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Ninety-Day Inhalation Study in Rats, Comparing Smoke from Cigarettes That Heat Tobacco with Those That Burn Tobacco[1]

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Ninety-Day Inhalation Study in Rats, Comparing Smoke from Cigarettes That Heat Tobacco with Those That Burn Tobacco. COGGINS, C. R. E., AYRES, P. H., MOSBERG, A. T., SAGARTZ, J. W., BURGER, G. T., AND HAYES, A. W. (1989). *Fundam. Appl. Toxicol.* 13, 460–483. Eight groups of 30 male and 30 female rats were exposed 1 hr per day, 5 days per week for 13 weeks, to smoke from reference (tobacco burned) or test (tobacco only heated) cigarettes, at nicotine concentrations of 5, 15, or 30 μg/liter of air. Similar smoke concentrations of wet total particulate matter and carbon monoxide were produced in each of the test/reference comparisons. There was a pronounced depression of minute ventilation of animals in the reference groups, but not in the test animals. Blood carboxyhemoglobin concentrations were similar in animals exposed to smoke from test and reference cigarettes. Plasma concentrations of nicotine and cotinine in the test groups were higher than in the reference groups. There were no differences between the smoke-exposed groups in terms of body weight or feed consumption. At necropsy, an increase in heart weight was noted in both high exposure groups. There were notable differences in histopathology, with fewer and less-pronounced changes in the test groups than in the reference groups. Many of the histopathological responses induced in the reference groups were absent in the test groups. Overall, the study demonstrated a substantial reduction in the biological activity of smoke from the test cigarette when compared with the reference. © 1989 Society of Toxicology.

A novel concept in cigarette design is the introduction of technology which allows tobacco to be heated rather than burned (RJRT, 1988). Since the tobacco does not burn, many of the compounds produced by burning tobacco are eliminated or greatly reduced.

The objective of the study described here was to compare in the rat the biological changes observed after repeated nose-only exposure to smoke from the test cigarette, with those changes observed in animals exposed to smoke from a cigarette which burned tobacco (the reference cigarette), at comparable concentrations of nicotine in the smoke presented to the animals. Many of the inhalation studies performed with cigarette smoke in the past have based exposures on the amounts of wet total particulate matter (WTPM) presented to the animals. Since the WTPM yielded by the test cigarette is chemically very different from that yielded by the reference cigarette (RJRT, 1988), it was decided to make the comparison using nicotine, one component of the smoke that is comparable for cigarettes which burn or only heat tobacco.

The experimental design was similar to that used in other inhalation studies of ciga-

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rette smoke in rats (Wehner *et al.*, 1981; Coggins *et al.*, 1982a). However, because there was no difference between the histopathology results obtained in these chronic studies (duration, 18–24 months) and data obtained in experiments with much shorter durations (6–12 weeks) (Walker *et al.*, 1978; Coggins *et al.*, 1980), the duration of the present study was limited to 90 days.

An additional objective of the study was to study the reversibility of any changes induced by exposure to smoke from test and reference cigarettes. To study this recovery process, groups of animals were kept for an additional 7 weeks, without treatment, at the end of the 90-day exposures.

MATERIALS AND METHODS

Experimental design. There were 30 male and 30 female animals in each of eight groups. Six groups were exposed to smoke (three each to test and reference cigarettes), one group was sham-exposed to air only, and there was one room control group (OECD, 1981). The six smoke-exposed groups were divided into low, medium, and high exposures for both test and reference cigarettes. The mainstream smoke from test and reference cigarettes was diluted to provide concentrations of 5, 15, and 30 μg of nicotine per liter of air at the animal breathing ports for low, medium, and high exposure groups, respectively.

Animals in the sham control group were placed in nose-only restraint tubes and exposed to filtered, humidified air only; the room control animals were kept in the animal room. Animals were exposed to smoke for one hour per day, 5 days per week (Monday–Friday), for 13 consecutive weeks. To ensure that animals were always exposed to smoke on the 2 days prior to necropsy, they were exposed for an additional 3 days in Week 14, to give a total of 68 exposures. Of the 30 animals per sex in the two high exposure groups and in the sham controls, 20 were killed at the end of the 90-day period. The remaining 10 animals per sex in each of these three groups received no further treatment for 7 weeks.

Cigarettes. Cigarettes were puffed under the conditions used by the Federal Trade Commission (FTC): a 35-ml puff of 2-sec duration, taken once per minute. The FTC condition of smoking to a fixed butt length is not applicable to the test cigarette, which does not shorten in length during smoking. Consequently, a fixed number of puffs (see below) was taken instead from each cigarette.

The test cigarette (Fig. 1) is based on new technology that heats rather than burns tobacco. The cigarette is lit in the conventional way. A highly refined carbon heat source in the tip of the cigarette generates warm air that passes through tobacco, tobacco extract, flavorings, and glycerol to form smoke. The reference cigarettes were manufactured in house and consisted of blended Turkish, Burley, flue-cured, and reconstituted sheet tobaccos. They were air-diluted by both paper and filter ventilation to yield, under the FTC conditions described above, quantities of WTPM, carbon monoxide (CO), and nicotine that were similar to those yielded by the test cigarette. The reference cigarettes were, therefore, unlike any commercially available cigarette. On an analytical smoking machine, the FTC yields were approximately 10 mg WTPM, 11 mg CO, and 475 μg nicotine. Standard filters and cigarette papers were used. The relative humidities (RH) used in conditioning test and reference cigarettes were approximately 10% for the former and 50% for the latter, at room temperature for at least 48 hr prior to use in the animal studies.

While full analytical chemistry was not performed on the smoke generated for inhalation exposures, such analysis has been performed on cigarettes similar to those used in this study (RJRT, 1988).

Experimental animals. A total of 540 (270 male and 270 female) Sprague–Dawley derived rats (Crl:CD) were purchased from Charles River Laboratories (Raleigh, NC). The Sprague–Dawley was chosen over the F-344 because of the larger blood volume (useful for dosimetry: see below) and because of the authors' experience with this strain. Animals were separated according to sex and acclimated to laboratory conditions for 3 weeks prior to their first exposure. At the time of receipt of the animals, after 6 weeks of exposures, and at 90-day and reversibility necropsies, sera were collected from 5 male and 5 female sentinel animals. The sera were tested by Microbiological Associates (Bethesda, MD) for the following antibodies to rodent pathogens: reovirus Type 3, mouse encephalomyelitis, Kilham's rat virus, Toolan's H-1 virus, pneumonia virus of mice, Sendai, rat coronavirus/sialodacryoadenitis virus, lymphocytic choriomeningitis virus, and mouse adenovirus.

Males and females were randomized separately into groups that were homogeneous for body weight, using a computerized random number generator. Records were kept of quarantine number, weight at randomization, and permanent identification number. The mean (±SD) weight of male animals was 152.8 ± 8.0 g at delivery and 355.5 ± 8.1 g at randomization; females weighed 141.9 ± 7.6 g at delivery and 236.4 ± 13.4 g at randomization. Animals were ear-tagged on the day after randomization, and exposures began on the following day.

The rats were housed and cared for in accordance with the Animal Welfare Act of 1970 and amendments. The rooms had controlled lighting (12-hr dark and 12-hr light), temperature (20–22°C), and RH (40–60%). Rats were housed individually in transparent polycarbonate

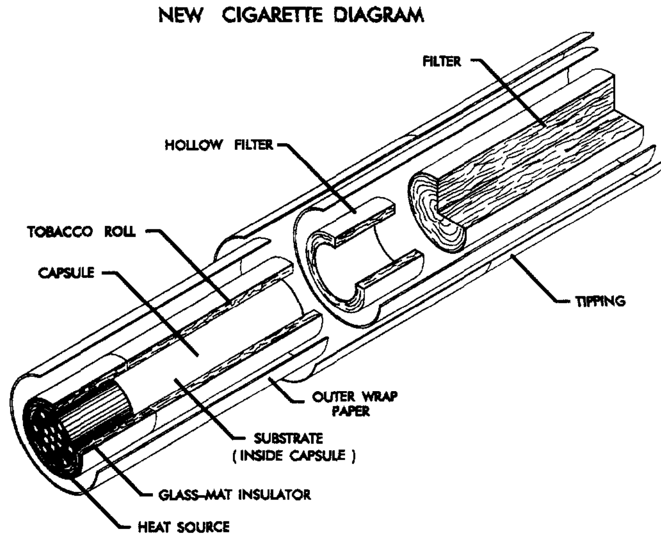

FIG. 1. Construction of the test cigarette. The carbon heat source fits into the aluminum capsule, which is surrounded by the tobacco roll. The filter is attached directly to the tobacco roll/aluminum capsule combination.

cages, which were cleaned thrice weekly and refilled with clean cellulose bedding (Alpha-Dri, Shepherd Speciality Papers, Kalamazoo, MI). Animals had free access to commercially available certified feed (Purina Rat Chow 5002) and tap water, except during inhalation exposures. No analyses of feed or water were conducted. During the weeks when consumption was being measured, feed was presented as a meal. Feed was withheld overnight prior to necropsy.

Exposure regimen. Smoke dilutions with HEPA-filtered, humidified air were established for equi-nicotine concentrations in both test and reference groups for low (5 μg nicotine per liter of air presented), medium (15 μg/liter), and high (30 μg/liter) exposures. These concentrations were confirmed before any animals were exposed to smoke. The CO concentrations were compared with levels previously determined to be lethal for this smoke component, to ensure that animals did not die from CO-induced hypoxia.

Inhalation exposure system. Two 30-port smoke generators (Baumgartner and Coggins, 1980) were used, at the FTC conditions (except butt length) described above. In this device a carousel rotates at 1 rpm over a fixed puffing port, connected to a peristaltic pump. Each port on the carousel is attached to a cigarette holder, positioned horizontally. The holders have a latex ring that provides a leakproof seal around the filter of the cigarette. The output from each generation device was delivered to an adjustable air dilution apparatus (volume 100 ml), designed to mix the smoke with HEPA-filtered air to achieve the desired nicotine concentrations.

Separate generation and dilution devices were used for test and reference cigarettes, and for the sham controls (total of three devices). The smoke generation devices were computer controlled: cigarettes were loaded and lit automatically and were extracted after a fixed number of puffs had been taken from them. The computer (Commodore 64, Commodore Business Machines, Inc., West Chester, PA) ensured an even distribution of "current puff" among the 30 cigarettes on the rotating carousel, to minimize the changes resulting from variations in smoke yields per puff. Loading of the cigarettes at the beginning of a run was "staggered," taking approximately 15 min to reach equilibration (30 cigarettes ignited on the carousel).

An additional "preignition" port was added to one smoke generator, to warm the tips of the test cigarettes and thereby improve subsequent ignition at the normal port. At the preignition port (three positions before the normal puffing port), a puff of 2-sec duration and a volume of 65 ml was taken and exhausted (i.e., this puff was not distributed to the animal exposure device). The preignition port was not operational for subsequent, nonignition puffs on the test cigarettes. After preignition, a further seven puffs were taken for this cigarette, the same number total as was taken from the reference cigarette.

The nose-only exposure apparatus consisted of two vertically concentric cylinders equipped with 64 animal exposure ports. The device was constructed as described earlier (Cannon *et al.*, 1983). The ports were arranged in a radial configuration with 8 ports on each of eight layers. At each port there was a continuous flow of smoke: continuously presented in the breathing zone of each animal and continuously removed by the outer annulus of the port. This arrangement provided a constant supply of

fresh smoke to each animal. The volume of the internal cylinder was less than 1 liter and the age of the smoke at the animal ports was less than 1 sec.

Animals were restrained in tubes fitting directly into the ports. Each animal was thus exposed only to fresh smoke, as the extraction lines were arranged so that there was no rebreathing of exhaled smoke. The design of the restraint tube permitted the tail of the animal to be positioned open to the atmosphere, minimizing stress associated with overheating.

The supply of fresh smoke to each exposure port was 500 ml/min, greater than the estimated peak inspiratory minute volume of individual animals (Coggins *et al.*, 1982b). Such a system minimizes breath-holding by the animals during exposure. Air flow and exhaust through the exposure device was maintained at 32.0 liters per minute. Before the initial animal exposures began, satisfactory achievement of uniformly distributed target concentrations was documented for the test and reference cigarettes at each of the three dilutions studied. The characterization of each machine included temporal and spatial variations in WTPM and nicotine, along with assessments of particle size distribution. The CO concentration was only analyzed temporally.

During animal exposures, four ports were used for monitoring the smoke composition. One port on levels one and eight was used to measure WTPM concentrations, using gravimetric analyses of 44-mm glass fiber filter pads (Model 1021215P1, Cambridge Filter Corp., Syracuse, NY). Filters were preconditioned at 40–60% RH before use. Weights of pad plus WTPM were obtained immediately after completion of sampling, using a Cahn C-31 balance (Cahn, Cerritos, CA). Weights were recorded after the pads had been on the balance pan for 3–4 min, to standardize for any evaporation of materials from the pad. Three gravimetric determinations were made during the 1-hr exposure to verify actual WTPM concentrations. Two filter pads were extracted with methanol to provide gas chromatography data on the nicotine and glycerol content of the smoke. The glycerol assay was not available during the first half of the experiment; samples were taken once per week during the remainder of the study.

One exposure port was used for monitoring CO concentrations, using a Horiba PIR-2000 CO analyzer (Horiba, Irvine, CA) calibrated with certified gas mixtures (AIRCO, Research Triangle Park, NC). The WTPM was monitored "on line" by a RAM-1 instrument (MIE Corp., Bedford, MA), which sampled smoke presented to the fourth exposure port. Chart recorders attached to the RAM-1 and PIR-2000 instruments served as general records of exposures; CO data were recorded at 0, 15, 30, 45, and 60 min of exposure.

Particle size distributions, as characterized by mass median aerodynamic diameter (MMAD) and associated geometric standard deviation (sigma g), were measured once per week by using a cascade impactor (In-Tox Products, Albuquerque, NM) (Mercer *et al.*, 1970). With a flow of 1 liter/min (250 ml/min of smoke from the animal port and 750 ml/min of diluting room air), the calculated effective cut-off diameters for the seven stages were 2.00, 1.53, 1.17, 0.89, 0.68, 0.52, and 0.40 μm. Calculations of MMAD and sigma g were made using probit analysis.

Temperature and RH measurements were made once per week. Relative humidity was determined by comparing the dry bulb temperature and the dew point temperature. The RH was controlled by regulated passage of a portion of the air dilution stream through an air humidifier. This humidified portion then joined the nonhumidified air prior to mixing with the smoke.

In-life biological measurements and observations. Animals were observed two to three times per day for each of the study days; times of onset and nature of clinical observations or death were recorded. Individual body weights were determined within 48 hr of receipt of the animals, during the randomization process, once per week during the study, and at necropsy (fasted). Feed consumption was determined during a 24-hr period in Weeks 1, 6, and 12 of the study, and the week before necropsy for the reversibility groups.

Blood for determination of carboxyhemoglobin (COHb) was drawn with heparinized micropipettes from the retroorbital sinus, under anesthesia with 70% carbon dioxide (CO_2) in air. Samples were taken from five animals per sex per group within 3 min of removal from the exposure system, after 55 min of exposure in Weeks 1, 4, 8, and 12. Samples were also taken at necropsy, to determine if any residual amounts of COHb were present 16–24 hr after the end of the exposure. Blood COHb concentrations and other hematology parameters were determined with a Model 282 CO-Oximeter (Instrumentation Laboratories, Hartford, CT), using the method described by Malenfant *et al.* (1968). Blood samples were drawn similarly for assays of plasma nicotine and cotinine concentrations, after 55 min of exposure during Weeks 2, 5, 9, and 13 of the study and at necropsy. It was necessary to pool blood from several animals within the same group in order to have a sufficiently large sample volume. Individual animal data were obtained at necropsy. The anticoagulant in the plasma nicotine assay was disodium edetate (EDTA), and samples were analyzed using the method of Davis (1986).

Tidal volume, respiratory rate, and minute volume were derived from respiratory flow measurements made continuously during smoke exposures and during the 10- to 15-min preexposure. Measurements were also made in the sham-exposed group of animals. Measurements were made on four animals per sex in each of the groups during Weeks 1, 5, 9, and 13.

The determination of respiratory airflow was made by whole-body plethysmography (Coggins *et al.*, 1981). The animals were enclosed in sealed exposure tubes with a double latex neck seal to minimize leakage of air. Air

flow from the plethysmograph passed through a Fleisch 0000 pneumotachograph (O.E.M., Richmond, VA) attached to a model MP-45 pressure transducer (Validyne, Northridge, CA). Flow was transduced into an electrical signal which was directly proportional to the volume inhaled by the animal enclosed in the plethysmograph. Tidal and minute volumes were not corrected for the difference between body volume change and inhaled volume. The authors' experience is that this difference is a constant and would result in values approximately 10% lower than those given.

The electrical signal was processed by an upper airways irritation monitor (Buxco Electronics, Inc., Sharon, CT) and pulmonary physiology software (Branch Technologies, Inc., Detroit, MI) for the final determination of respiratory rate, tidal volume, and minute volume. These parameters were recorded once per minute during the evaluation period. Parameter averages were determined for each 60-min exposure and group comparison made against the sham-exposed controls. Blood samples were also taken from each of the plethysmograph animals for additional assays of plasma nicotine and cotinine (see above).

Necropsy and histopathology. The Xybion Path-Tox System (Xybion Medical Systems, Cedar Knolls, NJ) was used to collate and analyze data. This software ran under the MPE-V operating system on an HP-3000 minicomputer.

Twenty males and 20 females from the high exposure and sham-exposed groups were selected randomly from survivors at the end of the 90 days and were euthanized on the day following their last exposure, along with the remaining animals in the other groups. The time interval between the last exposure and termination was 16–24 hr. The remaining animals in the high exposure and sham control groups were euthanized 7 weeks later, with no further exposure to smoke.

Animals were fasted overnight prior to necropsy. Animals were weighed and then anesthetized with 70% CO_2 in air and exsanguinated until cessation of heartbeat. Blood samples were collected from the vena cava for the different assays, using appropriate anticoagulants. The time of blood sampling was recorded.

The following assays were performed on serum obtained from blood taken at necropsy: calcium, phosphorus, chloride, sodium, potassium, glucose, alanine aminotransferase, aspartate aminotransferase, γ-glutamyl transpeptidase, urea nitrogen, albumin, creatinine, total bilirubin, and total protein. Serum samples were analyzed by Hazelton Labs America (Vienna, VA). A hematology profile, performed on whole blood samples by Roche Biomedical Laboratories (Burlington, NC), consisted of erythrocyte count, hemoglobin, hematocrit, erythrocyte volume, hemoglobin and hemoglobin concentration, total and differential white cell count, reticulocyte count, and platelet count. The anticoagulant used was disodium edetate.

Animals euthanized at scheduled necropsy were subjected to a complete gross examination with special attention being paid to the respiratory system. The lungs, liver, kidneys, brain, adrenals, spleen, and heart (excluding major vessels) were weighed. After removal, organs were kept in saline and were blotted dry prior to weighing. Organ to body weight and organ to brain weight ratios were calculated.

Tissues were fixed for 7–10 days in 10% neutral buffered formalin at a volume dilution of 1 part tissue to at least 15 parts formalin. After weights of lungs were obtained, they were infused with fixative via the trachea using gravity filling (25 cm water pressure) and the trachea was ligated.

The following tissues, and any gross lesions, were collected: adrenal glands, bone (femur and rib, with marrow), brain, cecum, colon, duodenum, eyes/optic nerve, heart, ileum, jejunum, kidneys, larynx, liver, lungs, lymph nodes (cervical, mediastinal, bronchial and mesenteric), mammary glands, nasal passages, ovaries, pancreas, parathyroids, pituitary gland, prostate gland, rectum, seminal vesicles, skeletal muscle, skin, spinal cord, spleen, stomach, testes, thyroid, thymus, trachea, urinary bladder, uterus, and Zymbal's glands. The carcass and remaining tissues were incinerated.

Respiratory tract tissues (and related lymph nodes), plus heart and gross lesions, were processed for histopathological examination. Additionally, histopathological examination was performed on the esophagus, liver, kidneys, spleen, pancreas, and urinary bladder of animals in the sham-exposed and high exposure groups. The nasal tissues were cut at four different levels, as suggested by Young (1981) who used I, II, III, and IV to describe transverse sections immediately posterior to the upper incisor teeth, at the incisive papilla, at the second palatal ridge, and at the level of the first upper molar teeth, respectively. The laryngeal sections were taken in the narrowly delineated base of the epiglottis, overlying glands in the ventral epiglottal area (Coggins *et al.*, 1980). Laryngeal sections in which the subepithelial glands were absent were considered to be in the wrong anatomical area for evaluations.

In the lung, the anatomical definitions of Reznik-Schuller (1983) were used. In this terminology, each primary bronchus branches to form, in order: lobar bronchi, segmental bronchi, subsegmental bronchi, bronchioles, terminal bronchioles, respiratory bronchioles, alveolar ducts, alveolar sacs, and alveoli. In the untreated rat, goblet cells are typically found only in the bronchi, as defined above. Consequently, in this experiment only those airways with goblet cells bearing periodic acid–Schiff/Alcian blue (PAS/AB)-positive cytoplasmic mucus droplets were considered as bronchi (primary, lobar, segmental or subsegmental). Those narrower airways in which PAS-AB-positive goblet cells are absent were considered to be bronchioles.

Tissues were stained with hematoxylin and eosin (H&E). Duplicate sections from nose (level III only),

TABLE 1

CHEMICAL AND PHYSICAL ANALYSIS OF SMOKE FROM TEST AND REFERENCE CIGARETTES

	Test			Reference		
	Low[a]	Medium[b]	High[c]	Low[a]	Medium[b]	High[c]
			Nicotine (μg/liter)			
Mean	5.23	15.2	30.6	5.18	15.2	29.8
SD	0.73	1.8	3.2	0.70	1.3	2.1
n	68	68	68	68	68	68
			Wet total particulate matter (μg/liter)			
Mean	115	325	604	138	411	795
SD	13	24	34	9	20	25
n	68	68	68	68	68	68
			Carbon monoxide (ppm)			
Mean	144	394	698	160	459	864
SD	27	75	133	14	35	60
n	68	68	68	68	68	68
			Glycerol (μg/liter)			
Mean	84.9	221	413	22.1	49.8	80.6
SD	18.0	13	29	17.5	15.0	16.7
n	6	7	8	6	7	8
			Mass median aerodynamic diameter (μm)			
Mean	0.88	0.83	0.82	0.81	0.79	0.80
SD	0.05	0.07	0.03	0.04	0.03	0.03
n	14	14	14	14	13	13
			Sigma g			
Mean	1.36	1.35	1.34	1.27	1.24	1.27
SD	0.05	0.03	0.02	0.05	0.03	0.01
n	14	14	14	14	13	13

[a] Target of 5 μg nicotine per liter.
[b] Target of 15 μg nicotine per liter.
[c] Target of 30 μg nicotine per liter.

lung, larynx, and trachea were stained with PAS/AB, to facilitate evaluation of goblet cells.

During a preliminary reading of the slides, the pathologist had access to the animal's exposure group and to previous findings. Once target organ(s) were identified, sections of those organs were randomized and reevaluated without knowledge of group or previous findings. The following abbreviations were used to describe the severity of the lesions: 0 = normal, 1 = minimal change, 2 = mild change, 3 = moderate change, 4 = marked change, 5 = severe change.

Statistical analyses. In-life functions and data from blood samples were analyzed separately for each sex by exposure group using analysis of variance techniques. Provided that the results of Bartlett's test for homogeneity of variance were not significant, group comparisons were made using the "AOVONEWAY" program in the MINITAB 5.1 series (MINITAB, State College, PA). If the results of the Bartlett's test were significant, comparisons with the sham control group were made by a t-test technique which made allowance for unequal variances ("TWO SAMPLE"). Organ weight data were analyzed

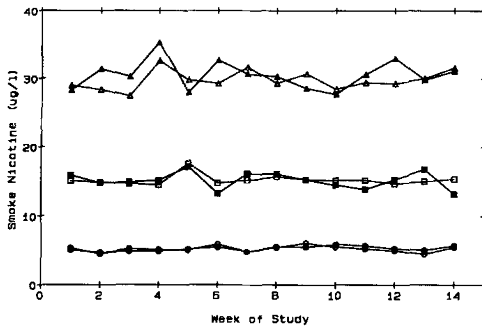

FIG. 2. Temporal variation in nicotine content of smoke produced by test and reference cigarettes. Data expressed as weekly means. Open circles: reference cigarette, low exposure; open squares: reference cigarette, medium exposure; open triangles: reference cigarette, high exposure; filled circles: test cigarette, low exposure; filled squares: test cigarette, medium exposure; filled triangles: test cigarette, high exposure.

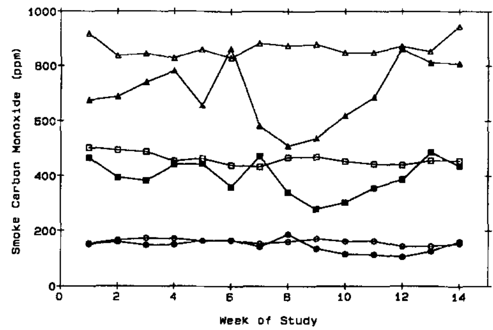

FIG. 4. Temporal variation in carbon monoxide content of smoke produced by test and reference cigarettes. Data expressed as weekly means. Open circles: reference cigarette, low exposure; open squares: reference cigarette, medium exposure; open triangles: reference cigarette, high exposure; filled circles: test cigarette, low exposure; filled squares: test cigarette, medium exposure; filled triangles: test cigarette, high exposure.

using Dunnett's test; a similar technique was used for the physiology data. Histopathology data were evaluated statistically by the nonparametric Kolmogorov–Smirnov test (Siegel, 1956). Statistical tests were carried out to 5%, two-sided criteria.

RESULTS

Inhalation Exposures

Results of the prestudy chamber characterization indicated a uniform distribution (co-

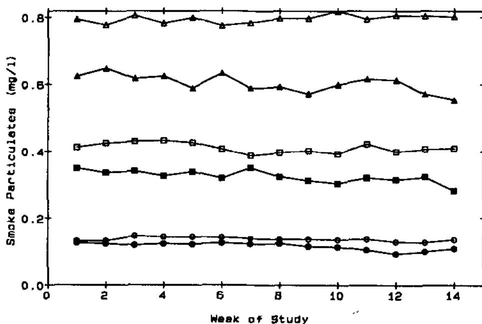

FIG. 3. Temporal variation in total particulate matter content of smoke produced by test and reference cigarettes. Data expressed as weekly means. Open circles: reference cigarette, low exposure; open squares: reference cigarette, medium exposure; open triangles: reference cigarette, high exposure; filled circles: test cigarette, low exposure; filled squares: test cigarette, medium exposure; filled triangles: test cigarette, high exposure.

efficients of variation less than 10%) of smoke for both spatial and temporal homogeneity in the test and reference exposure chambers.

The target exposures for nicotine were achieved, with overall mean values that were very close for test and reference cigarettes (Table 1). There was little temporal variation in mean nicotine concentrations produced by test and reference cigarettes (Fig. 2). There was no evidence that the nicotine yielded by the test cigarette was chemically any different from that yielded by the reference cigarette (RJRT, 1988). The mean WTPM concentrations in the test and reference groups (Table 1) were not as close as were the nicotine concentrations, and Fig. 3 shows the variation in WTPM concentrations. The CO concentrations showed approximately the same difference between test and reference cigarettes as did WTPM (Table 1), although CO showed a greater variability than did WTPM. Between Weeks 6 and 8 there was an "uncoupling" between CO and WTPM (see Fig. 4), possibly caused by interbatch variation. Glycerol concentrations were much higher in the test groups than in the reference groups (Table 1), because much of the WTPM in the test cigarette is glycerol.

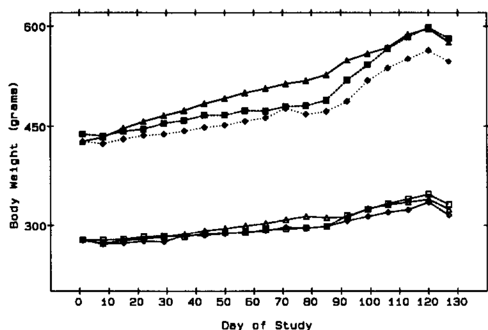

FIG. 5. Body weight change in sham controls and high exposure test and reference groups, for males and females. Filled diamonds: test cigarette, high exposure males; open diamonds: test cigarette, high exposure females; filled squares: reference cigarette, high exposure males; open squares: reference cigarette, high exposure females; filled triangles: sham control males; open triangles: sham control females.

The particle size distributions were very similar for the smoke produced by both test and reference cigarettes, with no effect of concentration of smoke (Table 1). The data obtained on MMAD and sigma g are similar to those reported for mainstream smoke by other workers (Chang *et al.*, 1985). Measurements of temperature did not show any significant deviation from approximately 20°C for each group; RH values were similar at 40–60% for each of the groups.

In-Life Observations

There was no positive serology and there were no treatment-related clinical observations or mortalities. Three animals were accidentally killed during the experiment. There were no significant differences in body weight between low, mid, and high exposure groups, or between responses in the test and reference groups. The changes in body weight for the two high exposure groups and the sham controls are given in Fig. 5.

At necropsy, each group of smoke-exposed male rats had a mean body weight that was approximately 8–10% lower than the mean in the sham controls (Table 2). Body weights of male rats in the sham controls were approximately 15% lower than were those in the room control animals. Female rats in the sham control group had body weights that were approximately 8% lower than in room controls. Females in each of the smoke-exposed groups showed similar weights as the sham controls, at each stage of the experiment. There were no significant differences between body weights or feed consumption in the comparable test and reference groups at any stage of the experiment (Table 2).

There were no significant differences between the groups for any of the CO-oximetry parameters except COHb (plus a reciprocal effect on oxyhemoglobin). For COHb, there were no significant differences between data obtained in Weeks 1, 4, 8, or 12, between sexes, or between groups exposed to smoke from test or reference cigarettes. Mean COHb values were around 10–12% in the low exposure groups, 22–25% in the medium exposure groups, and 38–40% in the high exposure groups. The mean COHb values correlated with the mean amounts of CO presented (Fig. 6).

Significant, exposure-dependent reductions in respiratory rate were observed in animals exposed to smoke from the reference cigarette ("reference animals": Table 3), as reported previously (Coggins *et al.*, 1982b).

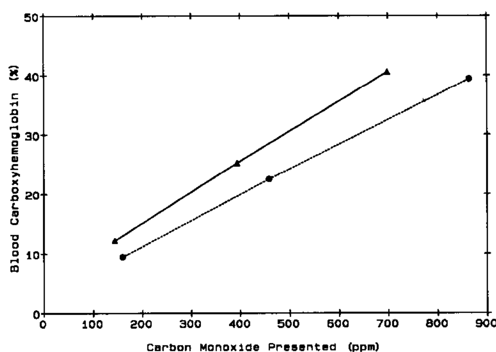

FIG. 6. Relationship between carbon monoxide presented and resulting blood carboxyhemoglobin, for test and reference cigarettes. Filled triangles: test cigarette; filled circles: reference cigarette.

TABLE 2

TERMINAL BODY WEIGHTS (g), IN ANIMALS EXPOSED TO SMOKE FROM TEST AND REFERENCE CIGARETTES

	Male			Female		
	Mean	SD	*n*	Mean	SD	*n*
			90-day necropsy			
Controls						
Sham	504.8	34.8	20	292.8	35.6	20
Room	577.7[a]	53.9	29	314.5[a]	31.8	30
Test cigarette						
Low	456.2[a]	43.0	29	276.3	28.9	30
Medium	442.9[a]	36.3	25	268.6[a]	20.6	30
High	451.3[a]	39.4	20	279.9	32.0	19
Reference cigarette						
Low	467.0[a]	43.0	30	279.0	23.7	29
Medium	459.1[a]	26.7	25	276.6	21.2	30
High	458.8[a]	40.6	20	280.6	26.3	19
			Reversibility necropsy			
Controls						
Sham	575.8	50.3	10	323.9	21.9	10
Test cigarette						
High	547.0	55.4	10	315.3	23.5	10
Reference cigarette						
High	582.1	59.2	10	330.9	32.8	10

[a] Significantly different from the sham control group.

Tidal volume was significantly less in the low exposure reference group than that in the control group. A significant exposure-dependent decrease in minute ventilation was observed in each group of reference animals: values were 63, 42, and 39% of sham-exposed values for the low, mid, and high groups. No reductions in tidal volume, breathing frequency, or minute ventilation were observed in groups of animals exposed to smoke from the test cigarette ("test animals").

There was no significant difference between plasma nicotine concentrations obtained in Weeks 2, 5, 9, or 13, or between sexes. Plasma nicotine concentrations correlated with nicotine presented (Fig. 7), but were higher in the test animals than in the reference animals. The overall mean values (Table 4) in the former at low, medium, and high exposures, respectively, were 2.5, 4.9, and 6.2 times the concentrations seen in the latter, when corrected for the nicotine content of the test and reference smoke. When corrections were also made for minute ventilation the ratios between test and reference cigarettes at low, medium, and high exposures were reduced to 1.4, 2.2, and 2.3, respectively, with an overall factor of 1.97.

For plasma cotinine, there was no significant difference between mean concentrations obtained in Weeks 2, 5, 9, or 13, or between sexes. The overall mean values (Table 4) in the test groups at low, medium, and high exposure were 3.3, 4.8, and 7.8 times the mean values in the reference groups. Corrections for minute volume reduced these factors to

TABLE 3

RESPIRATORY PHYSIOLOGY PARAMETERS IN ANIMALS EXPOSED TO SMOKE PRODUCED BY TEST AND REFERENCE CIGARETTES[a]

	Frequency[b]			Tidal volume[c]			Minute volume[d]		
	Mean	SD	n	Mean	SD	n	Mean	SD	n
Control									
Sham	133	28	30	1.26	0.40	30	164	61	30
Test cigarette									
Low	134	16	26	1.13	0.46	26	147	51	26
Medium	138	16	29	1.18	0.35	29	151	42	29
High	142	15	29	1.35	0.30	29	174	42	29
Reference cigarette									
Low	114[e]	16	27	0.93[e]	0.33	27	103[e]	36	27
Medium	71.7[e]	16.6	26	1.03	0.35	26	68.7[e]	25.4	26
High	54.4[e]	19.0	29	1.36	0.39	29	64.9[e]	24.9	29

[a] Data expressed as averages for 60 min of exposure; pooled from Weeks 1, 5, 9, and 13 and from both sexes.
[b] Breaths per minute.
[c] Milliliters (not corrected for the difference between inhaled volume and change in body volume.)
[d] Milliliters per minute.
[e] Significantly different from the sham control group.

2.0, 2.2, and 2.9 for low, medium, and high exposure, respectively, with an overall mean of 2.3. The mean values for the cotinine to nicotine ratio varied with the amount of smoke presented, with the highest values in the low exposure groups (Table 4). The ratio of plasma nicotine to cotinine was approximately similar in the test and reference groups, with large variability in the data.

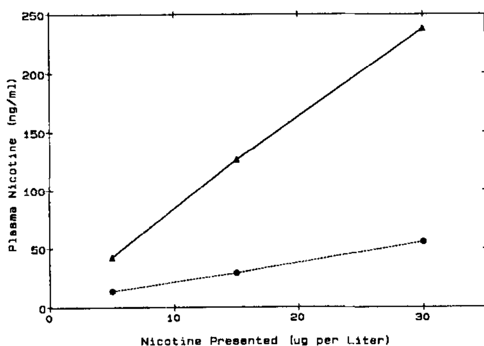

FIG. 7. Relationship between nicotine presented in smoke from test and reference cigarettes and resulting plasma nicotine concentrations. Filled triangles: test cigarette; filled circles: reference cigarette.

Necropsy Data

There were no significant differences between the groups for any of the hematology or clinical chemistry parameters, at either of the necropsy periods. Blood COHb, nicotine, and cotinine concentrations were negligible. In both of the high exposure groups there was a significant increase in heart weight (Table 5). Male test animals in the mid and high groups had adrenal weights that were significantly elevated when compared with the sham controls (Table 6). No other significant organ weight changes were observed. At the recovery necropsy, heart and adrenal weights were the same in each group (Tables 5, 6). The changes described above were similar when the organ weights were expressed as percentage of brain weight.

Congested and/or reddened lungs were noted at necropsy, in one to two female ani-

TABLE 4

PLASMA NICOTINE AND COTININE CONCENTRATIONS, FOR ANIMALS EXPOSED TO SMOKE PRODUCED BY TEST AND REFERENCE CIGARETTES[a]

	Nicotine (ng/ml)			Cotinine (ng/ml)			Cotinine/nicotine (%)		
	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*
Test cigarette									
Low	42.3	14.4	69	44.9	20.2	58	121	63.7	58
Medium	126	42	70	103	52.4	68	87.9	52.1	68
High	239	71	69	158	61	63	75.3	45.8	63
Reference cigarette									
Low	14.0	5.3	67	12.1	6.2	45	97.8	51.5	45
Medium	29.3	14.9	67	20.2	9.2	59	81.4	50.6	59
High	55.5	20.1	69	28.4	13.0	60	56.9	27.4	60

[a] Data pooled from several weeks and from both sexes. Samples were taken after 55–60 min of smoke exposure.

TABLE 5

HEART WEIGHTS (g), IN ANIMALS EXPOSED TO SMOKE FROM TEST AND REFERENCE CIGARETTES

	Male			Female		
	Mean	SD	*n*	Mean	SD	*n*
			90-day necropsy			
Controls						
Sham	1.64	0.14	20	1.04	0.11	20
Room	1.75	0.17	29	1.08	0.09	30
Test cigarette						
Low	1.52	0.16	29	0.98	0.11	30
Medium	1.66	0.21	25	1.06	0.08	30
High	1.85[a]	0.18	20	1.24[a]	0.14	19
Reference cigarette						
Low	1.59	0.16	30	1.01	0.09	29
Medium	1.65	0.14	25	1.06	0.09	30
High	1.76	0.20	20	1.16[a]	0.13	19
			Reversibility necropsy			
Controls						
Sham	1.78	0.13	10	1.10	0.08	10
Test cigarette						
High	1.85	0.19	10	1.15	0.09	10
Reference cigarette						
High	1.88	0.23	10	1.13	0.09	10

[a] Significantly different from the sham control group.

TABLE 6

ADRENAL WEIGHTS (g), IN ANIMALS EXPOSED TO SMOKE FROM TEST AND REFERENCE CIGARETTES

	Male			Female		
	Mean	SD	*n*	Mean	SD	*n*
			90-day necropsy			
Controls						
Sham	0.078	0.013	20	0.088	0.012	20
Room	0.075	0.013	29	0.091	0.012	30
Test cigarette						
Low	0.074	0.012	29	0.090	0.013	30
Medium	0.090[a]	0.011	25	0.096	0.012	30
High	0.092[a]	0.018	20	0.097	0.015	19
Reference cigarette						
Low	0.078	0.011	30	0.092	0.012	29
Medium	0.086	0.014	25	0.092	0.013	29
High	0.082	0.013	19	0.094	0.011	19
			Reversibility necropsy			
Controls						
Sham	0.062	0.011	10	0.090	0.013	10
Test cigarette						
High	0.066	0.007	10	0.089	0.009	10
Reference cigarette						
High	0.070	0.011	10	0.090	0.010	10

[a] Significantly different from the sham control group.

mals of each group except reference low exposure. In the reference medium exposure group, a total of eight female animals showed this change, which was not accompanied by corresponding microscopic changes such as increased congestion and/or hemorrhage. There were no such changes in males. The reddened lungs may have been caused by the anesthesia and blood collection techniques used at necropsy.

Histopathology

Pronounced differences were noted between responses in the test and reference animals, the appearance of the former being very similar to that of animals in both control groups. Reference animals showed changes in each section of the respiratory tract, changes which closely resembled those reported previously in smoke-exposed rats (Coggins *et al.*, 1980, 1982a; Kendrick *et al.*, 1976; Walker *et al.*, 1978; Wehner *et al.*, 1981). Most of the changes induced were reversible, within the 7-week recovery period.

In the reference animals, the *nasal passages* showed chronic active inflammation, epithelial hyperplasia, and squamous metaplasia (Fig. 8; Table 7). These changes were seen primarily in section I. In section II, atrophy of the olfactory epithelium was noted, in the high exposure reference cigarette group (Fig. 9). This change has been reported previously in the mouse (Matulionis, 1974); it

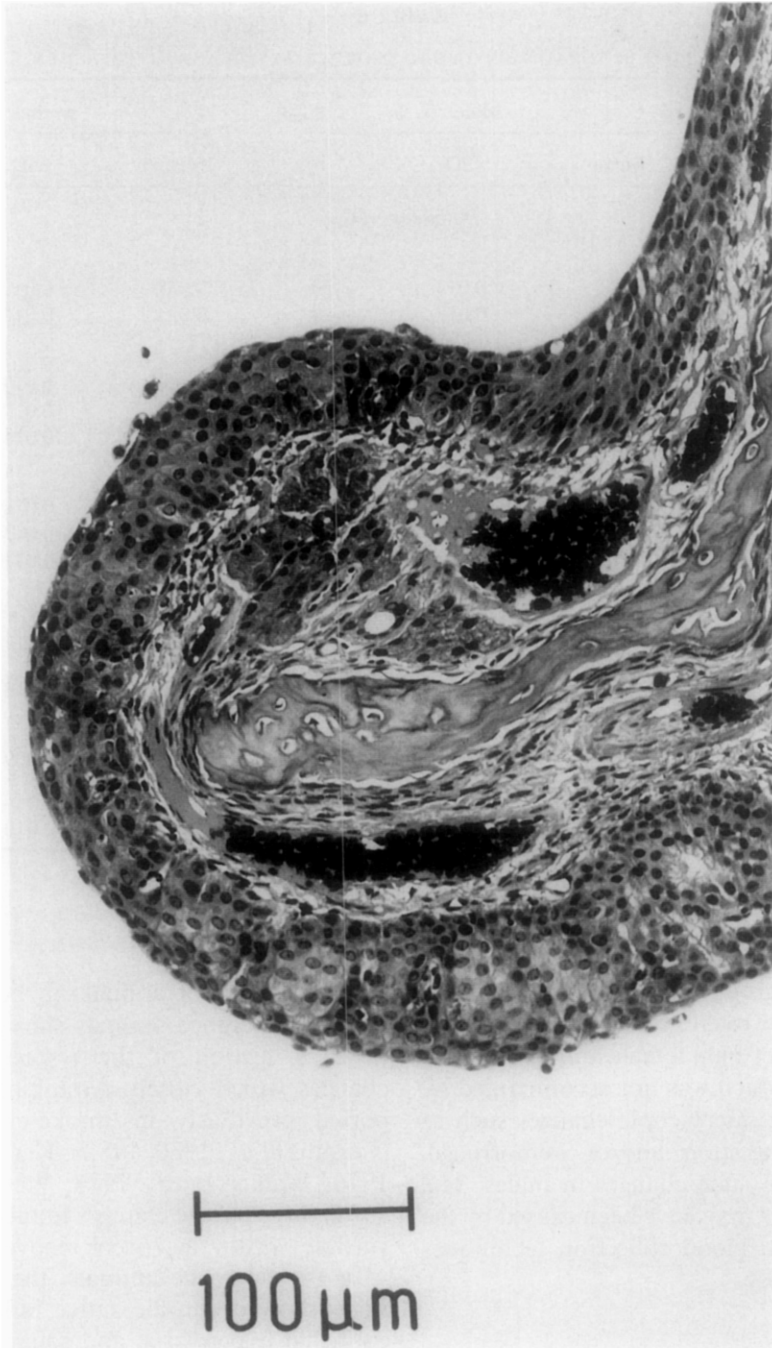

FIG. 8. Tip of nasoturbinate in Nasal I from high exposure reference animal, showing inflammation and epithelial hyperplasia. H&E stain; original magnification ×285.

was not seen in the test animals. The nasal changes induced in the high exposure reference groups were only partially reversed within the 7-week recovery period; the minimal changes induced in the test animals were completely reversed (Table 7).

TABLE 7

HISTOPATHOLOGY OF THE NASAL PASSAGES, IN ANIMALS EXPOSED TO SMOKE PRODUCED BY TEST AND REFERENCE CIGARETTES (HIGH EXPOSURE GROUPS ONLY)

	Male					Female				
	—[a]	1[b]	2[c]	3[d]	TL[e]	—	1	2	3	TL
Squamous metaplasia, Nasal I										
Control	18	0	2	0	20	20	0	0	0	20
Test	20	0	0	0	20	19	0	0	0	19
Reference	4	2	14	0	20[f]	2	7	9	1	19[f]
Squamous metaplasia, Nasal I, after reversibility										
Control	10	0	0	0	10	10	0	0	0	10
Test	10	0	0	0	10	10	0	0	0	10
Reference	8	1	1	0	10	8	1	1	0	10
Epithelial hyperplasia, Nasal I										
Control	6	8	4	2	20	11	9	0	0	20
Test	7	7	6	0	20	13	4	2	0	19
Reference	0	1	3	16	20[f]	0	0	9	10	19[f]
Epithelial hyperplasia, Nasal I, after reversibility										
Control	4	3	3	0	10	4	4	2	0	10
Test	0	3	7	0	10	4	3	3	0	10
Reference	0	0	8	2	10[f]	0	2	6	2	10[f]
Chronic, active inflammation, Nasal I										
Control	14	3	3	0	20	15	5	0	0	20
Test	14	5	1	0	20	15	4	0	0	19
Reference	3	9	7	1	20[f]	5	9	5	0	19[f]
Chronic, active inflammation, Nasal I, after reversibility										
Control	4	6	0	0	10	4	4	2	0	10
Test	2	4	4	0	10	3	6	1	0	10
Reference	0	2	7	1	10[f]	1	1	7	1	10[f]
Atrophy of olfactory epithelium, Nasal II										
Control	20	0	0	0	20	20	0	0	0	20
Test	20	0	0	0	20	19	0	0	0	19
Reference	14	0	3	3	20[f]	13	0	1	5	19[f]
Atrophy of olfactory epithelium, Nasal II, after reversibility										
Control	10	0	0	0	10	10	0	0	0	10
Test	10	0	0	0	10	10	0	0	0	10
Reference	10	0	0	0	10	8	0	1	1	10

[a] Normal.
[b] Minimal change.
[c] Mild change.
[d] Moderate change.
[e] Total examined.
[f] Significantly different distribution from the sham control group.

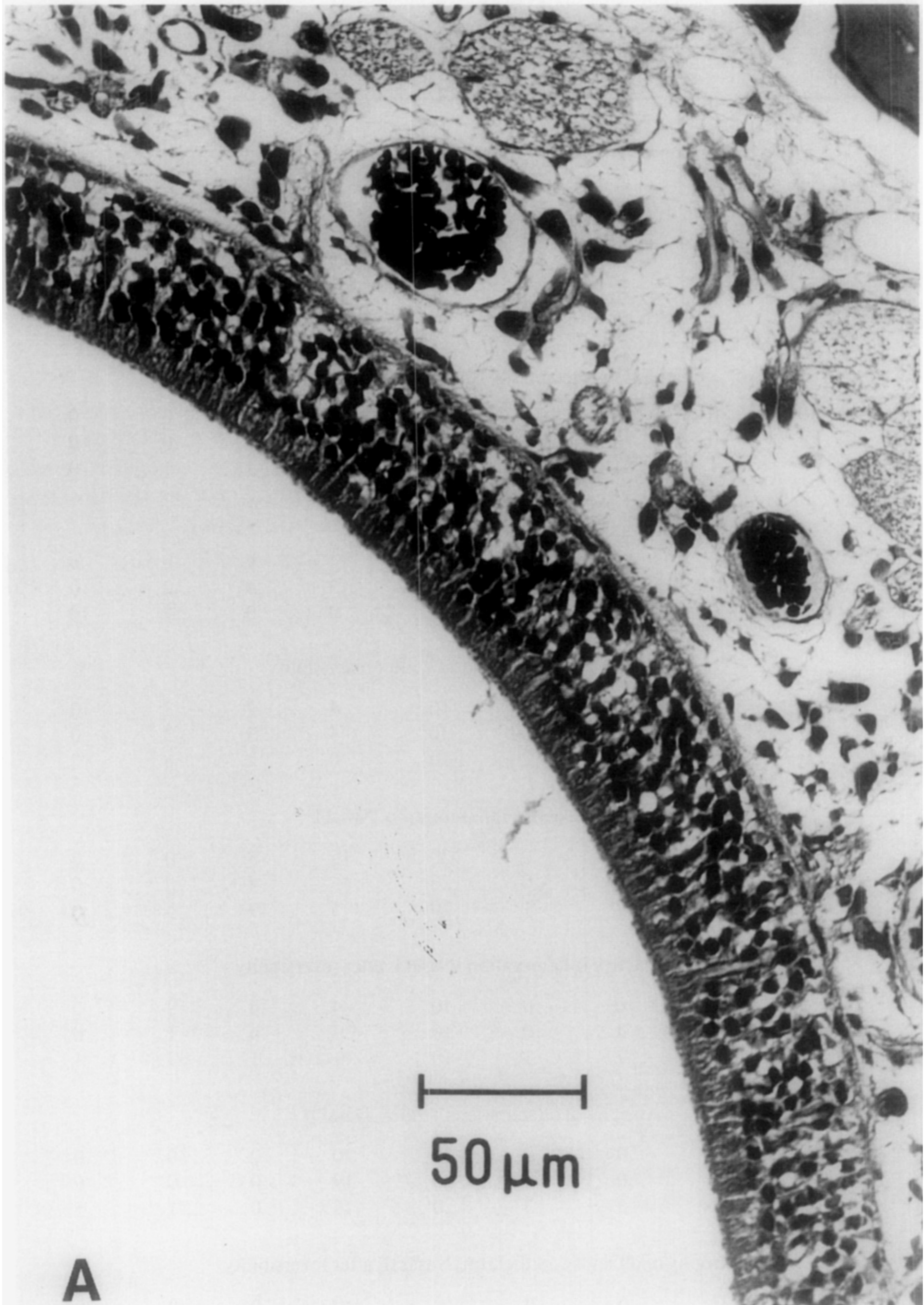

FIG. 9. Olfactory epithelium in Nasal II, from control (A) and high exposure reference (B) animals. In (B), note the decrease in thickness and shrunken cells which have lost their typical parallel orientation. H&E stain; original magnification ×450.

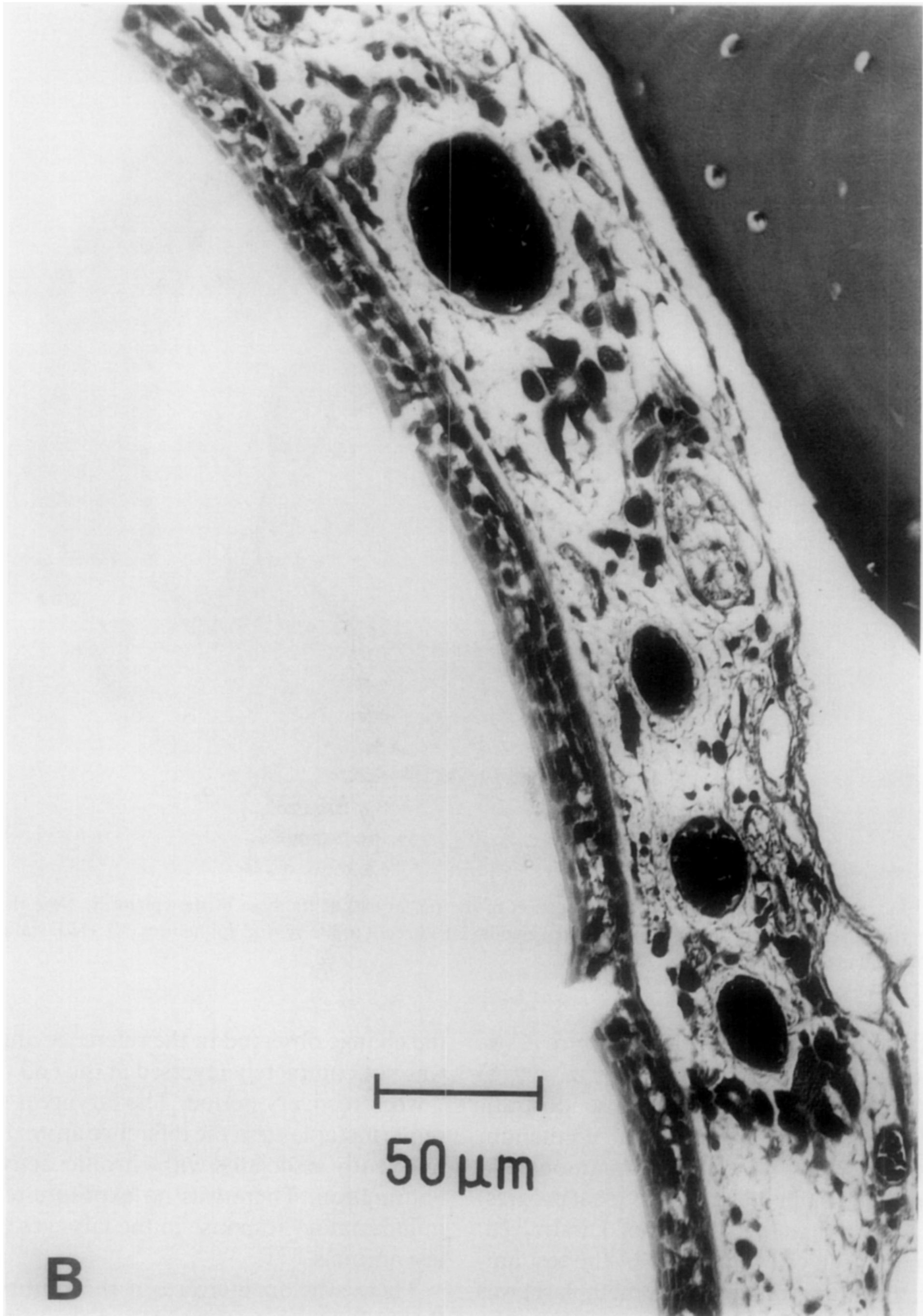

FIG. 9—*Continued*

The *larynx* was sectioned at or near the base of the epiglottis (Fig. 10). Squamous metaplasia of the ventral epithelium (Fig. 11) was seen in each of the reference animals. This change was seen throughout the organ and was similar in incidence and severity (Ta-

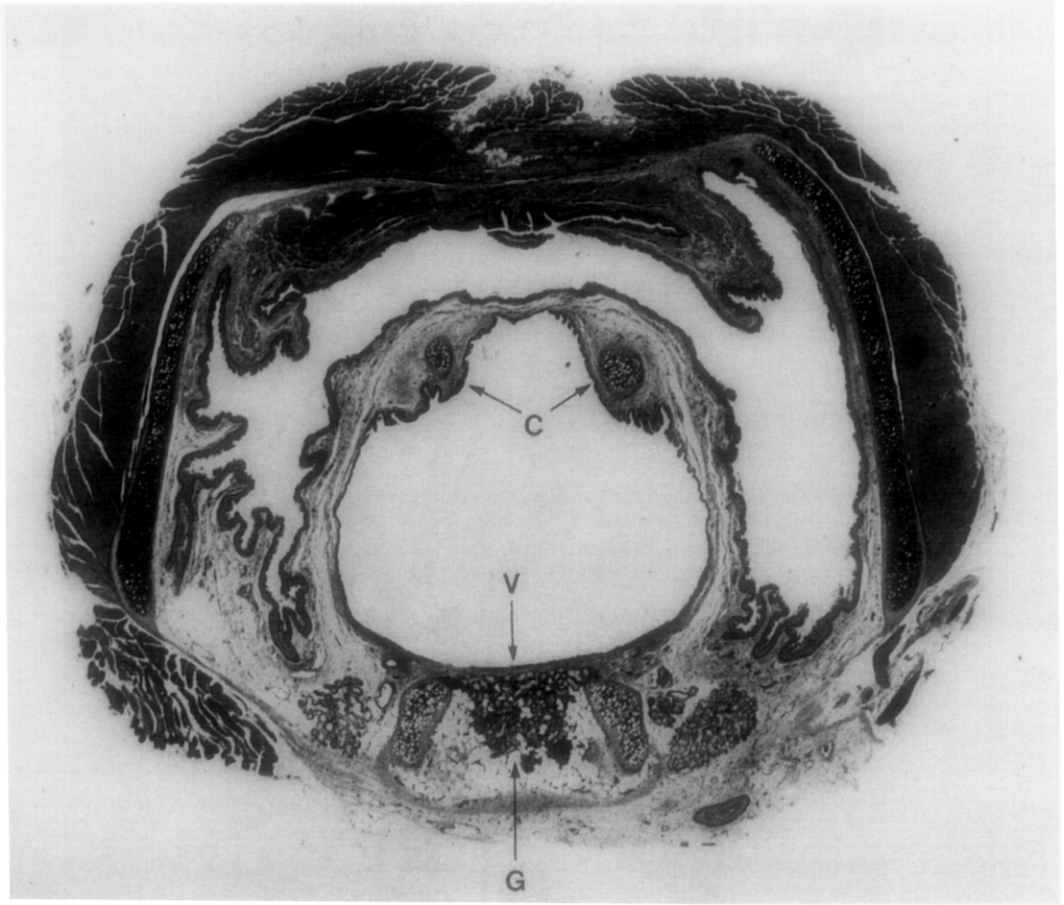

FIG. 10. Low power view of a cross-section of the rat larynx, at the base of the epiglottis. Note the arytenoid cartilages (C) and the submucosal glands (G) adjacent to the ventral epithelium (V). H&E stain; original magnification ×25.

ble 8) to that reported in the literature (Coggins *et al.,* 1980, 1982a; Walker *et al.,* 1978) for this organ. The normally "pseudostratified" cuboidal to low columnar epithelium was transformed to stratified squamous epithelium, in which there was a typical progression toward flattened layers of keratinized, desquamating cell remnants. In the test animals, the laryngeal squamous metaplasia was minimal to mild and was only noted in the medium and high exposure groups. The change was only seen in a very precise area of the ventral larynx, shown as V in Fig. 10. This change in the test groups was reversed completely, except in one high exposure animal; the change observed in the reference animals was not completely reversed at the end of the 7-week recovery period. The laryngeal squamous metaplasia in the reference animals was frequently associated with chronic, active inflammation. There was no exposure-related inflammatory response in the larynges of the test animals.

There was an increase in the number of PAS-AB-positive goblet cells in the primary, lobar, segmental, and subsegmental *bronchi* in the reference groups (Table 9), similar to that described previously (Coggins *et al.,* 1980; Walker *et al.,* 1978). After reversibility, there were no differences between the groups.

TABLE 8

HISTOPATHOLOGY OF LARYNX, IN ANIMALS EXPOSED TO SMOKE PRODUCED BY TEST AND REFERENCE CIGARETTES

	Male						Female					
	—[a]	1[b]	2[c]	3[d]	4[e]	TL[f]	—	1	2	3	4	TL
				Laryngeal squamous metaplasia								
Controls												
Sham	14	2	0	0	0	16	14	1	0	0	0	15
Room	21	2	0	0	0	23	20	2	0	0	0	22
Test cigarette												
Low	20	3	0	0	0	23	15	4	0	0	0	19
Medium	5	9	1	0	0	15	15	6	0	0	0	21
High	1	9	1	0	0	11[g]	3	8	3	0	0	14[g]
Reference cigarette												
Low	0	0	6	15	1	22[g]	0	0	7	11	1	19[g]
Medium	0	0	2	8	4	14[g]	0	1	5	14	4	24[g]
High	0	0	0	8	3	11[g]	0	0	0	12	1	13[g]
				Laryngeal squamous metaplasia, at reversibility								
Control	7	2	0	0	0	9	9	0	0	0	0	9
Test	7	1	0	0	0	8	7	0	0	0	0	7
Reference	0	0	5	2	0	7[g]	0	3	5	0	0	8[g]

[a] Normal.
[b] Minimal change.
[c] Mild change.
[d] Moderate change.
[e] Marked change.
[f] Total examined, excluding larynges for which diagnostic term "Subepithelial glands absent—precludes evaluation of epithelium" was applied.
[g] Significantly different distribution from the sham control group.

A limited amount of congestion (abnormal intravascular accumulation of blood) was noted as a very subtle change in the *lungs*, in the high exposure reference animals only. Increased intraalveolar brown-gold macrophages were noted, in the high exposure reference groups only. Occasionally, these macrophages formed aggregates within an alveolus (Fig. 12), as described previously (Kendrick *et al.*, 1976; Coggins *et al.*, 1980). There were no significant group differences in the histopathology seen in the *trachea*, or in any of the other organs examined.

In summary, after 90-days the following histopathological changes were seen in the reference groups but were either absent or substantially reduced in the test groups: epithelial hyperplasia in Nasal I, squamous metaplasia of Nasal I and larynx, inflammation in Nasal I and larynx, olfactory atrophy in Nasal II, increased goblet cells in the conducting airways, pulmonary brown-gold macrophages, and pulmonary congestion. Many of the changes observed in the reference animals reversed completely by the end of the recovery period. The exposure-related changes which did not reverse completely in the reference animals were squamous meta-

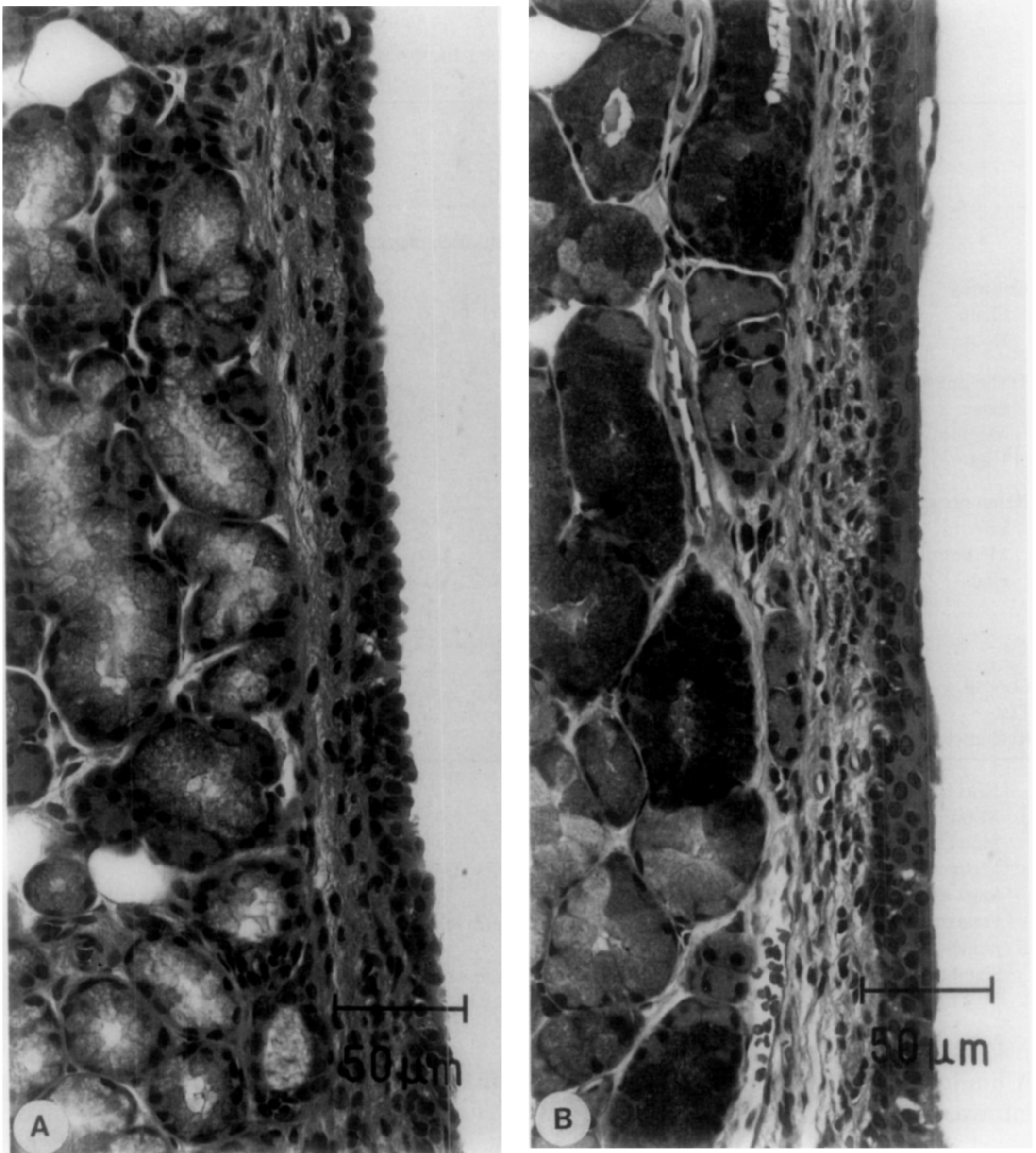

FIG. 11. Ventral laryngeal epithelium, from control (A), high exposure test (B), and high exposure reference (C) animals. In (C), note the presence of sub- and intraepithelial infiltrations of leukocytes, and squamous metaplasia of the epithelium. The normally "pseudostratified" cuboidal to low columnar epithelium and ciliated epithelium are transformed to stratified squamous epithelium in which there is progression of flattened layers of keratinized, desquamating cell remnants. H&E stain; original magnification ×380.

plasia of the ventral laryngeal epithelium, brown-gold pulmonary macrophages, atrophy of the olfactory epithelium in Nasal II, and increased numbers of goblet cells in the bronchi.

DISCUSSION

The exposure of test and reference groups of animals was directly comparable for the target compound, nicotine. This is a prereq-

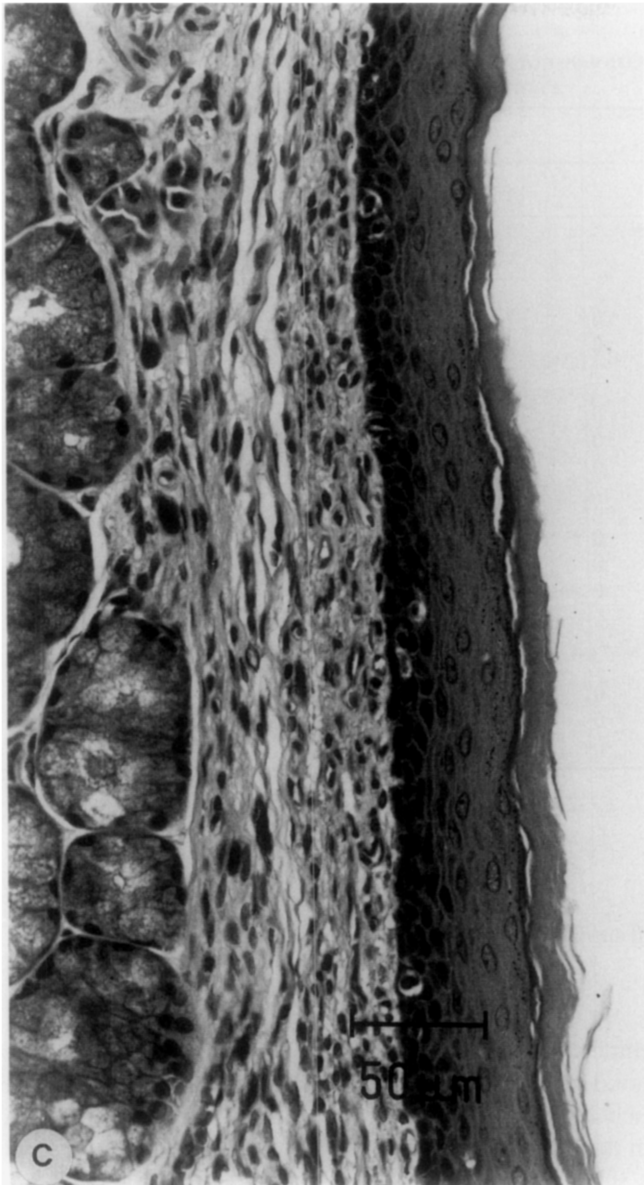

FIG. 11—*Continued*

uisite for valid comparisons to be made of any biological responses subsequently produced after cumulative exposures. The blood COHb and plasma nicotine concentrations confirm that the animals were not only exposed to the smoke, but that large amounts of CO and nicotine were in fact inhaled and retained, a further prerequisite for biological comparisons with cigarette smoke. Although the smoke presented to the animals was similar for selected chemical components and also for physical attributes, it was inhaled differently by the animals in the test and reference groups, because of exposure-related

TABLE 9

HISTOPATHOLOGY OF THE CONDUCTING AIRWAYS, IN ANIMALS EXPOSED TO SMOKE PRODUCED BY TEST AND REFERENCE CIGARETTES

	Male					Female				
	—[a]	1[b]	2[c]	3[d]	TL[e]	—	1	2	3	TL
					Goblet cells					
Controls										
Sham	0	17	3	0	20	0	20	0	0	20
Room	0	25	4	0	29	0	29	1	0	30
Test cigarette										
Low	0	26	3	0	29	0	27	3	0	30
Medium	0	25	0	0	25	0	28	2	0	30
High	0	19	1	0	20	0	15	3	1	19
Reference cigarette										
Low	0	20	8	2	30	0	20	9	0	29
Medium	0	5	12	8	25[f]	0	6	9	15	30[f]
High	0	2	9	9	20[f]	0	4	7	8	19[f]
					Goblet cells, after reversibility					
Control	0	10	0	0	10	0	9	1	0	10
Test	0	9	1	0	10	0	10	0	0	10
Reference	0	5	5	0	10	0	8	2	0	10

[a] Normal.
[b] Few.
[c] Moderate.
[d] Many.
[e] Total examined.
[f] Significantly different distribution from the sham control group.

decreases in the minute ventilation of the reference animals. Smoke from the test cigarette was inhaled in much larger quantities than was the smoke from the reference cigarette.

The COHb data show that the exposures used were very high: acute toxicity is likely to occur in animals with COHb concentrations only 10–15% higher than those reported here. The COHb concentrations attained in the present study are comparable to published data on this topic (Kendrick *et al.*, 1976; Coggins *et al.*, 1982a; Loscutoff *et al.*, 1982). The lack of a difference between the COHb concentrations in the test and reference groups, when there was a large difference in the amounts of smoke inhaled, probably reflects the very high affinity of hemoglobin for CO (Douglas *et al.*, 1912). It is unlikely that equilibrium COHb concentrations were achieved in the low or medium groups during their 1-hr exposures (Mosberg *et al.*, 1987).

The increased heart weights are similar to increases reported in guinea pigs exposed to cigarette smoke (Lough, 1978), where it was suggested that the cardiomegaly was caused by the CO content of the smoke. It has also been reported (Penney *et al.*, 1974; Ayres *et al.*, 1987) that CO alone at concentrations as high as those seen here in the smoke will also produce cardiomegaly. The transient increases in heart weights reported here were probably caused by the very high CO concen-

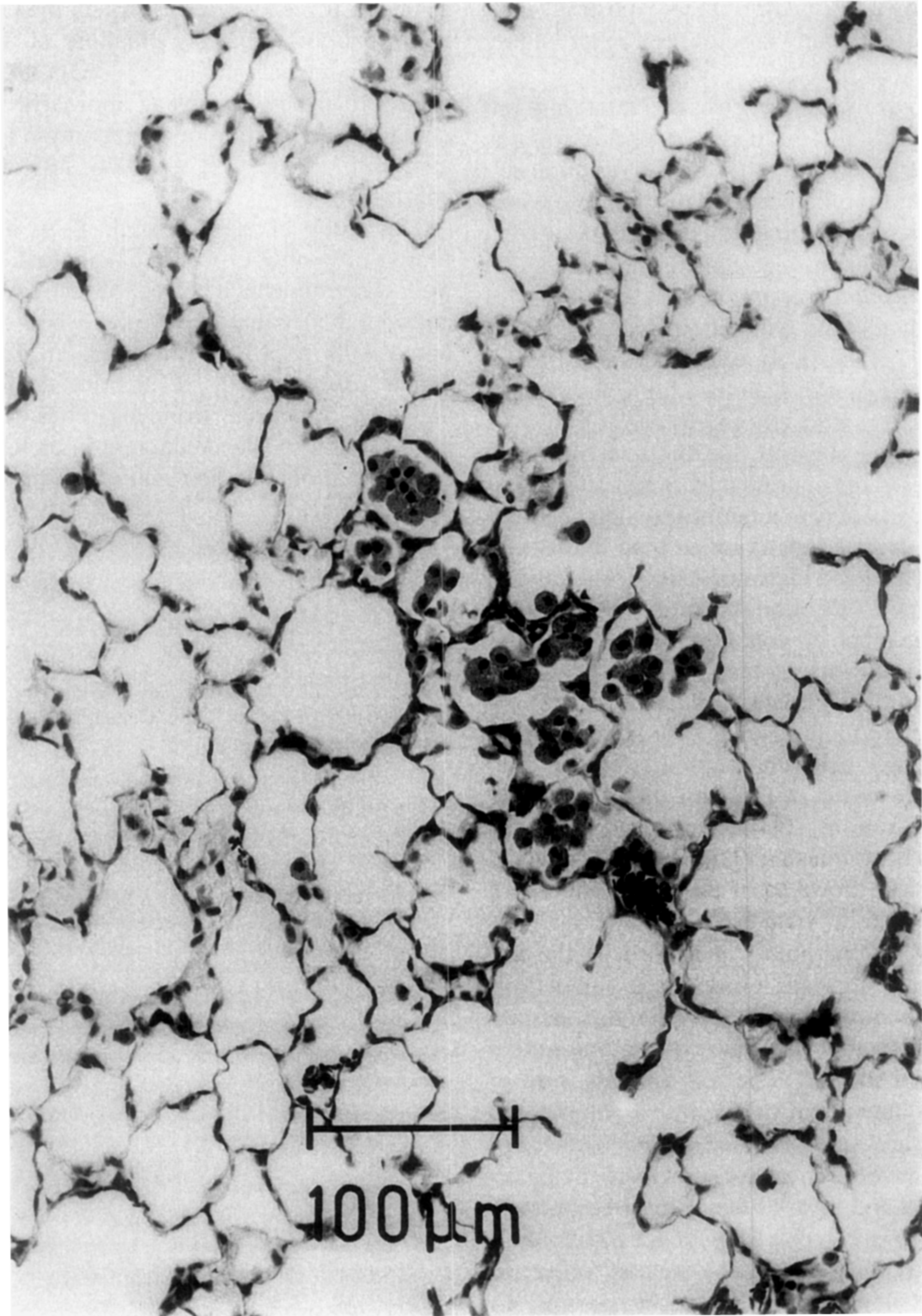

FIG. 12. Lung tissue from high exposure reference animal. Note the aggregate of intraalveolar pigmented macrophages. H&E stain; original magnification ×285.

trations used in the smoke exposures: their rapid reversibility has not been reported previously.

Part of the difference between test and reference groups for plasma nicotine was the smoke-induced depressions in minute volume in the reference groups. However, correction for minute ventilation still resulted in the reference animals having plasma nicotine concentrations approximately half those of the test animals. Differences in the "bioavailability" of the nicotine yielded by test and reference cigarettes have not been observed in subsequent work (Mosberg *et al.*, 1989). This latter work suggests that the reference cigarette used in the present work had a relatively low availability of nicotine (possibly, because of the special design), rather than the test cigarette having a high availability. The ratio of cotinine to nicotine indicates that the nicotine metabolism was approximately similar in the test and reference groups, with large variability in the data. Recent work in human subjects (deBethizy *et al.*, 1988) has shown no differences between test and reference cigarettes in terms of resultant plasma nicotine concentrations, plasma cotinine, and the plasma cotinine to nicotine ratio. Plasma nicotine was shown to be directly proportional to the nicotine yield of the cigarette.

The histopathology produced by the test cigarette showed pronounced reductions when compared with the reference, despite the substantial difference in the amount of material inhaled. The test animals were in many cases identical to untreated controls. All of the responses noted in the reference animals have been reported previously in the literature, and most of the changes completely reversed in 7 weeks. Many of the histopathology responses observed in the reference groups were not seen in the test groups; no novel lesions were noted in the test animals.

The lack of an effect of very high exposures of smoke (both cigarette types) on widely used parameters of clinical pathology means that little comparative information can be gained in this experiment through the use of these endpoints. The reduction of body weight in the male smoke-exposed groups indicates that these groups all approached the operational definition of a "maximum tolerated dose" (body weight 90% or less of the control group).

This study of the potential effects of repeated nose-only exposure of rats to smoke from cigarettes which heated tobacco showed considerably reduced responses when compared with responses produced, at similar smoke concentrations of nicotine, WTPM, and CO, by smoke from cigarettes which burned tobacco. This reduction in biological activity is probably the result of the substantial simplification of the smoke chemistry through the elimination of burning tobacco.

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