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EFFECTS OF CONCENTRATED CIGARETTE SMOKE ON RESPIRATORY TRACT CLEARANCE IN THE FERRET

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This study was performed to assess the effects of two high concentrations (about 380 mg/m^3 and about 38 mg/m^3) of inhaled cigarette smoke on tracer particle clearance from the head airways region and the thoracic (primarily deep lung) region of ferrets exposed during postnatal respiratory tract development. Ferrets were exposed 2 h/days, 5 days/wk to purified air or to the smoke aerosols starting at 5 wk of age, for a total of 15 wk of exposure. Three weeks prior to the end of the 15-wk exposure regimen, radiolabeled tracer particles were deposited by inhalation, and the clearance rates from the head airways and thoracic regions were monitored for 18 days. The head airways counting data were accurately fit by a double exponential function, reflecting fast-clearing and slow-clearing components. The thoracic counting data were adequately fit by a single exponential function. Statistically significant accelerations of particle clearance rates were observed for the slow-clearing and fast-clearing phases of clearance from the head airways region for both the higher and lower concentration groups; the results implied a dose-response relationship. Only in the case of the higher concentration group was significant slowing observed in the thoracic region analysis, indicating that the head airways region was perhaps the area of greater impact of the smoke aerosol.

Many children are exposed to environmental tobacco smoke (ETS) in the home from earliest childhood (Greenberg et al., 1991). There is presently controversy concerning the significance of risks to children from this exposure, which possibly include increased susceptibility to respiratory disease, and even persistent lung injury (Crawford, 1988). Twenty-four-hour mean particulate smoke concentrations in homes have been measured and found to be about 35 μg/m^3 of air per smoker (Samet et al., 1991); shorter term concentrations are expected to be much higher. Coggins et al. (1992) have defined 100 μg/m^3 as a "worst-case" level of indoor ETS. Animal studies for assessing the risks of exposure have employed both mainstream tobacco smoke (that has passed through the cigarette) and sidestream smoke (issuing between puffs). Environmental tobacco smoke consists of a mixture of both components, but sidestream smoke appears to be the larger contributor (First, 1985). Consequently, it is useful to perform animal studies specifically

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of use in estimating the effects of mixed sidestream and mainstream smoke exposure. This study used such a mixture and employed the ferret as an animal model for the developing mammalian lung. This model was chosen because the ferret lung structure in many ways resembles that of the human more than do the lung airway structures of other species used in such studies (Phalen and Oldham, 1983; Oldham et al., 1990; Mannix et al., 1991). Also, in a previous study the ferret particle clearance pattern was similar to that of humans (Mannix et al., 1991). In addition, the ferret undergoes rapid body growth from about 15 g at birth to as much as 1 kg at 20 wk of age. Therefore, an exposure regimen performed during the period of rapid lung development can be accomplished within a reasonable time frame, and the effects of exposure to very high levels of tobacco smoke on particle clearance can be determined.

The kinetics of clearance of radiolabeled polystyrene latex particles from both the head airways and thoracic regions were assessed. The head airways region was studied because environmental tobacco smoke is primarily inhaled by humans via the nasal route, and thus clearance from this region may be altered. Clearance from the slower clearing structures of the thoracic region (beyond the tracheobronchial region) was monitored because a significant portion of the smoke aerosol passes through the head airways region into the lungs, and repeated exposure to smoke during lung development may modify the immature terminal bronchiolar region, and thus affect the rate of clearance from the deep lung. This study was part of a longer term project that involves an early concentration-response phase, to be followed by longer exposures to lower levels of smoke. Although it would have been better to involve additional smoke concentrations in this phase of the study, our ferret housing limitation and other procedural problems combined to restrict our study to two smoke-exposed groups and one purified air-exposed group. This assessment of particle clearance was performed early in the project, so the exposure concentrations are much higher than those found in the air of smokers' homes. However, the concentrations are similar to those inhaled during active smoking (Davies, 1988), and such concentrations are possible transiently near a smoker.

EXPERIMENTAL METHODS

Animals

Prior to performing the study, a research protocol was submitted to the on-campus animal research committee, whose primary responsibility involves ensuring that all animal studies are performed in accordance with prevailing animal welfare guidelines. The protocol was deemed to be acceptable, with no violations of animal handling or treatment standards identified.

Eight pregnant female European ferrets (*Mustela putorius furo*) were obtained from a supplier of research-quality laboratory ferrets (Marshall

Farms; North Rose, NY). Their pregnancies were timed such that they would give birth approximately 2 wk after delivery to the laboratory vivarium. Prior to shipment, the female ferrets (jills) were maintained in a sanitary (but not barrier) environment, and each ferret was examined by a Marshall Farms veterinarian. The jills were certified to be clinically free of any contagious, infectious, or communicable diseases, but were not designated to be specific pathogen free. In our laboratory the ferrets were housed one jill per cage in a facility (accredited by AAALAC) supplied with purified air. After parturition, newborn ferrets (kits) were kept with the jills until they were weaned (at approximately 5 wk of age), at which time they were ready for use in the tobacco exposure portion of the study. After weaning, the mixed-sex juvenile ferrets were separated from the jills, metal tags were placed on one ear for identification purposes, and ferrets from the same litter were segregated by sex and placed up to four to a cage for the duration of the study. The ferrets were provided food (ferret chow, Purina Mills, St. Louis, MO, and IAMS Kitten Food, Dayton, OH) and water ad libitum, and were kept on a 24-h light/dark cycle (illumination provided 7 a.m.–7 p.m.). Personnel involved in handling the ferrets wore clean lab coats, head covers, masks, and gloves to prevent transmitting infections to this susceptible species (Fox, 1988). These preventative measures were adequate for our studies, since we experienced no disease problems. The ferrets were not maintained on individual laminar air barrier isolators, but the room air was HEPA (high-efficiency particulate air) filtered and supplied at 15–20 air changes per hour. At 6 wk of age the average body mass of the mixed-sex ferrets was 189 ± 45 (SD) g; at the end of the exposure (20 wk of age) the average body mass was 845 ± 270 (SD) g, indicative of a rapid growth rate of near 50 g per week between 6 and 20 wk of age. An analysis of the body mass data indicated that there was no statistically significant difference among the 3 exposure groups of ferrets at either the beginning or the end of the 15-wk exposure regimen.

Smoke Exposure

Each study group was originally designed to consist of 10 ferrets; however, due to parturition-associated mortalities, this number was reduced to a minimum of 5 per group. The weanling ferret kits were exposed head-only (using a polycarbonate manifold fabricated for this purpose) to a mixture of mainstream and sidestream tobacco smoke generated by a Walton Cigarette Smoking Machine (Process and Instruments, Inc.; Brooklyn, NY). The Walton smoke generator, which was designed to be used in mainstream smoke studies, was modified such that the sidestream smoke was mixed with the mainstream smoke before passing into the exposure manifold. The Walton machine was a cost-effective means of providing the desired mixture. The separate contributions to the overall smoke mass concentration were measured, and the mainstream smoke was determined to account for about 80% of the smoke mass present in the ferret's breathing zone (approximately 20% for the sidestream smoke). Three groups of ferrets

were exposed to one of the following atmospheres for 2 h daily: purified air, higher smoke concentration, or lower smoke concentration. Exposures for each group were 5 days/wk—1 h in the morning and 1 h in the afternoon, with at least a 2-h rest period when ferrets were returned to their cages for food and water in between exposure sessions. Ferrets for each study group were selected randomly from 8 litters, and each group consisted of about 50% females and 50% males wherever possible. The relative humidity in the exposure chamber was not controlled, but it was monitored periodically during the study, and it averaged approximately 60%. University of Kentucky 2R1 unfiltered research cigarettes (Tobacco and Health Research Institute, Lexington, KY) were lit and smoked at the rate of 6 per hour (1 cigarette per 10 min, 1 puff/min). This cigarette was chosen because of its high nicotine content. In order to achieve the higher smoke concentration, 3 cigarettes were placed in the machine and smoked simultaneously for a total of 10 puffs per 10 min per cigarette. For the lower smoke concentration, one cigarette was similarly smoked. In this case, more dilution air was also added in order to reduce the smoke to the desired concentration. The higher concentration smoke-exposed ferrets were exposed to smoke from 36 cigarettes a day, and the lower concentration smoke-exposed animals were exposed to smoke from 12 cigarettes a day. Particulate smoke samples were collected on pre-weighed fluorocarbon-coated glass fiber filters (Pallfex Filters; Putnam, CT) sampling at a rate of 10 L/min from an otherwise unused exposure manifold port. Samples were collected for 10 min during each hour of exposure, after which they were air-dried to allow evaporation of water and weighed to the nearest microgram for the determination of the particulate concentration (mg/m^3). The average higher smoke concentration for the duration of the study (15 wk) was 381 ± 97 (SD) mg/m^3, and the average lower smoke concentration was 38.2 ± 13.3 (SD) mg/m^3, but due to the puffing regime, instantaneous concentrations were highly variable.

Tracer Particle Deposition

Immediately following the exposures to purified air or cigarette smoke on the first day of wk 13 of exposure, the ferrets were placed while fully awake into plastic nose-only exposure tubes, which were then plugged into a wall of a tracer particle deposition system (Raabe et al., 1973; In-Tox Products, Albuquerque, NM). For 30 min the ferrets inhaled the ^{51}Cr-labeled microspheres (sized with a calibrated Mercer-type impactor: activity median aerodynamic diameter = 1.7 μm; geometric standard deviation of about 1.2). These tracer particles, which were labeled at this laboratory employing the technique of Hinrichs et al. (1978), were generated from a 0.1% (by volume) aqueous suspension of the particles using a Lovelace-type nebulizer (In-Tox Products). Immediately following tracer-particle deposition, the ferrets were removed from their nose-only tubes and their muzzles were washed twice with premoistened disposable baby wipes in order to reduce to a very low level the quantity of externally deposited particles. The animals were subsequently placed fully awake into plastic counting tubes and were inserted

into a two-detector counting system (Mannix et al., 1991; Fig. 1) that separately detected radiation emitted by particles in the head airways and thoracic regions. The head airways counting region covered the anatomical structures between the tip of the nose and the middle of the trachea, while the thoracic counting region included the lower half of the trachea and the lungs. The ferret's unusually long trachea permitted excellent separation of the counting regions. However, the thoracic counter was not sufficiently collimated to exclude radioactivity present in the gastrointestinal (GI) tract. For this reason, the starting point of the thoracic clearance phase (for data analysis purposes) was 48 h postdeposition—a time at which the GI tract was experimentally determined to be nearly free of radioactivity.

Each of the ferrets received its first head airways and thoracic counts within 15 min of the end of the deposition. The ferrets were then counted at each of the following time points postdeposition: 1, 2, 3, 4, 5, 7, 9, 24, 48, 96, 168, 240, 340, and 432 h. The counting times ranged from 100 s per ferret for the early counts (0–9 h), to 200 s for the later counts (24–432 h). While most of the animals remained relatively still and properly positioned in the counting tubes, occasionally a ferret would twist sideways in its tube, or rotate and turn upside down. When this occurred, the ferret was returned to its proper position and recounted.

Data Analysis

Graphical representations of the head airways radiation counting data obtained for the groups of ferrets between 0 and 48 h postdeposition indicated that the clearance curves were not well described by single exponen-

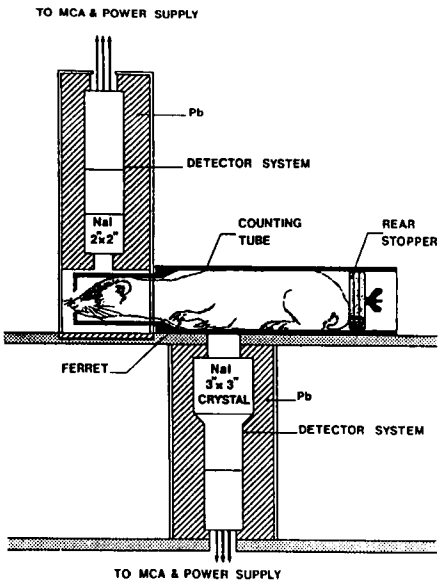

FIGURE 1. Gamma-ray detection system used to measure particle clearance in ferrets. Adapted from Mannix et al. (1991).

tial functions; log-linear regression analyses confirmed this fact. In all animals there appeared to be very rapid clearance of tracer particles soon after the deposition, followed by a period during which the rate of clearance was considerably slower. For this reason curve-stripping techniques were employed to separate the data into rapid clearing (0–2.5 h) and slower clearing (4–48 h) components. The data for each animal in each of these time intervals were subsequently fitted to single exponential functions such that each of the two phases of clearance was describable in terms of characteristic slope and intercept values. The resulting combined double exponential fits of the data were then plotted and compared with the actual observed data—both on an animal-by-animal basis, and for the groups of animals. Acceptable agreement was noted, giving us confidence in the fitting procedures. In contrast, the data obtained from counts of the thoracic region of individual and groups of ferrets between 48 and 432 h postdeposition were adequately fitted to single exponential functions; therefore, the thoracic clearance of each ferret was characterized in terms of the slope and intercept of its single exponential fit. Only thoracic counting data obtained after 48 h postdeposition were included in the analysis because the detector for this region did not adequately discriminate between thoracic and gastrointestinal radioactivity. The slopes and intercepts for the head airways region and the thoracic region from individual animals were combined in relation to exposure, and group means and standard deviations were calculated. Graphical analyses of the slope and intercept values had demonstrated that they were approximately normally (as opposed to log-normally) distributed around a mean, which indicated that the use of standard statistical analyses and assumptions would be appropriate.

The results for the groups were compared using an analysis of variance, and two-tailed *t*-tests using the F.S.D. assumption for multiple-comparison tests (O'Neill & Wetherill, 1971). Analyses were performed using BMDP Statistical Software (BMDP Statistical Software, Inc., 1988 version). Two critical values of p were used: .1 for a basic level of significance, and .05 as a more stringent level of confidence.

RESULTS

As mentioned, some of the kits died from causes not related to our study procedures; therefore, group sizes were less than originally planned. However, because the effects of cigarette smoke were quite striking, statistically significant effects on the rates of clearance of labeled particles were observed for both levels of smoke. As was previously noted, the smoke exposures did not produce any significant changes in growth rate of the ferrets.

Head Region Clearance Curves

Group clearance curves normalized to the initial radioactivity are shown in Figure 2. The data points are the means of net counts for all animals at each time point. The lines are not fits to these points. Rather, they represent

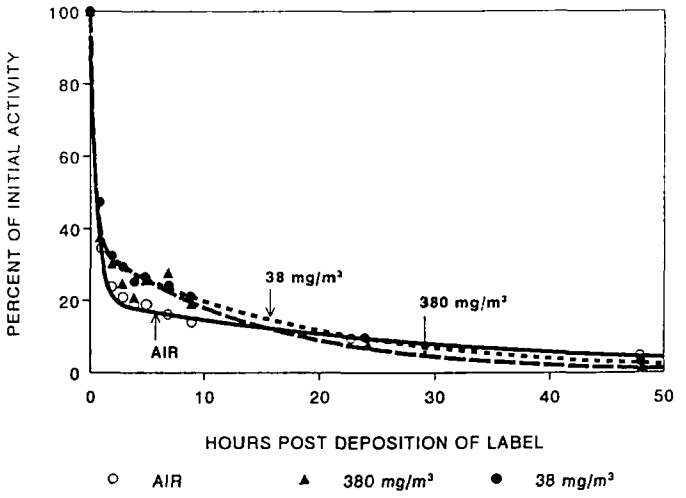

FIGURE 2. Head-airways clearance curves normalized to initial activity for the three groups.

averages of the individual slopes and intercepts of exponential curves fitted to each member of the group. Clearance from the head airways region is well fitted by a double exponential curve. The purified-air exposed group had a clearance curve that was nearly identical to that seen in older control ferrets in a previous study (Mannix et al., 1991). In purified air-exposed ferrets in both studies, most of the radioactivity (about 80%) deposited on epithelium that had rapid clearance. However, the remaining label appears to have deposited on inefficiently cleared epithelium. Material was detectable in some ferrets in this slowly cleared compartment for as long as 10 days post-deposition of the tracer particles. In the previous study, we carefully shaved the snouts of several animals and verified that the slowly cleared tracer was not due to external contamination of the fur. Therefore, this material apparently deposited on, or translocated to, nonciliated surfaces (olfactory or anterior, for example), or regions of damaged epithelium that did not clear particles efficiently.

Both the higher and lower level exposures to cigarette smoke significantly accelerated both short- and long-term clearance rates in the head airways region (see Table 1). Both rates exhibited apparent concentration-response relationships. The time-zero intercepts for the long-term components were significantly increased by the tobacco smoke exposures. This could be produced by either the slow-clearing head airways compartment being expanded by exposure (possibly due to epithelial changes) and/or the particle deposition being shifted into more slowly-clearing regions.

Thoracic Region Clearance Curves

Group clearance data (means of the slopes and intercepts from exponential data fits for each of the animals in the groups) are also shown in

TABLE 1. Group Clearance Data

Smoke (mg/m^3)	n	Head airways region				Thoracic region	
		Short-term component		Long-term component			
		Intercept[a]	Slope[b]	Intercept[a]	Slope[b]	Intercept[a]	Slope[b]
0	10	73.27 (8.23)	1.66 (0.29)	20.0 (10.9)	0.031 (0.007)	100.5 (5.0)	0.00066 (0.00026)
38	7	68.84 (9.90)	2.42 (1.23)[d]	34.0 (11.4)[c]	0.053 (0.019)[c]	105.5 (9.6)	0.00056 (0.00033)
380	5	68.88 (17.34)	2.75 (1.14)[c]	36.6 (15.9)[c]	0.070 (0.020)[c]	100.6 (5.3)	0.00034 (0.00024)[c]

Note. Values are given as mean (SD).

[a]Intercept expressed as a percent of first count for head airway region and percent of 48-h count for thoracic region.

[b]Slope expressed as a percent of remaining activity cleared per hour (isotope decay corrected).

[c]$p < .05$.

[d]$p < .1$.

Table 1. The use of a single exponential fitting procedure appeared to be acceptable, at least over the clearance time span examined in the study, as shown in Figure 3. Had the clearance of labeled polystyrene latex particles been followed for a longer time, it is likely that a simple single exponential fitting technique would have been inadequate. In fact, a pilot study with three ferrets indicated that radioactivity in the thoracic region clears at a steadily decreasing rate.

Tobacco smoke exposure appeared to affect the rate of clearance from the thoracic airways in a concentration-dependent manner. It was not possible to determine whether this represented impaired mucus movement, a shifting of deposition deeper into the lung, or an effect of the smoke on macrophage-mediated clearance from the gas-exchange airways. The effect reached statistical significance in the higher concentration group.

Concentration-Response Analysis

The clearance-rate (slope) and intercept data are shown in Figure 4, normalized to the purified air-exposed animals. It appears that concentration-response effects were observed for each of the clearance rates that were measured. The effects were not linear with respect to concentration; the higher concentration exposure was about 10 times greater than the lower concentration exposure, but the effects were not 10-fold greater. For nearly all of these measures of clearance, smoke exposure increased the standard deviations of the population.

DISCUSSION

Normal clearance of insoluble particles depends on a variety of factors (Pavia et al., 1980; Vincent, 1990). The airways of the head and neck and

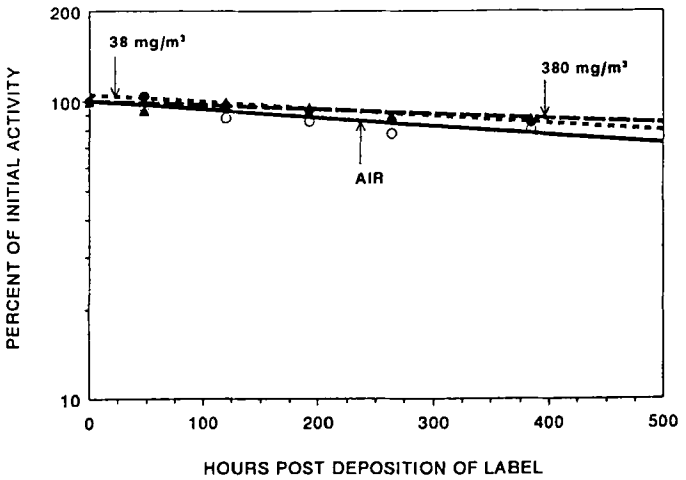

FIGURE 3. Thoracic airways clearance curves and data points for the three groups.

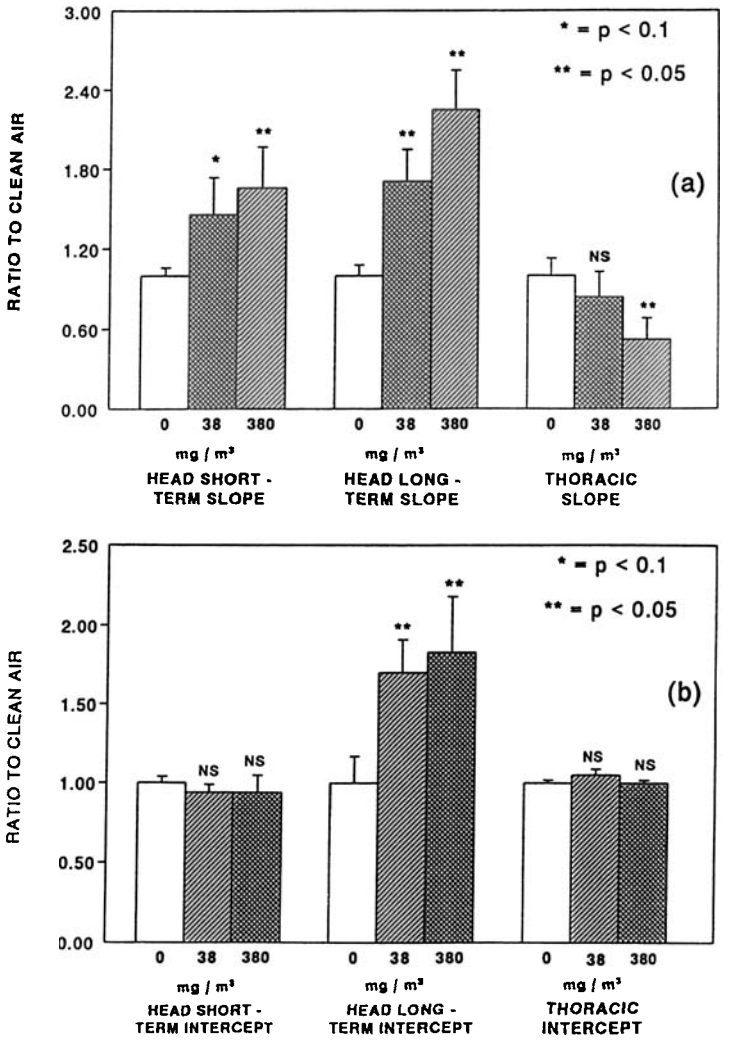

FIGURE 4. Clearance curve fit constants normalized to the purified air group: (a) slopes and (b) intercepts.

the tracheobronchial airways are primarily dependent upon mucociliary clearance. This implies that healthy ciliated cells in sufficient numbers are acting in synchrony, and that mucus-secreting glands and cells are likewise present in normal quantities and are functioning in a coordinated fashion. In the alveolar airways, particle clearance mechanisms are less well understood, but a normal-size population of healthy macrophages is most likely a key factor in the clearance of micrometer-size insoluble particles. Also, in both the head and thoracic airways, areas of damaged epithelium could possibly become sites of clearance stasis, and even regions of long-term buildup of particles.

The effects of cigarette smoke on the respiratory tract epithelium and on particle clearance have been examined in a very large number of studies over the past 35 yr, so only highlights are presented here. Rivera (1962) reviewed the acute effects of a variety of materials, including cigarette smoke, on isolated mucociliary epithelium. Among other work, Rivera cited earlier studies by Krueger and Smith in which cigarette smoke was seen to reversibly, but drastically, depress ciliary activity in vitro. In an early study of human volunteers, Sanchis et al. (1961) studied the lung clearance of inhaled radiolabeled albumin in both smokers and nonsmokers. They found a "slowing of the normally fast-clearing airways, and at the same time, a relative speeding of the second clearance phase" in smokers. Since their data were collected over a period of only 25 h (involving measurement of the fast-clearing components of lung clearance), and our thoracic clearance analysis involved data obtained at times greater than 48 h postdeposition (involving measurement of the slower-clearing components of lung clearance), it is difficult to directly compare the results of our study to their results. However, their study indicated that an acute exposure to cigarette smoke can, at least transiently, impair mucus movement, which is consistent with the early in vitro findings. Pavia et al. (1980) reviewed the published literature on the effects of tobacco smoke on particle deposition and clearance. This review underscores the complex responses of mucociliary function (in some studies clearance was slowed; in others, either it was accelerated or it did not change).

The differential effects of high versus low concentrations of cigarette smoke may be due to its chemical complexity; one or more components may dominate at high concentrations, and others at low concentrations. As an example, Albert et al. (1974) examined the effects of cigarette smoke exposure on bronchial clearance rates in donkeys. In their investigation a stimulation of clearance was seen at low levels of exposure (the animals smoked 2 cigarettes via nasal catheters), while a striking depressant effect was seen after higher level exposures (10–30 cigarettes smoked). Although these studies were acute, they demonstrate the complex manner in which a gross measure such as particle clearance can respond to cigarette smoke exposure. High levels of cigarette smoke appeared to impair mucus movement, while lower levels stimulated it. Our slowed thoracic clearance in ferrets exposed to high concentrations is consistent with this finding.

Vastag et al. (1986) observed a dose-response impairment of bronchial mucociliary clearance of radiolabeled human red blood cells in smokers. Effects were greatest in smokers who also exhibited bronchitis, but they were also present in an asymptomatic group. Churg et al. (1992) examined the autopsy particle burdens in the lungs of cigarette smokers (without evidence of emphysema at autopsy) in comparison to those of 12 lifetime nonsmokers. They found a "markedly decreased particle load retained in the smoker's (large) airways," but overall similar particulate levels in the deep lung. Again, the complex nature of the response of respiratory tract clear-

ance is evident, with the implication that clearance may not always be impaired in relatively healthy smokers.

There is evidence that cigarette smoke can radically alter the mechanisms involved in particle clearance from the deep lung. Huber et al. (1981) found that chronic exposure of rats to cigarette smoke led to an inflammatory response that included the activation of respiratory tract macrophages, increasing their rates of secretion and phagocytosis, and causing them to cluster in pigmented foci at the proximal alveolar duct region. Presumably, such macrophages would be able to engulf microorganisms, but they may not be able to effectively transport foreign material out of the lungs. In fact, modern mechanistic models of macrophage-mediated particle clearance indicate that increasing particle burdens may be expected to be associated with impaired clearance (Morrow, 1988; Yu et al., 1989; Stöber et al., 1989).

Cigarette smoke is known to produce alterations in nasal functions. Willes et al. (1992) examined 18 nonsmokers identified as "ETS-sensitive" after 15 min exposures to tobacco smoke. They found large increases in nasal resistance to airflow (primarily in the upstream flow-limiting region), and the symptoms of rhinitis (irritation, congestion, and runny nose). Similar symptoms were observed in many of our ferrets during exposure to tobacco smoke; such symptoms could stimulate the clearance of particles deposited in the nose. In a study of nasal mucociliary clearance in children that lived in homes with or without smokers, Corbo et al. (1989) failed to find any consistent effects on clearance that were attributable to passive smoking. Stanley et al. (1986) concluded that human smokers had normal nasal ciliary beat frequencies when compared to nonsmokers, but that the clearance of tracer particles from the nasal region of smokers was impaired. One interpretation of this study is that the area occupied by normal epithelium in the nose was decreased by the long-term smoke exposures, and that the coverage was not sufficient to provide normal clearance. In a study involving 14-d exposures of rats to aged, diluted sidestream smoke from 1R4F (filtered) research cigarettes, Coggins et al. (1992) evaluated tissue changes, organ and body weights, and clinical pathology. The highest particulate exposure group (10 mg/m^3 of air) was the only group demonstrating effects. These rats exhibited mild epithelial hyperplasia and inflammation in the nasal turbinate region. Zwicker et al. (1978) examined the clinical and pathological effects of 5 mo of daily exposure of young adult dogs to 6 or 12 cigarettes per day. Tracheobronchial inflammation, rhinitis, and turbinate epithelial hyperplasia were all seen in exposed animals, with the higher exposures generally producing more severe effects. Such events could be expected to produce changes in particle clearance (not assessed in the Zwicker study) similar to those seen in our ferrets.

Our results imply that enhanced clearance rates for the head region, and decreased clearance rates for the thoracic region, may occur as a result of exposure to tobacco smoke during the period of respiratory tract development. They are consistent with what would be expected in other mammalian

species, including humans, exposed to high concentrations of cigarette smoke. The finding that smoke exposure shifted additional tracer particles into a slow-clearing compartment in the head airways (as evidenced by the significantly elevated head long-term intercept values) is, to our knowledge, novel, and it warrants further study. The changes in particle clearance seen in this study were substantial, implying that important respiratory tract defense systems were affected. The individual variability in the responses to smoke was also a striking observation that should be investigated further. Although the concentrations of smoke used were very high, and thus not directly applicable to human children exposed to smoke in the home, these concentrations could be experienced for very short periods near a smoker, or by an individual who is smoking. Further studies are needed to determine the possible smoke concentration and exposure duration thresholds of the observed effects, the persistence of the effects, and the risks attendant to long-term exposures of human children in the home to realistic smoke concentrations. Finally, because only the clearance of tracer particles was measured, these data do not shed light on the fate of the inhaled smoke particles themselves.

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