THE TOXICOLOGY OF ENVIRONMENTAL TOBACCO SMOKE

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ABSTRACT

It has by now become obvious that environmental tobacco smoke (ETS) may pose a health risk to nonsmokers. Epidemiological data suggest that exposure to ETS may increase the risk of developing lung cancer, cardiovascular disease, intrauterine growth retardation, predisposition to chronic lung disease, and sudden infant death syndrome. The human populations most at risk from ETS exposure appear to be neonates, young children, and possibly the fetus while in utero. Experimental studies with cigarette sidestream smoke (SS) have successfully duplicated several of these disease conditions in laboratory animals, particularly the effects of SS on fetal growth, lung maturation, and altered airway reactivity. The availability of animal models may open the way to fruitful experimental studies on mechanisms that help us to better understand disease.

THE HEALTH EFFECTS OF INVOLUNTARY SMOKING

General

During the past two decades, public health officials have warned that environmental tobacco smoke (ETS) might constitute more than just a nuisance to nonsmokers (1, 2). The results of many epidemiological studies imply that ETS adversely affects the health of nonsmokers. A causal association between ETS exposure and lung cancer appears to exist, and nonsmokers married to smokers have a statistically significant (20–30%) increased risk of developing
lung cancer (3, 4). In the United States, an estimate of ETS-attributable lung cancer mortality was derived from a meta-analysis of 11 epidemiological studies; researchers concluded that ETS might be responsible for approximately 3000 lung cancers per year in nonsmokers aged 35 and over. ETS has been classified as a human carcinogen (5). What remains less certain for the time being is whether exposure of the fetus and of young children to ETS increases their risk of developing cancer in adulthood. Although such an association is plausible, more studies will be needed to yield conclusive evidence (6).

Cancer is not the only adverse health effect that may be linked to passive smoking. In adults, an association has been found between passive smoke exposure at the workplace and chronic respiratory symptoms such as wheezing and cough; the severity of the signs was dependent upon hours per day of smoke exposure (7). However, to what extent ETS contributes overall to chronic obstructive pulmonary disease and asthma in adults remains uncertain (8). Involuntary smoking may be a critical factor in the development of cardiovascular disease (9, 10), and in the United States approximately 35,000 to 40,000 deaths a year may be attributable to exposure to ETS. It has been suggested that passive smoking during pregnancy may result in a small, although significant reduction of birth weight (11, 12), and smoking in the same room where small infants are kept appears to increase the risk of sudden infant death syndrome (13). Whether ETS may compromise immune function in humans appears to be less certain (14). However, an ever growing body of evidence suggests that ETS may adversely affect the health of many nonsmokers. Undoubtedly, ETS is a substantial public health problem.

Children
Perhaps the most obvious untoward effects of passive smoking are found in children and are a major concern (5, 15). Epidemiological studies have clearly shown that children raised in homes with smokers have more coughing (15–17), wheezing (16), sputum production (16), and respiratory illnesses (15, 17–19); decreased FEV1 (20, 21), FEV1/FVC (22), FEF25–75 (21); and increased airway reactivity (24–26) compared with children raised in homes without smokers. Children exposed to smoke also have an increased rate of asthma (23, 27), an increased likelihood of using asthma medications, and an earlier (first year of life) onset of asthma (27). For children with asthma, smoke exposure is associated with more severe asthma and greater airway reactivity to histamine (28), cold air (21), and exercise (26).

Exposure to the mother’s active smoking rather than the father’s correlates best with pulmonary problems in children (19–21, 25, 27, 28). One explanation for this is that maternal smoking affects the lungs of the fetus as it develops in utero. Another explanation is that mothers are physically closer to their
children while providing for their care postnatally, thus exposing the children to a larger dose of ETS. In support of the in utero hypothesis, Martinez et al (25) showed that airway hyperreactivity was present in 70% of children whose mothers smoked during pregnancy, as compared with only 29% of children whose mothers did not smoke during pregnancy. However, the effect of the mother’s current smoking status could not be statistically separated from the prenatal effects. Hanrahan et al (29) showed that expiratory flow at FRC was 74.3 ml/s in 4-week-old infants born of continuous smokers vs 150 ml/s in infants born of nonsmokers. This was a highly statistically significant finding. Using multiple regression models, Hanrahan et al (29) found that postnatal ETS exposure was not related to the pulmonary function of these infants. However, their definition of ETS exposure involved such a small exposure (exposure to smokers at least 2 h twice a week) that a postnatal ETS effect may have been missed. Cunningham et al (30) studied 8863 children and found that their lung function at 8–12 years of age was lower if the mother smoked during pregnancy. After adjusting for smoking during pregnancy, they found that current maternal smoking was not associated with lower lung function. The 75 children of mothers who smoked during pregnancy but not after their birth were found to have an 11% lower FEF$_{25-75}$ than the never-exposed children. Children whose mothers did not smoke during pregnancy but who smoked during their children’s first 2 years of life had a 2.8% lower FEF$_{25-75}$. These data suggest that lung function is most compromised by in utero exposure to mainstream smoke, although early postnatal exposure to ETS may play a role.

If such is the case for in utero mainstream smoke exposure from the mother, perhaps the same is true for in utero ETS exposure through the mother. Scherer et al (31) estimated that a 20 cigarette/day mainstream smoker as compared with a 8 h/day passive smoker receives only a 1.5- to 4-fold higher dose of certain gaseous phase constituents of cigarette smoke such as carbon monoxide, formaldehyde, volatile nitrosamines, and benzene. Thus substantial doses of ETS constituents may be absorbed by the mother and passed to the fetus. Changes in respiratory status of infants have indeed been shown in situations where the mother is not a smoker but is exposed to ETS throughout pregnancy followed by direct exposure of the child to ETS after birth. In a study of 3285 infants in Shanghai whose mothers were nonsmokers, there was a 4.5-fold increase in respiratory hospitalizations in low-birth-weight infants during their first 18 months of life if they were raised in a household where more than 20 cigarettes/day were smoked. It is not known whether it was the in utero ETS exposure or the postnatal ETS exposure that was responsible for the changes in respiratory status of the infants (31a).

The conclusions reached by regulatory agencies concerning the health effects of ETS have not gone unchallenged. Epidemiological studies have been
criticized for incorrectly dealing with confounding factors such as misclassification of smokers and nonsmokers, recall bias, dietary and socioeconomic factors, and correct assessment of exposure conditions (32–35). The debate pitches officials concerned with public health against the interests of the tobacco industry. It seems to have reached an impasse mainly because of substantial gaps in the available data base, particularly on exposure to ETS, and also because of substantial differences in opinion on how to interpret available data (34, 36–38).

EXPERIMENTAL TOXICOLOGY OF ENVIRONMENTAL TOBACCO SMOKE

General

To toxicologists, ETS poses a challenging problem. Experimental toxicology can explore mechanisms of toxicity. It can also establish an experimental basis for definition of boundaries of exposure, i.e. design experiments that eventually can delineate conditions of exposure not likely to produce certain toxic effects. For carcinogenicity, no such boundaries can be defined; it is generally accepted that even exposure to small amounts of a potential human carcinogen is associated with a finite risk. On the other hand, for noncancer effects, such as reduction in birth weight or enhanced development of cardiovascular disease, exposure thresholds may exist. Within the limits of its methodology, experimental toxicology can provide some guidance that might help to estimate the impact of ETS exposure on human health.

In order to conduct experimental studies with ETS, it is necessary to have proper exposure systems and facilities. Essentially, ETS is a mixture of cigarette sidestream smoke (SS) and mainstream smoke (MS). MS constitutes 15% of total ETS and is smoke first inhaled by an active smoker and then exhaled; while being retained for a few moments in the lung, the smoke is scrubbed of some of its constituents, most notably nicotine and CO, as well as much of the particulate matter. ETS contains about 85% SS, the smoke curling between puffs off the end of a lit cigarette. SS is generated at lower burning temperatures than is MS and has a different chemical composition than has MS. Most notably, it is richer in certain carcinogens than is MS (5, 39–41). It also undergoes rapid aging, a process that may change its physico-chemical properties (41).

Exposure of nonsmokers to ETS is difficult to estimate. Particulate matter is a commonly measured indoor air pollutant. It is generated in part from MS and SS. Indoor measurements show a wide range of total suspended particulates (TSPs), and it is usually estimated that in an environment where people smoke, about half of the TSPs originate from SS and MS. Indoor air concentrations of TSPs may range from 10 to 1000 \( \mu g/m^3 \); personal exposure of nonsmokers to particulate matter associated with ETS has been estimated to range from 18 to
Table 1 Exposure conditions in animal studies with cigarette SS

<table>
<thead>
<tr>
<th>Total suspended particulates (mg/m³)</th>
<th>Nicotine (µg/m³)</th>
<th>CO (ppm)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>Not detected</td>
<td>3.6</td>
<td>57</td>
</tr>
<tr>
<td>0.3</td>
<td>88</td>
<td>4–5</td>
<td>107</td>
</tr>
<tr>
<td>1.0</td>
<td>350–400</td>
<td>5–6</td>
<td>81</td>
</tr>
<tr>
<td>2.5</td>
<td>87–128</td>
<td>4–5</td>
<td>67</td>
</tr>
<tr>
<td>4.0</td>
<td>1,011</td>
<td>17</td>
<td>53</td>
</tr>
<tr>
<td>10</td>
<td>1,708</td>
<td>57</td>
<td>59</td>
</tr>
<tr>
<td>87</td>
<td>16,000</td>
<td>246</td>
<td>54</td>
</tr>
</tbody>
</table>

64 µg/m³ (5, 41). Exposure to airborne nicotine is usually 10 µg/m³ or less; however, under special conditions, such as in badly ventilated rooms or in cars, it may be five to ten times higher (41). As far as CO is concerned, exposures of CO to the general public usually do not exceed 4 ppm.

Because it is not feasible to expose animals to a mixture of 85% SS and 15% human lung–modified MS, SS alone or reinforced with puffs of MS is used as an experimental substitute for ETS. Systems that can properly generate cigarette SS for animal inhalation experiments have been described (42, 43). Concentrations of TSPs, nicotine, and CO that are usually observed in such studies are listed in Table 1, as a comparison with what may be encountered in the “real world.” In this section, the results of studies obtained by exposing laboratory animals to properly generated SS are reviewed and are summarized in Table 2.

Carcinogenesis

It would seem that one of the most pressing problems would be to test SS in long-term animal inhalation studies in order to obtain an estimate of its carcinogenic potency. Such data might reinforce the conclusions drawn from epidemiological studies. SS might even be a more potent carcinogen than is MS. Cigarette SS condensates are considerably more efficient in producing mouse skin tumors than are MS condensates (44). On the other hand, there seems little incentive to conduct such expensive studies. It is most plausible that SS, a human carcinogen, can produce lung tumors in experimental animals. However, it might be more difficult to actually provide unequivocal proof, since it has been notoriously difficult to produce lung tumors in experimental animals with cigarette smoke. The available data on studies with MS conducted between 1960 and 1985 were critically evaluated in 1986 by the International Agency for Research on Cancer (IARC) (45). Out of four rat studies that were judged to be adequate for critical analysis, only one yielded unequivocal evidence for tobacco smoke being a respiratory tract carcinogen. Tumor incidence in the exposed
Table 2

<table>
<thead>
<tr>
<th>Exposure concentration (mg TSP/m³)</th>
<th>Duration</th>
<th>Species</th>
<th>Effects</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>Up to 90 days</td>
<td>Rat</td>
<td>No observable effects in respiratory tract tissues</td>
<td>59</td>
</tr>
<tr>
<td>0.3</td>
<td>From birth up to 120 days</td>
<td>Rat</td>
<td>Increased activity of CYP1A1 in airway epithelia</td>
<td>107</td>
</tr>
<tr>
<td>0.5</td>
<td>From birth up to 120 days</td>
<td>Rat</td>
<td>Increased activity of CYP1A1 in alveolar cells</td>
<td>107</td>
</tr>
<tr>
<td>1.0</td>
<td>5 days</td>
<td>A/J mice</td>
<td>Increased cell proliferation in airways</td>
<td>63</td>
</tr>
<tr>
<td>1.0</td>
<td>Pregnancy</td>
<td>Rat</td>
<td>Intrauterine growth retardation</td>
<td>75</td>
</tr>
<tr>
<td>1.0</td>
<td>From birth up to 15 weeks</td>
<td>Rat</td>
<td>Altered airway reactivity, decreased pulmonary compliance</td>
<td>96, 97</td>
</tr>
<tr>
<td>1.0</td>
<td>8 to 43 days</td>
<td>Guinea pigs</td>
<td>Decreased airway reactivity of C-fiber system</td>
<td>98</td>
</tr>
<tr>
<td>1.0</td>
<td>1 week</td>
<td>Hamster</td>
<td>Increased cell proliferation in nasal septum</td>
<td>64</td>
</tr>
<tr>
<td>2.5</td>
<td>16 weeks</td>
<td>Cockerels</td>
<td>Promotion of arteriosclerotic plaque development</td>
<td>67</td>
</tr>
<tr>
<td>4.0</td>
<td>90 days</td>
<td>Rat</td>
<td>Hyperplasia/metaplasia nasal epithelium</td>
<td>62</td>
</tr>
<tr>
<td>60.0</td>
<td>10 weeks</td>
<td>Rabbit</td>
<td>Enhanced formation of arteriosclerotic plaques in cholesterol-fed animals</td>
<td>68</td>
</tr>
<tr>
<td>90.0</td>
<td>20 weeks</td>
<td>A/J mice</td>
<td>Enhanced lung tumor formation</td>
<td>54</td>
</tr>
</tbody>
</table>

More recently, Finch et al (47) reported that in female rats kept for at least 12 months in an atmosphere containing 250 mg/m³ of tobacco smoke particulate matter, lung tumor prevalence was about 7%, not exactly an overwhelming response. Hamsters developed laryngeal tumors only, but no tumors in the deep lung (45). The 1986 IARC monograph also analyzes six chronic mouse studies. The aggregated data of these studies show that out of an overall of total of 1703 mice exposed to tobacco smoke, only 108 animals (6.3%) developed lung tumors. Strains examined were C57Bl, BLH, C57Bl/Mil, Snell’s, and BALB/c. In the corresponding control groups, tumors were found in 39 animals (3.9%) out of 998 animals. In another large study conducted in one single laboratory, some 1600 BC3F1/Cum mice were exposed to tobacco smoke for 110 weeks. Lung tumor incidence in the exposed animals was again comparatively low, 5%, and in controls was 4.3% (48). An overall evaluation of the animal carcinogenicity data reported in the IARC document leads to the inevitable conclusion that under usual conditions of experimental exposure and in most rat and mouse strains, tobacco smoke is a comparatively weak carcinogen and produces few tumors.
Strain A/J mice are much more sensitive to carcinogens than other mouse strains and readily develop multiple lung adenomas and adenocarcinomas when challenged (49). Early studies with A/J mice on the effect of MS on lung tumor development were inconclusive (49–52). Furthermore, these early experiments lacked good exposure data, i.e. they failed to report precise measurements of the concentrations of smoke constituents within the exposure chambers. This makes it difficult to relate the results of the previous studies to contemporary protocols. A recent experiment reexamined the effects of SS on lung tumor development in strain A/J mice. Male mice were exposed, for 6 h/day, 5 days/week, to a chamber concentration of 4 mg/m$^3$ of TSPs generated from burning Kentucky 1R4F reference cigarettes. Six months later, lung tumor incidence and lung tumor multiplicity was identical in the lungs of the SS-exposed mice compared with the controls. This study failed to provide evidence for a tumorigenic effect of SS in strain A/J mice (53). In a second experiment, the A/J mice were exposed to a much higher concentration of TSPs (87 mg/m$^3$). After five months of smoke exposure, tumor incidence and multiplicity was slightly, but not significantly, higher in the SS-exposed animals. It was only after an additional four-month recovery period in fresh air that SS-exposed animals showed a significant increase in tumor incidence and in tumor multiplicity (54). The data seem to indicate that under appropriate conditions of exposure, SS does produce pulmonary tumors in mice, although the SS concentrations required to have a demonstrable effect are high. Finch et al (55) exposed strain A/J mice for 26 weeks to 248 mg/m$^3$ of TSPs and gave them a smoke-free recovery period of only 5 weeks, but this did not have any effect on lung tumor initiation or promotion (55).

**Pulmonary Effects in Adult Animals**

A few studies examined the effects of prolonged exposure to SS on the respiratory tract tissues of adult animals. A recent study describes that passive inhalation of tobacco smoke produced, within three months, emphysematous changes in rat lungs (56). The data are difficult to evaluate because no information on SS concentrations within the exposure chamber is available. Proper characterization of exposure conditions is provided in the studies by Coggins and coworkers (57–61). They exposed rats to three different concentrations of SS: 0.1, 1.0, and 10.0 mg/m$^3$ of TSPs. Exposure was 6 h/day, 5 days/week for 2 weeks and for 13 weeks. A complete histopathological evaluation was done, and additional studies included measurement of replicative DNA synthesis and of DNA adducts in respiratory tract tissues and cytogenetic evaluation of alveolar macrophages. NOELs (no observed effect levels) for histopathology, cell replication, and DNA adduct formation were found to be at least 1 mg/m$^3$ of TSPs, and for chromosomal aberrations in the macrophage population at least
10 mg/m$^3$. The only notable change was in the nasal turbinates of rats exposed for 90 days to the highest SS concentration, characterized by slight epithelial hyperplasia in the rostral nasal cavity (59). The authors concluded that a NOEL for SS inhalation was likely to be higher than 1 mg/m$^3$ of SS. Essentially no effect of SS was also found in a 90-day study in which rat and hamsters were exposed to 4 mg/m$^3$ of TSPs (62). Hyperplasia and metaplasia in the epithelium of the dorsal nasal turbinates in rats were the only changes found; no signs of smoke toxicity were observed in the hamster respiratory tract. On the other hand, we found that short-term (5 days) exposure of A/J mice to SS enhanced cell proliferation in the airway epithelia, and no effect was seen when the particulate phase of SS was removed with a filter (63). Increased cell proliferation was found in the nasal epithelia of Syrian golden hamsters exposed to 1 mg/m$^3$ of TSPs for up to three weeks (64). The observations show that in A/J mice and in hamsters, 1 mg/m$^3$ does not necessarily constitute a NOEL.

**Cardiovascular Changes**

Passive smoking is a risk factor in the development of cardiovascular disease. The fact that nonsmokers do not seem to have “adapted” to the many toxic agents in smoke makes ETS a serious threat to many people (9, 10, 65). Cardiovascular changes resulting from exposure to SS have been successfully demonstrated in several animal models. In a series of studies, Penn and coworkers (66, 67) found that a 16-week–long exposure of cockerels (6 h/day, 5 days/week) to 2.2 to 2.8 mg/m$^3$ of TSPs is sufficient to significantly increase the size of arteriosclerotic plaques in the aorta. Increased development of arteriosclerotic lesions was also found in rabbits fed a cholesterol-rich diet. After 10 weeks of exposure to 4 and 33 mg/m$^3$ of TSPs (6 h/day, 5 days/week), a dose-dependent increase in the size of arteriosclerotic lesions was found in the aorta and the pulmonary artery (68). Platelets of the SS-exposed animals displayed increased stickiness. The development of the lesions could not be attenuated by metoprolol (69). SS also increased infarct size in a rat coronary artery ligation–reperfusion model (70). Although in these experiments the exposure concentrations to TSPs were quite high (60 mg/m$^3$), the authors emphasized that similar concentrations of ETS might be easily obtained inside passenger cars carrying smokers.

**Intrauterine Growth Retardation**

There is overwhelming evidence that active maternal smoking during pregnancy has harmful effects on the fetus (71, 72). On average, babies born to women who smoke during pregnancy are 200 g lighter than those born to women who do not smoke. There is a clear dose-response relationship for this effect, and the reduced birth weight is due to retardation of intrauterine growth. It is less certain whether exposure of pregnant women to ETS has a similar effect. The
average birth weight of babies in mothers exposed to cigarette smoke from their smoking husbands was reduced approximately 120 g for each pack of cigarettes smoked per day by the father (11); exposure during pregnancy for at least 2 h/day to ETS at home or at work reduced the average birth weight by 24 g (12). Although the conclusions drawn from these studies have occasionally been questioned (73), it is plausible that exposure to ETS during pregnancy may produce a small degree of intrauterine growth retardation.

Some animal data support this conclusion. Pregnant Sprague-Dawley rats were exposed to SS for 6 h/day, at a concentration of 1 mg/m$^3$ of TSPs, on days 3, 6–10, and 13–17 of pregnancy and killed on day 20 of gestation. No differences were found in maternal body weight gain or average daily food consumption between smoke-exposed and pair-fed controls, and the number of fetuses and of implantation sites per litter were comparable. However, there was a small but significant reduction in mean pup weight. This was not accompanied by any significant decrease in fetal ossification, an index of gestational age. This suggests that exposure to SS under the present conditions produced intrauterine growth retardation (74, 75). In another study, rats were exposed for 2 h/day on days 1 through 20 of pregnancy to the smoke given off by 10 king-size cigarettes. The average litter weight ($n = 8$) in the SS-exposed animals was reduced to 91% of the control values (76). Unfortunately, the study does not contain any information on the concentrations of SS constituents within the inhalation chamber, but from the fact that the animals were kept in small (6 liter) plexiglass chambers, it might be inferred that smoke concentrations must have been higher than 1 mg/m$^3$ of TSPs. In several other studies, decreased overall fetal weight in rats exposed to MS has been found. The extent of body weight reduction ranged from being comparable to what was found in (75) experiments (approximately 5% weight reduction) to a fetal weight reduction of 30% (to 70% of control values) in one study that used extreme exposure conditions (77). A direct comparison between the different experiments is not possible, however, because of the different exposure regimens (days of exposure, length of exposure to MS) and because, as in the Leichter study (76), chamber atmospheres were either not or only incompletely characterized.

**Effects of SS on Lung Maturation**

It is well established that active and possibly passive smoking adversely affect pre- and postnatal development. Increased risk of premature delivery and spontaneous abortions associated with tobacco smoke exposure during pregnancy has been reported (78). Particularly, in utero exposure to tobacco smoke may reduce lung volume at term, reduce formation of saccule partitions (79), and increase the volume of interstitium in the lung parenchyma (80). These findings, however, could not account for several observations made in epidemiological
studies such as the relationship between ETS exposure in young children and increased risk of developing lung cancer as adults. This makes it necessary to more closely examine possible effects of SS in neonatal animals in which the lungs undergo rapid growth and cellular differentiation.

The effects of SS (1 mg/m³ of TSPs, 6 h/day, 5 days/week) on bronchiolar epithelial cell development and the expression of cytochrome P450 isozyme 1A1 protein in the developing postnatal rat lung were recently studied (81). In control animals, the labeling indices for epithelial cells in proximal bronchi and terminal bronchioles at 7 days of age were 2.3% and 4.9%, respectively, and decreased to 0.1% and 0%, respectively, by 100 days of age. With exposure to SS from birth, the labeling index of epithelial cells in distal airways of rats was significantly reduced at 7 and 14 days of age, but not in epithelial cells of proximal bronchi. SS was also found to modulate the activities of pulmonary mixed function oxidases. The expression of P450 isozyme 1A1 antigen in bronchiolar epithelial cells of control rats reached the maximal level observed at 21 days of age and subsequently decreased to low levels at 50 and 100 days of age. In contrast, exposure to SS significantly increased the distribution and intensity of staining for 1A1 antigen in bronchiolar epithelial cells of proximal and distal airways as early as 7 days of age and maintained elevated levels of 1A1 protein in these cells through 100 days of age. At 21 and 50 days of age, NADPH reductase protein expression was higher in the airway epithelium of rats exposed from birth to SS than that noted in the airways of controls. In contrast, cytochrome P450 isozyme 2B and Clara cell secretory protein (CCSP) expression were unchanged. The studies show that exposure to SS from birth interferes with proliferation of the epithelial cells in the terminal bronchioles but not in the proximal bronchi, accelerates and maintains expression of cytochrome P450 isozyme 1A1 protein in Clara cells during postnatal lung development, and increases bronchiolar expression of NADPH reductase but does not increase expression of cytochrome P450 isozyme 2B or CCSP. Postnatal exposure to SS may thus interfere, in the developing lung, with airway epithelial cell development.

While several earlier studies (81a–c) have shown that both MS and SS increase the activities of cytochrome P4501A1 and 2E1 in the lungs of adult animals, little information is available on the influence of SS on cytochrome P450 monoxygenase activity in the developing lung. The effects of SS on the developmental profiles of cytochrome P450 monoxygenases 1A1 and 2B1 in rat lung and liver were examined in animals exposed to filtered air or SS (1 mg/m³ of TSPs) from birth to 7, 14, 21, 50, and 100 days of age (82). Pulmonary P4501A1 activity (measured as ethoxyresorufin-O-dealkylase (EROD)] in control rats was not detected until 14 days of age. It then increased threefold between 14 and 21 days of age and remained unchanged to 100 days of age.
In contrast, in animals exposed to SS from birth, pulmonary EROD activities were detected as early as 7 days postnatal and were elevated three- to fourfold above control at all other ages examined. Hepatic EROD activities were unaltered by SS exposure. Short-term (4 day) SS exposure was as effective in up-regulating pulmonary microsomal EROD activities as were 100-day exposures. Induction of pulmonary EROD activities and the associated increases in mRNA levels were dependent upon the particulate fraction. Microdissection of the lung airways also allowed measurement of enzyme activities in anatomically defined regions of the respiratory tract. In the major and minor daughter airway subcompartments of the bronchial tree, EROD activities were three- to fourfold higher in SS-exposed animals compared with controls. In contrast, there was no enzyme induction in the trachea and less than a twofold increase was found in the parenchyma (Figure 1). Pulmonary pentoxyresorufin-O-dealkylase (PROD) activities, indicative of cytochrome P4502B1, developed more slowly than EROD and did not reach adult levels until day 50. SS did not alter pulmonary or hepatic PROD activities. These studies show that P4501A1 and P4502B1 develop at different rates in rat lung and liver and that exposure to SS markedly increases P4501A1 activities in the lung at all ages examined.

The significant effect on enzyme activity in the bronchial tree is most likely mediated by profound effects of SS on Clara cells. Nonciliated bronchiolar epithelial cells or Clara cells are characterized by electron-dense secretory granules and large amount of smooth endoplasmic reticulum in their cytoplasm.

Figure 1  The effect of SS on EROD activity in trachea, major daughter and minor daughter airway, and lung parenchyma from 50-day-old rats exposed to filtered air or whole SS for 6 h/day for 4 days. *, Significant difference between exposed and control values ($p < 0.05$). Values are means ±SD from four animals for each group. (Data from Reference 82, with permission.)
They are associated with several functions in adult lung including: (a) a progenitor role for ciliated cells and Clara cells; (b) synthesis, storage, and secretion of protein components found in the lining layer of the airways; and (c) metabolism of xenobiotic materials via the cytochrome P450 monooxygenase system. Unlike many other cell types in the lung, nonciliated bronchiolar epithelial (Clara) cell development is a postnatal phenomenon in many species, including mice (83), rabbits (84), rhesus monkeys (85), and humans (86).

Clara cells are targets for several toxicants because of their ability to generate reactive metabolites, by high levels of P450 monooxygenases found in these cells (87). Isozymes 1A1, 2B, and 4B and NADPH reductase (88) have been identified in the lungs of rabbits and rats. Boyd (89) proposed that Clara cells represent sites of cytochrome P450–dependent activity, as evidenced by the metabolic activation of the pulmonary toxin 4-ipomeanol. Subsequent enzyme activity measurements and immunocytochemical studies support this hypothesis. Domin et al (90) measured P450 monooxygenase activities of different isolated cell types of the rabbits and found that the activities of isozymes 2B and 4B and NADPH reductase were highest in Clara cell fractions when compared to type II cell fractions or whole lungs. Plopper et al (91) used antibodies raised against P450 monooxygenase isozymes 2B and 4B and NADPH reductase and demonstrated that these isozymes were distributed throughout the tracheobronchial airways of the rabbit in a similar pattern to that of the Clara cell. These proteins have been shown to be associated with smooth endoplasmic reticulum (SER) and plasma membranes of Clara cells (92).

It is not surprising that SS may affect Clara cells. Actually, this particular cell population may play a crucial role in SS toxicity. Clara cells are selectively damaged by several toxicants such as naphthalene and 4-ipomeanol in a species-selective manner. The mechanism(s) for species and cell selectivity is quite complex, but the presence of the cytochrome P450 monooxygenase system in Clara cells has been known to play a crucial role. Because this cell type is involved in metabolism of chemicals by the lung, it represents a likely target for a number of inhaled and systemically delivered agents in the lung. Various components in ETS could undergo metabolism by the P450 system to increase or decrease the relative toxicity of parent compounds. P450b (2B) and P450c (1A1) are involved in the bioactivation of NNK (nicotine-derived nitrosamine ketone) to a carcinogen (93). It has also been found that P450c is exclusively located in Clara cells in untreated rats (94), while P450b is much more concentrated in the Clara cells than in other cells of rat lung (95). Some ETS constituents may also function as inducers or inhibitors of P450 isozymes 1A1 and 2B and consequently alter the metabolic pathways of other compounds. In fact, isozyme 1A1 activity in the rat can be elevated twofold by SS exposure
(82). Clearly, the effects of ETS on Clara cells in the developing lung need further clarification.

**Effects of SS on Lung Function in Developing Organisms**

As we discussed above, prenatal and postnatal exposure to ETS may compromise the health of children. The relative importance of prenatal and early postnatal exposure on pulmonary symptoms, function, and airway responsiveness is difficult to evaluate by epidemiologic methods. Also, epidemiological studies can only show a relationship between smoke exposure and lung function; other variables such as early viral infections or exposure to other indoor and outdoor pollutants and antigens may confound the data. Animal studies may help to determine if in utero MS exposure, and/or postnatal ETS exposure, and/or postnatal ETS exposure is responsible for the pulmonary changes seen in children of smokers.

One such animal study examined the effects of postnatal ETS exposure on immature rats exposed postnatally to SS under controlled conditions (96). In rats exposed daily to SS (1 mg/m\(^3\) of TSPs, 6 h/day, 5 days/week) from age 2 days of life to 8–15 weeks of life, SS exposure did not change lung resistance, dynamic compliance, lung weight/body weight ratio, pulmonary artery pressure, or body weight. Airway responsiveness to increasing doses of methacholine, as a measure of nonspecific airway reactivity, also was not affected by SS exposure during postnatal development. Similarly, lungs from 15-week-old rats exposed to SS for 3 h or for 4 days showed no change in dynamic compliance, lung resistance, lung weight/body weight, pulmonary artery pressure, or airway reactivity to methacholine. Thus the study showed that exposing rats to SS in the early postnatal period did not change baseline pulmonary function or increase airway responsiveness as is seen in children raised in the homes of smokers. Interestingly, however, the study showed that rats exposed to SS from day 2 to week 11 of life developed airway hyporesponsiveness to serotonin. Since pulmonary artery responsiveness to serotonin was not changed by SS exposure, the effect seemed to be an airway phenomenon, perhaps representing down-regulation of serotonin receptors due to chronic serotonin secretion.

Pulmonary neuroendocrine cells were considered to be a likely candidate for the serotonin-secreting cell because they (a) are located in the airway epithelium; (b) contain serotonin as well as other bronchoconstrictors such as endothelin and bombesin; (c) are regulated perinatally such that they are greatest in number during late fetal life and decrease after birth; (d) are increased in number in diseases associated with reactive airways such as bronchopulmonary dysplasia, cystic fibrosis, and asthma; and (e) are stimulated to grow by nicotine. Thus, Joad et al (97) determined the effects of SS exposure on the number
of pulmonary neuroendocrine cells, baseline lung function, and airway responsiveness and extended the SS exposures to include the in utero as well as the postnatal period. In this study, pregnant Sprague-Dawley rats were exposed to filtered air or to SS (1 mg/m³ of TSPs, 6 h/day, 5 days/week) under controlled conditions from day 3 of gestation until birth. The female pups were then further exposed to either filtered air or SS for 7–10 weeks postnatally. Exposure to SS both pre- and postnatally resulted in lungs that were less compliant, much more reactive to methacholine (Figure 2), and had a 22-fold greater number of pulmonary neuroendocrine cells (Figure 3). Exposure to SS either just prenatally or just postnatally did not change lung compliance or airway responsiveness to methacholine (Figure 1). Although there was a trend toward an increase in neuroendocrine cell number with prenatal SS exposure alone and with postnatal SS exposure alone, the effects were not statistically significant (Figure 3).

Thus in the rat, SS exposure throughout the perinatal period, i.e. both pre- and postnatally, was required to reproduce the changes in lung function seen...
Figure 3  Number of pulmonary neuroendocrine cells (identified by neuron-specific enolase immunostaining) per centimeter basal lamina in lungs from rats exposed to in utero filtered air (FA) followed by postnatal FA (FA/FA), in utero sidestream smoke (SS) followed by postnatal FA (SS/FA), in utero FA followed by postnatal SS (FA/SS), and in utero SS followed by postnatal SS (SS/SS) (n = 3 in each group). SS/SS exposure resulted in more pulmonary neuroendocrine cells per centimeter basal lamina (p = 0.0025 ANOVA, SS/SS differs from all other groups as indicated by the brackets, p < 0.01 Fisher PLSD; values are means ±SEM). (Data from Reference 97, with permission.)

in children raised in the homes of smokers. A possible effector cell for these changes, the neuroendocrine cell, was identified. Postnatal exposure to SS alone did not mimic the lung changes seen in children raised in the homes of smokers, although a significant decrease in airway responsiveness to serotonin suggests that SS exposure postnatally alone can change lung function.

Researchers also examined whether SS exposure would affect the neural control of airways. The hypothesis studied was that SS exposure would attenuate the activity of the nerve fibers responsible for the defense reflex, leaving lungs more vulnerable to noxious agents. The first of these studies (98) evaluated whether chronic exposure of developing guinea pigs to SS would impair lung function and morphology and/or change the activity of the local bronchopulmonary C-fiber system. When bronchopulmonary C-fibers are activated, the nerve impulses travel to the central nervous system, resulting in rapid shallow breathing, cough, and a cholinergically mediated bronchoconstriction. The nerve impulses also cause a local release of tachykinins: (α) substance P, which interacts with NK₁ receptors to cause mucus secretion, airway microvascular
leak, and (in guinea pigs) bronchoconstriction and (b) neurokinin A, which interacts with NK<sub>2</sub> receptors to cause bronchoconstriction (99). C-fibers are known to be stimulated by MS (100, 101), and by components of ETS such as nicotine (102), acrolein (103), and oxidants (104). Duncan Hartley guinea pigs were exposed to filtered air or to SS (1 mg/m<sup>3</sup> of TSPs for 6 h/day, 5 days/week) from 8 to about 43 days of life. Their lungs were then studied in an isolated buffer perfused system in which increasing doses of capsaicin (a C-fiber stimulant found in hot peppers) or substance P (a C-fiber neurotransmitter) were injected into the pulmonary artery. SS exposure significantly increased baseline dynamic compliance by 17% but did not change baseline pulmonary resistance. SS exposure reduced the capsaicin-induced change in lung resistance (Figure 4) but did not change lung responsiveness to substance P. SS exposure did not change fixed lung volume, surface area, mean linear intercept length, or elastin deposition. Thus, SS exposure to developing guinea pigs (a) increased lung compliance without affecting alveolar size or elastin deposition and (b) decreased the airway reactivity of the C-fiber system without changing reactivity to one of its neurotransmitters, substance P.

Figure 4  Capsaicin-induced changes in pulmonary resistance (R<sub>L</sub>) in isolated lungs from guinea pigs exposed to filtered air (FA, open circles) or sidestream smoke (SS, solid circles) from age 8 to about 43 days of life, n = 6–8 in each group. Capsaicin injected into the pulmonary artery increased R<sub>L</sub> (p = 0.0001). However, the increase in R<sub>L</sub> was significantly diminished by SS exposure (p = 0.02). Statistics one-way multivariate repeated measures ANOVA on the log-transformed data. (Data from Reference 98, with permission.)
The second study (105) evaluated whether chronic exposure of developing guinea pigs to SS would impair the activity of the other pulmonary defense nerve fibers, the rapidly adapting receptors (RARs), or “irritant” receptors. When RARs are stimulated, the nerve impulses travel to the central nervous system, resulting in augmented inspirations, tachypnea, cough, and a cholinergically mediated bronchoconstriction and mucus secretion (106). RARs are known to be stimulated by, for example, MS, ozone, pulmonary venous congestion, and bronchoconstriction (106). In this study, Duncan Hartley guinea pigs were exposed to filtered air or to SS (1 mg/m³ of TSPs for 6 h/day, 5 days/week) from 8 to about 43 days of life. The animals were then anesthetized, and single lung RAR fiber activity and peak tracheal pressure were examined in response to a known stimulant of RARs, three breaths of MS. SS exposure did not alter baseline RAR activity. Low-nicotine MS increased RAR activity in the filtered air but not in the SS group. High-nicotine MS increased RAR activity in both groups but more so in the filtered air than in the SS group (Figure 5). Baseline peak tracheal pressure was lower in the SS group. Both low- and high-nicotine MS increased peak tracheal pressure but more so in the filtered air than in the SS group. The increase in RAR activity preceded the increase in peak tracheal pressure, suggesting that the RARs were responsible for the increased tracheal pressure rather than the other way around. Thus, exposing guinea pigs to SS during their postnatal development diminished the responsiveness of RARs to acute inhalation of MS.

These two studies demonstrated that exposing postnatal guinea pigs to SS diminished the defense reflexes of the lungs. C-fibers and the RARs were less responsive to stimulation by capsaicin and MS, respectively. If further studies confirm that this nervous system hyporesponsiveness is generalizable to other stimulants, then it may be possible that the lungs of children raised in the homes of smokers are more vulnerable to a number of other air pollutants and/or infectious agents.

These observations made in animals are a first step toward exploration of potential mechanisms that underlie etiologies for the increased cough, wheeze, sputum production, respiratory illnesses, airway reactivity, and decreased FEV₁, FEV₁/FVC, and FEF₂₅₋₇₅ reported in children raised in the homes of smokers. They suggest that ETS exposure both in utero and postnatally may be required for the maximal development of airway hyperresponsiveness and that neuroendocrine cell hyperplasia may play a role in its development. Early postnatal ETS exposure without prenatal ETS exposure may result in subtle changes in lung function such as hyporesponsiveness to serotonin. Postnatal ETS exposure may chronically stimulate the defensive sensory nerve fibers of the lungs, the C-fibers, and RARs, thus producing cough, wheeze, and sputum production. Of greater concern, however, C-fibers and RARs may become hyporesponsive to other irritants such as ozone, leaving the lungs of children more vulnerable to injury by these agents.
Figure 5  Mainstream smoke (MS) effects on lung rapidly adapting receptor (RAR) activity from guinea pigs exposed to filtered air (FA) (hatched bars) or sidestream smoke (SS) (solid bars) from age 8 to about 43 days of life. Values are means ±SEM. (A) Baseline activity of 14 RARs recorded in FA-exposed guinea pigs and 11 RARs recorded in SS-exposed guinea pigs was not different, but activity after low-nicotine (LN) MS was greater in the FA-exposed group (*p < 0.03 by unpaired t-test). LN smoke inhalation significantly increased activity of RARs in FA- but not in SS-exposed group. RAR activity in both groups recovered. (B) High-nicotine (HN) MS increased activity of RARs in both groups, but RAR activity in FA-exposed group was significantly higher than that in SS-exposed group (*p = 0.04 by unpaired t-test). CON I, CON II, and CON III are first, second, and third control periods, respectively. (Data from Reference 105, with permission.)

CONCLUSIONS

An interpretation of the available experimental data on the toxicity of SS shows the following. Cigarette SS, even at comparatively low concentrations, undoubtedly poses a risk to developing organisms. Although a concentration of 1 mg/m³ of TSPs may represent a NOEL for pulmonary effects in adult rats, the same concentration certainly has profound effects on the fetus and the newborn. Pre- and postnatal exposure to SS produces intrauterine growth retardation, changes the pattern of metabolic enzymes in the developing lung, results in hyperplasia
of the pulmonary neuroendocrine cell population, and adversely affects pulmonary compliance and airway responsiveness to pharmacological challenges. Experimental studies with SS have thus successfully duplicated what is a major concern in human health: The populations most at risk from ETS exposure are neonates, young children, and possibly the fetus while in utero. With newly developed animal models now available, mechanisms can now be studied. Eventually, this will lead to a better understanding of disease.

In adult organisms, SS also has been found to produce changes that may be attributed to ETS exposure of humans, such as inflammatory changes in the airways, accelerated formation of arteriosclerotic plaques, and even development of lung tumors. Although the changes are often comparatively minor and require exposure to rather elevated concentrations of SS, they certainly support the conclusions that were drawn from human epidemiological studies. Cessation of all exposure to ETS, achieved by a ban of active smoking, would be the best way to deal with the human health problem. It is doubtful whether this goal is realistically achievable. In the meantime, we will have to continue to rely on additional animal studies in order to develop appropriate therapeutic and preventive strategies that should help to mitigate some of the serious, widespread, and costly health problems of our times.

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