of a brief analysis of the literature up until a couple of years ago, giving brief, representative examples of inhalation studies with the five main species of laboratory animals that have been used: rat, mouse, hamster, dog, and nonhuman primate. A brief examination of the various technologies used to expose laboratory animals is given, along with an analysis of the histopathology and related toxicology data (specifically, biomarkers of exposure) that have been reported. The paper concludes by briefly mentioning the most recent studies, where positive results have been reported.

Keywords Cigarettes, Inhalation, Laboratory Animals, Lung Cancer

This work was performed to verify whether the measured response to cigarette smoke in the five most commonly used animal species for assessing carcinogenic potential in humans reflects the strong epidemiological evidence in human smokers (Doll et al. 2005).

CRITERIA USED

Rigorous criteria by which to evaluate the results of studies published in the peer-reviewed literature were selected in accord with accepted standards of toxicology, pathology, and carcinogenesis.

The overall duration was to be consistent with the induction of carcinogenesis, including those studies where details on gross pathology, subsequent histopathology or both were lacking. In addition, there was a fundamental requirement for a detailed histopathological description, no matter what the duration of the experiment.

CIGARETTES, SMOKING CONDITIONS, SMOKE CHEMISTRY

The cigarettes used by many of the reviewed papers were unfiltered and had very high yields; as such, they are very different from the cigarettes commercially available today. Nearly all cigarettes were from the University of Kentucky (Diana and Vaught 1990). These reference cigarettes contain the same tobacco blends and are made with the same processing aids (such as humectants) as those used in commercial cigarettes. The reference cigarettes do not however contain the small quantities of flavoring materials used in commercial products (Carmines 2002).

Most studies used the Federal Trade Commission (FTC)/International Standards Organisation (ISO) standard of 35-ml puffs of 2-s duration, taken once per minute (ISO 1991). A few studies included radiolabeled markers of deposition, because minute ventilation is known to be markedly affected by smoke (Coggins, Musy, and Ventrone 1982). Many studies used nose-only smoking machines (Baumgartner and Coggins 1980; Henry et al. 1985), others used whole-body exposures with known disadvantages (Langård and Nordhagen 1980). Some studies used single cigarettes; others had rotating carousels. Some allowed aging of smoke or rebreathing of exhaled smoke. The highly invasive tracheotomy technique was used in many of the dog studies.

Smoke chemistry was rarely measured, apart from smoke particulates, nicotine, and carbon monoxide. Particle size was occasionally measured; mass median aerodynamic diameter (MMAD) values were generally found to be submicron and so highly rat respirable. The most appropriate biomarkers of exposure are blood carboxyhemoglobin (COHb), which reflects uptake of the vapor phase of smoke, and plasma nicotine reflecting uptake of the particulate phase of smoke. The major metabolite of nicotine, cotinine, has also been measured in plasma. Blood COHb concentrations were often >60%.

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PATHOLOGY

Any neoplasms reported at necropsy and/or with full histopathological description were included. Many studies not included simply stated that "full necropsies were performed," with no data presented on neoplasms (so presumably, there were none observed).

MOUSE

Henry and Kouri (Henry and Kouri 1986) performed a chronic inhalation study in male mice exposed to smoke from the 2R1 unfiltered reference cigarette (yields, 37 mg tar and 2.4 mg nicotine). The publication is a distillation from a much larger report from the Council for Tobacco Research (CTR) in 1984 (Council for Tobacco Research 1984). Mice were exposed 5 days per week for 110 weeks, and followed for up to 4 years. Animals were inoculated at the beginning of the study with Sendai virus. Animals were exposed sequentially, rather than in a parallel manner: animal number 2 received the exhaled smoke from animal number 1. As there were over a thousand animals being exposed simultaneously, there could be significant differences between the animals "downstream": the smoke these animals received could have been inhaled and exhaled many times previously (Henry et al. 1981, 1985). No chemical analysis of the smoke actually presented to the animals was made; assumptions were made as to the physical composition of this smoke.

A full analysis of the histopathology noted in the study is missing: there is no accurate description of the alveolar adenocarcinoma. Additional problems with this study include the unknown effects from the inoculation with the Sendai virus, and also the physical restraint causing large numbers of mortalities (neck lesions) in the study.

The major neoplasms observed were hepatic tumors, sarcomas, fibrosarcomas, lung adenocarcinomas, liver carcinomas, and mammary carcinomas. A total of 19 of 978 smoke-exposed mice and 7 of 651 sham-exposed mice were observed with alveolar adenocarcinomas. The authors stated that "*the difference between the smoke- and sham-exposed animals was not statistically significant at $P < 0.05$*"; additional statistical analyses were reported by the authors (see below).

The tumor incidence data in the CTR book were analyzed in a number of different ways; however, under no circumstances could a statistically significant difference be noted between sham- and smoke-exposed groups. At no time in this study was the incidence of tumors in the smoke-exposed mice higher than that in the sham-exposed animals. Nevertheless, the authors concluded that the 2R1 cigarette smoke has "*weak carcinogenic activity,*" apparently the result of the statistical analyses of the animals that died during the experiment. The statistical analyses at necropsy quite clearly show that there is no difference in response between smoke-exposed and sham animals.

As stated earlier, a larger set of data on this experiment was presented in the CTR Final Report. Upon examination of this larger data set, I found that there were histopathology data on a group of mice, namely a shelf control group, and that these data were not present in the published paper. This paper presents survival and body weight data for three groups, but comparative histopathology data are only given for two groups (the histopathology data for the shelf controls are inexplicably excluded).

The CTR report presents data for the number of animals with alveolar adenocarcinoma in each of three groups of mice. For shelf control, smoke-exposed, and sham-exposed groups, these numbers were 18, 11, and 7 animals, respectively. The report then gives the number of cumulative animals "diagnosed" by histopathology after 1, 2, or 3 years (including 110 weeks of exposure); for shelf control, smoke-exposed, and sham-exposed, there were 369, 985, and 659 animals, respectively. These data result in lung carcinoma rates of 4.88%, 1.12%, and 1.06 % for shelf control, smoke-exposed, and sham-exposed groups, respectively.

The shelf control animals thus have a lung carcinoma rate 4.4 times greater than that of the smoke-exposed animals, a finding that is not mentioned in either the published paper or the CTR report. Stress and/or nutrition could be the reason for these findings, since "*the rate of weight gain of the untreated, shelf control animals were significantly greater ($p < 0.05$) than that of the smoke and sham exposed mice,*" and "no difference between the mean body weights of the smoke and sham exposed mice was found over the course of the study." I estimate from the published paper (Henry and Kouri 1986) that at 96 weeks the shelf control animals were approximately twice as heavy as were the smoke-exposed and the sham-exposed animals (Coggins 2000).

The strain A mouse was developed in the 1920s and has been widely used in cancer and immunology testing. The exceptionally high propensity of the strain A mouse to develop spontaneous lung neoplasms and its exquisite sensitivity to chemically induced lung tumors renders it most useful as a research tool to investigate the mechanisms of lung tumorigenesis (Maronpot et al. 1983, 1986).

The information on A/J mice provided by the original breeder (Jackson Laboratories, Bar Harbor, ME) include a primary lung tumor background incidence rate of 32% in male and 26% in virgin female A/J mice. Spontaneous lung tumors reportedly occur at a rate of 0.21 tumors per mouse at 24 weeks of age. Lung tumors may be found in strain A as early as 3 to 4 weeks of age, with a steady increase to almost 100% by 24 months of age (Shimkin and Stoner 1975).

Finch and colleagues (Finch et al. 1996) exposed A/J mice to mainstream smoke for 6 h/day, 5 days/week for 26 weeks at a mean total particulate matter concentration of 248 milligrams per cubic meter (mg/m^3), to test the hypothesis that chronically inhaled mainstream cigarette smoke would either induce lung cancer or promote lung carcinogenicity induced by the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK).

A biologically significant level of smoke exposure was achieved, as indicated by body weight reductions, lung weight

increases, and COHb concentrations of about 17%. Lung nodules observed at necropsy were used as the primary measure of tumorigenicity. The authors justified this by stating that *"even though a substantial number of focal alveolar epithelial hyperplasias were observed histologically, and some may have been counted grossly as nodules (and therefore counted as tumors), the hyperplasias can justifiably be classified as tumors because of the progression of hyperplasias to adenomas and ultimately carcinomas in the A/J mouse." The authors also considered that "our efforts probably represent an approximate upper bound of the tolerable level of cigarette smoke exposure"* (Finch et al. 1996).

Despite the very high dosages used and the sensitivity of the experimental animals, the authors reported that cigarette smoke exposure neither induced lung tumors nor promoted NNK-induced tumors.

There are a large number of reports in the literature by Witschi and colleagues on the use of the A/J mouse (Witschi 2005c). These will not be reviewed here, but are reviewed in detail in an adjacent article (Witschi 2007). Some recent work from Dr. Witschi on a surrogate for environmental tobacco smoke indicates that the "carcinogenic activity" resides in the vapor phase of the smoke. Compounds such as the polyaromatic hydrocarbons (PAHs), tobacco-specific nitrosamines (TSNA), and a large range of other compounds can therefore be ruled out as being the responsible agent (Witschi et al. 1997; Witschi 2005a), with 1,3-butadiene currently being suggested instead (Witschi 2005b). The phenomenon of no difference between smoke-exposed and control animals at the end of the smoke exposures, but a large difference occurring several months after the end of the experiment, has not been adequately explained, nor has the kinetics of the difference or the overall mechanism been completely established (Curtin et al. 2004; Stinn et al. 2005b; Yao et al. 2005; Witschi 2005, 2007).

RATS

Wehner and colleagues (Wehner et al. 1981) exposed groups of 80 female rats to smoke from three types of cigarette, the aim being to determine whether inhalation bioassays in rats are a suitable technique to determine the biological effects of such smoke. Additional end points to this study were published: these two ancillary studies (Loscutoff et al. 1982; Phelps et al. 1984) will be considered within the following analysis of the study (Wehner et al. 1981).

Animals were exposed to the partially diluted smoke from one cigarette (10 puffs, one puff per minute) eight times per day, 7 days per week, for up to 24 months. Exposures were made in smoking machines (Maddox et al. 1978) in which up to 10 rats can receive nose-only exposures to the smoke from one cigarette. Cigarettes were unfiltered, with tar yields up to 25 mg per cigarette and nicotine yields up to 1.9 mg/cigarette.

Loscutoff (Loscutoff et al. 1982) examined a number of physiological variations in these animals, and showed that mean blood COHb concentrations at the end of the 10-min exposures ranged from 22% to 55%. The experimental design was replicated in a subsequent study, in which deposition in the respiratory tract was assessed using cigarettes spiked with ^{14}C-radiolabeled dotriacontane (Phelps et al. 1984). These exposures were made after a 3-week exposure to nonlabeled smoke from each of the three cigarette types. The authors (Phelps et al. 1984) showed that the result of smoke exposure was deposition of smoke particles deep in the lungs and throughout the lung lobes. The lung exhibited approximately 20% to 30% of the total label burden. The head and the larynx contained 5% to 10% of the material, and less than 4% was in the trachea. Over half of the material deposited was on the rat's pelt. The authors commented that although smoke particles were deposited in the lower respiratory system, there was some transfer of the inhalation chamber contents into the (sealed) animal containment tubes, followed by deposition on the animals' pelts.

After 12 and 18 months in the main study, rats were killed by ether anesthesia and exsanguination; and survivors were killed 24 months after initiation of smoke exposure. Body weight and survival data showed minor differences between the groups. The most common pathological changes were smoke granulomas in the lung. These smoke granulomas consisted of disseminated foci of macrophages in alveolar spaces and adjacent interstitial areas of all lobes of the lung. When close to terminal bronchioles, blood vessels and subpleural areas, the foci of macrophages were more numerous. Macrophages were distended with granular brownish pigment mixed with small cytoplasmic vacuoles. The alveolar epithelium adjacent to the macrophages was hyperplastic and showed glandular metaplasia, and the alveolar septa occasionally had areas of fibrosis. Between 12 months and sacrifice time, the granulomas did not progress significantly in severity (Wehner et al. 1981).

In one smoke-exposed rat (from the group exposed to the smoke from a low-"tar," medium-nicotine cigarette), an epidermoid carcinoma was noted. The carcinoma was composed of sheets and strands of stratified squamous epithelium forming several large keratin-filled cavities. The stratified epithelium was extended into adjacent pulmonary arteries and replaced large areas of myocardium. Metastases were seen in the mediastinal lymph nodes and the contralateral lung lobes. A total of seven other rats showed small nodules of squamous metaplasia (including keratin pearl formation). There was a slight squamous metaplasia of the bronchioles (no nodule formation) seen in one of the sham exposed rats. Three rats developed adenomatous hyperplasia of the alveolar epithelium (tendency for non-neoplastic nodule formation) (Wehner et al. 1981).

Smoke exposed rats had a significant increase of squamous metaplasia of the laryngeal and tracheal epithelium. Smoke exposed rats also exhibited basal hyperplasia of the laryngeal and tracheal epithelium, but with a lower incidence and group average severity than for squamous metaplasia. Lesions were rare in the nasal cavity, but squamous metaplasia and basal cell hyperplasia were seen occasionally there in the smoke exposed rats.

There were no other microscopic changes in the respiratory tract clearly related to smoke exposure.

The authors concluded that although a statistically significant carcinogenic effect of cigarette smoke was not observed, differences in the severity of smoke granulomas, degree of pulmonary interstitial reaction, body weight, and survival rate made it possible to differentiate, "*to a certain degree,*" between the biological effects of different cigarette types (Wehner et al. 1981).

SYRIAN-GOLDEN HAMSTER

Several inhalation studies with Syrian-golden hamsters *(Mesocricetus auratus)* were performed in the 1970s (Dontenwill et al. 1974; Homburger, Bernfeld, and Russfield 1974; Wehner, Stuart, and Sanders 1979). In virtually every study the most responsive organ was the larynx, anatomically somewhat different in the hamster than in the rat or the mouse (Renne and Miller 1996; Renne and Gideon 2006).

Dontenwill (Dontenwill et al. 1974) described an extremely large experiment where a total of 4400 male and female hamsters were used: 3610 were exposed for their entire lifetime and 830 were exposed for no longer than 52 weeks. A total of 20 experimental groups were used, with between 80 and 300 animals per sex per treatment.

Seven different cigarette types were used, with tar yields ranging from 20 to 34 mg per cigarette and nicotine yields ranging from 0.4 to 1.6 mg per cigarette. Cigarette variables included the addition of sodium nitrate, reconstituted tobacco sheet, a combination of the latter two, cigarettes with acetate, cellulose or charcoal filters, and a cigarette ("*black*") with a higher content of burley and various cigar and dark tobaccos. Other variables superimposed on the (unfiltered) reference cigarette group included intratracheal instillations of saline/carboxymethycellulose solutions containing dimethylbenzanthracene (DMBA; a synthetic, nonenvironmental agent), diethylnitrosamine (DEN), or "*blue cape*" asbestos. One group was exposed to smoke passed through a particulate filter (vapor phase only). Animals were exposed in groups of 10, nose-only, to the smoke of 30 cigarettes, 1, 2, or 3 times per day, using for each cigarette type an approximately 40% dilution of the mainstream smoke. No reports of dosimetry were presented (Dontenwill et al. 1974).

Mean survival times varied from 40 weeks (females, added sodium nitrate, twice daily) to 85 weeks (cage control males). There was a dose-dependent reduction in survival time in males but not in females. Minimal data were presented on body weight changes but there were clear differences between at least some of the treatments. There were some increases in both erythrocyte count and hemoglobin in the smoke-exposed animals (Dontenwill et al. 1974).

The authors considered that the major effects of the smoke exposures were changes in the larynges, and they presented a 6-point scale for describing these changes (Dontenwill et al. 1974). The lower scores (1 to 2) represented slight epithelial metaplasia, stages 3 and 4 were considered comparable to skin papillomas of mice, and stage 5 was a *"pseudoepitheliomatous leukoplakia,"* although the authors recognized that this definition could be considered differently by different investigators. Stage 6 was described as "early invasive carcinomas" *in which "infiltrative growth was found."* Note, however, that the authors state that the "*early invasive carcinoma*" did not in fact invade cartilage, and no metastases were observed.

Because the animals were exposed to a fixed dilution of smoke from cigarettes with very different yields, the authors accepted that not all of the groups could be directly compared. Using only the scores of 5 and 6 described above, virtually every treatment that could be realistically compared with the reference cigarette showed a major reduction in the overall response. The greatest reduction in biological response resulted from cigarettes with acetate, cellulose, and charcoal filters, cigarettes with reconstituted tobacco sheets with and without sodium nitrate, and black cigarettes. The laryngeal changes for the reference cigarette were 30% for stage 5 and 10.6% for stage 6; the lowest scores of all the other treatments were 10% for stage 5 and 1.25% stage 6 for the black cigarettes (Dontenwill et al. 1974).

Laryngeal effects of the other treatments were absent: the added asbestos could not be evaluated "*because the amount of asbestos applied was too small*" even though pigment-free macrophages and asbestos bodies were prominent in lungs of animals pretreated with asbestos. DEN treatment did not affect the laryngeal response: possibly because "*nitrosamine is not an important factor in tobacco smoke carcinogenesis*." The animals exposed the vapor phase only did not show any stage 5 or 6 laryngeal responses, leading the authors to state "*the carcinogenic factors were assumed to be mainly in the particulate phase*."

There was a single lung carcinoma: in the DMBA plus smoke group. There were no effects of smoke exposure on pulmonary emphysema, pneumonia or bronchitis.

DOGS WITH TRACHEOTOMY

Hammond and coworkers (Hammond et al. 1970) exposed 89 male "pedigree" beagle dogs to cigarette smoke via tracheotomy, with a further 8 animals receiving tracheotomy but no smoke. The aim of this work was to determine whether "*dogs smoking cigarettes equipped with efficient filters would develop pulmonary emphysema and fibrosis to a greater degree, if at all, than dogs smoking the same number of non-filter cigarettes*" (pulmonary neoplasia was not a planned end point). The unfiltered cigarettes used produced 35 mg of tar and 1.85 mg of nicotine; the filter cigarettes had yields approximately half this. Animals were "*habituated to smoke*" over a 56-day period, using cigarettes with filters. After this period animals were exposed to smoke twice daily for up to 875 days. The following groups were used: F: filter cigarette, 7 cigarettes/day, 17 mg tar /cigarette, 12 animals; L: nonfilter cigarette, 3.5 cigarettes/day, 35 mg/cigarette, 12 animals; and H: nonfilter, 7 cigarettes/day, 35 mg/cigarette, 24 animals. Group h was a replicate of group

H, where the 38 animals were used "*for a preliminary long-term experiment on the effects of smoking non-filter cigarettes*" (see below) (Hammond et al. 1970).

Animals in the F, H, and h groups were exposed to the smoke from approximately 6400 cigarettes over the 875 days; in the L group the number was 3100 cigarettes. From these numbers a calculation was made of the amount of tar "delivered" to a dog over the 875 days: around 100 g/dog in the F and L groups and 200 g/dog in the H and h groups. Comparable calculations for nicotine were 5 to 7 g/dog in groups F and L, and 11 g/dog in groups H and h. No analysis was made of smoke chemistry, and no measurement of blood COHb was made.

Twenty-eight of the 86 long-term smoke-exposed dogs (30%) died during the course of the exposures, and the death rates "*paralleled the dosage of tar and nicotine*." The principal causes of death reported were pulmonary edema, bronchial pneumonia, pulmonary fibrosis, emphysema, and cor pulmonale. At necropsy, bronchioloalveolar tumors were found in 16 of the 28 mortalities: 12 noninvasive tumors and 4 invasive tumors. The main conclusion of this paper was that "*smoking cigarettes equipped with efficient filters produces less pulmonary fibrosis and emphysema in male beagle dogs than smoking the same cigarettes with the filters removed, duration of smoking and number of cigarettes smoked per day being the same*."

The pulmonary neoplasms were described in a second paper, the well-known "*Auerbach beagle study*" (Auerbach et al. 1970). In this paper noninvasive bronchioloalveolar tumors (not "carcinomas") were reported in both smoke-exposed and control animals (2 of the 8 animals in the nonsmoking control group had tumors). The lesion was described as nests of neoplastic foci, invariable associated with a bronchiole, with apparent communications between the bronchiolar lumen and the surrounding acini in the tumor. In some of the higher-dose animals the tumor was termed "*invasive*": here there was an "*extension of tumor cells through the basement membrane into the underlying stroma, accompanied by destruction of alveolar architecture and formation of confluent tumor masses*." Many of the bronchioloalveolar tumors were found by microscopic analysis, rather than by gross examination. Auerbach showed clearly in other work (Auerbach and Garfinkel 1991) that the bronchioloalveolar tumor is unrelated to smoking, with a possible involvement of viral oncogenes. Such an involvement could explain the presence of the tumors in each of the groups, including the controls.

Two animals showed invasive squamous cell carcinomas in a bronchus. Both animals had been exposed for approximately 880 days, to smoke from 6200 cigarettes. In no animal was any metastasis reported.

It is surprising that the fate of the group h animals (26 animals alive after 875 days of exposure, or 48% of the total study survivors) is not given in this article (or apparently, in any other article), except to say that "*no Group h dogs were killed*" (Auerbach et al. 1970). Other dog studies have also not been published, including a large 2-year study on atherogenesis (Hazleton 1981).

DOGS WITHOUT TRACHEOTOMY

Cross and colleagues (Cross et al. 1982) exposed male and female beagle dogs to smoke from 10 or 20 cigarettes per day, 7 days a week, for up to 65 months. Other groups of animals were also exposed to radon, radon daughters and uranium ore dust, plus a combination of these with cigarette smoke. The 1R1 reference cigarette was used. A deposition study was performed, using ^{14}C-labeled dotriacontane. The dotriacontane data confirmed that about 30% of the inhaled smoke was, in fact, deposited in the lung, in approximate agreement with the COHb concentrations (around 5%).

Dogs exposed to 10 cigarettes/day had no significant respiratory lesions. However, three of the dogs exposed to smoke from 20 cigarettes per day had severe respiratory tract changes, including focal areas of pleural thickening, alveolar fibrosis and sub-pleural inflammation. The authors stated, however, that "*the quantity of smoke from that number of cigarettes was very high when compared on an organ or body weight basis and, if inhaled may be unparalleled in all but the most avid of human cigarette smokers*." In the animals exposed to radon daughters and uranium ore dust alone, the histopathological changes were much more prevalent and severe than those in the combined exposures plus cigarette smoke groups, termed "*mitigation*." Some of these dogs had adenomatous lesions that progressed to squamous metaplasia of the alveolar epithelium, epidermoid carcinomas (associated with large cavities noted within the lung parenchyma), and bronchioloalveolar carcinoma (Cross et al. 1982).

The overall incidence of lung tumors was 37% in the animals exposed to radon, radon daughters, and uranium dust, but only 5% (one animal) in the group with added cigarette smoke. Neoplastic changes were also prominent in the nasal mucosa, where again the radon daughters plus cigarette smoke group showed a lower incidence of nasal carcinomas than did the radon daughters alone. There were no tumors (pulmonary or nasal) in the group of animals exposed to smoke alone.

The results of the experiment indicated that cigarette smoke had a mitigating or "beneficial" effect on radon daughter–induced respiratory tract cancer. This difference was statistically significant, and the authors suggest two reasons for this. First, that smoking could cause an increase in mucus production that would result in a smaller radiation dose to bronchial and bronchiolar epithelial cells, and second, the amount of cigarette smoke inhaled could have a net stimulatory effect on mucociliary clearance (Cross et al. 1982).

NONHUMAN PRIMATE

Rogers and coworkers (Rogers et al. 1988; Rogers, McCullough, and Caton 1981) exposed baboons *(Papio cynocephalus anubis)* to the smoke from unfiltered 2R1 reference cigarettes (37 mg tar, 2.5 mg of nicotine) per day for periods of up to 3.3 years. A total of 30 male and 25 female baboons were used in separate experiments, with subgroups of animals placed on

an atherogenic diet. Animals were taught (operant conditioning with water rewards) to inhale smoke through the mouth, using in some cases a double-puff technique to "*simulate the smoking pattern of many humans*." In one experiment the baboons took an average of 311 puffs per day, and in the other the average was 511 puffs per day. Animals had puff volumes that were close to the standard of 35 ml; puff durations were approximately 4 s. Blood COHb concentrations were approximately 1% in the smoke-exposed animals and 0.3% in the controls. Urinary cotinine and serum thiocyanate concentrations were also used to demonstrate smoke inhalation.

A number of different parameters were examined in this study, including hematologic variables and atherosclerotic lesions (Rogers et al. 1988). The latter were obtained through histologic sections of the abdominal aorta. Cigarette smoking for 1.6 or 2.8 years did not increase the extent of diet-induced experimental atherosclerosis. There were no differences between smoke-exposed and control animals in the extent of fatty streaks or in the prevalence of fibrous plaques. The authors stated "*it seems unlikely that the failure to observe a substantial effect of cigarette smoking on experimental atherosclerosis is due to use of an ineffective method of smoke exposure*." The necropsy and histopathology results were restricted to the cardiovascular system.

SUMMARY OF OLDER STUDIES

Significant increases in the numbers of malignant tumors of the respiratory tract were not seen in rats, mice, hamsters, dogs, or nonhuman primates exposed for long periods of time to very high concentrations of cigarette smoke (Coggins 1998, 2001).

As such, the results are clearly at variance with the epidemiological evidence in smokers (Doll et al. 2005), and it is difficult to reconcile this major difference between observational studies in humans and controlled laboratory studies in five different animal species.

MORE RECENT WORK

Recent studies from the Lovelace Respiratory Research Institute have been more successful than those described above. Two of these studies are described in considerable detail in an adjacent article (Hahn 2007).

Briefly, a 30-month inhalation study in F-344 rats showed a statistical increase in the incidence of neoplastic lung lesions, in females only (Mauderly et al. 2004). The incidence of bronchioloalveolar carcinomas in female rats was 0% in controls and 4.9% for the high-smoke-exposure groups; incidences in males were 2.5% and 6.1%, respectively.

In the second study, whole body exposures of female $B6C3F_1$ mice to mainstream smoke produced statistical increases in the incidence of focal alveolar hyperplasias, and pulmonary adenomas, papillomas, and adenocarcinomas (Hutt et al. 2005). The incidence of pulmonary adenocarcinomas was 2.8% in controls and 20.3% in the smoke-exposure groups.

Work with ferrets (*Mustela putorius furo*) is showing promise but so far only very small numbers of animals have been used. In a 6-month study, 6 out 12 ferrets exposed to both NNK injection and cigarette smoke developed grossly identifiable lung tumors, whereas none of nine ferrets from the sham treatment group developed any lung lesions (Kim et al. 2006). The histopathological types of these tumors (squamous cell carcinoma, adenosquamous carcinoma and adenocarcinoma) in ferret lungs are very similar to those in humans. In addition, 10 out of 12 ferrets exposed to both NNK and cigarette smoke developed preneoplastic lesions (squamous metaplasia, dysplasia, and atypical adenomatous hyperplasia) with complex growth patterns, whereas the sham group did not show any of these lesions. The development of both preneoplastic lesions and gross lung tumors in ferrets may provide a "*model for studying lung cancer chemoprevention with agents such as carotenoids, and for studying the molecular mechanism of carcinogenesis in the earlier stages of smoke-related lung cancer*" (Kim et al. 2006).

Nose-only exposure of male and female Wistar rats to a surrogate for environmental tobacco smoke, termed room-aged sidestream smoke (RASS), to diesel engine exhaust (DEE), or to filtered, fresh air (sham exposure), was performed 6 h/day, 7 days/week, for 2 years, followed by a 6-month postexposure period (Stinn et al. 2005a). The particulate concentrations were 3 and 10 mg/m^3. Markers of inflammation in bronchoalveolar lavage fluid showed that DEE (but not RASS) produced a dose-related and persistent inflammatory response, as noted elsewhere (Friedrichs, Miert, and Vanscheeuwijck 2006). Lung weights were increased markedly in the DEE (but not RASS) groups and did not decrease during the 6-month postexposure period. Bulky DNA adducts increased in the lungs of RASS groups, but not in the DEE groups. Cell proliferation in the lungs was unaffected by either experimental treatment.

Histopathological responses in the RASS groups were minimal and almost completely reversible; lung tumors were similar in number to those seen in the sham-exposed groups. Rats exposed to DEE showed a panoply of dose-related histopathological responses: largely irreversible and in some cases progressive. Malignant and multiple tumors were seen only in the DEE groups; after 30 months, the tumor incidence (predominantly bronchiolo-alveolar adenomas) was 2% in the sham-exposed groups, 5% in the high RASS groups, and 46% in the high DEE groups (sexes combined) (Stinn et al. 2005a).

The results suggest that in rats exposed to DEE, but not to RASS, the following series of events occurs: particle deposition in lungs → lung "overload" → pulmonary inflammation → tumorigenesis, without a significant modifying role of cell proliferation or DNA adduct formation (Stinn et al. 2005a).

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