

# DRAFT FINAL REPORT

## *Location specific systemic health effects of ambient particulate matter*

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Check if applicable:

Animal subjects

Human subjects

## Disclaimer

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## Abstract:

This project evaluated pulmonary inflammation and systemic inflammatory and platelet responses to fine (PM2.5) and ultrafine (UFP) ambient particulate matter collected from an urban (Sacramento) and rural (Davis) location. The objective of these experiments was to determine whether prior chelation of transition metals with Deferoxamine mesylate (DFM) or binding of bacterial source endotoxin by polymyxin B reduced pulmonary and systemic responses to PM2.5. We hypothesized that responses to a more transition metal rich urban source PM2.5 would be more inhibited by metal chelation while rural source PM2.5 would be more affected by endotoxin binding. Adult mice were given intratracheal instillations of collected PM2.5 and pulmonary pathology, systemic cytokine concentrations, and platelet activation evaluated 24 hours later. Our results support the following conclusions:

- 1) Urban source PM2.5 was significantly more pro-inflammatory than equivalent doses of rural source PM2.5.
- 2) DFM pre-treatment of urban source PM2.5 was without effect on pulmonary inflammation.
- 3) Treatment with DFM alone lead to significant systemic platelet activation
- 4) Pulmonary inflammatory responses to both urban and rural source PM2.5 were inhibited by pre-incubation of PM2.5 with polymyxin B.
- 5) While several prior experiments with two-week field exposures to concentrated ambient particulates where platelet function was measured at 24 hours following the last exposure day demonstrated significant activation of circulating platelets in treated animals, this study with a single exposure dose found no evidence of platelet activation in response to instilled PM2.5 at 24 hours after exposure..
- 6) Urban source PM2.5 elicited transcription of genes associated with polycyclic aromatic hydrocarbon (PAH) metabolism, reactive oxygen species (ROS) response elements and inflammation in small airways, pulmonary arterioles and alveolar parenchyma.

## **Executive Summary:**

### **Background**

Environmental particulate matter (PM) particularly that less than 2.5 microns in diameter (PM<sub>2.5</sub>) is a significant public health concern due to its strong correlation with exacerbation of both pulmonary and cardiovascular disease. While regulation of PM levels is principally done on the basis of mass concentration within specific size ranges, there is much interest in the relative contribution of PM components and sources to PM-induced health effects. Classes of chemicals thought to contribute to PM toxicity include transition metals that generate reactive oxygen species (ROS), polycyclic hydrocarbons that alter cellular metabolism of xenobiotics and inflammatory molecules, and pro-inflammatory biologic material such as endotoxin.

While ROS generation resulting from transition metal catalysis as well as polycyclic aromatic hydrocarbon (PAH) metabolism has wide acceptance as a mechanism leading to pulmonary injury resulting from urban sources of PM such as vehicular exhaust and power generation, the role of endotoxin in eliciting inflammation has primarily been addressed in PM from agricultural sources. Our previous concentrated ambient particulate field studies demonstrated significant systemic platelet activation in 2 week mouse exposures to both rural and urban source PM. Cell culture studies with PM collected from these exposures showed that responses to urban source PM were more inhibited by ROS scavengers while binding of endotoxin was a more effective inhibitor of rural source PM induced cellular inflammatory responses.

We hypothesized that similar differences in endotoxin and ROS mediated responses would be evident in animals treated with urban or rural source PM. The present study tested that hypothesis using oropharyngeal aspiration of urban or rural source PM pretreated with a transition metal chelator, deferoxamine mesylate (DFM) or polymyxin B, an antibiotic that specifically binds endotoxin. A corollary objective of this study was to compare pulmonary and systemic inflammatory responses to urban and rural source PM using equivalent gravimetric doses, something not possible in CAPs experiments.

### **Methods**

We compared the pulmonary and systemic responses to collected ambient PM from rural (Davis CA) and urban (Sacramento) sampling locations. Mice were given gravimetrically equal amounts (200 ug) by oropharyngeal aspiration. We used laser capture microscopic dissection to characterize and compare gene responses of airways, pulmonary arteries and alveolar parenchyma using probes for genes associated with responses to PAH, ROS and Inflammation. We will determine the relative contribution of endotoxin to pulmonary and systemic responses by pre-incubating PM with polymyxin B, an antibiotic that specifically binds endotoxin. Similarly, compared responses with PM pre-treated with the transition metal chelating agent deferoxamine (DFM). We evaluated endpoints including nature and extent of pulmonary inflammation by histopathology, regionally specific pulmonary gene responses by LCM and rt PCR, systemic cytokine concentrations by bead based ELISA assays, and platelet activation using flow cytometry and probes for platelet surface molecules altered in pro-thrombotic states. Platelet studies used expression of GP41b, LAMP-1 and P-Selectin, surface markers of activation in both unstimulated platelets and in response to the platelet agonists ADP and thrombin. In addition, we evaluated platelet-monocyte aggregates, a pro-thrombotic and pro-atherogenic consequence of platelet activation.

## **Results**

While pulmonary inflammatory responses were present 24 hours after instillation of PM from both sources, these responses were more intense in mice treated with urban source PM. Results of experiments inhibiting urban source PM responses with DFM did not demonstrate any effect of DFM pre-treatment. These results were further complicated by evidence that DFM treated controls had altered systemic platelet responses. In consequence, we limited further experiments to endotoxin binding studies with polymyxin B. Contrary to expectations, polymyxin B inhibited pulmonary inflammatory responses to both rural and urban source PM.

To determine whether pulmonary inflammation initiated a systemic inflammatory response, we evaluated serum concentrations of a panel of pro-inflammatory cytokines and immune response proteins. In summary, of the few cytokine changes analyzed as statistically significant, all but GM-CSF in the urban source experiment were decreased relative to control animals, and there was no effect of rural source PM<sub>2.5</sub> on any serum cytokines.

Platelet activation studies similarly showed little response to PM instillation at 24 hours. Experiments using DFM chelation did show an effect of DFM to increase platelet activation parameters while polymyxin B had the opposite effect, decreasing the percent of activated platelets in both control and PM treated mice. There were no changes in the numbers of platelet monocyte aggregates in any treatment group.

Only urban source PM<sub>2.5</sub> induced transcription of genes probed in these experiments. Untreated urban source PM<sub>2.5</sub> increased expression of the PAH response element CYP1A1 in airways and parenchyma. Aldehyde dehydrogenase 3a, another PAH response element, was increased in airways of animals given untreated urban PM<sub>2.5</sub>. ATF3, a transcription factor activated by the ROS sensitive Stress activated MAP kinase and the ROS response element HOX-1 were both increased in the polymyxin B experiment and their expression was suppressed by polymyxin B pre-treatment. Untreated urban source PM<sub>2.5</sub> induced increased airway expression of CCL-20, a cytokine associated with immune responses. In the experiment investigating polymyxin B inhibition, IL-1beta, a central pro-inflammatory cytokine, was increased by urban source PM<sub>2.5</sub> and inhibited by polymyxin B treatment. GM-CSF, a cytokine important in bone marrow activation, was similarly upregulated by urban PM and inhibited by polymyxin B pre-treatment.

## **Conclusions**

Experiments in this study examined the relative role of transition metals and bacterial derived endotoxin in inducing pulmonary and systemic inflammation in response to PM<sub>2.5</sub> derived from urban vs. rural sources. Our findings confirm that urban source PM<sub>2.5</sub> induces greater responses on a mass equivalent basis than PM<sub>2.5</sub> 2.5 collected from rural areas. Pulmonary inflammation and gene responses were diminished by pretreatment to bind endotoxin in PM<sub>2.5</sub> but not by pretreatment to bind transition metals. In contrast to prior studies demonstrating platelet activation in longer term field exposures to concentrated ambient particles, no PM<sub>2.5</sub> induced platelet activation was detected in these single dose studies. We conclude endotoxin is a key factor in both urban and rural PM<sub>2.5</sub> toxicity.

## **Recommendations:**

- 1) This study provides additional evidence for the importance of PM 2.5 composition in regulatory decision making. Experiments with equal gravimetric doses of PM from differing regions led to markedly different inflammatory responses
- 2) Endotoxin must be considered an important constituent in both rural and urban source PM
- 3) The nature and time course of systemic responses to PM 2.5 inhalation deserves further investigation, particularly relative to the length of post-exposure period necessary to induce these responses.

## Detailed Report:

### Introduction:

Environmental particulate matter (PM) particularly that less than 2.5 microns in diameter (PM<sub>2.5</sub>) is a significant public health concern due to its strong correlation with exacerbation of both pulmonary and cardiovascular disease. While regulation of PM levels is principally done on the basis of mass concentration within specific size ranges, there is much interest in the relative contribution of PM components and sources to PM-induced health effects. Classes of chemicals thought to contribute to PM toxicity include transition metals that generate reactive oxygen species (ROS), polycyclic hydrocarbons that alter cellular metabolism of xenobiotics and inflammatory molecules, and pro-inflammatory biologic material such as endotoxin.

Generation of ROS through both endogenous inflammation and exogenous sources is often considered a key mechanism in PM induced injury<sup>1</sup>. ROS generation from PM is a consequence of redox active organic hydrocarbons and can also be catalyzed by transition metals<sup>2</sup>. Both of these PM components are derived from products of fuel combustion in vehicles and power generation, and thus are associated with urban sources of PM. Metal chelators decrease PM induced *in vitro* responses including arachidonic acid release<sup>3</sup> and ROS generation<sup>4</sup>. Inclusion of ultrafine iron particles in exposures of rats to soot markedly increased evidence of inflammation and cytotoxicity in broncho-alveolar lavage<sup>5</sup>.

Metals of particular importance to PM toxicity include iron, nickel, copper, zinc and vanadium<sup>6</sup>. Iron is most frequently cited as catalyzing production of ROS through a chemical process known as the Fenton reaction. Iron, copper and zinc are present in relatively similar concentrations in PM<sub>2.5</sub> collected in California<sup>7</sup>. Seasonal variation in iron concentration has been demonstrated and was correlated with increased markers of systemic inflammation in a two week CAPs study<sup>7</sup>. Iron concentrations are generally higher in urban source particulates with an expected two fold difference from rural source atmospheric particles<sup>8</sup>.

Increases in pulmonary antioxidant systems are the key findings suggesting that ROS contributes to responses to PM<sub>2.5</sub> inhalation<sup>1</sup>. While it is commonly thought that transition metal catalyzed ROS generation is the key mechanism leading to ROS mediated injury, other sources of ROS generation related to PM<sub>2.5</sub> toxicity are recognized. These alternative means of ROS generation complicate the justifications for regulatory decisions based on reducing transition metal generation. Polycyclic aromatic hydrocarbons (PAH), particularly the quinone derivatives, are highly capable of redox activity and have been proposed as significant contributors to PM<sub>2.5</sub> mediated ROS generation in the lung<sup>9</sup>.

While ROS-related responses are often attributed to metal-based generation through catalysis of Fenton-like redox activity, endogenous sources of ROS as part of inflammatory responses may also contribute to ROS mediated injury. Experiments with inhibitors of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase), a key enzyme in endogenous ROS generation, shows significant reduction in inflammatory cytokine generation in response to various sources of PM<sub>2.5</sub> *in vitro* and *in vivo*.<sup>10</sup>

Despite much interest in transition metal-induced ROS in the mechanisms of PM2.5 mediated health effects, epidemiologic evidence comparing source specific composition with adverse effects of PM2.5 shows inconsistent correlations between transition metals and physiologic alterations, such as extend of inflammation in the lung, altered heart rate variability and increases in inflammatory proteins in blood<sup>11,12</sup>. Markers of inflammation in blood from elderly humans with cardiovascular disease were associated with ultrafine PM concentrations of PAHs and hopanes, but not other organics or transition metals<sup>2</sup>. Individuals with polymorphisms enhancing the iron sequestration activity of the hemochromatosis (HFE) gene product were protected from PM2.5 -induced decrease in the high-frequency component of heart rate variability<sup>11</sup>. In contrast, a study evaluating pulmonary inflammation resulting from instillation of mice with PM2.5 collected from several sites in Europe demonstrated correlations with oxidized organic compounds and transition metals, but an inhibitory effect was associated with PAH concentration<sup>12</sup>.

Given the contrast between *in vitro* studies implicating transition metals and ROS generation in cellular responses and the variability of findings from epidemiologic studies, the role of transition metals and ROS in PM2.5 induced health effects remains uncertain. This project used a transition metal chelator, Deferoxamine mesylate (DFM) to examine the effect of selective inhibition of metals on pulmonary and systemic responses to urban source PM2.5.

DFM is a siderophore derived from bacteria that effectively binds ferric iron and prevents oxidative tissue damage. DFM also binds aluminum and calcium, and exhibits iron-independent antioxidative effects on hydroxyl radicals and tyrosine nitrosylation<sup>13</sup>. DFM chelation abrogated 90% of *ex vivo* hydroxyl radical formation in collected ambient particulate matter<sup>6</sup>. Fly ash particle-induced formation of ROS, liberation of AA and PGE2/TXB2 and activation of stress-associated MAP kinase pathways was decreased in macrophages pretreated with DFM<sup>14</sup>. Similarly, DFM (but not polymyxin B) pretreatment of particles collected in a subway inhibited PM2.5 induced TNF production in murine macrophages<sup>15</sup>. While DFM has been used in clinical trials as an anti-inflammatory agent, its effect on PM2.5 toxicity *in vivo* has had limited study. In one experiment, decreases in whole blood clotting time in response to water soluble metals extracted from PM2.5 were prevented by DFM treatment<sup>16</sup>. Exposure to the water-soluble fraction of particulate matter (PM2.5), at doses as low as 50 ng/ml original particle, significantly diminished the whole-blood coagulation time. Inclusion of DFM prolonged coagulation time following the exposures to the water-soluble fraction<sup>16</sup>. The present project investigated the effect of DFM chelation on mice treated with PM2.5 collected from an urban region in Sacramento California. We evaluated pulmonary and systemic inflammation, as well as the effect on platelet activation 24 hours after intratracheal instillation.

Multiple lines of evidence suggest a significant role for endotoxin in pro-inflammatory responses to inhaled particulates. Many of these studies emphasize agricultural occupational exposures<sup>17</sup>. Endotoxin concentrations are high in both dairy and swine confinement operations<sup>18</sup> and workers in these areas have decreased forced expiratory volume in 1 second and forced vital capacity during and after their shifts<sup>19</sup>.

Because of the difficulty in assay specificity, the role of endotoxin in PM2.5 induced health effects has been little studied. Reduction in endotoxin levels was a more consistent factor than transition metal concentrations driving differences in *in vitro* toxicity of PM2.5 collected after stringent controls were applied in preparation for the

2008 Olympic games<sup>20</sup>. Similarly, endotoxin was strongly associated with cytokine secretion in nasal fluid of humans exposed to PM2.5 from a variety of locations, while oxidant potential was not correlated with inflammation<sup>21</sup>. Principal component analysis of PM2.5 collected from multiple sites in Mexico City correlated *in vitro* secretion of the inflammatory cytokines TNF $\alpha$  and IL-6 with elements derived from soil, including endotoxin, but not with PAH or most anthropogenic transition metals<sup>22</sup>. Endotoxin concentrations are likely higher in rural source PM2.5, with one study demonstrating three to ten-fold higher endotoxin levels in PM2.5 collected from rural agricultural regions compared with urban sources<sup>23</sup>.

Polymyxin B is commonly used to bind endotoxin for *in vitro* experiments<sup>24</sup> and is proposed as a treatment for septic shock<sup>25</sup>. Polymyxin B treatment selectively attenuated the effects of coarse urban PM to reduce acetylcholine-mediated relaxation of isolated pulmonary arteries<sup>26</sup> and reduced cytokine responses in coarse PM but not PM2.5 treated monocytes<sup>24</sup>. In contrast, previous studies in our laboratory showed polymyxin B to selectively inhibit monocyte cytokine secretion and adhesion to endothelial cells in response to treatment with urban PM2.5 from Fresno California<sup>27</sup>. In preliminary studies we also found that *in vitro* responses to rural source PM2.5 were much more attenuated by polymyxin B treatment than responses to urban source PM2.5 (unpublished data). While these cell culture studies implicate endotoxin as an important pro-inflammatory component of PM2.5, confirmation of this in animal exposures has not previously been done. Our preliminary studies combined with the reported higher concentrations of endotoxin in rural source PM2.5 led to our hypothesis that polymyxin B treatment would be much more effective in attenuating pulmonary and systemic inflammatory responses to rural source PM2.5 compared with urban sources.

While many animal studies of PM2.5 are done with real time, concentrated ambient particles (CAPs) comparison between studies is complicated by variability in the overall dose. Our prior CAPs study demonstrated upregulation of inflammatory and xenobiotic metabolism genes in specific regions of lung that varied by season and urban or rural location<sup>7</sup>. We also found that intratracheal instillation of collected PM2.5 elicits pulmonary inflammation and generates a similar upregulation of inflammatory and xenobiotic metabolism genes in specific regions of pulmonary parenchyma. Using treatment with equivalent mass, we showed that these responses varied in intensity and specificity between PM2.5 collected from urban or rural sites but with opposite site-specific intensity relative to responses in CAPs studies (manuscript in preparation). The present study asked whether location specific differences in responses to PM2.5 could be related to differences in transition metal or endotoxin content of PM2.5. We hypothesized that inflammation resulting from instillation of urban source PM2.5 is primarily related to metal components in the PM2.5 mixture that would be inhibited by DFM treatment, while inflammation induced by rural source PM2.5 would be primarily dependent on endotoxin content that would be inhibited by polymyxin B.

## **Methods:**

### *Project Design:*

Our original project design included four experiments, each consisting of an animal exposure to urban or rural source PM2.5, either as collected, or pre-treated with either Polymyxin B to remove endotoxin, or Desferoxamine, to chelate out metals. Health-related endpoints included several pulmonary and systemic responses. Our proposal

was modified from the original contract through consultation with the ARB contract manager in two ways. First, we changed the source of PM<sub>2.5</sub> originally proposed because there proved to be an insufficient amount of archival samples from urban and rural Fresno to complete the study. Therefore, we used more recently collected samples from an urban and a rural site close to Sacramento. Secondly, based on the negative results using Deferoxamine pretreatment of urban source PM<sub>2.5</sub>, we eliminated Task 2 (Deferoxamine pretreatment of rural source PM<sub>2.5</sub>). Tasks 1, 3, and 4 were completed as outlined below. Funds originally intended for Task 2 were redirected to expanding the number of endpoints and enlarging the sample sizes in the remaining three experiments.

Task 1: Intratracheal instillation of rural source PM<sub>2.5</sub> +/- Polymixin B.

- a. Animal study, Flow Cytometry
- b. Cytokine analysis, Histopathology
- c. Laser Capture Microdissection RT-PCR

Task 2: Intratracheal instillation of rural source PM<sub>2.5</sub> +/- Deferoxamine.

- a. Animal study, Flow Cytometry
- b. Cytokine analysis, Histopathology
- c. Laser Capture Microdissection RT-PCR

Task 3: Intratracheal instillation of urban source PM<sub>2.5</sub> +/- Polymixin B.

- a. Animal study, Flow Cytometry
- b. Cytokine analysis, Histopathology
- c. Laser Capture Microdissection RT-PCR

Task 4: Intratracheal instillation of urban source PM<sub>2.5</sub> +/- Deferoxamine.

- a. Animal study, Flow Cytometry
- b. Cytokine analysis, Histopathology
- c. Laser Capture Microdissection RT-PCR

*Collection of ambient particulate matter:* Experiments in this study used archival samples of ambient particulate matter collected by the UC Davis Air Quality Research Center at their urban site in Sacramento and at rural sampling sites near Davis, CA. The urban site is located in a high traffic area near the junction of three major urban freeways. The rural site is located at the University of California Institute for Toxicology and Environmental Health, and is adjacent to the University's animal science research facilities. The facility actively operates as an agricultural research station with mixed crop plantings. Ambient PM<sub>2.5</sub> from both sites was collected using a high-volume sampler combined with a PM<sub>2.5</sub> cutoff cascade impactor in the winter of 2011. The sampling was done during a typical winter temperature inversion associated with high PM<sub>2.5</sub> concentrations in the California's central valley. Bulk PM samples were collected with 3 co-located Reference Ambient Monitor (RAAS) filter samplers loaded with prebaked (48 hrs at 550°C) Teflon membrane filters. Samples were collected for six hours a day for five days each week for two weeks. Filters were stored sealed in an argon atmosphere at -80°C until analyzed or used for biologic experiments.

*Particle composition and preparation for use:* Details of collected PM<sub>2.5</sub> composition have previously been described<sup>28</sup>. Particles were collected over 6 daytime hours of 10 days during the winter season in an urban location adjacent to multiple freeways in Sacramento California and a rural location adjacent to both animal and plant agricultural fields south of Davis, California. Collection was done with Micro-Orifice Uniform Deposit

Impactors (MOUDIs) with particle aerodynamic diameter size cuts  $D_p = 0.056, 0.1, 0.18, 0.32, 0.56, 1.0,$  and  $1.8\mu\text{m}$ . A cyclone separator was used upstream of each MOUDI to remove coarse PM. MOUDIs were loaded with clean Teflon membrane substrates that were used for both extraction for animal experiments and analysis. The particle elemental carbon (EC) and organic carbon (OC) concentrations were determined by thermo-optical analysis following the NIOSH temperature protocol<sup>29</sup>. Water-soluble ions were quantified with Ion Chromatography (IC) analysis and trace elements were quantified with Inductively coupled Plasma Mass Spectrometry (ICP-MS) analysis<sup>29</sup>. Polycyclic aromatic hydrocarbons (PAHs) were extracted from samples via sonication in organic solvents followed by concentration by nitrogen evaporation and quantification using gas chromatography-mass spectrometry (GC-MS). PAH concentrations were quantified using isotopically labeled internal standards.

PM<sub>2.5</sub> was extracted from filters by probe sonication in saline as previously described<sup>30</sup>. Particles for intratracheal instillation were obtained by probe sonication of collected filters followed by filtration through Quiagen spin filters (Quiagen, Valencia, CA) to remove fibers derived from collection filters. The resulting PM suspension was frozen to  $-80^\circ\text{C}$  and then lyophilized to obtain dry PM. Total extracted mass was determined by pre- and post-weighing final storage vial. For intratracheal instillation, PM was resuspended in 30ul phosphate buffered saline (PBS). Control Mice were treated with similarly prepared extract of clean filters. Estimates of concentration were based on gravimetric determination of PM<sub>2.5</sub> mass per unit area for each filter used with extraction concentration based on the area of filter sonicated. Preliminary experiments demonstrated close reconstitution of PM size distribution with similarly treated defined size iron oxide particles (data not shown).

*Animal studies:* Male C57BL/6 mice were obtained from Jackson laboratories at 8 weeks of age. Mice were acclimated in AALAC accredited facilities for one week before experiments were begun. All experimental protocols were approved by the UC Davis IACUC committee. Groups of six mice were anesthetized with ketamine/xylazine and given 200 ug of PM<sub>2.5</sub> from either urban or rural archival samples by pharyngeal aspiration of PM<sub>2.5</sub> suspensions in 30 ul phosphate buffered saline (PBS). Additional groups of mice received urban PM<sub>2.5</sub> pretreated for one hour with DFM (500  $\mu\text{M}$ , EMD Biosciences), urban PM<sub>2.5</sub> pretreated with Polymyxin B (100  $\mu\text{g}/\text{ml}$ , Sigma-Aldrich) or rural source PM<sub>2.5</sub> treated with Polymyxin B. The relatively high dose of PM<sub>2.5</sub> is comparable to that used in several prior studies<sup>31-33</sup>. It is approximately equivalent to the total exposure of an adult human breathing a high ambient concentration of  $20\mu\text{g}/\text{m}^3$  for 24 hours (calculated based on assumptions of 0.5 l tidal volume and 15 breaths/min.). Control animals were similarly instilled with PBS alone. Mice were monitored during recovery from anesthesia (approximately 60 min.) and kept for 24 hours before euthanasia after which blood and tissue samples were collected.

A tracheal cannula was inserted and secured by ligation with suture material. The lungs were removed from the thorax. The left lung lobe was ligated at the mainstem bronchus and removed. The left lung lobe was inflated with poly ethylene glycol and polyvinyl alcohol cyosectioning matrix (OCT) (50% OCT: PBS) and three transverse sections flash frozen in 100% OCT by immersion in liquid nitrogen cooled isobutane. These three sections were cryosectioned for use in laser capture microdissection experiments. The remaining right lung lobes were inflated with 10% neutral buffered formalin at 20 cm H<sub>2</sub>O pressure. The formalin instilled right lung lobes were fixed overnight and transverse sections of each embedded in paraffin using standard histotechnology approaches and

an automated processor. These blocks were then used to prepare standard hematoxylin and eosin stained slides.

*Histologic Evaluation:* Sections of lung were evaluated by a veterinary pathologist blinded to treatment group assignment. Subjective lesion scores were generated for overall inflammation and epithelial hypertrophy/hyperplasia in small airways, parenchymal inflammation and alveolar wall thickening, and arteriolar inflammation and mural thickening. Lesions were scored relative to severity on a 0-4 scale with 4 representing the most prominent or intense lesion in the study.

*Laser Capture Microdissection (LCM):* The left lung lobe from experimental mice were perfused with 50% polyethylene glycol and polyvinyl alcohol (OCT) in saline and flash frozen in labeled plastic cassettes. 15  $\mu$ m sections were cut using a cryostat, mounted on a glass slide coated with a thin layer of polyethylene naphthalate foil (Leica), and preserved with RNAlater ICE (Ambion) at -20°C. LCM was performed at the UC Davis Center for Health and the Environment using a Leica LMD6000 system equipped with an ultraviolet laser and an upright microscope with moving prisms to guide the laser over a stationary sample. Based on the location of histologically evident inflammatory responses, specific tissue regions including terminal bronchioles, venous and arterial blood vessels, and surrounding parenchyma were microdissected from each mouse and sorted into separate microfuge tubes.

*Quantification of gene expression levels:* Total RNA was immediately isolated from pooled samples of each tissue type from individual lung lobes using an RNeasy Micro Kit (Qiagen), including a DNA digestion step, following manufacturer's instructions. Total RNA was quantified and mRNA was reverse transcribed into cDNA using a Superscript III First Strand Synthesis System (Invitrogen). TaqMan assays (Applied Biosystems) specific for genes of interest were used to quantify gene expression changes using qRT-PCR according to the following amplification parameters: initial denaturation for 10 min at 95°C, followed by 40 cycles at 95°C for 15 s (melting) and 60°C for 1 min (annealing and extension). Transcript levels were measured using the ABI Prism 7700 system (Applied Biosystems) and normalized to  $\beta$ 2-microglobulin expression levels. Results were expressed as fold changes using the comparative  $C_T$  method for relative quantification. Target genes were selected based on *in vitro* experiments with collected particulate matter (Wong, 2011, Aung, 2011, Wilson, 2010).

*Bioplex Assay:* Blood samples were collected from the inferior vena cava of sodium pentobarbital anesthetized mice at euthanasia, under institutionally approved protocols, and allowed to clot. Using the resulting serum, cytokine assays were carried out using a commercial immuno-bead based analytical system and mouse specific assay kits (Bioplex, Biorad Life Science, Hercules CA). Growth factors assayed included Eotaxin, Fibroblast Growth Factor (FGF), Granulocyte Colony Stimulating Factor (G-CSF), Granulocyte Monocyte Colony Stimulating Factor (GM-CSF), Interferon- $\gamma$  (IFN- $\gamma$ ), Interleukin-1 $\alpha$  (IL-1 $\alpha$ ), Interleukin-1 $\beta$  (IL-1 $\beta$ ), Interleukin-2 (IL-2), Interleukin-3 (IL-3), Interleukin-4 (IL-4), Interleukin-5 (IL-5), Interleukin-6 (IL-6), Interleukin-9 (IL-9), Interleukin-10 (IL-10), Interleukin-12 protein 40 (IL-12p40), Interleukin-12 protein 70 (IL-12p70), Interleukin-13 (IL-13), Interleukin-15 (IL-15), Interleukin-17 (IL-17), Interleukin-18 (IL-18), Keratinocyte chemotactic factor (KC), Leukemia Inhibitory Factor (LIF), Monocyte Chemotactic Protein-1 (MCP-1), Monocyte Colony Stimulating Factor (M-CSF), Monokine Induced by Gamma Interferon (MIG), Macrophage Inflammatory Protein-1 $\alpha$  (MIP-1 $\alpha$ ), Vascular endothelial growth factor (VEGF), Macrophage

Inflammatory Protein-1 $\alpha$  (MIP-1 $\alpha$ ) Macrophage Inflammatory Protein-2 (MIP-2), Platelet Derived Growth Factor (PDGF), Regulated Upon Activation, Normally T-Expressed, and presumably Secreted (RANTES), and Tumor Necrosis factor- $\alpha$  (TNF $\alpha$ )

*Flow Cytometry Studies:* Blood samples were collected into acid citrate dextrose (ACD) coated syringes with a ratio of 1:6 ACD:blood from the inferior vena cava of sodium pentobarbital anesthetized mice, under institutionally approved protocols. Following the addition of 10 $\mu$ g/ml PGE<sub>1</sub> whole blood was incubated at 37°C for 30 min prior to analysis. All cell counts were determined using an automated blood counter (ActDiff, Beckman-Coulter, Miami, FL).

*Platelet alpha granule proteins and integrins:* Mouse platelet activation was analyzed in ACD-A anticoagulated whole blood (collected from the vena cava at euthanasia) by flow cytometry using the following anti-mouse antibodies, along with appropriate isotypes for each: a biotin conjugated monoclonal antibody to the  $\alpha_{2b}$  subunit for the major platelet integrin  $\alpha_{2b}\beta_{3a}$  (CD41, BD Pharmingen) followed by Streptavidin Alexa 633 (in vitrogen), a FITC-conjugated monoclonal antibody to P-selectin for alpha granule secretion (CD62P, BD Pharmingen), and a PE-conjugated monoclonal antibody to LAMP-1 for lysosomal granule secretion (CD107a, eBioscience). A resting (unstimulated) sample and samples stimulated with either 10 $\mu$ M ADP or 0.1U/ml thrombin were examined for each animal. After stimulation of whole blood, platelets were labeled with the preceding antibodies for one hour and fixed in 1% (final) paraformaldehyde prior to analysis by flow cytometry (FC500, Beckman-Coulter, Miami, FL). Platelets were defined by forward (FSC), and side scatter (SSC) characteristics and ten thousand events were collected within the platelet gate for each animal and each condition. Platelet-derived (CD41positive) membrane microparticles were identified based on FSC with the threshold set at 10<sup>1</sup> representing the lower FSC boundary for resting platelets.

Platelet activation was assessed by the presence of either alpha granule secretion – resulting in P-selectin expression on the platelet surface or by lysosomal granule secretion – resulting in LAMP-1 expression on the platelet surface. Generally resting platelets express very little of either protein. Stimulation by agonist (either ADP or thrombin) results first in alpha granule secretion, followed by lysosomal granule secretion. Platelets were evaluated using two parameters: (1) the percent positive cells – the number of cells present within the pre-defined platelet gate – which express the protein of choice on their cell surface, and (2) the mean fluorescence intensity – the relative number of molecules on the platelet surface. Below are general definitions and information regarding platelets, which should clarify interpretation of the data.

When considering flow cytometric data there are two things collected for each antibody studied: the percent positive and the mean fluorescence intensity (MFI). The percent positive are the number of events (cells) that are positive for the antibody. The mean fluorescence intensity (MFI) is an indicator of how many molecules of the particular antigen are present on the surface of the cells.

Unstimulated or “resting” platelets have large amounts of the major platelet integrin alpha2b beta3a on their surface – and this should be reflected by the high percent positive cells (between 85-99%) and the high MFI – which is arbitrary, as it is based on the titration of the antibody. The MFI generally runs between 100-300 arbitrary fluorescence units (AFUs). Resting platelets should not have either alpha or lysosomal granule membrane proteins on their surface, as they should not have secreted their

granules provided they are truly in a resting state. Thus they should have little P-selectin both with regard to %+ and MFI, as well as little LAMP-1.

When platelets are stimulated with thrombin or ADP, both physiological agonists, there is usually secretion both of alpha granules and lysosomal granules with resultant increase in both %+ and MFI for both P-selectin and LAMP-1. With regard to CD41b there is a more complicated phenotype. Not only is CD41b present in large numbers on the platelet surface but it is also present on the membranes of alpha granules. When platelets are stimulated by agonist one of three things can happen and those events are not mutually exclusive. 1. CD41b (along with its beta subunit) can be shed on microvesicles. That would result in a decrease in %+ and decrease in MFI of the platelets. 2. CD41b and its beta subunit can be recycled (internalized) – also resulting in a decrease in %+ and MFI. It should be noted that it is very difficult to differentiate between these processes without evaluating the number of CD41b positive microvesicles. In either case, however, the decrease in %+ and decrease in MFI are strong and well recognized indicators of platelet activation. Lastly, when the platelets are only slightly activated – there can be an increase in CD41b on the platelet surface, simply reflective of alpha granule secretion and the CD41b associated with those alpha granules

*Leukocytes and Monocytes:* Leukocytes were identified with a monoclonal antibody to CD11b (eBioscience) and monocytes were specifically labeled with antibody to CD115 (eBioscience). Separate samples were prepared to evaluate platelet-monocyte and platelet-leukocyte interactions. After processing with antibodies, blood was fixed in 1% paraformaldehyde and analyzed by flow cytometry. Platelet-monocyte or -leukocyte aggregates were defined as populations that were positive for both CD41 and either CD11b or CD115. All flow cytometric data were analyzed in FloJo (TreeStar, Oregon)

*Statistics:* For cytokine and PCR assays, comparisons between control and particle-instilled animals were evaluated by Student's T-test. Subjective histologic scores were compared using the Kruskal Wallance non-parametric ranking statistic. Differences in platelet populations and platelet monocyte aggregates were compared using a Student's T-test. Statistical calculations were done with GraphPad Prism. All tests used a significance level of  $p \leq 0.05$ .

## **Results:**

### *Particle Composition:*

Urban source particles had greater proportions of both elemental and organic carbon, iron, copper and zinc and overall polycyclic hydrocarbons (PAH) than did rural source PM 2.5 (appendix 1). Rural source PM had greater amounts of water soluble ions (27 +/- 0.5% vs 16 +/- 0.6% in urban source PM).

### *Histology:*

To compare responses to urban and rural PM2.5, untreated, and pre-treated with DFM or polymyxin B on pulmonary inflammation, sections of lung were subjectively scored for the extent of small airway hyperplasia and inflammation in the terminal bronchiole, alveolar parenchyma and surrounding the pulmonary arterioles at the terminal bronchiole. Compared with control animals (Fig 1A), mice instilled with urban source PM2.5 had marked infiltration of neutrophils surrounding aggregates of homogenous fine black particulates admixed with lesser refractile crystalline or amorphous brown particles

(Fig 1B). Particles were both extracellular and within macrophages. Neutrophils also infiltrated the walls and adventitia of bronchiolar arterioles. Mice given urban source PM2.5 pretreated with DFM had no difference in the location or extent of pulmonary inflammation (Fig 1C), while mice given urban source PM2.5 pretreated with polymyxin B had similar amounts of histologically evident PM2.5 but markedly reduced inflammatory cell infiltrates (Fig 1D).

Subjective scores for pulmonary inflammation are presented in Fig 2. There was a significant increase in airway and parenchymal inflammation resulting from urban PM2.5 treatment compared with DFM alone and equivalent increases in all regions from animals given DFM pretreated PM2.5 (Fig 2A). Similar increases in inflammation scores were evident in a separate experiment using polymyxin B inhibition (Fig 2B). Polymyxin B pre-treatment of PM2.5 prevented much of the inflammatory infiltrate in response to both urban (Fig 2B) and rural (Fig 2C) source PM2.5.

Histologically, there was a significant difference in the nature of inflammatory infiltrates between mice treated with urban vs. rural source PM2.5. Mice treated with urban source PM2.5 had marked neutrophilic infiltration surrounding PM2.5 particles that were both extracellular and intracellular in macrophages. Inflammation in lungs of mice treated with rural source PM2.5 was predominantly composed of macrophages with most PM2.5 located intracellularly. The proportion of neutrophils in rural source induced exudates was markedly less than that urban source treated animals (Fig 3).

Histologic findings demonstrate that DFM pretreatment had little effect on pulmonary inflammation induced by urban source PM2.5, while polymyxin B pretreatment significantly reduced pulmonary inflammation. In addition, there were significant differences in the nature and extent of neutrophilic inflammation in response to equivalent mass treatments of urban vs. rural source PM2.5 with urban source being much more pro-inflammatory, as evidenced by higher subjective inflammation scores in the mice treated with urban PM, and a relatively less prominent neutrophil infiltrate in response to rural PM treatment.

#### *Systemic Cytokines:*

To determine whether pulmonary inflammation initiated a systemic inflammatory response, we evaluated serum concentrations of a panel of pro-inflammatory cytokines and immune response proteins. Results are presented in Table 1. In summary, of the few cytokine changes analyzed as statistically significant, all but GM-CSF in the urban source experiment were decreased relative to control animals, and there was no effect of rural source PM2.5 on any serum cytokines. There were few changes more than 25% of control values and no consistent effect of pre-inhibition with either DFM or polymyxin B.

#### *Deferoxamine effects on platelet activation by urban source PM 2.5*

Resting platelets did not show any differences, either in the percent positive or MFI for CD41b or for LAMP-1. However, There was a significant increase in the percent positive platelets for P-selectin in the PBS+PM2.5+DFM group relative to control (PBS+DFM) (p,0.05). There were no differences in the P-selectin MFI for any condition.

Platelets were stimulated with one of two physiologic agonists: either ADP or thrombin. ADP is a weaker agonist than thrombin, however there were differences in both P-selectin and CD41b. There were no differences in the percent positive or the MFI for expression of LAMP-1 under any conditions. There was a significant increase in P-

selectin percent positive of PBS+PM2.5+DFM+ADP vs. control (PBS+DFM+ADP ( $p < 0.05$ ), but no differences for MFI. With regard to the expression of CD41b, both the control and PM samples showed high expression of the integrin, while the PBS+PM+DF sample had greatly reduced expression of CD41b. There were significant differences in the percent CD41b positive between PBS+DFM+ADP and PBS\_PM2.5\_DFM+ADP and PBS+PM2.5+ADP and PBS+PM2.5+DFM+ADP (both  $p < 0.001$ ), however there were no changes in MFI.

Thrombin is a potent physiological agonist and incubation with the agonist resulted in increases in P-selectin and LAMP-1 expression in all groups for both the percent positive as well as the MFI. There was decreased percent positive and integrin expression (MFI) in all groups as well. None of these data were statistically significant. Thus, it can be concluded that there is no alteration of the effect of agonist by incubation either with DF or with PM and/or PM+DF.

Our prior CAPs studies had shown an interaction between platelets and monocytes, thus we determined platelet-monocyte and platelet-leukocyte interactions by flow cytometry. There are no differences in platelet-leukocyte or platelet-monocyte aggregates under any conditions

#### *Polymyxin B effects on Platelet activation by urban source PM2.5*

Similarly to our DF studies, we saw no significant differences in any of the conditions in the number of platelet-monocyte aggregates nor the number of platelet-leukocyte aggregates.

Resting Platelets showed no evidence of alpha granule secretion, as there was no difference either the percent positive or the MFI of P-selectin for any of the conditions. There was, however, a significant difference between the percent positive for LAMP-1 between PM2.5 and PB+PM2.5 ( $p < 0.05$ ), but there were no differences in MFI. All conditions had roughly equal numbers of platelets that were positive for CD41b. The MFI of the control (PBS) was significantly lower than PB (0.001), and was significantly lower than PB+PM (0.01). In addition the MFI of PM was significantly decreased when compared with the MFI from PB (0.05).

ADP stimulation showed no differences in either the percent positive cells or MFI for both P-selectin and LAMP-1. Additionally there were no differences in the percent positive cells for CD41, but similarly to resting platelets, there were differences in CD41b MFI. The control MFI (PBS) was significantly decreased relative to the PM 2.5 ( $p < 0.01$ ), as well as being decreased compared with polymyxin ( $p < 0.001$ ) and decreased compared with PM2.5+PB ( $p < 0.0001$ ). The increased expression of CD41b on the surface is very similar to that seen in the resting platelets.

When platelets were stimulated with thrombin there were changes seen in all three parameters. There were significant differences in the percent positive P-selectin cells between PBS and PB ( $p < 0.05$ ) and PBS and PM+PB ( $p < 0.05$ ). There were no differences in P-selectin MFI. There were significant changes in percent positive LAMP-1 cells or the MFI under any condition. There were no significant changes in the percentage of CD41b positive cells, however there was a difference of  $p = 0.042$  between all of the conditions for the CD41b MFI.

### *Polymyxin B effects on Platelet activation by rural source PM2.5*

Resting PB treated platelets (both PB and PM+PB) had decreased expression of P-selectin, both with regard to percent positive cells ( $p < 0.05$ ) as well as the MFI ( $p < 0.001$ ). Interestingly, PB had absolutely no effect on LAMP-1 expression, nor did it effect CD41b expression or the percentage positive cells..

ADP stimulation resulted in increased numbers of P-selectin positive cells, however there was no difference between any of the conditions. There was, however, a slight decrease in MFI for PM2.5, PB and PM2.5+PB expression ( $p < 0.01$  for all conditions). There were no difference seen for either parameter of LAMP-1, nor were there changes seen in CD41b percent positive or MFI.

Thrombin stimulation resulted in small changes with both granular membrane proteins. There was a significant decrease in percent positive P-selectin in PM2.5+PB, ( $p < 0.05$ ) no effect on MFI. There were no significant differences for any LAMP-1 parameters. There is a significant reduction in the percent positive CD41b cells in the PM2.5 samples ( $p < 0.001$ ) when compared with PBS controls (consistent with platelet activation) There are also significant decreases in CD41b percent positive PM2.5 compared with PB ( $p < 0.05$ ) as well as a significant decrease in percent positive PM2.5 compared with PM2.5+PB ( $p < 0.01$ ). There is a significant difference in MFI between PBS and PM2.5+PB (decreased) ( $p < 0.01$ ).

There are no significant differences in any of the conditions in the number of platelet-monocyte aggregates nor the number of platelet-leukocyte aggregates.

Results of polymyxin B effects on platelet activation suggest the following conclusions: 1) PB knocks down the platelet response, 2) There is no evidence of platelet activation in circulating blood 24 hours after intratracheal instillation of PM2.5 from either urban or rural sources and 3) no platelet-leukocyte or platelet-monocyte interactions of significance were found in these studies.

### *Laser Capture Microscopy and rt-PCR.*

Since changes in specific lung regions are often diluted in evaluations of whole lung gene responses, we isolated terminal bronchioles, alveolar parenchyma and pulmonary arterioles and probed each with a set of gene markers of PAH, ROS, and inflammatory response elements (Figure 5). Only urban source PM2.5 induced transcription of genes probed in these experiments while mice treated with rural source PM had no differences in pulmonary gene responses relative to controls. Untreated urban source PM2.5 increased expression of the PAH response element CYP1A1 in airways and parenchyma in both the DFM and Polymyxin B experiments. Aldehyde dehydrogenase 3a, another PAH response element, was increased in airways of animals given untreated urban PM2.5 in the polymyxin B inhibition experiment. ATF3, a transcription factor activated by the ROS sensitive Stress activated MAP kinase and the ROS response element HOX-1 were both increased in the polymyxin B experiment and their expression was suppressed by polymyxin B pre-treatment. Untreated urban source PM2.5 induced increased increased airway expression of CCL-20 in both experiments. In the experiment investigating polymyxin B inhibition, IL-1beta, a central pro-inflammatory cytokine, was increased by urban source PM2.5 and inhibited by polymyxin B treatment. GM-CSF, a cytokine important in bone marrow activation, was similarly upregulated in the polymyxin B experiment and inhibited by polymyxin B pre-treatment.

NOX-2, an NADPH dependent oxidase and source of endogenous reactive oxygen species, was probed in parenchyma and arterioles. While no PM2.5 treatment effect was evident, interestingly both DFM treatment and polymyxin B decreased NOX-2 expression relative to controls. This suggests that DFM and polymyxin B both have some protective effect on background inflammation induced by the instillation process.

Despite significant periarteriolar inflammation in PM2.5 treated animals, transcription of the endothelial cell adhesion molecules E-selectin and ICAM-1 were not significantly upregulated in PM2.5 treated arterioles or parenchyma in any of these studies.

#### **Discussion:**

Our findings that pulmonary inflammation and the pattern of gene responses to instilled PM2.5 differs between urban and rural sources correlate with our previous study demonstrating location and season specific differences in responses to exposure to PM2.5 from another region of California<sup>7</sup>. The principal findings of the present study are that DFM chelation of metals in PM2.5 had no effect on pulmonary inflammation resulting from instillation with urban source PM2.5. In contrast, instillation with PM2.5 pretreated with polymyxin B significantly reduced pulmonary inflammation and gene responses in animals instilled with particles from both urban and rural sources.

Subjective histology evaluation showed a trend towards more pulmonary inflammation in animals exposed to urban source PM2.5 relative to exposure to equivalent doses of rural source PM2.5. This was further substantiated in gene response studies where urban source PM2.5 elicited robust increases in both xenobiotic metabolism and inflammatory genes in all three lung compartments sampled, while there were no significant gene responses to rural source PM2.5. These findings correlate well with chemical analysis of the PM used in these same studies. The urban PM2.5 used in these studies had 2 fold more iron and copper and 2 fold greater concentrations of PAH compounds relative to the rural PM2.5 (appendix 1, unpublished data).

Previous studies by ourselves and others demonstrate increases in serum cytokines and activation of the clotting system in response to field exposures to concentrated ambient particulates<sup>28, 34</sup>. These changes were not recapitulated in the present single dose aspiration study. The reasons for this remain unclear. However, there were several differences in the protocols used by the two studies that could have influenced the results. Both studies sacrificed the animals about 24-hr following exposure. But, the field studies involved exposure to concentrated ambient particles for several consecutive days, in contrast to the single exposure used in the present study. It is possible that we may not have collected tissues for analysis at the most appropriate time post-exposure, in that epidemiologic evidence in humans suggests that there is a 2-3 day lag between exposure to PM2.5 and onset of morbidity and mortality.

Genes selected for evaluation in this study were chosen based on previous *in vitro* gene response screening studies and represent pathways associated with xenobiotic metabolism (CYP 1A1, AldH2ase), ROS generation or response (NOX-2, ATF3, HOX-1), and inflammation (CCL2, CCL20, GM-CSF, ICAM E-Selectin). Urban source PM2.5 exposure elicited consistent upregulation of CYP1A1 in both airways and parenchyma. A similar upregulation of AldH2ase occurred in airways. These findings correlate with our previous demonstration of CYP 1A1 as a signature of environmental particulate exposure *in vitro*<sup>30, 35</sup>.

While there were no trends towards upregulation of the endogenous ROS generating gene NOX-2, ROS sensitive genes ATF3 and HOX-1 were both up regulated in airways with similar trends in parenchyma and arteries. ATF3 is a transcription factor activated by the ROS sensitive JNK subset of MAP kinases. HOX-1 acts as an antioxidant through catabolism of free heme. It is transcriptionally regulated in response to cellular oxidative stress. These findings suggest that oxidative stress does occur in PM2.5 exposed lungs but is not a consequence of endogenous ROS generated by NOX-2. Interleukin-1beta (IL-1b) a master cytokine stimulating diverse pro-inflammatory responses and CCL-20, a chemokine enhancing mucosal immune function, and the general leukocyte growth factor GM-CSF were all upregulated in airways by urban PM2.5 treatment and not decreased by metal chelation or endotoxin binding. Upregulation of pro-inflammatory genes correlates with PM2.5-induced airway diseases such as asthma<sup>36</sup>.

Upregulation of xenobiotic metabolism, ROS response, and inflammatory genes was not evident in response to rural source PM2.5. Histologic evaluation of lungs from rural PM2.5- treated animals showed slightly less but still significant inflammation relative to urban PM2.5 treated animals. Estimates of particle density based on visual density of carbon particles in histologic sections suggested an equivalent particle burden in lungs of rural and urban PM2.5 treated animals. This suggests that the gene responses in lung compartments are driven by particle composition rather than simple inflammatory responses.

In contrast to expectations, DFM treatment had little effect on any parameters evaluated. There was no difference in subjective histology scores between PM2.5 and DFM pretreated PM2.5 animals, and only a moderate influence on gene transcription. In the limited number of serum cytokines with significant differences between groups, the majority had higher cytokine levels in animals treated with DFM alone than in either PM2.5 treated group. Polymyxin B pretreatment of PM2.5 did, however, significantly blunt pulmonary inflammation and gene responses. While equivalent particle density was present in Polymyxin B/PM2.5 treated animals, the histologic inflammatory response was markedly reduced in response to both urban and rural PM2.5 instillations. Gene responses in urban source treated animals suggest the polymyxin B response is not limited to inflammation alone but also influences the upregulation of xenobiotic metabolism and oxidative stress genes. Overall, results of these experiments implicate endotoxin as a key element in both urban and rural source PM2.5 toxicity. Combined with the lack of response to iron chelation by DFM, the decrease in multiple pulmonary gene responses and subsequent pulmonary inflammation suggest that endotoxin may be a more significant component of PM2.5 than transition metals.

This project demonstrates the utility of intratracheal instillation of collected ambient PM2.5 in comparative biologic response studies in laboratory animals. Evaluation of selected regions of pulmonary parenchyma greatly enhances sensitivity in characterizing tissue specific gene responses. Controlling dose controls for differences in environmental conditions that are inherent in CAPs studies. In contrast, there remain potential artifacts in particle collection and processing relative to particle size and extraction of soluble PM components. Given the nature of particle concentrator processes, similar critiques relative to size and composition can be applied to both exposure approaches. Intratracheal exposures are practically limited to single doses so the cumulative effects that can potentially occur in longer term CAPs exposures may not be seen. The extent of pulmonary inflammation induced by intratracheal instillation is

significantly greater than that seen in CAPs studies where often little pulmonary inflammatory cell infiltrate is evident. Thus the lack of systemic responses in these studies was unexpected. Our studies were limited to a 24 hour time point post dosing. The lack of systemic platelet and cytokine responses may reflect a delay between induction of pulmonary inflammation and onset of systemic disease similar to that seen in epidemiologic studies of human populations. While the clear responses to polymyxin B inhibition provide significant evidence for an endotoxin effect in pulmonary inflammatory responses to PM2.5, decreased platelet reactivity in polymyxin B treated controls suggests consideration for additive effects of endotoxin present in animal housing must be given in future studies. Despite a record of safe use of DFM in humans, we found DFM treated animals had evidence of platelet activation even in controls. This was unexpected and future experiments with this drug should include untreated controls.

## **Conclusions**

Experiments in this study examined the relative role of transition metals and bacterial derived endotoxin in inducing pulmonary and systemic inflammation in response to PM2.5 derived from urban vs. rural sources. Our findings confirm that urban source PM2.5 induces greater responses on a mass equivalent basis than PM2.5 collected from rural areas. Laser capture microscopic isolation of target pulmonary structures demonstrated activation of pro-inflammatory, oxidant metabolism and xenobiotic metabolism consistent with known biologic functions of airways, pulmonary parenchyma and vasculature. Pulmonary inflammation and gene responses were diminished by pretreatment to bind endotoxin in PM2.5 but not by pretreatment to bind transition metals. In contrast to prior studies demonstrating platelet activation in longer term field exposures to concentrated ambient particles, no PM2.5 induced platelet activation was detected in these single dose studies. We conclude endotoxin is a key factor in both urban and rural PM2.5 toxicity.

## **Recommendations:**

- 1) This study provides additional evidence for the importance of PM 2.5 composition in regulatory decision making. Experiments with equal gravimetric doses of PM from differing regions led to markedly different inflammatory responses
- 2) Endotoxin must be considered an important constituent in both rural and urban source PM
- 3) The nature and time course of systemic responses to PM 2.5 inhalation deserves further investigation, particularly relative to the length of post-exposure period necessary to induce these responses.

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Table 1: Cytokine analysis in serum of mice given intratracheal instillations of urban or rural source PM2.5 pretreated with either polymyxin B or DFM.

Serum	Urban			Urban				Rural			
	Value (pg/ml)			Value (pg/ml)				Value (pg/ml)			
Mediator	PBS+DF	PBS+PM	PBS+DF+PM	PBS	PBS+PoIB	PM	PM+PoIB	PBS	PoIB	PM	PM+PoIB
Basic FGF	505	529	405	822	686	276	795	551	397	591	353
Eolaxin	522	317	370	ND	ND	ND	ND	ND	ND	ND	ND
G-CSF	516	1251	2585	ND	ND	ND	ND	ND	ND	ND	ND
GM-CSF	231	140	174	76	121	132	95	95	57	55	73
IFN- $\gamma$	21	11	13	11	14	14	12	9	10	9	10
IL-1 $\alpha$	ND	ND	ND	9	9	11	8	7	5	7	7
IL-1 $\beta$	253	214	206	179	179	164	163	138	137	159	142
IL-2	24	23	26	22	23	20	20	15	ND	19	19
IL-3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IL-4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IL-5	39	34	35	20	23	20	22	19	14	17	15
IL-6	9	15	20	ND	ND	ND	ND	ND	ND	ND	ND
IL-9	554	424	481	201	250	184	203	193	147	197	118
IL-10	77	58	55	30	33	32	26	28	21	29	25
IL-12p40	73	52	58	75	77	95	86	67	75	67	64
IL-12p70	128	75	101	56	50	54	61	47	36	52	50
IL-13	205	149	153	120	117	112	116	99	83	104	86
IL-15	1220	1485	1064	952	677	182	858	505	280	533	252
IL-17	246	173	186	88	97	84	100	89	97	95	109
IL-18	988	976	978	1661	1473	739	1557	1247	927	1391	939
KC	121	157	193	23	18	29	28	30	30	61	21
LIF	140	165	117	85	68	22	77	44	26	50	25
M-CSF	2312	2044	1868	1065	968	860	992	1107	967	913	797
MCP-1	316	264	253	162	164	158	148	139	118	145	124
MIG	2860	3085	2528	3059	2488	926		1822	1371	2142	1193
MIP-1 $\alpha$	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MIP-1 $\beta$	50	33	40	45	37	32	37	32	24	35	27
MIP-2	87	98	75	46	37	11	42	28	17	28	15
PDGF $\beta$	12674	13906	12487	17438	16286	7504	12343	7245	6404	9952	3297
RANTES	34	32	34	32	31	18	32	16	13	15	11
TNF $\alpha$	777	496	464	423	440	486	377	362	291	328	304
VEGF	743	810	605	629	489	130	575	349	201	380	183

Table 2: Statistical analysis of cytokine results from mice treated with urban source PM2.5. Changes in black represent decreased concentration. Changes in red are increased concentration.

Serum	Significant Changes	
Mediator		
Basic FGF	PBS+PM vs PBS+DF+PM	
Eotaxin		
G-CSF		
GM-CSF	PBS+DF vs PBS+PM	PBS vs PM (Urban, PoIB) Upreg
IFN-g	PBS+DF vs PBS+PM	
IL-1a		
IL-1b		
IL-2		
IL-3		
IL-4		
IL-5		
IL-6		
IL-9		
IL-10		
IL-12p40		
IL-12p70		
IL-13		
IL-15	PBS+PM vs PBS+DF+PM	
IL-17		
IL-18		
KC		
LIF	PBS+PM vs PBS+DF+PM	PBS+DF vs PBS+DF+PM
M-CSF	PBS+DF vs PBS+PM	
MCP-1		
MIG	PBS+PM vs PBS+DF+PM	PBS+DF vs PBS+DF+PM
MIP-1a		
MIP-1b		
MIP-2	PBS+PM vs PBS+DF+PM	
PDGFbb		
RANTES	PBS vs PM Urban (poIB)	PM + PoIB vs PM (Urban)
TNFa	PBS+DF vs PBS+DF+PM	PBS+DF vs PBS+PM
VEGF	PBS+PM vs PBS+DF+PM	

## Figure Legends

Figure 1a: Histopathology of terminal bronchiolar junction with pulmonary arteriole from mice given intratracheal instillations of 200 ug/mouse of A) PBS only B) Urban source PM2.5 C) Urban source PM2.5 pretreated with DFM or D) Urban source PM2.5 pretreated with polymyxin B. PM2.5 alone treatment elicited significant neutrophil accumulation around evident PM2.5 in proximal alveolar ducts and in the adventitia of pulmonary arterioles. DFM pre-treatment did not alter the location or extent of inflammatory response but polymyxin B pre-treatment significantly reduced the neutrophil influx.

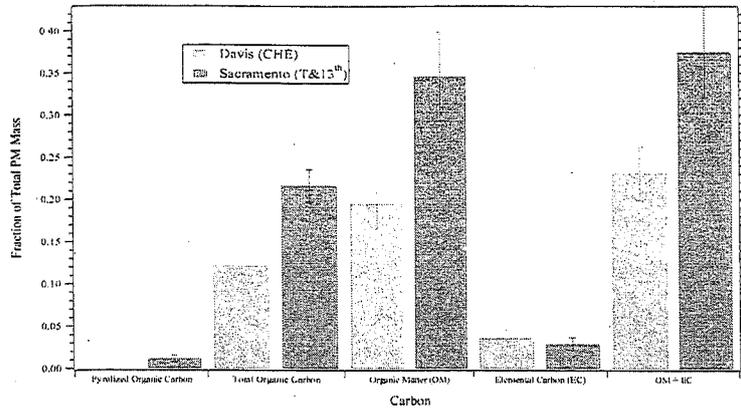
Figure 1b: Subjective histologic analysis of airway, alveolar and arteriolar inflammation comparing responses to intratracheally administered urban source PM2.5 pre-treated with polymyxin B(A) or DFM (B) or rural source PM2.5 pretreated with polymyxin B (C).

Figure 2: Histopathology of terminal bronchiolar junction with pulmonary arteriole from mice give intratracheal instillations of 200 ug/mouse of A) urban source or B) rural source PM2.5. While there is little difference in visual PM2.5 densities, urban source PM2.5 elicits a significantly greater neutrophil response while rural source PM2.5 is more evident intracellularly in macrophages. Similarly, periarteriolar inflammatory cell infiltrates are more prominent in lungs of mice treated with urban source PM2.5.

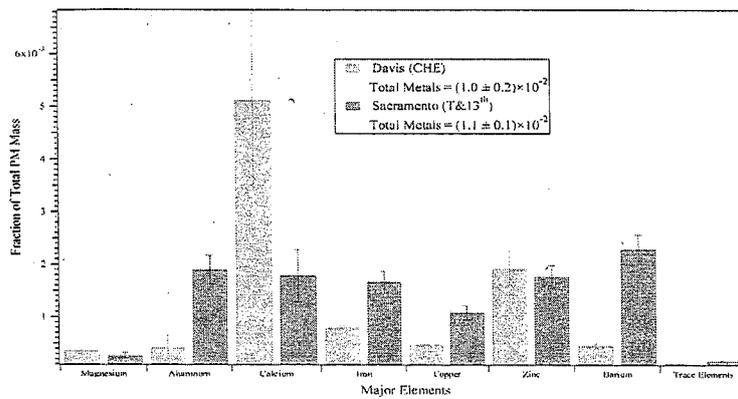
Figure 3: Results of rt-PCR measurement of transcripts from terminal bronchioles, alveolar parenchyma or pulmonary arterioles isolated by Laser capture microscopy. Significant responses were only evident in tissues isolated from mice treated with urban source PM2.5. Significant increases in PAH response elements (CYP 1A1, Aldh3a1) ROS response elements (ATF3, HOX-1) and Inflammatory markers (CCL-20, IL-1 beta and GM-CSF) were present in urban PM2.5 treated mice. While DFM pretreatment had little effect on responses, polymyxin B pretreated PM2.5 inhibited transcription of CYP 1A1 in airways and parenchyma and ATF3, HOX-1, IL-1beta and GM-CSF in airways.

Appendix 1: relative composition of urban (Sac) and rural (Davis) PM2.

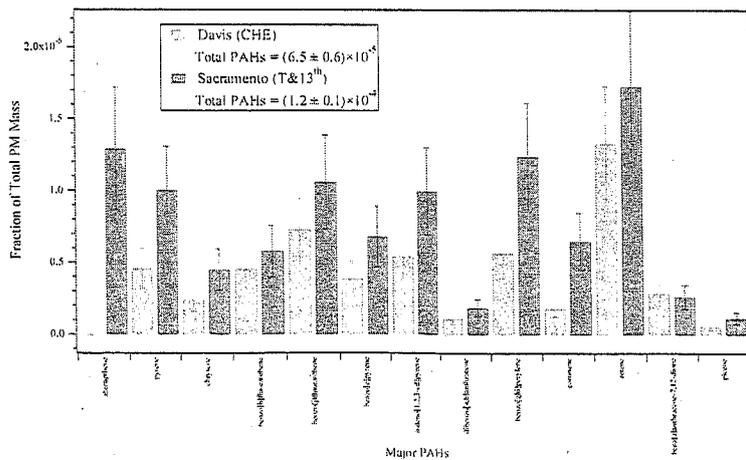
Carbon



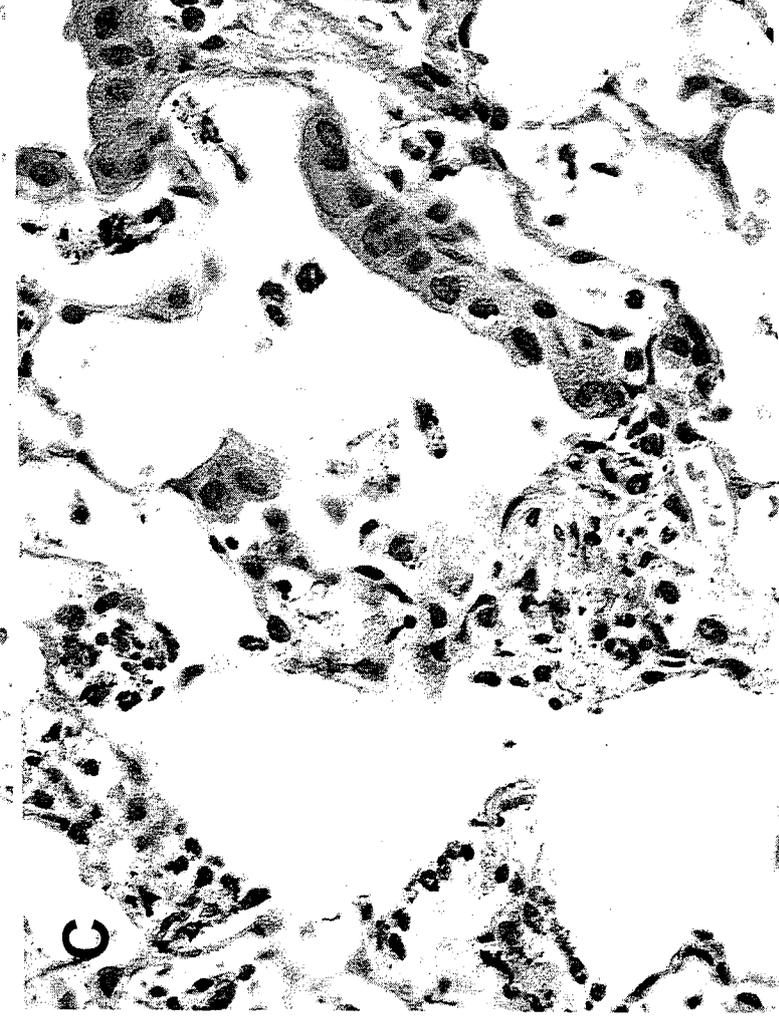
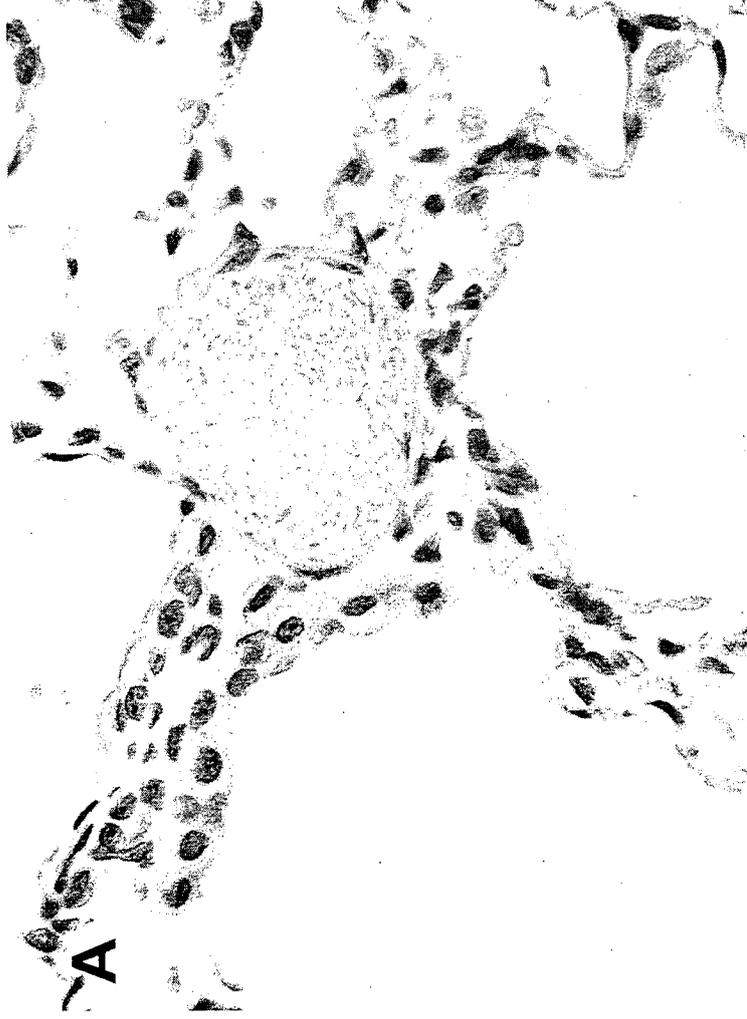
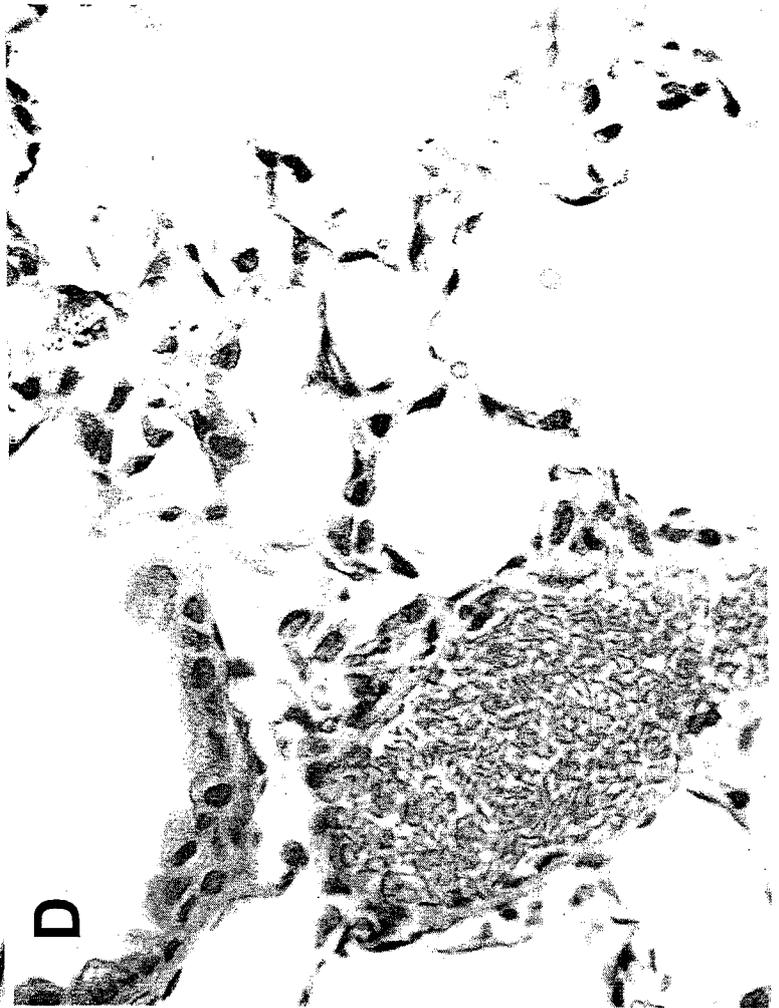
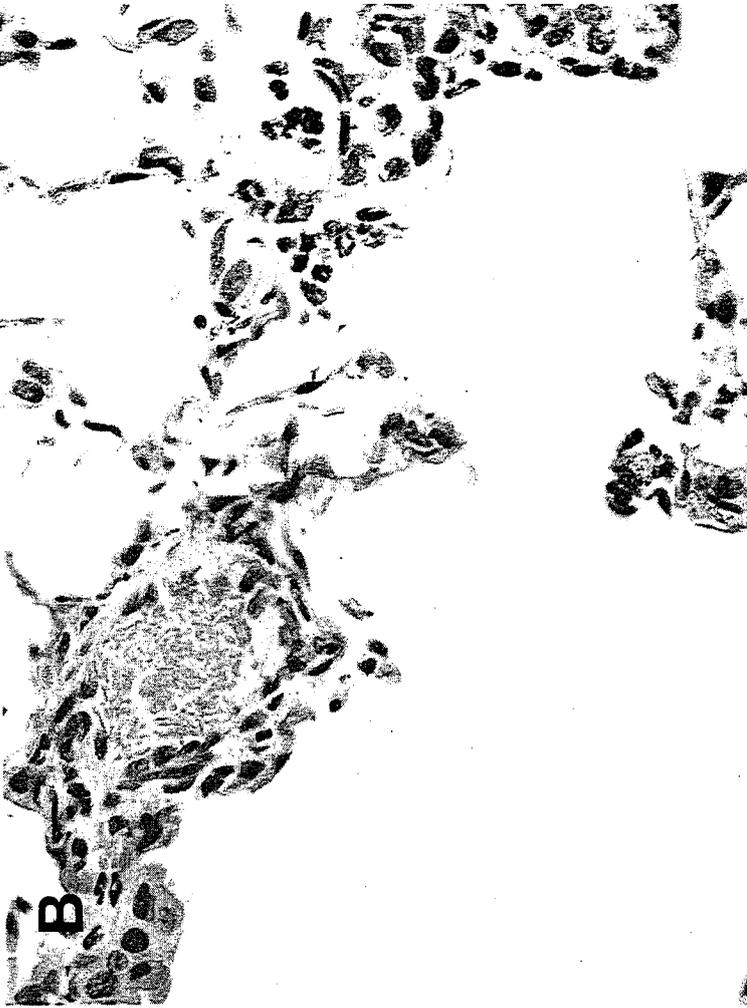
Major Metals

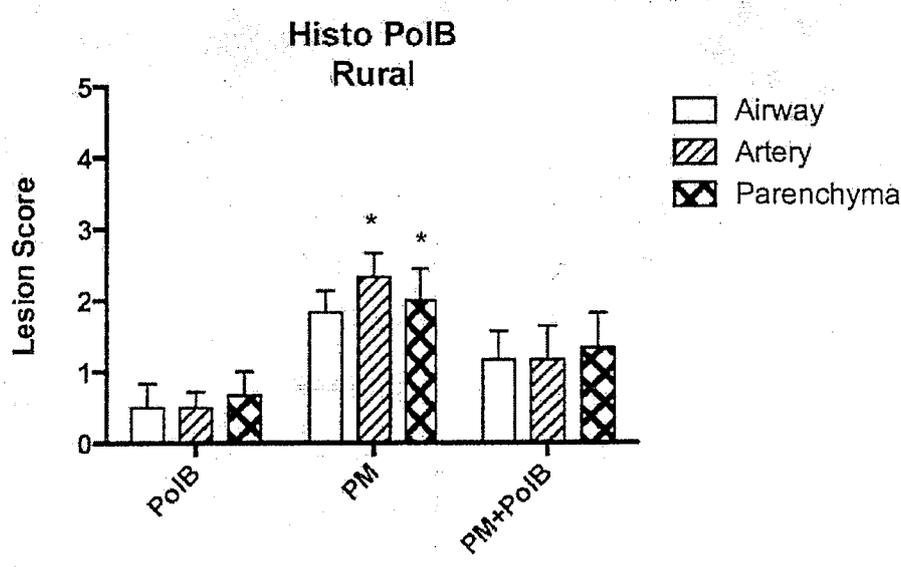
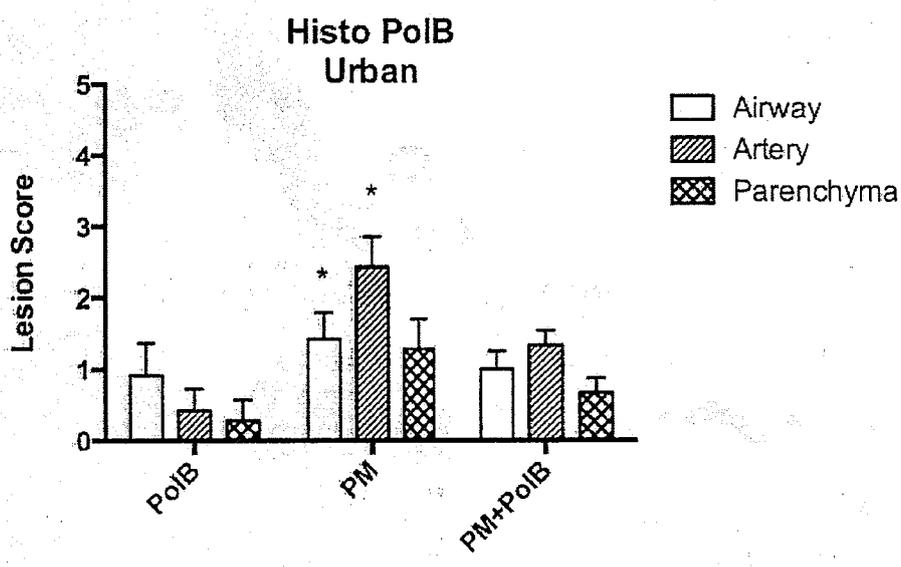
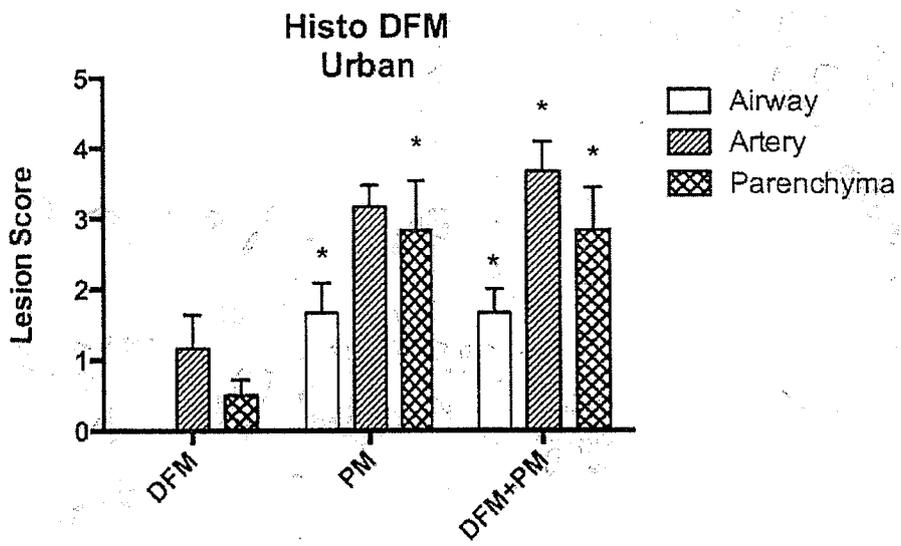


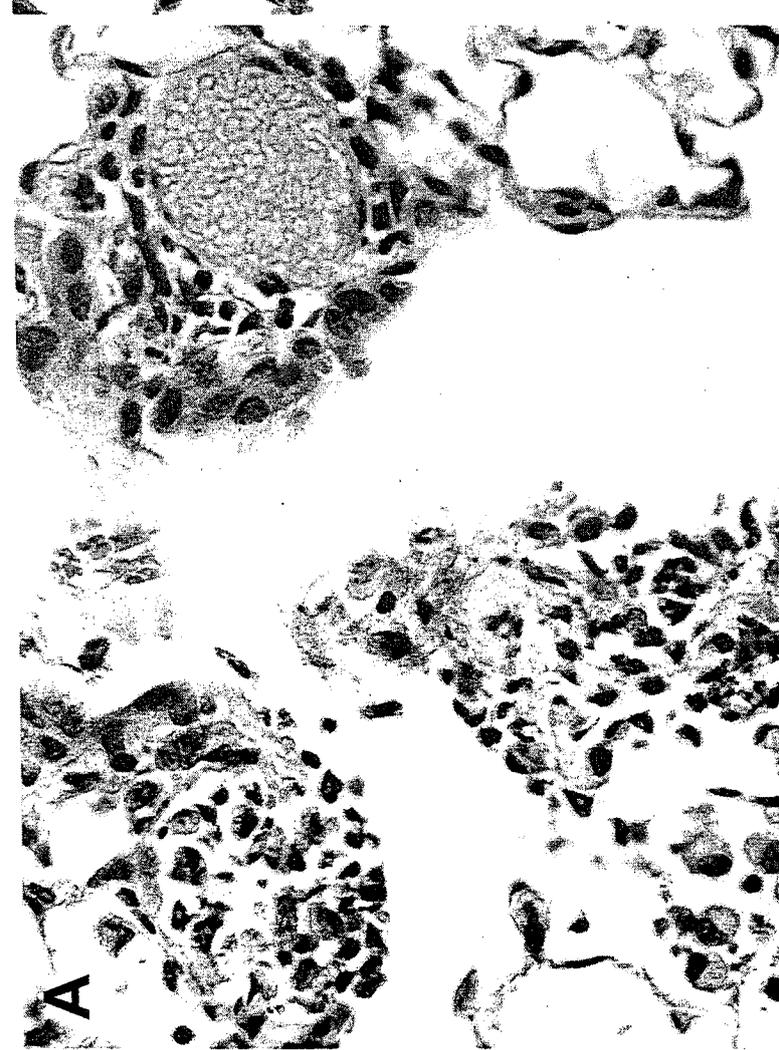
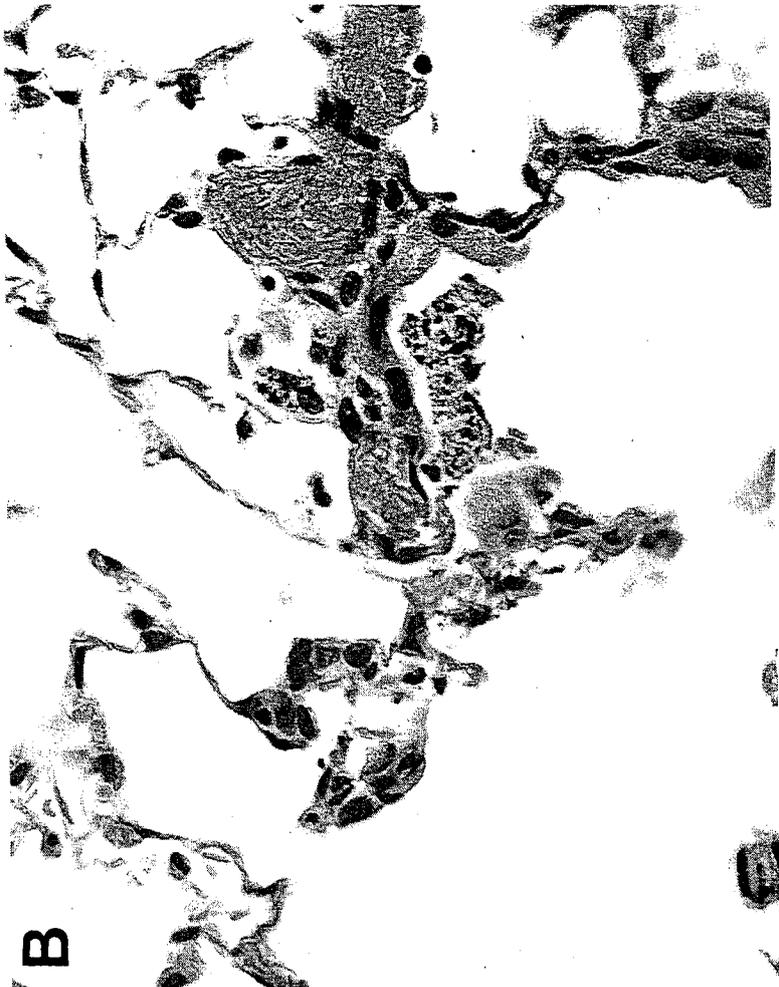
Major PAHs



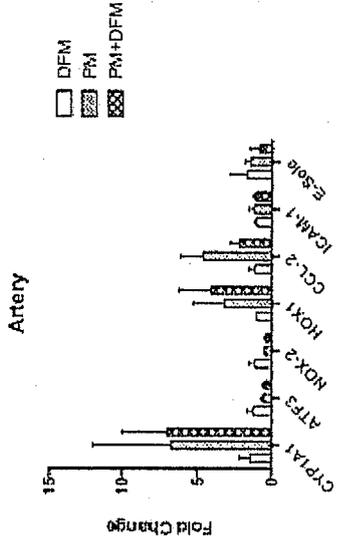
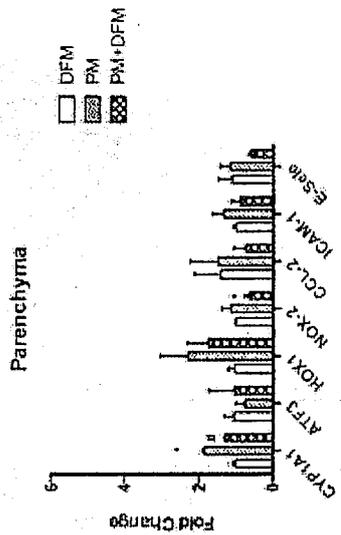
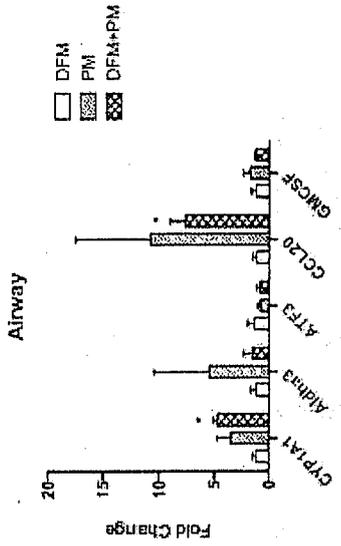




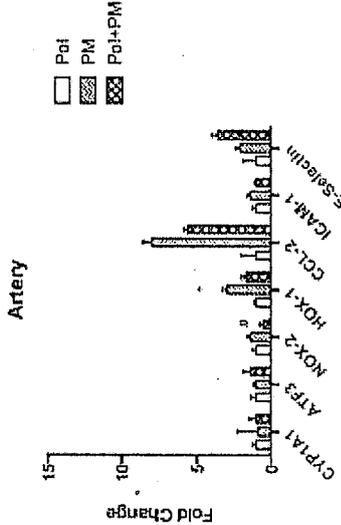
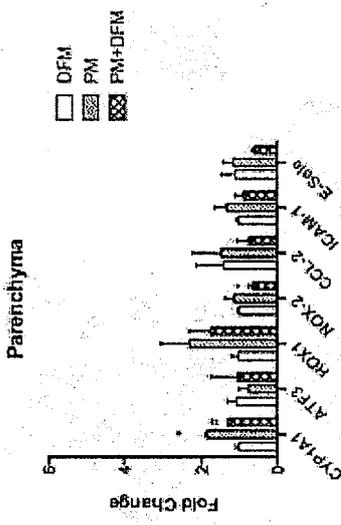




# Urban



# Urban



# Rural

