Canines as Sentinel Species for Assessing Chronic Exposures to Air Pollutants: Part 1. Respiratory Pathology


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A complex mixture of air pollutants is present in the ambient air in urban areas. People, animals, and vegetation are chronically and sequentially exposed to outdoor pollutants. The objective of this first of 2 studies is to evaluate by light and electron microscopy the lungs of Mexico City dogs and compare the results to those of 3 less polluted cities in Mexico. One hundred fifty-two clinically healthy stray mongrel dogs (91 males/61 females), including 43 dogs from 3 less polluted cities, and 109 from southwest and northeast metropolitan Mexico City (SWMMC, NEMMC) were studied. Lungs of dogs living in Mexico City and Cuernavaca exhibited patchy chronic mononuclear cell infiltrates along with macrophages loaded with particulate matter (PM) surrounding the bronchiolar walls and extending into adjacent vascular structures; bronchiolar epithelial and smooth muscle hyperplasia; peribronchial fibrosis, microthrombi, and capillary and venule polymorphonuclear leukocytes (PMN) margination. Ultrafine PM was seen in alveolar type I and II cells, endothelial cells, interstitial macrophages (M0), and intravascular M0-like cells. Bronchoalveolar lavage showed significant numbers of alveolar macrophages undergoing proliferation. Exposure to complex mixtures of pollutants—predominantly particulate matter and ozone—is causing lung structural changes induced by the sustained inflammatory process and resulting in airway and vascular remodeling and altered repair. Cytokines released from both, circulating inflammatory and resident lung cells in response to endothelial and epithelial injury may be playing a role in the pathology described here. Deep concern exists for the potential of an increasing rise in lung diseases in child populations exposed to Mexico City’s environment.

Key Words: dogs; air pollution; lungs; particulate matter; ultrafine particulate matter; ozone; endothelial and epithelial lung dysfunction; chronic lung inflammation; lung remodeling.

Air pollution produces adverse health effects. A complex mixture of gases, chemicals, and particulate matter (PM) is present in the ambient air in polluted urban and industrial areas. Epidemiological studies strongly suggest that children and adults have increased morbidity and mortality from photochemical smog and PM (Abbey et al., 1999; Dockery et al., 1989; U. S. EPA, 1992; 1996; Woodruff et al., 1997). Mexico City (MC) is a 20 million-person megacity with severe air pollution problems (Bascom, 1996; Edgerton et al., 1999). The consequences of lifelong daily exposures to atmospheric pollutants to the respiratory and cardiovascular apparatus of healthy children are of considerable clinical and epidemiological importance. A significant association between a lifetime exposure to southwest Mexico City’s (SWMMC) atmosphere and chest X-ray abnormalities in healthy children has been reported (Calderón-Garcideneas et al., 2000). An excess of 6.9% in infant mortality in SWMMC is associated with an increase of 10 µg/m3 in the concentrations of fine PM 3–5 days prior to the children’s demise (Loomis et al., 1999). Domestic and wild animals living in polluted environments represent an important biological source to obtain data useful for assessing risks to human health (Schilderman et al., 1997). Canines are often the species of choice as an experimental model for the study of pulmonary responses to long-term exposure to air pollutants (Heyder and Takenaka, 1996). There are multiple similarities between the canine and the human lungs in terms of size, anatomy, patterns of development, pulmonary function, and cellular composition (Pinkerton et al., 2001). Further, dogs live long enough to study them and to ensure that histopathological findings are not confounded by aging (Heyder and Takenaka, 1996; Pinkerton et al., 2001). Several long-term environmentally controlled chamber canine studies with single or combined air pollutants have been done (Heyder and Takenaka, 1996). Long-term retention of PM at the bronchiolar level has been shown in beagles given shallow bolus inhalation of 2.5 μm polystyrene particles (Kreylings et al., 1999, Kreyliong and Scheuch, 2000), and healthy adults show 100% retention at 24 h, of 6, 8, and 10 μm particles deposited in gener-
TABLE 1
Study Population

<table>
<thead>
<tr>
<th>City</th>
<th>No. of animals</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mexico City*</td>
<td>SWMMC: 74</td>
<td>36 M; 38 F</td>
<td>1.7 ± 1</td>
<td>13.8 ± 7.6 kg</td>
</tr>
<tr>
<td></td>
<td>NEMMMC: 35</td>
<td>20 M; 15 F</td>
<td>1.9 ± 1</td>
<td>18.4 ± 11 kg</td>
</tr>
<tr>
<td>Cuernavaca</td>
<td>29</td>
<td>26 M; 3 F</td>
<td>2.2 ± 1.1</td>
<td>23.1 ± 6.4 kg</td>
</tr>
<tr>
<td>Tlaxcala</td>
<td>7</td>
<td>5 M; 2 F</td>
<td>1.8 ± 0.2</td>
<td>11.8 ± 2.8 kg</td>
</tr>
<tr>
<td>Tuxpan</td>
<td>7</td>
<td>4 M; 3 F</td>
<td>1.6 ± 1</td>
<td>10.8 ± 4.4 kg</td>
</tr>
<tr>
<td>Total</td>
<td>152</td>
<td>91 M; 61 F</td>
<td>1.68 ± 0.4</td>
<td>15.5 ± 5.1 k</td>
</tr>
</tbody>
</table>

*Included 19 dogs > 4 years (7.2 ± 2.1 years).

The rationale behind selecting the SW and the NE geographical areas in MC lies in the spacial distribution of pollutants such PM_{10}, O_3, sulfur dioxide (SO_2), and nitrogen dioxide (NO_2) within the city, a reflection of the higher concentrations of particulate and gaseous emissions in the northern part of the city where most industries are located. Ozone is higher in the south, a residential area, as the result of wind transport of the mass precursor pollutants emitted in the industrial north and central parts of MC. Northeast MC, on the other hand, is characterized by pollutant emissions from light and medium industries and high vehicular traffic, both on paved and unpaved roads (Edgerton et al., 1999), with high PM values. PM_{10} averages on the order of 235 μg/m³ (Cicero-Fernandez et al., 1993) and annual averages of PM_{2.5} are 44 μg/m³ (Edgerton et al., 1999). This article is the first of 2 dealing with the histopathological changes in the respiratory apparatus and the heart in canines exposed naturally to a complex mixture of air pollutants in urban areas. The objective of this study is to identify and characterize the histopathology in the target lung structures involved for each geographical cohort, and correlate the findings with ambient air pollutants. The objective of the second article (Calderón-Garcidueñas, 2001) is to characterize the cardiac histopathology in the different geographical cohorts, and to make a correlation between the cardiac and the major lung pathological findings. The overall goal of this work is to provide data to build future hypothesis-driven mechanistic studies that would explain the biological plausibility of the epidemiological data suggesting increased cardiorespiratory morbidity and mortality in susceptible populations (children and the elderly) associated with air pollutants.

**MATERIALS AND METHODS**

**Canine population.** One hundred fifty-two clinically healthy stray mongrel dogs (91 males/61 females) including 7 dogs from Tuxpan, 7 from Tlaxcala, 29 from Cuernavaca, and 109 from southwest (SWMMC) and northeast Mexico City (NEMMC) were studied. Their average weight was 16.8 ± 5 kg. Animals were divided into 2 age groups: 6 months–4 years (n = 132) and > 5 years (n = 19; Table 1). The study protocol was reviewed and approved by the Basic Research Committee at the Instituto Nacional de Pediatría in MC. Work was conducted on tissues obtained from pound dogs euthanized for reasons other than this study (urban control stray dog program). Euthanasia was conducted in accordance with established guidelines and in accordance with applicable animal care and use regulations (Panel on Euthanasia, 1993). Samples were collected between 1 October 1997 and 4 January 2000.

**Study areas.** Metropolitan MC extends over 2000 km² and is located in an elevated valley, 2250 m above sea level. It is a megacity with 20 million residents and associated production of air pollutants from automobiles, leakage of petroleum gas, and industrial activity. The climate is mild, with year-round sunshine, light winds, and temperature inversions. Each of these factors contributes to create an environment in which complex photochemical reactions produce oxidant chemicals and other toxic compounds. Air quality data is provided by an automated surface network of 33 monitoring stations in and around MC; hourly near-surface measurement of monitored pollutants include: ozone (O_3), particulate matter (PM_{10}, SO_2, NO_2, NO_3, carbon monoxide (CO), and lead. MC’s main pollutants are PM and ozone, which exceed their respective U. S. National Ambient Air Quality Standards (USNAAQS) most of the year. The maximal concentrations of ozone precursors appear downwind from the emission zones, toward the southern part of the urban area, southwest and southeast MC (Garcia-Gutierrez et al., 1991). Other pollutants with relevant concentrations include: volatile organic compounds (VOC), formaldehyde, and acetaldehyde (Baez et al., 1995, Fast, 1998, Edgerton et al., 1999). According to Fast and Zhong (1998), the highest particle concentrations regularly occur in the vicinity of the peak ozone concentrations during the afternoon, and daily maximum ozone concentrations vary among individual stations by as much as 0.150 ppm on a given day, evidence of the spacial inhomogeneity of ozone within the basin. Ozone concentrations as high as 0.48 ppm have been measured during severe air pollution events (Blake, 1995). The SWMMC atmosphere is characterized by average maximal ozone daily concentrations of 0.250 ppm. An average of 4 ± 1 h/day with ozone > 0.08 ppm...
is recorded in SWMMC year-round (83.9% of days, Garcia-Gutierrez et al., 1991). NO\textsubscript{2} concentrations do not usually exceed the annual arithmetic mean of 0.053 ppm (4.6% of days), while SO\textsubscript{2} levels exceed the 24-h primary standards for O\textsubscript{3} (71.9% of days), NO\textsubscript{2} (15.8% of days), and CO; VOC et al., mutagenic particulate matter (Villalobos-Pietrini et al., 1995); metals, e.g. vanadium, manganese, and chromium (Riveros-Rosas et al., 1997); and peroxycetyl nitrate (Edgerton et al., 1999).

Northeast MC, on the other hand, is characterized by pollutant emissions from light and medium industries and high vehicular traffic, both on paved and unpaved roads (Edgerton et al., 1999), with high PM values. PM\textsubscript{10} averages on the order of 235 \mu g/m\textsuperscript{3} (Cicero-Fernandez et al., 1993); the PM\textsubscript{10} annual average is 44 \mu g/m\textsuperscript{3} (Edgerton et al., 1999). Concentrations are above the standards for O\textsubscript{3} (71.9% of days), NO\textsubscript{2} (15.8% of days), and CO; VOC averages 3130 ppbC (Edgerton et al., 1999). The high persistent PM concentrations in the NE are due to both entrainment and near-surface convergence (Fast and Zhong, 1998); these authors make a point that is interesting to emphasize regarding comparisons of health effects between MC and other midlatitude polluted cities: ozone exceedances in MC occur anytime during the entire year regardless of the season because of its subtropical latitude and high altitude. In MC, chlorotic banding and motting of 2 pine species native to the area have been well documented in the past, and have persisted up to the present time. The chlorotic banding likely represents a response to periodic random and/or episodic exposure to oxidants, while the motting is considered a response to chronic oxidative stress (de Bauer and Krupa, 1990).

Cuernavaca is the capital city in the state of Morelos. It extends over 31 km\textsuperscript{2} and is located 60 km south, downwind from MC, at 1154 m above sea level. It has a temperate climate, an average year-round temperature of 10–20°C, and receives 1200 mm of rain annually. Its 316,782 inhabitants are mostly dedicated to service, business, industry, and commerce sectors. Twenty-four percent of the 4086 industries in the state of Morelos are located in Cuernavaca; air pollution is related to vehicular traffic and the generation of PM by the cement and brick industries in town, as well as the open field burning of garbage. A clear oxidant-induced vegetation injury gradient has been established between MC and Cuernavaca (de Bauer and Krupa, 1990). Tlaxcala is the capital city of the state of Tlaxcala, located 2252 m above sea level. It has a tropical climate with average temperatures in the range of 23–36°C, and a relative humidity above 70%. Its 127,622 inhabitants are above sea level. It has a temperate climate, an average year-round temperature of 16°C and receives 700 mm of rain annually. It has 63,423 inhabitants and various industries including textile, plastics, foods, and drinks. Tuxpam is a small port on the Gulf of Mexico, 390 km northeast of MC. It is 14 m above sea level. It has a tropical climate with average temperatures in the range of 24–36°C, and a relative humidity above 70%. Its 127,622 inhabitants are dedicated to commerce, service, fishing, and business sectors.

**Atmospheric pollutant data.** For the purpose of this work, MC’s atmospheric pollutant data was obtained from 2 representative pollutant monitoring stations located in the SW and NE areas, and from the available literature. In the case of the less polluted cities, data were obtained from the Subsecretaria de Ecología. The data are representative of the air pollution patterns corresponding to the collection period of the dogs (1997–2000).

** Necropsy and tissue preparation.** Animals were examined by a veterinarian before they were euthanized. A brief clinical examination included cardiac and lung fields auscultation, and abdominal and peripheral lymph node palpation. A gross external description was done after their demise, with special focus on age and nutritional status. The animals were weighed and a restricted autopsy was done. Immediately after death, the trachea, extrapulmonary bronchi and lungs, along with the heart, were excised intact from the thoracic cavity. The abdominal cavity was opened and examined; samples of liver, spleen, and kidneys were obtained, and immediately immersed in 10% neutral formaldehyde. The cardiorespiratory block was transported on ice to the laboratory where it was inspected for gross lesions and photographed when necessary. In a group of 50 animals (M, n = 43; Tuxpam, n = 7), a bronchoalveolar lavage (BAL) was performed immediately upon arrival at the laboratory (average 2 h after death). The lungs were filled by intratracheal instillation of warm (37°C) calcium and magnesium-free Dulbecco’s phosphate buffered saline (Sigma Chemical Co., St. Louis, MO), at a ratio of 35 ml/kg body weight. The saline was aspirated and re-injected twice more, and the BAL fluid was recovered in cold centrifuge tubes. BAL fluid was centrifuged at 350 g for 15 min at 4°C. The BAL procedure in these animals was followed by formaldehyde fixation. The lungs were inflated via the trachea with 10% neutral formaldehyde at a pressure of 25 cm H\textsubscript{2}O of a column of fixative solution. The pressure was maintained for a minimum of 4 h following which the trachea was ligated and the lungs were immersed in 10% neutral formaldehyde solution an average of 1 week prior to taking sections. In a group of 60 dogs from the 4 selected cities, electron microscopic samples were taken from unavlagel right lungs in the fresh state. A fragment of the caudal right lobe was cut in small pieces, immersed in glutaraldehyde (2.5%), embedded in plastic, and conventionally processed for electron microscopy. A 0.1 M cacodylate buffer was used in the tissue processing. For light microscopy (LM), blocks of tissue 2 × 2 × 1 cm were taken from trachea, carina, right and left main bronchi, and right caudal and cranial lobes. Peribronchial and peritracheal lymph nodes were macroscopically described and sections for LM taken. Paraffin sections 5 \mu m thick were cut and routinely stained with hematoxylin and eosin. Special stains included: Masson’s trichrome for collagen, Prussian blue for the detection of Fe\textsuperscript{3+}, periodic acid-Schiff and luxol fast blue (PAS/LFB) for the detection of polysaccharides and mucosubstances containing hexoses or deoxyhexoses, and elastic Verhoeff stain. The histological parameters evaluated in each section included: tracheal and bronchial epithelial and smooth muscle hyperplasia, terminal and respiratory bronchiolar epithelial hyperplasia, bronchial smooth muscle hyperplasia, dilatation of terminal and respiratory bronchioles, alveolar macrophage hyperplasia, peribronchial fibrosis, sepal thickening, microthrombi, PMN margination in capillaries and venules, and peribronchial and perivascular chronic inflammatory infiltrates. Sections were scored from 0–3; absent corresponded to 0, and the most severe findings to 3. For the microthrombi we evaluated 0 negative and 1 positive finding. All sections were read blindly by experienced observers.

**BAL cell pellet studies.** Cell viability was assessed by the trypan blue exclusion method and the propidium iodide (PI) exclusion assay. Fifty micro liters of the cell pellet suspension were taken to perform a cell count with differential and trypan blue dye exclusion. An aliquot of 100 \mu l was used to test viability with the PI assay. 200 \mu l was used for DNA cell cycle analysis in an EPICS Profile II Coulter flow cytometer and a 200 \mu l sample was used for electron microscopic studies.

**BAL DNA cell cycle analysis.** The PI method was used for DNA cell cycle analysis (Darzynkiewicz et al., 1994). Processed samples were analyzed using the Multicycle analysis software, a cell cycle analysis program that is based on a procedure of curve fitting that separates the G\textsubscript{0}/G\textsubscript{1}, S, and G\textsubscript{2}/M phases of a cell cycle in one DNA histogram. The DNA synthesis phase (%S or the portion of the cell cycle in which DNA is replicated) was determined for each BAL sample. The cell cycle analysis model used was a zero-order S phase with sliced nuclei debris correction, which results in increased reproducibility (Bergers et al., 1997).

**BAL differential cytology.** Cells obtained by lavage (1 \times 10\textsuperscript{7}) were sedimented on glass slides using a cytocentrifuge (Cytospin-3, Shandon, Pittsburgh, PA) at 350 g for 8 min. The slides were stained with Papanicolaou stain (Sigma Chemical Co., St. Louis, MO). Differential counts for M\textsubscript{6}, white blood cells (WBC), and epithelial cells were determined by microscopic examination (Mayet et al., 1990).

**Compturized axial tomography of lungs.** Computed tomography (CT) was done in isolated infection-fixed, air dried, postmortem lungs of MMG dogs (n: 11), including n: 5 from SW and n: 6 from NE. The lungs were visualized in toto and then sectioned along the plane of the CT image. The CT findings were correlated with histological changes in these sets of lungs.
Statistics. Statistical analyses were performed using the Instat program (Graph Pad, San Diego, CA). The following statistical procedures were used: (1) one-way ANOVA. The Tukey-Kramer multiple comparisons test was used to establish the differences in values of synthesis phase (%S), and the % viabilities for BAL cells for control vs. MC dogs. (2) Pearson correlation was used to assess the strength of association between the lung perivascular mononuclear cell infiltrates, and the bronchiolar epithelial and smooth muscle cell hyperplasia. (3) Unpaired t-test was used to compare the differences in the numbers of cells per ml in BAL, and the significance of the histological endpoints among the different geographical locations. Data are expressed as mean values ± SD. Significance was assumed at $p < 0.05$.

RESULTS

Air quality data. People and animals in MC are chronically and sequentially exposed to a complex mixture of air pollutants. The numbers of h/year SWMMC residents have been exposed to ozone above the USNAAQS for the years 1984 – 1998 are: 40, 30, 740, 959, 1224, 1403, 1561, 1395, 1146, 1061, 1249, 1080, 1123, 1203, and 1342 respectively. Figure 1 illustrates the concentrations of ozone for SW, NEMMC, and Cuernavaca; Figure 2 shows the concentrations of PM$_{10}$ for SW and NEMMC. The atmosphere in Cuernavaca is characterized by maximal ozone values of 0.330 ppm, with a third of the days in a year with ozone above the standards. The months with the higher ozone values in 1998 and 1999 were: April, May, and June. SO$_2$ levels exceed the 24-h primary standard of 0.14 ppm in the Tlahuapa monitoring station located in the industrial area. Total suspended particles have an average value of 120 $\mu$g/m$^3$ (Oswald, 1999). Tlaxcala and Tuxpam are in compliance for all major pollutants.

BAL differential cytology. Lavage cells from MC and Tuxpam dogs ($n = 50$) were 95% $\mu\theta$, 3% PMN, and 2% epithelial cells. In all groups the frequency of PMN was always less than 3%. Basophils could be readily identified in the majority of MC dogs. EM showed evidence of basophil degranulation while $\mu\theta$ displayed features of activation with lysosomal bodies containing PM and RBC fragments.

BAL viability and cell numbers. There were no differences in cell viability between SW and NE dogs (92.8 ± 8 vs. 92.5 ± 6.6) and the control dogs (93.5 ± 4.1). MC dogs had 27 ± 3.4 × 10$^6$ cells per ml of lavage fluid, while control dogs had 13.4 ± 5.2 × 10$^6$ cells ($p < 0.0001$).

DNA cell cycle analysis. We analyzed all BAL samples available with a CV $G_0/G_1$ peak < 5. The average of % S phase cells was defined as the average S-phase value of a DNA-diploid cell cycle. A significant difference was seen in the % S of alveolar macrophages between lower and higher polluted cities ($p < 0.0001$). Dogs from the lower polluted city (Tuxpam) had an S% value of 4.9 ± 4.4, while SW and NEMMC dogs had similar values of 22.5 ± 4.5 and 22.06 ± 7.3 respectively.

Clinical and gross pathological observations. All the animals in this study were apparently healthy without evidence of overt respiratory or cardiovascular disease. Approximately 16% of animals included in each geographical location had a low weight for size, without evidence of severe malnutrition or clinical sickness. There were no overweight dogs in this study.

Gross morphology of the lungs. No gross abnormalities were seen in the lungs of animals from Tuxpam. Lungs from Tlaxcala and Cuernavaca displayed discrete areas of pleural thickening and anthracotic macules. Lungs from MC dogs displayed patchy whitish pleural areas with prominent anthracotic macules. These pleural changes were seen in every lobe and had no apparent relationship with the ribs or the lung apices. The changes were particularly prominent in older dogs. Enlarged, congested, anthracotic pulmonary hilar lymph nodes were observed in all MC dogs and in NE dogs, lymph nodes were larger and completely replaced by black pigmen-

FIG. 1. Representative 24-h ozone concentrations (ppm) for NEMMC, SWMMC, and Cuernavaca.

FIG. 2. Representative 24-h particulate matter < 10 $\mu$m in diameter (PM$_{10}$) in $\mu$g/m$^3$ for SWMMC and NEMMC.
tation. No evidence of intercurrent lung disease was seen in any of the animals.

Lung histopathology. The normal histologic and ultrastructural morphology of the dog lung has been described by several investigators (Pinkerton et al., 2001; Plopper and Hyde, 1992; Takenaka et al., 1998). A summary of relevant lung findings is seen in Tables 2 and 3. Relevant findings were confined to pleura, terminal and respiratory bronchioles, centriacinar regions, and lung parenchymal blood vessels.

Pleura. Areas of fibrosis associated with particle-laden macrophages were common in MC and Cuernavaca dogs. In a few cases, the thickness of the pleura was considerable (0.3–0.5 cm) and fibrous tissue extended into the lung parenchyma (Fig. 3A). In Tlaxcala dogs, the pleura showed patchy areas of mild fibrosis with small clusters of PM-laden θ. In Tuxpam, only scattered macrophages with a few particles were present.

PM location in the different cohorts. At low magnification, coal-like and non-coal-like dust PM were present in MC and Cuernavaca dogs in: (1) alveolar macrophages (AM), and within multinucleated giant cells in alveolar spaces; (2) within the alveolar septae; (3) within the interstitium of respiratory bronchioles, and in the cytoplasm of bronchiolar epithelial cells; (4) within clusters of Mθ in the submucosa of small and medium size bronchi; (5) within the adventitia surrounding venules and arterioles in the pulmonary parenchyma; (6) within endothelial cells in arterioles, venules and capillaries; and (7) in the pleura (Figs. 3B–F). Tlaxcala dogs exhibited PM in AM, visceral pleura, and around a few respiratory bronchioles, as well as in the adventitia of scattered venules and arterioles vessels. Tuxpam dogs had a few AM with PM, and a few Mθ with PM in the visceral pleura.

Conducting airways. In MC and Cuernavaca dogs, trachea and bronchi exhibited focal ciliated cell loss, patchy areas with basal and goblet cell hyperplasia, and scattered epithelial and submucosal mononuclear cell infiltration. Submucosal fibrosis was present in MC dogs, while a moderate increase in the smooth muscle layer around large bronchi was prominent in NE dogs and in a small number of older SW dogs.

Terminal and respiratory bronchioles. A predominant finding in MC and Cuernavaca dogs was respiratory bronchio-
lar epithelial hyperplasia. The hyperplastic bronchiolar lesions consisted of either diffuse epithelial cell proliferation or focal micronodular epithelial projections (Figs. 4A and 4B). The hyperplasia was predominantly composed of nonciliated Clara cells (Fig. 4C). In the samples with focal hyperplasia there was a close relationship between the epithelial changes and the presence of macrophages laden with PM (Fig. 4B). Neovascularization was prominent in terminal bronchioles from MC dogs and to lesser extent Cuernavaca dogs (Fig. 3B). Smooth muscle hyperplasia was salient in terminal and respiratory bronchioles of SWMMC, NEMMC, and Cuernavaca dogs (Figs. 4A–4C). Chronic mononuclear cell infiltrates along with macrophages loaded with PM were commonly surrounding the bronchiolar walls and extending into adjacent vascular structures (Fig. 4B). Patchy proximal acinar air space enlargement was centered on respiratory bronchioles and alveolar ducts, and was accompanied by an apparent loss of interalveolar septae and emphysematous-like irregular areas. It was more frequent in NE dogs regardless of age (p < 0.01).

**Alveolar macrophage hyperplasia.** AM hyperplasia consisted of increased numbers of alveolar macrophages that contained coal dust-like particles or a mixture of particles with large needle-like material, brown pigment, golden iron pigment, and birefringent particles. Every dog in the study displayed θ in the alveolar lumen (Figs. 4E and 4F). It was mild for Tlaxcala and Tuxpan dogs, and mild to moderate for Cuernavaca and MC dogs. In focal areas, conglomerates of macrophages laden with PM occupied most of the alveoli. By EM these macrophages contained thin lamellar-like bodies (Fig. 5A).

**Alveolar type II hyperplasia.** Alveolar type II hyperplasia was seen predominantly in MC dogs, but it was also present in a Cuernavaca dog and Tlaxcala dogs. The epithelial hyperplasia was mostly focal and irregularly distributed. It consisted of an increased number of cuboidal, alveolar type II epithelial cells lining alveolar septae (Figs. 5B and 5C). Type II alveolar cells displayed scattered and thin lamellar surfactant bodies in MC dogs (Fig. 5C). In MC dogs, free electron dense ultrafine PM was observed in the cytoplasm and in close association with the abnormal lamellar surfactant bodies (Fig. 5C).

**Alveolar type I cells.** An increased luminal and abluminal pinocytotic activity in type I alveolar cells was noticed in MC and Cuernavaca dogs. Ultrafine electron-dense particles were present in association with the pinocytotic vesicles (both on the luminal and abluminal sides), in filopodia engulfing the particles, and free in the cytosol (Figs. 5D and 5E).

**Septal fibrotic reaction.** Septal focal fibrosis was present mostly in MC dogs, but to a lesser extent also in Cuernavaca dogs. It was mostly associated with alveolar type II and macrophage hyperplasia, and was more prominent in older dogs. Trichrome staining revealed fibroconnective tissue in alveolar septae; these findings were confirmed by electron microscopy (Figs. 5B and 5C). An increase in the numbers of cells displaying metachromatic granules was seen in association with the fibrotic areas stained with toluidine blue. EM showed that these cells corresponded to partially degranulated mast interstitial cells. MC dogs displayed numerous interstitial macrophages with cytoplasmic processes surrounding clusters of PM (Fig. 5F). PM in interstitial MΦ was a striking finding in NEMMC dogs, the internalized condensed ultrafine particles were mostly free in the cytoplasm (Fig. 5F). Foci of osseous metaplasia were seen in 2 NE and 1 Cuernavaca dogs.

**Inflammation.** A striking finding in MC and to lesser degree in Cuernavaca dogs, was the presence of moderate to marked infiltration by mononuclear cells of vascular walls and perivascular spaces of pulmonary veins and arterioles. Mononuclear cells were seen along MΦ laden with coarse PM. Chronic inflammatory cells were present primarily around terminal and respiratory bronchioles and in adjacent vascular structures (Figs. 4B and 4D). Low-grade focal pneumonitis was confined to end-airway structures. Aggregates of AM, scattered PMN, and fibrinocellular debris were seen within a few alveoli.

**Vascular changes.** Alongside the scattered infiltration of small pulmonary veins and arterioles by mononuclear cells and macrophages (SW, 83%; NE, 78%; Cuernavaca, 36%), MC dogs displayed platelet micro-thrombi, and PMN endothelial margination, particularly prominent in septal capillaries and venules (Figs. 4D–4F). The changes were particularly striking in SW dogs (Fig. 4F). EM showed the presence in septal capillary lumen of PMN with a decreased amount of intracytoplasmic granules (Fig. 6D). Endothelial cells displayed numerous pinocytic vesicles both in the luminal and abluminal surfaces. Ultrafine PM was present both in relation with the pinocytic vesicles as well as free in the cytosol. The endothelial cells send multiple thin projections of cytoplasm into the lumen (anemone-like), as well as larger amounts of cytoplasm with striking pinocytic activity (Figs. 5E and 6C). Interestingly, the anemone-like cytoplasmic projections mostly originated at the zonula occludentes (Fig. 6A). Large macrophage-like cells with irregular shape, abundant cytoplasm containing lysosomal bodies, and free ultrafine PM in the cytosol displayed extensive areas of interaction with the capillary endothelium (Figs. 5E, 6B, and 6C). No fused membranes were seen between the macrophage-like cells and the endothelial surface, however membrane adhesive complexes were present, with intercellular separations between 12–14 nm (Fig. 5E). The macrophage-like cells were seen on the thick side of the alveolar septum. Endothelial cell junctions were intact.

**Lung-associated lymph nodes.** In lung-associated lymph nodes, a distortion of the normal architecture with dilatation of peritrabecular and subcapsular sinuses and a mixture of coal-like particles, brown-yellow, and birefringent particles was present in macrophages (Fig. 3E). Germinal centers were prominent in some animals, but in the majority were very small or nonexistent. In older dogs there was almost complete nodal...
replacement by abundant particle-laden Mφ. Clusters of Mφ with PM could be seen in the lumen of the subcapsular marginal sinuses (Fig. 3E).

Postmortem CT. CT scanning was performed on formaldehyde-fixed lungs. Formaldehyde’s density is nearly the same density as the lung parenchyma by CT, limiting the evaluation.
to the presence or absence of hyperdense nodules. Small hyperdense areas were seen in the lung parenchyma, and subpleural regions. Histologically, the hyperdense areas corresponded to conglomerates of densely packed macrophages loaded with gold-brown and coal-like particles.

**DISCUSSION**

Exposure to complex mixtures of air pollutants, predominantly PM and ozone, is causing lung histopathological changes both in young and older canines in MC, as well as in...
young dogs exposed to lower concentrations of pollutants, similar to the ones seen in noncompliance areas in the United States (Southern California, the Texas Gulf coast, and the Northeast corridor). The crucial lesion in these dogs with lifelong exposures to air pollutants seems to be the epithelial and endothelial injury leading to persistent chronic parenchymal lung inflammation. PM is playing a crucial role in the lung histopathology observed in MC and Cuernavaca dogs. On one hand, coarse PM is seen within lysosomes in the numerous, highly proliferating AM, and in the macrophages taking part in the perivascular and peribroncholar chronic inflammatory process; on the other hand, ultrafine particulate material is present in type I and II alveolar cells, endothelial cells, interstitial macrophages and in macrophage-like intravascular cells. There is a significant ultrafine particulate transport from the epithelium to the interstitium, to the endothelial and intravascular compartments in these exposed dogs. This is an important observation since it has been shown that ultrafine particles are associated with decreased recognition and phagocytosis by alveolar macrophages, subsequent endocytosis by epithelial cells and preferential translocation into the interstitium, stronger inflammatory responses, prolongation of their lung retention, induction of procollagen expression and increases in airway fibrosis (Baena-Squiban et al., 1999; Churg et al., 1999; Churg, 2000; Ferin et al., 1992; Kreling and Scheuch, 2000; Nemmar et al., 1999). Simultaneous exposure to pollutants such as ozone and nitrogen dioxide are also likely contributing to the epithelial particle uptake and their translocation into the interstitium by increasing epithelial permeability (Bhalla and Crocker, 1987; Hubbard et al., 1994). Adamson et al. (1999) suggested that any lung with preexisting epithelial injury is more susceptible to severe PM damage, even to PM doses that by themselves cause little lung change. Other relevant lung findings in MC and Cuernavaca cohorts included bronchiolar epithelial and smooth muscle hyperplasia, the formation of lung microthrombi, PMN capillary and venule margination, and the indirect evidence of lung capillary leakage. The presence of ultrafine PM free in the cytoplasm of the M cells and preferential translocation into the interstitium, strongly affects the expression of adhesion molecules and chemokines (IL-8) (Kawanami et al., 1995; McGuirre et al., 1981; Vaillant et al., 1996). Impairment of pulmonary vessel’s structure and function has been described in patients with mild chronic obstructive pulmonary disease (COPD), suggesting the potential involvement of an inflammatory process in the pathogenesis of pulmonary vascular abnormalities in the early stage of COPD (Peinado et al., 1999). Our findings of vascular pulmonary and myocardial prominent PMN margination and microthrombi formation, as well as fibrin vascular deposition and breakdown of RBC could be related to endothelial dysfunction in these highly exposed dogs (Bevilacqua and Gimbone, 1987; Chen and Manning, 1995; Sibille and Reynolds, 1990; Zomas et al., 1998). Activated endothelium induces the presence of pro- and anticoagulant factors, and expression of a variety of adhesion molecules (Bombeli et al., 1997; Krishnaswamy et al., 1999; Ward and Hunninghake, 1998). Circulating PMN adherent to endothelial cells can damage these endothelial cells and produce the capillary leak that is central to the evolution of a systemic inflammatory response to multiple organ failure (Chen and Christou, 1998). Evidence of lung

Pulmonary vascular endothelial and epithelial cells are crucial barriers that keep lung tissue functional and play a dynamic role in the lung inflammatory responses (Castranova et al., 1988; Driscoll et al., 1997; Majno and Joris, 1996; Ryan, 1986; Saffiotti, 1996; Simon and Paine, 1995; Ward and Hunninghake, 1998). Damage of the capillary endothelial cells and type I alveolar cells are the earliest changes of lung toxicity by diesel exhaust particulates; these cell injuries lead to alveolar edema and a subsequent inflammatory response (Ichinoe et al., 1995). Endothelial damage is a main feature of acute lung injury and in microangiopathies where the endothelial injury is accompanied by the production of cytokines such as IL-10, IL-12, tumor necrosis factor (TNF-α) and interferon-γ, in turn affecting the expression of adhesion molecules and chemokines (IL-8) (Kawanami et al., 1995; McGuirre et al., 1981; Vaillant et al., 1996). Impairment of pulmonary vessel’s structure and function has been described in patients with mild chronic obstructive pulmonary disease (COPD), suggesting the potential involvement of an inflammatory process in the pathogenesis of pulmonary vascular abnormalities in the early stage of COPD (Peinado et al., 1999). Our findings of vascular pulmonary and myocardial prominent PMN margination and microthrombi formation, as well as fibrin vascular deposition and breakdown of RBC could be related to endothelial dysfunction in these highly exposed dogs (Bevilacqua and Gimbrone, 1987; Chen and Manning, 1995; Sibille and Reynolds, 1990; Zomas et al., 1998). Activated endothelium induces the presence of pro- and anticoagulant factors, and expression of a variety of adhesion molecules (Bombeli et al., 1997; Krishnaswamy et al., 1999; Ward and Hunninghake, 1998). Circulating PMN adherent to endothelial cells can damage these endothelial cells and produce the capillary leak that is central to the evolution of a systemic inflammatory response to multiple organ failure (Chen and Christou, 1998). Evidence of lung
capillary leakage is present in MC dogs; RBC are seen in alveolar spaces and in BAL macrophages.

A striking finding in the lung capillaries of MC and Cuernavaca dogs was the presence of partially degranulated PMN attached to the endothelial walls. Leukocytes within the circulation are in dynamic equilibrium with a marginated pool, thought to reside mainly within the pulmonary capillaries (Downey et al., 1990). Environmental factors such as inhalation of cigarette smoke delay and activate PMN traveling through the lung capillaries (Brown et al., 1995; Hogg, 1994). As in the rat model of cigarette smoke, where electron microscopy shows lung capillary damage with adherent PMN, in canines PMN could also contribute to the alveolar wall damage (Terashima et al., 1999). The result of capillary leukocyte

FIG. 6. (A) Close-up of an interstitial Mφ. Clusters of PM (*) are wrapped by the cell processes; however, no lysosomal limiting single membrane bound structures are identified. The Mφ also contains free PM in the cytoplasm. Caveola are numerous on the Type I alveolar cell, the endothelial cell, and the interstitial θ (arrowheads). An anemone-like projection of the endothelial cells is also shown (arrow). Original magnification ×20,000. (B) A NEMMC dog with an intravascular macrophage-like (M) cell packed with free ultrafine PM. The endothelial cell shows numerous pinocytic vesicles (*). Red blood cells are seen in the capillary lumen (RBC). There are several points of contact between the Mφ-like cell and the endothelial surface. (C) A macrophage-like (M) cell is seen in the lumen of a capillary in a SWMMC dog. Note the multiple cytoplasmic projections and the close contact of these projections with the endothelial cell (E). The endothelial cell shows numerous pinocytic vesicles and sends projections (arrow) into the lumen (L). There is abundant connective tissue in the interstitium (*). A fibroblast is seen (F) in the midst of the interstitium. Original magnification ×7000. (D) An electronmicrograph of an alveolar capillary in a SWMMC young dog. A PMN (arrow) is seen occupying the lumen; note its partial degranulation and the close contact with the endothelial cell (white *). Bundles of collagenous fibers are seen in the interstitial space (black *). The alveolar type I cell is marked with arrowheads. An activated alveolar macrophage (AM) with PM is seen. Original magnification ×7000.
sequestration appears to be impaired alveolar capillary perfusion (Kuebler et al., 2000).

In the model of alveolar fibrosis and capillary alteration in rat silicosis (Kawanami et al., 1995), peribronchial vessels were suggested as likely sources for renewal of damaged alveolar capillary endothelium, an observation that coincides with the neovascularization in terminal bronchioles in MC canines. In sharp contrast with the histopathological picture of acute lung injury, where early in its course large numbers of PMN are sequestered in and then migrate from the pulmonary capillaries towards the alveolar spaces, in our material we were unable to visualize the migration of these adherent PMN into the alveolar space, and further the numbers of PMN in BAL were low. Thus, migration from the lung capillaries to the alveolar spaces is not taking place in these chronically exposed dogs, an observation that coincides with the increased numbers of macrophages, but not PMN, in the sputum-induced samples obtained from healthy children and adults living in MC (L. Calderón-Garcidueñas, personal observation).

The EM findings in the surfactant profiles of type II alveolar cells are also important to point out, since injury to type II cells can alter their ability to synthesize, secrete, and recycle surfactant (Griese, 1999; Simon and Paine, 1995). Differentiation of alveolar type II to I cells implies that type II cells lose their apical microvilli and surfactant-containing lamellar bodies (Smith, 1983). Further, during cell division many of the surfactant-containing lamellar bodies are also lost (Smith, 1983). In relation to surfactant we observed alveolar macrophages with numerous phagocytized lamellar-like bodies, suggesting that ingestion of potentially recyclable surfactant by macrophages could contribute to the slow loss of surfactant lipids from the recycling pool, as pointed out by Miles et al. (1985).

The accumulation of PM in lymph nodes draining conducting airways is also worth comment (Adamson and Prieditis, 1998). PM is in contact with highly proliferating lymphoid cells and potential mutagenic materials are likely present. There has been a rise in the incidence of non-Hodgkins lymphomas in Europe, Canada, the U.S., and Mexico; workers in wood, petroleum, solvent, plastic, and ceramic industries have an excess risk (Cartwright et al., 1999; Mohar et al., 1997; Scherr et al., 1992). Relevant to this work in canines, the highest incidence of malignant neoplasms in MC corresponded to boys living in the southern areas of metropolitan MC (Fajardo-Gutierrez et al., 1997). Lastly, cell proliferation in BAL cells, as determined by the percentage of cells in S phase was \( \bar{x} \) 22% in MC dogs vs. \( \bar{x} \) 4.9% in dogs from less polluted locations, suggesting that the marked increase in the numbers of alveolar macrophages is due to in situ cell division (Spurzem et al., 1987). These marked increments in macrophage cell proliferation are further evidence of an ongoing pulmonary injury (Evans and Shami, 1989).

In summary, dogs naturally exposed to PM and ozone, as well as other ambient air pollutants, in concentrations at or above the current standards are displaying evidence of lung epithelial and endothelial pathology along with lung remodeling and altered repair with focal fibrosis. Therefore, the studies presented here may be useful to build future hypothesis-driven mechanistic studies to explain the biological plausibility of the epidemiological data suggesting increased cardiorespiratory morbidity and mortality in susceptible populations, and to gain a better understanding of the effects of air pollution on the respiratory system in children in both developing and developed countries.

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