V. RELEVANT PM PROPERTIES-RELATED PAPERS (SESSION 2)

Is SO_2 a Causative Factor for the PM Associated Mortality Risks in the Netherlands?

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Abstract

Associations between serious health risks and PM have been found in numerous studies, including studies in the Netherlands (Verhoeff et al., 1996). More recent European studies have also found associations with gaseous components (Katsouyanni et al., 1997, Hoek et al., 1997), of which SO_2 is one of the gasses. A recent report in the UK (COMEAP, 1998) concludes that in ambient air SO_2 leads to an increase in total mortality of 0.6% per 10 $\mu g/m^3$.

Although these statistical associations have been found, it remains questionable as to whether or not the associations are causal. A careful analysis of a nine-year Dutch time series (Hoek et al., 1997) by successive exclusion of the highest concentrations indicates that SO_2 is probably not causally associated with the health effects, but that it is correlated. A separate analysis of the mortality over different three-year periods indicates that in the first three years SO_2 lead to a significantly lower relative risk than in the last three years, which had the lowest SO_2 concentrations. The conclusion that in the Netherlands SO_2 does not seem to be a causative factor for PM associated health effects is substantiated by further circumstantial evidence, in combination with biological arguments, indicating that a factor correlating with SO_2 (probably PM) might explain the observed associations with total mortality.

Introduction

Associations between serious health risks and PM have been found in numerous studies, including studies in the Netherlands (Verhoeff et al., 1996). More recent European studies have also found associations with gaseous components, of which SO_2 is one of the gasses. For total mortality Katsouyanni et al., (1997) report a RR of 1.023 (95% CI 1.017 - 1.028) per 50 $\mu g/m^3$ increment in air concentration, which is comparable to the RR of 1.028 (95% CI 1.015 - 1.041) per 40 $\mu g/m^3$ reported by Hoek et al., (1997). A recent report in the UK (COMEAP, 1998) concludes that in ambient air SO_2 leads to an increase in total mortality of 0.6% per 10 $\mu g/m^3$ and it assumes no threshold for this effect. Associations of health effects and SO_2 have been found at concentrations well below the Dutch limit values for SO_2 (median of daily averages of 75 $\mu g/m^3$) and the EU target values of yearly averages (of 40-50 $\mu g/m^3$). Although the associations are statistically sound, it remains questionable as to whether or not the associations are causal. If SO_2 is causal for the health effects that have been found in epidemiological studies and the concentration response relationships are linear as is implicitly assumed in most studies (COMEAP, 1988) an exclusion of higher concentrations would not alter the slope of the regression coefficient. In this study the evidence for SO_2 being a causative factor for the PM associated health risks is weighed and the plausibility of SO_2 as a causal factor is discussed.

Method

A nine-year Dutch time series has been reported by Hoek et al., (1997). The relationship between daily mortality and air pollution was modelled using Poisson regression analysis. All pollution mortality associations were adjusted for potential confounding due to long time trends, seasonal trends, influenza epidemics, ambient temperature, ambient relative humidity, day of the week and holidays. Non-parametric smoothers were used as a flexible approach for specifying relationships between mortality and confounders. Recently Hoek and Brunekreef (1999) have reported a further analysis of the same time series. This time series covers the period 1986-1994. For the whole Dutch population of approximately 15 million people total daily mortality and daily mortality for various causes of death have been associated with gaseous and particulate air pollution measured on a daily basis in the Dutch National Air Quality Monitoring Network. The material collected for these studies is so extensive that it also allows a separate analysis of the role of SO_2 as a potential causative factor for the population health effects. This is done by studying the influence of successive exclusion of the highest concentrations of the running weekly averages of SO_2 on the RR and its significance. This analysis is done separately for the whole of the nine-year period and for different three-year periods. Because of the implementation of abatement policies leading to a reduction of SO_2 emissions and concentrations at a national and European level, the ambient concentrations of SO_2 in the Netherlands have decreased during the study period.

Results

For the period 1986-1994 the RR depends on the concentration ranges of SO_2, leading to an increased risk at lower concentrations. Table 1 presents the influence of excluding high running weekly average SO_2 levels on the attributive risk of total

mortality of the Dutch population; the results are presented as percentage total mortality increase per 10 $\mu g/m^3$ of SO_2. During this period the 90th percentile of the daily SO_2 concentrations in the Netherlands was 22 $\mu g/m^3$.
The separate analysis in each period of three years is presented in Table 2.

Table 1. Influence of excluding high running weekly average SO_2 levels in the Netherlands during 1986-1994 on the total mortality increase per 10 $\mu g/m^3$ SO_2, the 95% confidence intervals and number of daily measurements,
(Source: Hoek et al., 1997).

SO_2 avg.	Mortal. Incr.	95% CI	N=
All	0.7%	0.4-1.0	3068
<100	0.8%	0.4-1.1	3062
<50	1.4%	1.0-1.9	3039
<25	2.1%	1.5-2.8	2917
<10	4.3%	2.1-6.6	1424

Table 2. Total mortality increase per 10 $\mu g/m^3$ running weekly average SO_2 in the Netherlands during three different three year periods, the 95% confidence intervals, number of daily measurements and average SO_2 levels in $\mu g/m^3$.

Period	SO_2 $\mu g/m^3$	Mortal. Incr.	95% CI	N=
86-88	16	0.7%	0.3-1.0	963
89-91	12	1.7%	1.1-2.4	1051
92-94	9	2.8%	1.8-3.9	1054

Table 3. Influence of excluding high running weekly average SO_2 levels in the Netherlands during 1986-1994 on the total mortality increase per 10 $\mu g/m^3$ SO_2, during different periods of three years

SO_2 avg.	Mortal. Incr. 1986-88	Mortal. Incr. 1989-91	Mortal. Incr. 1992-94
All	0.7%	1.7%	2.8%
<100	0.4%	1.7%	2.8%
<50	1.6%	2.0%	2.8%
<25	1.8%	2.4%	3.7%
<10	n.s.	n.s.	7.3%

n.s.= coefficient becomes non significant

Discussion and Conclusion

Table 1 seems to lead to the conclusion that lower ambient concentrations of SO_2 might be more toxic because they are associated with a higher risk of total mortality for the same amount of 10 $\mu g/m^3$ SO_2. This conclusion cannot be substantiated with a

geographical analysis by differentiating the Netherlands into two regions: an urban and a rural region. The urban region is the combination of the four largest cities in the Netherlands (approximately 3 million inhabitants) and the rural region is the rest of the country (approx. 12 million inhabitants). The average SO_2 concentration during the total study period was 18 $\mu g/m^3$ for the urban region and 10 $\mu g/m^3$ for the rural region. If a lower SO_2 concentration coincided with a higher RR for total mortality, the mortality increase should have been greater in the rural area than in the urban region. In reality the urban mortality increase per 10 $\mu g/m^3$ SO_2 was 1.1%, while it was 0.7% for the rural area. Also the results presented in Table 3 point to a direction that does not substantiate a higher relative toxicity of SO_2 for lower concentrations. The average SO_2 concentration during the first three years of the study period was 16 $\mu g/m^3$ associated with a mortality increase of 0.7%, while during the last three years the average SO_2 concentration was decreased to 9 $\mu g/m^3$ with 2.8% mortality increase per 10 $\mu g/m^3$ of SO_2.

Table 2 shows a significant difference in the relative mortality of SO_2 in the first period of three years compared to the second or third. There has been no change in the SO_2 monitoring during the years of the study period. Since 1985 only one type of SO_2 monitor has been in use in the Dutch National Air Quality Monitoring Network. Also the number and location of the SO_2 measuring points has not changed substantially during this period. It may be concluded from Table 2 that a period of three years seems to be too short for a reliable estimate of the health effects of SO_2.

Table 3 shows the influence of excluding high running weekly average concentrations on the total mortality increase per 10 $\mu g/m^3$ SO_2. At low concentrations SO_2 (< 10 $\mu g/m^3$), the number of days decreases from 953 to 256 in the first three-year period and from 1051 to 453 for the second three-year period, which gives such wide confidence intervals that it makes the estimate of the coefficient become non significant. Because of the general lowering of the SO_2 levels during the study period, the last three-year period in Table 3 did not have any days with running weekly average values of SO_2 > 50 $\mu g/m^3$.

In principle an explanation of this puzzling higher toxicity of SO_2 at lower concentrations is that it might be an artifact. Such an artifact might be caused by some other ambient pollutant concentration(s) being higher in the summer when generally speaking the SO_2 concentrations are lower in the Netherlands (on average in summer 9 $\mu g/m^3$ and in winter 16 $\mu g/m^3$). This hypothesis was tested by Hoek and Brunekreef (1999), in some further analyses of daily mortality in relationship to air pollution. These authors concluded that high air pollution effect estimates in the summer season are not an artifact of insufficient adjustment for high temperatures. In the analysis they also concluded, that air pollution effect estimates in the summer season were unchanged when pollen counts were added as additional confounders. Finally they concluded that high air pollution effect estimates for pollutants that have their highest concentration in the winter season in the Netherlands (particles, SO_2, NO_2, CO) are not due to interaction between these pollutants and ozone.

A process of "harvesting" of the susceptible population has been suggested as a hypothesis to explain the counterintuitive results of Table 1. However "harvesting" would probably result in some other relationship between mortality increase and SO_2

concentrations than can be found in Table 1. One would expect a higher mortality at higher concentrations because these higher concentrations affect those that are very susceptible; in the days following these higher concentrations harvesting leads to slightly lower mortality because of a lack of a susceptible population. "Harvesting" is an acute phenomenon and probably can not be discerned in the mortality rates estimated for periods as long as seasons or years. Table 3 indicates that "harvesting" can not be the general answer to the results found in this analysis, as the relative mortality during the first period of three years is considerably less than during the last period of three years.

Apart from these epidemiological considerations there also is supporting biological evidence against a causal role of SO_2 in total mortality in the Netherlands at the current levels. The Air Quality Guidelines of the WHO (1987) do not present any human clinical evidence for health effects at the current ambient SO_2 levels in the Netherlands. There is also no animal evidence or no plausible mechanism for mortality caused by SO_2 at these concentrations. SO_2 is a known noxious agent, but at much higher levels than are currently encountered in the ambient environment. In our laboratory SO_2 is used in concentrations that are more than 10,000 fold higher than ambient levels in the Netherlands, to create chronic bronchitis animal models in order to test the toxicity of ambient PM. For the development of those models in rats the animals are exposed for 4 weeks, during 5 days to concentrations of 150-200 PPM.

In the Netherlands SO_2 is highly correlated with PM_{10} and other gaseous pollutants. The Spearman correlation coefficient of the daily SO_2 and PM_{10} concentrations is 0.65 for the entire country. The correlation coefficient of SO_2 with the gaseous components NO_2 and CO is 0.71 and 0.65 respectively. RIVM (1997) reports that in 1985 the Dutch emissions of SO_2 by traffic were 9% of the national emission of 255 kilo Tons of SO_2. In 1995 the traffic emission of SO_2 had risen to 22% of a substantially lower total emission of 142 kilo Tons in the Netherlands. The Dutch definition of traffic also includes shipping, which is an important source of traffic related SO_2. The industrial and refinery emissions of SO_2 in the Netherlands are mostly from tall stacks and they contribute relatively less to population exposure than the emissions of traffic at lower heights. In the Netherlands especially, the use of diesel and fuel oil contributes to the traffic emissions of SO_2, therefore SO_2 might be seen as a proxy for the exposure of the population to the exhausts produced by the use of these fuels, however the numerical value of this proxy changes over time.

The conclusion remains that SO_2 is associated with health effects in the Dutch population. Despite the epidemiological associations it seems highly implausible that SO_2 is the causative factor for the health effects in the Netherlands. Probably SO_2 is only correlated with the causative factor for mortality in the population. Therefore it is advisable not to use SO_2 as a proxy for risk assessment and risk management of the PM related health effects in the Dutch population.

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Composition Of Particulate Matter As The Determinant Of Cellular Response.

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Abstract

We have previously reported that exposure of pulmonary epithelial cells to size-fractionated ambient particulate matter (PM) activates cell signaling pathways leading to expression of the inflammatory cytokine, interleukin-8 (IL-8). In this study, we investigated whether the composition of PM influences the cellular response. In Feb, 1998, 12-hr samples of coarse (2.5-10 μm) and fine (< 2.5 μm, corresponding to PM2.5) particles were collected on Teflon filters from 4 locations in southwestern Taiwan using dichotomous samplers. The concentrations of anions (SO_4^{2-}, NO_3^-) and 20 relevant elements were determined using ion chromatography and x-ray fluorescence, respectively. Elemental carbon (analyzed separately on particles collected on quartz filters) composed 40% of the PM mass regardless of size fraction. Nitrate contributed between 14 to 17% of the mass, while sulfate contributed between 10 to 14% of the PM mass. Aluminum, silica, sulfur, chloride, iron, and zinc were among the major elements detected. Confluent monolayers of A549 cells were exposed to coarse and fine particles for 2 hr at concentrations of 25, 50, and 100 μg/ml. Immediately after exposure, the intracellular pH (using the fluorescent dye BCECF), reactive oxygen intermediates production (using fluorescent dye DCF), and expression of IL-8 mRNA (using RT-PCR) were measured. For the fine particles, the cellular responses were correlated with the concentration of sulfur, but not with any metals. It was determined that PM from an upwind urban area was the primary contributor to the cellular response, while locally generated coarse particles had less of an effect.

Introduction

Numerous epidemiological studies have demonstrated significant associations between the airborne concentrations of ambient particulate matter (PM) and increases in mortality and morbidity. Ambient PM is a heterogeneous mixture that varies in physicochemical characteristics. The characteristics of particles which may be responsible for lung injury after exposure to air pollution particles is not known. However injury has been postulated to be mediated by ultrafine particles, biological agents (e.g., endotoxin), acid aerosols, polyaromatic hydrocarbons and transition metals associated with particles.

PM contains transition metals such as iron, copper, nickel, vanadium, and cobalt. These metals are capable of catalyzing the one-electron reductions of molecular oxygen necessary to generate

ROI. Oxidative stress is known to be involved in cardiac ischemia, and direct pulmonary effects. Soluble metals from inhaled PM dissolved into the milieu of the airway lumen could initiate lipid peroxidation of the cell membrane and result in cell death and subsequent lung injury.

To test the hypothesis that the biological effects associated with PM depend upon physicochemical characteristics of the particles, we exposed respiratory epithelial cells to size-fractionated ambient PM collected from several sampling sites in southwestern Taiwan. The production of reactive oxygen intermediates (ROI) and intracellular pH (pHi) (both using fluorescent techniques) as well as the expression of interleukin 8 (IL-8) were measured in respiratory cells exposed to these particles.

Methods

Collection and Characterization of Ambient PM in Taiwan. Ambient PM samples were collected in February, 1998. Samples of coarse (2.5-10 μm) and fine (< 2.5 μm, corresponding to PM2.5) particles were collected on Teflon filters from 4 locations in southwestern Taiwan using dichotomous samplers. The concentrations of anions (SO_4^{2-}, NO_3^-) and 40 elements were determined using ion chromatography and x-ray fluorescence, respectively. Elemental carbon (analyzed separately on particles collected on quartz filters) composed 40% of the PM mass regardless of size. Nitrate contributed between 14 to 17% of the mass, while sulfate contributed between 10 to 14% of the PM mass. Aluminum, silica, sulfur, chloride, iron, and zinc were among the major elements detected.

Cell Culture. Throughout the study, a human pulmonary epithelial cell line (A549, generations 77-121; American Type Tissue Culture, Rockville, MD), was used. This cell line was derived from an alveolar cell carcinoma of the lung of a 58-year-old male Caucasian patient and has alveolar type-II cell characteristics. A549 cells, at a density of $1x10^4$ cells per well, were cultured in Costar 96-well black plates with clear bottoms at 37°C and 5% CO_2 in F-12K medium, 10 % fetal bovine serum, and 1 % penicillin/streptomycin (equivalent to 100 U/ml and 100 μg/ml), respectively. Cells became confluent in 3-4 days.

In vitro Exposure of A549 Cells to Ambient PM. Ambient particles collected on Teflon filters were immersed in serum-free media (final concentration of PM were 100 μg/ml). Particles were scraped off with a rubber policeman and sonicated in an ultrasound water bath for 30 minutes.

ROI Measurement. To measure ROI production, the fluorescent probe DCF-DA (dichlorodihydrofluorescein diacetate) was used. DCF-DA was dissolved in DMSO (final concentration, 10μM). Cells were incubated with 100μl of 10 μM DCF-DA at 37°C for 30min, washed with serum-free F12 media before exposures to particles. Fluorescent intensities were measured using a Bioassay reader Perkin Elmer HTS 7000 with an excitation wavelength of 485nm, and an emission wavelength of 535nm. Fluorescent intensities of cells treated with ambient PM were normalized to the fluorescent intensities of cells treated with PMA (phorbol myristate acetate).

pHi Measurement. To measure pHi, the fluorescent probe BCECF-AM (2', 7'-bis (2-carboxyethyl)-5 (6)-carboxyfluorescein acetoxymethyl ester) was used. Cells were incubated with 100μl of 5 μM BCECF-AM at 37°C for 30min, washed with serum-free F12 media before

exposure to particles. Fluorescent intensities were measured as described above. Nigericin technique was used for pHi calibration.

Measurement of IL-8 mRNA. Confluent A549 cells grown on 25 mm petri dishes were exposed to ambient PM for 2 hr. Immediately at the end of the exposure, cells were lysed, and total RNA will be isolated via the RNAzol-B method. Northern blot analyses were performed to measure mRNA expression. All IL-8 mRNA levels were normalized to actin expression and expressed as fold induction over control mRNA isolated from control cells.

Data Analysis. Statistical comparisons were made using an one-way analysis of variance (ANOVA) in order to identify significant differences across group means. Principal component analysis was used to determine which groups of elements in ambient PM were associated with the cellular response.

Results

As shown in Figure 1, on an equal mass basis, fine particles produced greater cellular responses than did coarse particles for both ROI production and pHi in pulmonary cells. Particles collected

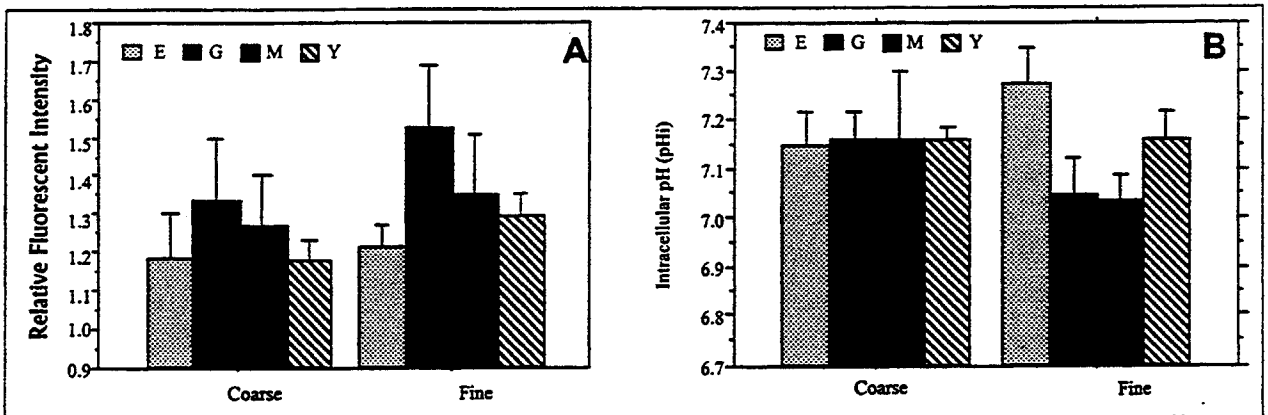

Figure 1. Effects of particles size and sampling sites on ROI production and pHi in A549 cells.

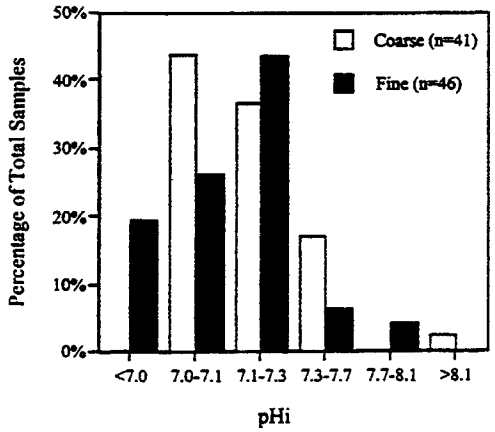

Figure 2. Distribution of pHi in pulmonary cells exposed to coarse and fine ambient paricles.

at Site E, which is not downwind from major emission sources, produced the least degree of cellular responses. In contrast, particles collected at Sites G and M, which are downwind from major metropolitan areas and major highways, produced the greatest cellular responses as compared to those collected at other sampling sites. As shown in Figure 1 B. only samples collected at sites G and M produced pHi levels below the control values (7.15). Further more, as shown in Figure 2, pHi values below 7.0 were measured only in cells that were exposed to fine particles.

Interestingly, as shown in Figure 3, ambient PM collected at nighttime at site G produced greater ROI with corresponding low pHi changes than that produced by samples of other sites. In contrast, day time PM samples produced the lowest pHi (at Site G) without corresponding changes in ROI. These results implied that local activities and geographic factors may affect cellular responses.

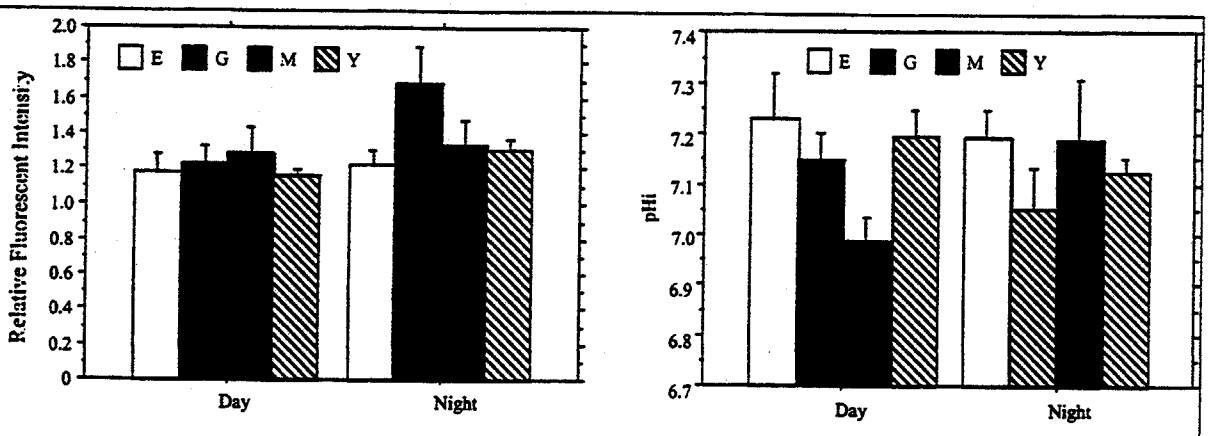

Fig.3 Effects of the sampling time on ROI production and pHi in pulmonary cells.

Table 1
Principal Component Analysis Of Coarse Mass Elemental Data (sample size = 41)

	Component 1	Component. 2	Component. 3
Na	0.035	0.089	**-0.125**
Al	**0.307**	0.046	**0.222**
Si	**0.906**	**-0.252**	-0.014
S	0.097	**0.628**	**0.718**
Cl	**0.181**	**0.715**	**-0.621**
Ca	**0.119**	**0.115**	**-0.143**
Fe	**0.132**	0.016	-0.005
Standard deviation	45.87	8.62	5.69
Proportion of Variance	94.5%	3.3%	1.5%
Cumulative Proportion	94.5%	97.8%	99.2%

Principal Component Analysis Of Fine Mass Elemental Data (sample size = 46)

	Component 1	Component 2	Component 3
Al	**-0.992**	**-0.101**	-0.044
Si	-0.023	**0.150**	0.088
S	-0.073	**0.872**	**-0.442**
Cl	-0.060	**0.414**	**0.838**
K	-0.018	**0.149**	0.002
Fe	-0.025	0.084	**0.107**
Pb	-0.074	0.021	**0.266**
Standard deviation	19.92	10.42	3.53
Proportion of Variance	74.9%	20.5%	2.4%
Cumulative Proportion	74.9%	95.3%	97.7%

To determine which groups of elements in ambient PM were associated with the cellular response the original 40 elemental concentrations in PM (as determined using XRF) were projected into orthogonal principal components. As shown in Table 1, for the fine particles, the first two orthogonal principal components accounted for more than 95% of the total variance. For the fine particles, aluminum alone contributes most of the loading for the first principal component indicating that resuspended road dusts were the major component (based on mass) of the fine particles. In addition, for the fine particles, sulfur and chloride contributed heavily to the 2nd principal component with additional contribution from aluminum, silica, and potassium. The

loading of these elements of each components were regressed against ROI and pHi. Linear regression analysis showed that the second component of the fine particles was correlated with ROI ($r^2 = 0.14$, $p = 0.01$) but not the first component ($r^2 = 0.01$, $p = 0.50$). These results showed that sulfur compounds in the fine particles produced by combustion of fossil fuels were capable of inducing ROI production in respiratory cells. Intracellular pH changes were not correlated with either components.

For the coarse particles, the first principal component alone accounted for 94% of the total variance, which was highly correlated with silica and aluminum, and also slightly correlated with iron, calcium, and chloride. Linear regression analysis showed that ROI production was correlated with this component ($r^2 = 0.33$, $p = 0.0001$). These results showed that coarse particles were also capable of inducing ROI production in the respiratory cells, perhaps due to the presence of iron in these particles. However, as described above, the magnitude of ROI productions induced by the coarse particles was much less than that induced by the fine particles. Similar to the fine particles, pHi changes were not related to the component of the coarse particles.

Another approach to delineate which element or group of elements in the ambient PM is responsible for the observed cellular responses is to group these variables (elemental concentrations and cellular responses) on the basis of their similarity using a cluster analysis technique. As shown in Figure 4, ROI is closely related to the profile of V, S, Ni, and Br; while pHi is related to Na, Cl, K, and other metals.

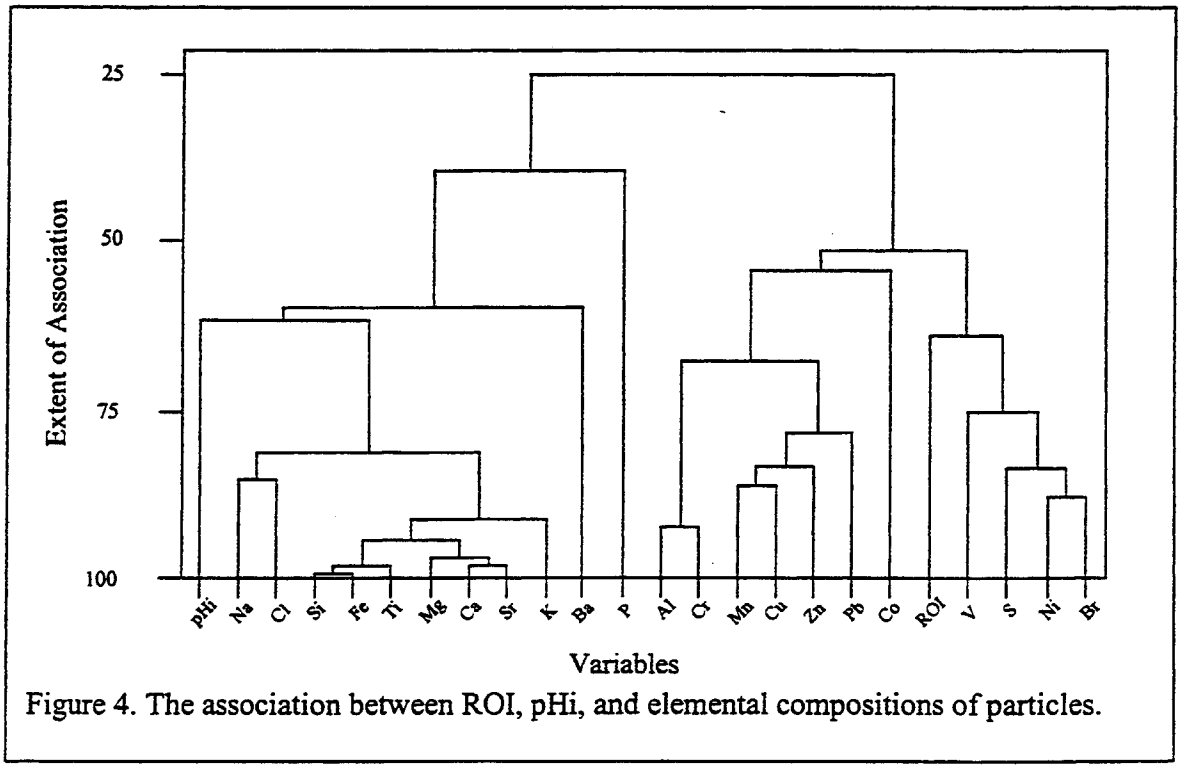

Figure 4. The association between ROI, pHi, and elemental compositions of particles.

Table 2. Multiple Regression Analysis

The regression equation is:

Log(ROI+1) = 0.285 + 0.049 Size(2) - 0.019 D/N + 0.061 Log(S+1)- 0.078 Log(Fe+1) + 0.381 Log(Cu+1) + 0.151 Log(Co+1)

Predictor	*Coef*	*StDev*	*T*	*P*	*VIF*
Constant	0.285	0.022	13.20	0.000	
Size(2)	0.049	0.016	3.09	0.003	4.2
D/N	-0.019	0.008	-2.37	0.020	1.3
Log(S+1)	0.061	0.018	3.36	0.001	2.9
Log(Fe+1)	-0.078	0.020	-3.84	0.000	3.4
Log(Cu+1)	0.381	0.118	3.23	0.002	2.0
Log(Co+1)	0.151	0.071	2.12	0.037	1.5

S = 0.03626 r^2 = 32.5% r^2 (adj.) = 27.6%

Analysis of Variance

Source	DF	SS	MS	F	P

Using a multiple regression model, the production of ROI in A549 cells was associated with size, time of the day, and elemental concentrations of sulfur, iron, copper and cobalt (Table 2). This model exhibits low collinearity and significant t and F-statistics with all predictors. The partial correlation coefficient of ROI with sulfur taking size, time of sampling, iron, copper and cobalt into account is 0.34 indicating that if time of sampling, PM size, cobalt, copper and iron are held fixed, 100% increase in sulfur will be associated with 34% increase in ROI.

Conclusions

In this study, we observed that on an equal mass basis, fine particles produced greater cellular responses than did coarse particles. Intracellular pH levels below 7.0 were measured only in cells that were exposed to fine particles. Particles collected downwind from major metropolitan areas and major highways produced the greater cellular responses as compared to those collected at other sampling sites. The results of principal component analysis showed that sulfur compounds in the fine particles produced by combustion of fossil fuels were associated with the ability of ambient PM in stimulating respiratory cells to produced ROI. Further analysis showed that several metals such as vanadium, cobalt, iron and copper were the major contributors to the cellular response induced by ambient PM. In addition, increasing sulfur concentrations in PM were associated with decreasing pHi and increasing ROI in respiratory cells. Taken together, these results showed that fine particles of ambient PM contained acids and the acidity of these particles could make transition metals in the ambient PM bioavailable to produce cellular response. However, none of the cations and anions measured using ion chromatography as well as elemental carbon were related to the cellular response measured in this study. In addition, expression of IL-8 mRNA in cells exposed to ambient PM was highly variable and was not correlated with any parameters.

Stronger Effects of Coarse Particles in Mexico City

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Abbreviations: AIC, Akaike's Information Criterion; CI, confidence interval; ICD, International Classification of Diseases; $PM_{2.5}$, particulate matter with aerodynamic diameter <2.5 $\mu g\ m^{-3}$; PM_{10}, particulate matter with aerodynamic diameter <10 $\mu g\ m^{-3}$; $PM_{10\text{-}2.5}$, difference of PM_{10} and $PM_{2.5}$; TSP, total suspended particulates.

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Abstract

Several recent epidemiologic studies suggest a stronger effect of fine particles ($PM_{2.5}$) than of coarser particulate matter. To examine the support for such a differential effect, we conducted a daily time-series analysis of mortality in relation to measurements of $PM_{2.5}$, PM_{10}, and $PM_{10\text{-}2.5}$ in southwestern Mexico City in the years 1992-1995. A generalized linear model based on Poisson regression was used to control for weather and periodic cycles. The mean concentrations of $PM_{2.5}$ and PM_{10} were 27.4 mg m^{-3} 44.6 mg m^{-3}, respectively, and the mean concentration of $PM_{10\text{-}2.5}$ was 17.2 mg m^{-3}. PM_{10} was highly correlated with both the fine and coarse fractions (correlation coefficients 0.89 and 0.84, respectively), but $PM_{2.5}$ and $PM_{10\text{-}2.5}$ were rather weakly correlated with each other (coefficient 0.52). We used the average concentration of the previous five days as the index of particle exposure because it was most strongly associated with mortality in analyses of lag structure. All three particle size fractions were associated individually with mortality: a 10 mg m^{-3} increase in PM_{10} was associated with a 1.83% increase in total mortality (95% CI -0.01-2.96), and an equal increment in $PM_{2.5}$ was associated with a 1.48% increase in deaths (95% CI 0.98-2.68%). The largest effect was observed for a 10 mg m^{-3} increment in $PM_{10\text{-}2.5}$; mean daily mortality increased 4.07% for each 10 mg m^{-3} (95% CI 2.49-5.66%). These patterns persisted after adjustment for O_3 and NO_2. To assess the independent contribution of $PM_{2.5}$ and $PM_{10\text{-}2.5}$, we added both variables simultaneously to the regression model. The effect of $PM_{10\text{-}2.5}$ was maintained at 4% per 10 mg m^{-3} (95% CI 1.96-6.02%) while the effect of $PM_{2.5}$ virtually disappeared (0.18% change). Our findings suggest that the relative effects of coarse and fine particles on mortality should be examined in more cities with a wider variety of climates, population characteristics, and air pollutants.

Epidemiologic studies around the world have shown increases in the level of ambient airborne particles associated with acute increases in mortality (Ostro, 1993; Schwartz, 1994; EPA, 1996; Thurston, 1996; Zmirou et al, 1998). Recent research (Dockery et al., 1993; Pope et al., 1995; Schwartz et al, 1996) has focused attention particularly on the role of $PM_{2.5}$ in producing the observed effects: Schwartz et al (1996) found stronger associations of mortality with fine particles than with coarse particles in five out of six selected cities in the United States. To further investigate whether there is a difference in response according to particle size, we reanalyzed a time series of air pollution and mortality data in an area of Mexico City from 1993 to 1995.

Methods

The study was conducted in an area of about 2.5 million people formed by six political jurisdictions in the southwest part of the Federal District of Mexico. We obtained electronic records of death certificates of residents of this area for 1993-1995 from the Instituto Nacional de Estadística, Geografía, e Informática.

Ambient air pollutant levels were monitored at a station in the study area, operated by Universidad Autonóma Metropolitana-Xochimilco and the Harvard School of Public Health with support from the U.S. Environmental Protection Agency Levels of thoracic particles (PM_{10}) and fine particles ($PM_{2.5}$) were recorded as 24-hour integrated particle mass and concentrations of O3, NO_2 , and SO_2 were measured hourly, using US Environmental Protection Agency reference methods.

The methods used to analyze the relationship of mortality and air pollution were the same as in an earlier study (Borja-Aburto et al., 1998).

Results

Descriptive data for air pollution and mortality are shown in Table 1. On average, 62% percent of PM_{10} was composed of $PM_{2.5}$, but the ratio of $PM_{2.5}/PM_{10}$ varied from 80% during the rainy season to 50% during the dry season. PM_{10} was highly correlated with both the fine and coarse fractions (correlation coefficients 0.89 and 0.84, respectively), but $PM_{2.5}$ and $PM_{10-2.5}$ were only moderately correlated (coefficient 0.52).

The lag structure of the relation of particle exposure and mortality was evaluated using 1-5 day exposure windows. The average concentration of the previous five days was associated most strongly with mortality and was used as the index of particle exposure in all subsequent analyses.

All three particle-size fractions were associated with mortality (Figure 1). Each 10 $\mu g\ m^{-3}$ increase in five-day mean of PM_{10} was associated with a 1.83 percent increase in total mortality, and an equal increment in $PM_{2.5}$ was associated with a 1.48 percent increase in deaths. The largest effect, a 4.07 percent in all-cause mortality, was observed for a 10 $\mu g\ m^{-3}$ increment $PM_{10-2.5}$ (Figure 1).

Adjustment for O_3 and NO_2 had only small effects on these associations (Table 2).

To assess the independent contribution of particles of different sizes to daily mortality, we considered $PM_{2.5}$ and $PM_{10-2.5}$ simultaneously in the regression model. The effect of $PM_{10-2.5}$ was maintained (3.99%, 95% CI 1.96-6.02), while the effect of $PM_{2.5}$ virtually disappeared (0.18%, 95% CI 1.72-2.08).

Discussion

Our data show a stronger association of mortality with coarse thoracic particles than with fine particles. Although previous time-series analyses have suggested that daily mortality is not strongly associated with coarse particle concentrations in general, there is evidence of associations with specific types of coarse particles. In the Six Cities study analysis of daily mortality, Schwartz and colleagues (1996) reported no consistent association with coarse particles in the pooled analysis across all communities. However, positive associations were found in Steubenville and in Knoxville, both locations dominated by large local sources of coarse combustion particles. Thus, coarse combustion particles were associated with increased daily mortality in these two cities.

On the other hand, Schwartz and colleagues (1999) reported no association between daily mortality and coarse particles during dust storms in Spokane, Washington. Thus, the evidence to date suggests that windblown crustal particles are relatively innocuous. This is not to suggest that all dust is innocuous, however. Biogenic materials in wind-blown dust can be a particular hazard. Recent research (Rosas et al., 1994; 1998) suggests that substantial quantities of both enteric bacteria and aeroallergens are associated with airborne particles in Mexico City.

Our findings suggest that the relative effects of coarse and fine particles on mortality should be examined in more cities with a wider variety of climates, population characteristics, and air pollutants. The associations we observed may be attributable to specific combustion or biogenic materials within the coarse particle mass. Understanding of these relationships will require analyses of the composition of these coarse particles.

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Table 1. Summary statistics for mortality, air pollutant concentrations, and weather indicators, Southwest Mexico City, 1993-1995. Deaths, pollutant levels, and meteorological parameters expressed as 24-hour means except as noted; observed pollutant levels given under local conditions.

	Total Mortality	$PM_{2.5}$	PM_{10}	$PM_{10-2.5}$	O_3	NO_2	Minimum temperature
		($\mu g\ m^{-3}$)	($\mu g\ m^{-3}$)	($\mu g\ m^{-3}$)	(ppb)	(ppb)	(^0C)
Valid observations	942	866	866	866	901	861	942
Mean	32	27.4	44.6	17.2	44.1	37.7	9.1
Standard deviation	6.4	10.5	16.8	8.7	15.7	11.3	3.2
Minimum	16	4	10	0?	4.1	12.8	-1.2
Lower quartile	27	20	32	10	33.7	29.2	6.7
Median	32	26	43	16	43.7	36.3	9.5
Upper quartile	51	34	56	23	54.2	43.9	12
Maximum	55	85	121	55	127.1	86.8	17.2

Table 2. Percent increase in total mortality associated with a 10 $\mu g\ m^{-3}$ increment in $PM_{2.5}$, PM_{10} and $PM_{10-2.5}$, estimated by Poisson regression controlling for temperature on the three days before death and nonparametrically-smoothed periodic cycles.

Outcome	Particle size fraction*								
	$PM_{2.5}$			PM_{10}			$PM_{10-2.5}$		
	%	95%CI	t	%	95%CI	t	%	95%CI	t
One pollutant models	1.48	-0.01, 2.96	1.95	1.83	0.98, 2.68	4.21	4.07	2.49, 5.66	5.03
Ozone adjusted†	1.28	-0.43, 2.99	1.47	1.85	0.82, 2.89	3.51	4.28	2.40, 6.15	4.47
Ozone and NO_2 adjusted†	1.25	-1.13, 3.63	1.03	2.47	1.14, 3.81	3.63	4.48	2.44, 6.51	4.32

*Exposure index for particles, mean concentration during the previous five days.
†Exposure index for ozone and NO2, 24 hr mean concentration.

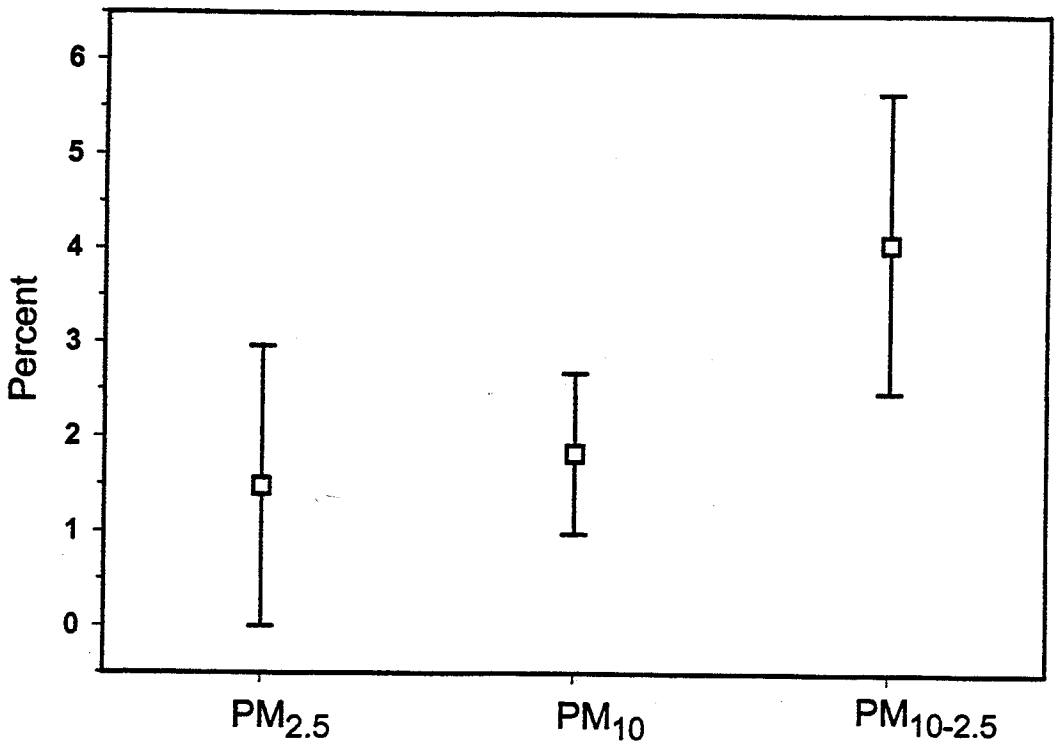

Figure 1. Percent increase in total mortality per 10 μg m^{-3} increase in mean $PM_{2.5}$, PM_{10}, and $PM_{10-2.5}$ during the previous 5 days.

A Preliminary Analysis of Air Pollution and Mortality Outcomes in Phoenix, Arizona

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Abstract

The associations between elderly mortality outcomes and PM_{10}, $PM_{2.5}$, coarse particle mass concentration, organic particulate carbon, elemental particulate carbon, and gaseous pollutants were evaluated in Phoenix, Arizona using 3 years of daily data (1995 – 1997). Located in the arid southwest, Phoenix is the 6th largest city in the US with a population of 1.2 million residents with 9.7% of the population older than 65. Particulate matter data were obtained from the EPA National Exposure Research Laboratory Platform in central Phoenix. Gaseous pollutant data were obtained from the EPA AIRS Database: carbon monoxide (CO), nitrogen dioxide (NO_2), ozone (O_3), and sulfur dioxide (SO_2). The three year average (± standard deviation) and maximum daily average concentration of $PM_{2.5}$ =12.0 ± 6.6 $\mu g/m^3$ and 32.6 $\mu g/m^3$; CO=1.5 ± 0.8 ppm and 3.7 ppm; NO_2=30 ± 10 ppb and 50 ppb; O_3=27.1 ± 10.4 ppb and 57.0 ppb, and SO_2=3.1 ± 2.2 ppb and 6.3 ppb. Correlation analyses show that $PM_{2.5}$ was correlated with the daily CO (r = 0.85) and NO_2 (r = 0.79), but less so with O_3 (r = -0.50) and SO_2 (r = 0.41). CO values were averaged over 4 monitoring sites, O_3 over 4 sites, NO_2 over 2 sites, however, only one monitoring site was used for SO_2. The average number of non-accidental deaths in Phoenix in the zip code region selected for this analysis for all residents and residents older than 65 years were 12 and 8.6 per day, respectively. Poisson regression analysis was used to evaluate the association between air pollution and total elderly, non-accidental mortality and mortality due to ischemic heart disease, respiratory disease, pneumonia, COPD and cardiovascular disease. Significant associations were found between PM_{10} and ischemic heart mortality, the RR was 1.09, (CI=1.01,1.18) with 0 lag days. The association was also significant with coarse fraction (PM_{10}-$PM_{2.5}$) with a RR of 1.09 (CI=1.01,1.17). For cardiovascular mortality, significant associations were found with PM_{10} and $PM_{2.5}$. For PM_{10} at 0 days lag, the RR was 1.05 (CI=1.01,1.09). For $PM_{2.5}$ the RR was 1.06 (CI=1.02,1.10) with 1 day lag, and the RR was1.05 (CI= 1.01, 1.09) at 4 days lag. No significant associations were found between COPD mortality and particulate matter.

Introduction

The associations between air pollution, especially particulate matter, and adverse human health effects have been well documented. However, the majority of US studies have been conducted in the eastern US, where particulate matter composition differs greatly from that on the West Coast. To date, few studies have addressed the effects of PM on mortality and morbidity in the Western US outside of California. The goal of this study was to assess the association between daily air pollution and mortality (total non-accidental and cause-specific) in Phoenix , AZ, an arid, Southwestern U.S. city. Phoenix, AZ has a population of 1.2 million residents, with an elderly population of approximately 9.7%. A unique aspect of this

study is that our pollution data includes *daily* information from 1995 to 1997 on particulate matter in various size fractions, and the general chemical components of PM. The primary sources of PM are motor vehicles, paved road dust, and biomass burning. Our mortality data includes daily mortality records for all of Maricopa County from 1995-1997.

Statistical Analysis

A Poisson regression model was used to evaluate the air pollution and health associations, controlling for day of week with indicator variables, and smooth functions for time trends, temperature and relative humidity. The degrees of freedom for the smoothing functions for temperature and relative humidity were chosen to minimize the Akaike Information Criterion (AIC). The AIC is the residual deviance plus 2 times the degrees of freedom used to fit the data. Degrees of freedom for the smooth function for time trend were based on minimizing autocorrelation as well as the AIC. Base models for each mortality outcome were constructed controlling for time trends, temperature and relative humidity. Air pollution exposure variables were evaluated by adding them individually to the base model. The assumption of a linear relationship was evaluated using a smooth function. This assumption was met if a straight line could be placed within the 95 percent confidence intervals. A p-value ≤ 0.05 associated with the pollution exposure variable was considered significant. All statistical analyses were conducted using S-PLUS 4.

Results

Daily and Annual PM Concentrations (1995-1997)

Range of Daily Values	$PM_{2.5}$ ($\mu g/m^3$) (gravimetric)	PM_{10} ($\mu g/m^3$) (TEOM)	$PM_{2.5}$ ($\mu g/m^3$) (TEOM)	coarse fraction*
1995	(4-37)	(9-129)	(1-40)	(5-104)
1996	(3-39)	(5-213)	(0-42)	(5-187)
1997	(2-35)	(7-186)	(1-34)	(5-159)
3 year mean± SD (1995-1997)	12.0±6.6	46.5±22.3	13.0±7.2	33.5±17.3

TEOM=Tapered Element Oscillating Microbalance

* Coarse fraction = TEOM PM_{10} - TEOM $PM_{2.5}$

Daily and Annual Gaseous Pollutant Concentrations (1995-1997)

Range of Daily Values	CO (ppm)	NO_2 (ppb)	O_3 (ppb)	SO_2 (ppb)
1995	(0.5-4.0)	(8-64)	(6-52)	(0-11)
1996	(0.3-4.0)	(9-59)	(8-57)	(1-17)
1997	(0.3-3.7)	(8-61)	(7-58)	(2-12)
3 year mean± SD (1995-1997)	1.5±0.8	30±10	27.1±10.4	3.1±2.2

Specific Mortality Counts for Ages ≥ 65 in Phoenix

Year	Ischemic Heart	Pneumonia	COPD	CVD	Respiratory	Total nonaccidental
1995	333	127	259	1391	726	3072
1996	320	135	232	1473	719	3201
1997	297	116	271	1318	726	3003
95-97	950	378	762	4182	2171	9276

Average Number of Daily Deaths by Disease for Ages ≥ 65

Disease	1995	1996	1997	1995-1997
pneumonia	0.34	0.38	0.63	0.35
ischemic heart	0.91	0.88	0.84	0.87
COPD	0.71	0.63	0.76	0.70
cardiovascular	3.86	3.98	3.73	3.85
respiratory	1.97	1.99	2.04	2.00
total non-accidental	8.45	8.74	8.45	8.55

Associations Between Air Pollutants and Total Non-accidental Mortality in Individuals ≥ 65

(significant results only)

Pollutants	Lag days	RR	Lower CI	Upper CI
Carbon monoxide	0	1.06	1.02	1.09
Carbon monoxide	1	1.05	1.02	1.09
Nitrogen dioxide	0	1.05	1.01	1.10
Nitrogen dioxide	1	1.07	1.02	1.12

Associations Between Air pollutants and Total Cardiovascular Mortality in Individuals ≥ 65

(significant results only)

Pollutant	Lag days	RR	Lower CI	Upper CI
Carbon monoxide	1	1.10	1.04	1.15
Carbon monoxide	2	1.07	1.02	1.12
Carbon monoxide	3	1.07	1.02	1.12
Carbon monoxide	4	1.08	1.03	1.13
Carbon monoxide	5	1.06	1.01	1.11
Nitrogen dioxide	1	1.10	1.04	1.17
Nitrogen dioxide	4	1.12	1.05	1.19
Nitrogen dioxide	5	1.08	1.02	1.15
Sulfur dioxide	4	1.07	1.02	1.12
PM_{10} (TEOM)	0	1.05	1.01	1.09
$PM_{2.5}$ (TEOM)	1	1.06	1.02	1.10
$PM_{2.5}$ (TEOM)	4	1.05	1.01	1.09
coarse fraction	0	1.05	1.01	1.09
elemental carbon	1	1.05	1.01	1.10

Associations Between Air Pollutants and Ischemic Heart Mortality in Individuals ≥ 65

(significant results only)

Pollutants	Lag days	RR	Lower CI	Upper CI
PM_{10} (TEOM)	0	1.09	1.01	1.18
coarse fraction	0	1.09	1.01	1.17
SO_2	2	1.12	1.02	1.23

Associations Between Air Pollutants and Respiratory Mortality in Individuals ≥ 65

(significant results only)

Pollutants	Lag days	RR	Lower CI	Upper CI
CO	0	1.11	1.04	1.19
NO_2	0	1.12	1.03	1.23

Associations Between Air Pollutants and Pneumonia Mortality in Individuals ≥ 65

(significant results only)

Pollutant	Lag days	RR	Lower CI	Upper CI
CO	1	1.21	1.03	1.41

Conclusions

The preliminary analysis of mortality and air pollution in Phoenix resulted in several significant associations of interest. A unique aspect of this analysis was the use of a comprehensive air monitoring platform in Phoenix, which included information on the chemical composition and size distribution of particulate matter. This allowed us to test for associations between cause specific mortality and chemical /physical specific metrics of particulate matter. In spite of a data set restricted to only zip code regions represented by the PM_{10} monitor, we found significant associations between mortality outcomes and air pollution. With a single pollutant model, the strongest associations were found between mortality from ischemic heart disease and PM_{10}. Cardiovascular disease was found to be strongly associated with PM_{10}, $PM_{2.5}$ and coarse fraction. Total non-accidental mortality, respiratory disease and pneumonia were found to be associated with only the gaseous pollutants (CO and/or NO_2). No associations were observed between ozone and mortality outcomes. Future analysis include multi-pollutant exposure metrics.

Particulate Matter (PM) In Minutes To Hours, Involving Immunological And Electrophysiological Mechanisms, Can Account For Epidemiological Associations Of Daily Morbidity And Mortality With 24-Hour-Average PM In Air

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Abstract.

Daily airborne PM-10 concentrations within the 150-μg/M3 NAAQS are associated with morbidity and mortality. The U. S. EPA has changed the size and concentration, but not the 24-hour averaging time, of PM addressed by the standard. We report on emerging evidence of shorter-term mechanisms of PM toxicity, justifying consideration of a shorter regulatory averaging time, such as one hour, in addition to 24-hour and annual averaging times in the NAAQS.

PM instilled intratracheally or inhaled caused morbidity in animals within minutes, including apnea and electrophysiological effects in dogs. PM killed rats within one hour to a few hours via electrophysiological mechanisms. In clinical settings, PM effects have occurred in asthmatics during brief exercise or, in one study, rest. Allergenic bioaerosols (fragments of mesquite pollen and dried alfalfa) incapacitated 300 people, many within minutes following exposure outdoors. Cockroach allergen in airborne PM indoors was identified as a major cause of asthma among urban children in the U. S. Daily asthma symptoms were most strongly associated with the maximum one-hour average airborne PM concentration during the day, whereas the association lost strength with dilution into eight-hour and then 24-hour averaging times. In Australia, daily mortality was associated with daily maximum one-hour, but not 24-hour, PM concentrations.

Documented PM excursions lted mortality and morbidity in communities (for example, see 15; 51, 52). Particle levels during the 1952 London fog; during which some 4,000 people died over a period of 10 days; have been estimated at 1,200 μg/M3 (54; PM-10 ² 1,200 μg/M3). Recent studies associate significant elevations of morbidity and mortality with 24-hour particle levels well within PM-10 = 150 μg/M3 [reviewed by EPA 1995 (59)], and below levels which have been explainable by known toxicological mechanisms. In addition to the absence of plausible mechanisms, epidemiologists have failed to observe a threshold for PM morbidity and mortality, yet all PM effects associated with 24-hour PM are non-cancer effects, which typically act via threshold mechanisms.

Notwithstanding the apparent absence of effect thresholds, and notwithstanding the absence of a plausible mechanistic understanding, the U. S. EPA based its decision to increase the stringency of the 24-hour PM NAAQS upon the premise that reported epidemiological associations of 24-hour PM with mortality and morbidity are causal (U. S. EPA 1995; 59). However, an alternative explanation is that excursions to high PM levels known to exert toxic effects might be causal (40-44). Such PM excursions may be of short duration, such that they exert little if any perceptible influence upon 24-hour average PM levels. Hence, no 24-hour threshold would be expected to be observable if excursions are causal.

Reports of the mechanisms by which short-term toxic actions exerted by excursions of airborne PM and PM constituents are emerging. The mechanisms are diverse, but have in common a short time frame of toxic action, from minutes to hours. In view of documented PM excursions also lasting minutes to hours, this study inquires whether such short-term mechanisms might contribute to explaining daily morbidity and mortality.

Methods

The methods consisted of evaluating available analytical data and literature.

Findings

Airborne Particle Excursions. Data sets were screened to identify those which 1. were obtained using the identified technologies, 2. exhibited short time resolution, 3. encompassed at least one 24-hour period, and 4. revealed 24-hour airborne particle levels well within the current NAAQS. Screening identified three locations, and 10 days, during which 24-hour average PM-10 < 150 mg/M3. At all three locations, and in six of the 10 identified cases meeting the screening criteria, excursions exceeding 150 μg/M3 were noted (39, 42-44).

Toxicological Significance of Excursions. Adverse PM Excursion Effects In Clinical Studies. Numerous studies of short-term effects exerted by PM during controlled acute human exposures were identified (4-10, 12, 16-19, 24, 25, 29-34, 36, 46, 60, 61, 64). Many of these reported positive results (7, 8, 10, 16-18, 24, 25, 29, 30, 36, 46, 60, 61, 64). Adverse particle-associated health effects have been demonstrated beginning at levels as low as 202 μg/M3 (64) and at exposure durations as short as 30 minutes (61, 64). These effects were documented in asthmatics at rest (64) or engaged in exercise (61). Participant ages in these studies varied from 19 to 50 (61) and 23 to 48 (64). Thus, no frail elderly, elderly, or infirm subjects were included in the studies. Other positive studies likewise involved asthmatic subjects exercising or at rest, of ages up to 57 years (10, 18, 24, 46). For ethical reasons, adverse effects, when observed, could not be allowed to progress to clinical significance. However, reported effects included increased airway resistance; reduced values of lung function parameters such as forced expiratory volume at one and three minutes (FEV1, FEV3), forced vital capacity (FVC), and peak flow; as well as such symptoms as throat irritation and coughing.

Adverse PM Excursion Effects In Epidemiological Studies. Epidemiological evidence of PM effects following exposure to PM within minutes to hours is emerging. In an early study in England, Lawther, et al. (1970; 35) employed a diary method, in which bronchitis patients recorded daily changes in the severity of their symptoms relative to their recollection of previous-day symptom severity. The diary method required participants to make an entry pertaining to a full day of symptom experience. Lawther, et al. reported a clear association of symptom severity with daily concentrations of smoke or sulfuric acid in London. They concluded, however, that this association could not be attributed to the measured 24-hour average concentrations, but more likely to The effects of brief exposures to the maximum concentrations occurring during the day (page 538), which may be several times the 24-hour average (63) which served merely as a surrogate parameter for such excursions. The basis for the Lawther, et al. conclusion included their (uncited) experimental work on normal subjects.

In Australia; Simpson, et al. (1997; 55) reported that daily mortality in Brisbane was associated with daily maximum one-hour, but not 24-hour, average PM concentrations. Table 1 indicates that PM was associated with mortality from cardiovascular but not respiratory conditions. In Sydney, Morgan, et al. (1998; 45) reported that admissions of people over age 65 into hospitals for treatment of chronic obstructive pulmonary disease (COPD) was associated more strongly with daily maximum one-hour PM concentrations (3.01-percent increase) than with 24-hour concentrations (2.41-percent increase). The rate at which people over age 65 were admitted to hospitals for treatment of heart conditions was associated with both maximum one-hour and 24-hour average PM concentrations. This latter association was reported to be of similar magnitude (2.7 to 2.8 percent increase in admissions), notwithstanding the longer duration of the 24-hour average exposure.

In the U. S., asthma among inner-city children was found in large part to be attributable to airborne cockroach allergen (53). Delfino, et al. (13, 14) found that the strength of the association of asthma symptom severity with PM depended upon the PM averaging time. The association increased in strength with increasing exposure duration, as would be expected. However, the direction of this trend was reversed when equivalent exposure metrics were used, specifically, inhaler use per unit of exposure time (per hour) rather than per total exposure time (which had varied between one and 24 hours). Measured via an equivalent exposure metric, the strength of the association increased as PM averaging time decreased from 24 hours to eight hours to one-hour (42).

A short time frame for human response to PM also was evident when 300 people were overcome shortly after wind lofted fine particles into the air during four-day outdoor music festival attended by an estimated 40,000 people in Queen Creek, Arizona in April 1997 (42). Arizona has a high concentration of residents with respiratory conditions. Examination of data reported by emergency medical personnel at an on-site treatment station revealed the approximate time course of visitations to the station by concert attendees experiencing respiratory distress. A significant elevation, indeed a peak, occurred in the rate of visitation for emergency medical treatment. A similar increase occurred in the rate at which individuals seeking medical attention were transported to area hospitals. Indeed, the rate increased so sharply that ambulances quickly saturated the local hospital, necessitating airlifting of patients to more distant health care facilities.

Adverse PM Excursion Effects In Bioassays. Two bioassays were conducted in which aerosols were delivered constantly at the average concentration or with excursions included. In both bioassays, rats were exposed for four hours to either of two aerosols using published methods (28). The rates at which the aerosols were delivered included one excursion or four excursions (28, 42-44). An excursion was defined as an elevation of at least 50 percent above an otherwise constant steady state aerosol concentration, lasting for at least five minutes. Groups of six rats were exposed to either of two sulfate aerosols, designated AMS and SAM. Rat lungs were examined microscopically 48 hours after exposure. The fraction of microscopically examined lung area exhibiting aerosol-induced lesions was evaluated in groups of rats exposed to excursions vs. no-excursion controls exposed to the same aerosol. With respect to both aerosols, rats exposed to excursions exhibited greater lesion area. However, in the case of AMS, the

increment over control rats was greater, absolutely and proportionately, reflecting the effect of four excursions, compared with only one excursion in the case of rats exposed to SAM aerosol.

Mechanisms of Action Educed from Animal Bioassays and In Vitro Studies. PM of Combustion Origin. Investigations into airborne PM-induced effects on rats and dogs have been conducted at Harvard University's School of Public Health (20, 21-23, 27, 47, 48, 50). The findings implicate short-term, electrophysiological and inflammatory (immunological) mechanisms. In one study; Pierce, et al. (1996; 50) reported pulmonary inflammation and release of chemokines and cytokines within one to four hours following intratracheal instillation of rats with dissolved or suspended vanadium compounds (Fig. 1). Carter, et al. (1997; 11) reported release of inflammatory cytokines within two hours of exposure of cultured normal human bronchial epithelial cells to residual oil fly ash (ROFA). Use of chelators revealed dependence of this response on metals present in ROFA, most notably vanadium (Fig. 2).

Fine-particle-induced cardiopulmonary malfunctioning also has been revealed via electrocardiograms (EKGs; 42). Nearing, et al. (1996b; 48) induced ST segment elevation and periods of apnea in dogs inhaling fly ash at 1,000 µg/M3 for 3 d, 4 h/d. Nearing, et al. (1996a; 47) also demonstrated increased amplitude of T-wave alternans, an electrophysiological indicator of vulnerability to ventricular fibrillation, in dogs inhaling fly ash at 3,000 µg/M3 for three hours per day over three days. ST segment elevation and T-wave alternans effects began soon after exposure and became most prominent by day three. Apnea for 20 to 30 seconds occurred during hour four on day three. As a frequent precursor to potentially fatal heart attacks, T-wave alternans represents a clinically serious cardiological predictor of PM lethality.

PM lethality also has been demonstrated in animal models. Some studies have involved rats exhibiting either monocrotaline-induced pulmonary inflammation or SO2-induced chronic bronchitis (20, 21-23, 27). Rats with such respiratory pathology then inhaled particles concentrated from Boston air at 228 to 288 µg/M3 for six hours per day over three days. Breathing rates declined from 17.1/min. in controls to 9/min. in exposed rats. Apnea for up to 22 seconds occurred. Bronchoconstriction occurred in 80 percent of rats exhibiting respiratory disease, but in only 25 percent of controls. Rats exhibited no signs of distress, but deaths occurred on all exposure days, possibly as a result of particle-induced arrhythmias or other EKG-related cardiopulmonary malfunction. Mortality also appears to have been consistent with systemic effects following particle induction of proinflammatory mediators, such as the cytokines interleukin-1 and tumor necrosis factor-alpha (TNFa; 20, 21-23, 27). Godleski also reported on directional PM collection by the PM concentrator: electrophysiological parameters varied in real time with the source direction of PM delivered to the test animals. Madl showed that PM accumulation in the lungs of rats treated with monocrotaline may be attributable to inhibition of alveolar macrophage (AM) chemotaxis and impairment of PM clearance rather than to alteration in PM fate upon entering the lungs (Madl 1998; 37).

PM of Botanical Origin. Veronesi, et al. (1999; 62) reported that irritant receptors sensitive to the prototype botanical irritant capsaicin were stimulated by exposure to ROFA. ROFA induced human bronchial epithelial cells cultured in vitro to release proinflammatory cytokines within minutes to hours following onset of exposure. Cytokine release was determined to be initiated by acidic, soluble components of ROFA. The mechanism by which bronchial epithelial cells

were induced to release cytokines involved activation of pH-sensitive, capsaicin-sensitive irritant receptors. Involvement of capsaicin-sensitive irritant receptors in the inflammatory response suggested that PM of botanical origin similarly might induce inflammation.

Johnston, et al. (1998; 26) reported evidence that the pulmonary inflammatory process in mice was evident within two hours following inhalation of lipopolysaccharide for 10 minutes. The inflammatory process was controlled by release of cytokines and chemokines by polymorphonuclear leukocytes (PMNs). Nessa, et al. (1997; 49) reported that human alveolar macrophage (AM) cells incubated for 30 minutes with Aspergillus fumigatus (fungus) conidia or with silica ingested the conidia more rapidly than the silica (Fig. 3). As Fig. 3 indicates, AM cells released metabolic oxygen radicals more rapidly when incubated with conidia than with silica. The authors concluded that increased production of reactive oxygen metabolites may represent a short-term defense mechanism against the conidia, notwithstanding possible lung tissue damage resulting from long-term exposure to Aspergillus conidia.

Regulatory Significance. Findings reported in bioassays, controlled clinical studies, and epidemiology studies in which PM exposure is uncontrolled must be evaluated relative to U S EPA policies and procedures for air pollutant standard setting (3, 58) based upon the weight of evidence for the existence, nature, and magnitude of health risks. The Agency requested the American Thoracic Society (ATS) to define toxicological effects, and degrees of effect, which might qualify as forming the basis for limiting air pollutants, and both ATS (3) and the EPA (58) have published these guidelines. According to U. S. EPA policy (1994; 58), air pollution limits must be set based upon a clinically significant adverse effect, not any effect. Documented PM effects clearly are within EPA's definition. Qualifying effects include mortality and morbidity in animals, and morbidity in humans; including symptoms of asthma exacerbation such as bronchoconstriction, increased airway resistance, reduced FEV1 and FEV3, reduced FVC, and reduced peak flow.

Conclusions

Excursions of airborne particle mass may elevate PM-10 by an order of magnitude relative to 24-hour average levels within the 150-μg/M3 NAAQS. PM levels comparable to those reached during documented excursions can exert toxicological effects upon humans and animals, including mortality in animals. PM of combustion and of biological origin may elicit responses of the types observed. Effects appear to begin as expected in the lungs, with initiation of immunological responses including pulmonary inflammation, influx of alveolar macrophage cells, release of inflammatory cytokines, and production of reactive oxygen species. Subsequent electrophysiological effects appear to follow, including EKG abnormalities such as ST-segment elevation and T-wave alternans. This sequence of pulmonary effects followed by cardiological effects is plausible and intuitively satisfying, inasmuch as the pulmonary veins represent an express trains to the heart. Specifically, the pulmonary veins quickly carry high volumes of newly oxygenated blood, along with inflammatory metabolites secreted by cells such as alveolar macrophages, from the lungs to the heart.

PM excursions might constitute a previously unrecognized cause of mortality and morbidity revealed by epidemiology studies based upon 24-hour average PM levels. Toxicological effects

reported in association with PM in the range of observed excursions are of types determined to be appropriate for air pollutant standard setting under the Clean Air Act, according to U. S. EPA criteria. Thus, EPA's decision to change the PM standard may be justified by residual health risks associated with the old standard. However, the time required for PM to cause adverse effects may be briefer than the time interval over which the effects commonly have been observed (Michael's 1997a). A corollary is that threshold effects exerted by PM may be caused by brief exposure to PM excursions exceeding effect thresholds. Such thresholds may be imperceptible, however, not because several non-cancer effects lack thresholds if they are caused by PM, but because the thresholds may be obscured by dilution within 24-hour PM averages routinely reported.

Thus, EPA's preference for managing 24-hour average PM concentrations, reflecting availability of 24-hour average PM data, is technically unjustified (41). Additional study of the epidemiology of PM excursions is needed. However, data already available and presented herein suggest that greater reduction of residual risks posed by PM within the old NAAQS may be achievable via control of one-hour PM averages than by control of 24-hour averages.

In many or most industries, controlling PM to a one-hour mass limit of, say, 300 μg/M3 can be achieved more economically than controlling 24-hour averages. Industrial processes often include brief periods of reduced PM control, when excursions occur. For example, plastics factories may produce several plastics using the same (extrusion) equipment operated with resins at different temperatures. Shifting to a new plastic type requires purging the previous resin, changing the temperature, and running the equipment with the new resin. PM emissions during these transitional phases of operation can be controlled more economically because they require improvements to only a small part of overall operations. For example, plastics factories can run resins requiring higher temperatures only after purging lower-temperature resins which may have been run earlier. To switch to lower-temperature resins, operators can assure adequate extruder temperature reduction prior to introducing the lower-temperature resins, thereby averting resin decomposition and potentially health-significant PM excursions.

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Air Pollution, Pollens, and Admissions for Chronic Respiratory Disease in King County

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Abstract

We analyzed the association between various indices of air pollution, pollens, and hospital admissions for chronic respiratory disease in King County over the period 1987-1995. Both air pollution and tree pollens were independently associated with hospital admissions. In single pollutant models, among the gases, we found the strongest association between carbon monoxide and hospitalization. The association with sulfur dioxide was weaker, and there was no evidence of an association with ozone. We also found association of hospital admissions with PM_{10}, and a suggestion of an association with an index of light scattering measured by nephelometry. In two-pollutant models, the effect of carbon monoxide remained stable, whereas the effect of particulate matter, measured either as PM_{10} or by nephelometry, was attenuated and became unstable. We examined also the association between air pollution, pollens, and hospital admissions in three broad age groups, 0 – 19 years, 20 – 64 years, and ages 65 and above. Whereas tree pollens were associated with hospital admissions in each of these age groups, the association between air pollution and hospital admissions was seen only in the youngest age group.

Introduction

A number of papers using regression methods for analyses of time-series have recently reported associations between hospital admissions for respiratory diseases and particulate air pollution and ozone (Bates, 1983; Schwartz, 1994a; Schwartz, 1994b; Burnett, 1994). These associations persisted even after adjustment for weather. Most of these papers have restricted attention to admissions for individuals over the age of 65 because data for these admissions are readily available from the Health Care Financing Administration (HCFA).

In this study, we undertook analyses of air pollution and daily hospital admissions for chronic pulmonary disease in the Seattle metropolitan area during the nine-year period 1987 – 1995. Because the State of Washington maintains detailed admissions records for each hospital in the state, we were able to obtain daily counts of admissions for chronic respiratory disease for all

hospitals located in King County. In particular, unlike previous studies, we did not have to restrict our analyses to the elderly. Additionally, we had air quality monitoring information on three particulate matter (PM) measures: PM_{10}, $PM_{2.5}$, and light scattering by nephelometry, which can be converted into a measure of either $PM_{2.5}$ or of PM_1. Finally, we obtained information on daily pollen counts in the Seattle metropolitan area so that we could also investigate the association between daily fluctuations in pollen counts and hospital admissions for chronic respiratory disease.

Methods

The information in CHARS includes the date of admission, and a primary and up to eight secondary discharge diagnoses, coded according to the *International Classification of Diseases*, 9th revision (ICD-9). We obtained information on the daily number of hospital admissions with a primary diagnosis of chronic respiratory disease (ICD-9, 490-496, includes chronic obstructive pulmonary disease and asthma) in all King County hospitals during the period January 1, 1987 to December 31, 1995, from CHARS.

Monitoring of PM_{10} has been done at three sites in King County since 1989. $PM_{2.5}$ has been routinely monitored at one (Duwamish) of these three sites since late 1986 and at another since early 1989. Daily nephelometry readings are also available for the entire study period at two of the three sites and since mid-1989 at the third. There are missing observations on a number of days (table 2), with nephelometry data being the most complete. For PM_{10} and nephelometry, we used data only for those days on which daily readings were available at all three sites. On these days we used the daily averages of the readings from all three stations. Thus, effectively, all analyses that include either PM_{10} or nephelometry measures are for the time-period mid-1989 to end 1995. This procedure led to too many missing data for $PM_{2.5}$, however. We therefore used the data from Duwamish for $PM_{2.5}$. Because the conversion of nephelometry readings into direct measures of $PM_{2.5}$ or PM_1 can be site-specific, we chose to analyze the data and report the results directly in terms of nephelometry units without conversion into one of the other PM measures. Carbon monoxide was monitored at four sites over the period of the study. We used the daily averages over all available sites for our analyses. Sulfur dioxide was measured at a single site (Duwamish) during the period of the study. Ozone was monitored at one site east of Seattle between April and October. For these two gases we used daily averages in our analyses. No monitoring information on the oxides of nitrogen was available.
We obtained information on daily pollen and mold spore concentrations from investigators at the Virginia Mason Hospital in Seattle. Pollen and mold spore concentrations were measured in units of grains/m^3, averaged over a 24 hour period at a Federal Way location in South King County by a rotating rod sampling technique. Counts were made on most weekdays from late-February through mid-to-late September over the period of this study. The pollen grains were classified as grass, weed or tree. Further partial classification was made by percentage of the principal component of tree pollen into cedar, birch, alder, maple, fir, hemlock, and other categories. We did not consider this sub-classification in our analyses.

We obtained information on daily temperature and dew point from the weather station at the Seattle-Tacoma airport.

Tables 1 and 2 show the distributions of hospital admissions, air pollution, tree pollen and temperature in King County over the period of this study.

Methods of Analysis

We used a series of semiparametric Poisson regression models (generalized additive models, GAM) for analyses of the data (Hastie & Tibshirani, 1990). All models included an intercept term, indicator variables for day of week, nonparametric smoothers for temperature and day of study, the latter to adjust for temporal trends and variations in hospital admissions not attributable to pollution or weather. We then added the pollutants and the pollens to the models. In most of our models we adjusted temporal trends by a 30 degree of freedom (between 3 and 4 degrees of freedom for every year in the period of the study) and investigated the impact on our conclusions of greater or less smoothing.

We first investigated the lag structure for temperature. We found that the association of respiratory admissions with temperature was strongest with a lag of 1 day. Subsequently we adjusted for temperature by using a 6 degree of freedom smoothing spline for the effect of temperature lagged by 1 day. We found that dew point was not associated with hospital admissions once temperature was accounted for. We did not consider dew point further. For models with pollutants or pollens, we first undertook a limited exploration of the effect of lag between exposure and hospital admissions. Specifically, we examined lags of 0, 1, 2, 3 and 4 days for each of the pollutants and pollens and picked the lag that yielded the strongest association with chronic respiratory admissions. We then considered models with multiple pollutants and pollens using the lag structure that maximized the effect of each pollutant or pollen.

We fit all models using the statistical software package S-PLUS. After the models were fit, the residuals were examined for autocorrelations.

Results

Tables 1 and 2 show the summary statistics for daily admissions for respiratory disease and for the pollutants. Table 3 shows the correlations among the pollutants. Note, in particular, the high correlations among the various particulate matter metrics and between particulate matter and CO. Tree pollens were only modestly correlated with the pollutants. Among the gases, the highest correlation was with CO (correlation coefficient = 0.23). The correlations with the various PM metrics were about 0.3.

There was little evidence of overdispersion after the models were fit to the data. Typically, the overdispersion parameter was approximately 1.1. Our confidence intervals were not corrected for overdispersion. Such a correction would have increased the length of the confidence intervals by about 5 – 10 %. There was also little evidence of autocorrelations of the residuals of the fitted models.

Figure 1 shows the association of the various components of particulate matter with admissions for chronic respiratory disease in single pollutant models. Note that PM_{10} shows the most

consistent effect, with coefficients for all lags positive. Despite the high correlation between PM_{10} and $PM_{2.5}$ and nephelometry, the latter two show no consistent association with hospital admissions for chronic respiratory disease. We note, however, that, because of missing data on different days for the different PM metrics, the coefficient estimates were not derived from the same data sets. This problem is endemic to most epidemiological studies of air pollution.
Figure 2 shows the associations between the gaseous pollutants and chronic respiratory admissions. The strongest association was found with CO, with the estimated coefficients positive for all lags. Recall that our definition of chronic respiratory disease includes asthma. A recent paper (Sheppard et al., 1999) has reported associations between CO and admissions for asthma among the non-elderly in the Seattle metropolitan area. We found little evidence of any consistent association with ozone. Sulfur dioxide appears to show a modest association with hospital admissions for chronic respiratory disease.

Figure 3 shows the results of analyses with two pollutants, a component of PM and a gas, considered simultaneously. In this figure, the lag used for specific pollutants is the one that exhibited the strongest association in the single pollutant models.

The distribution of tree pollens is highly skewed with 45% of days reporting no measurable tree pollen. The median was 5 grains/m^3, and the maximum count over the period of observation was over 6800 grains/m^3. We found strong associations between tree pollen and hospital admissions for chronic respiratory disease with the estimated coefficients positive for all lags. This association is shown in figure 4. Because of the skewed distribution of pollen counts, we repeated the analyses with the top 10% of days excluded (days with pollen counts above 300 grains/m^3 excluded). We also restricted our analyses to days on which the tree pollen counts were between 10 and 300 grains/m^3. The positive association between tree pollens and hospital admissions persisted in these analyses.

Figure 4 also shows the results of analyses with both tree pollen and a pollutant considered simultaneously. We note that the analyses for figure 4 were restricted to the period March 1 – August 31 because measurements of pollen were available only during this six month period each year. In figure 4, all effects are shown for this restricted data set. The results of joint analyses suggest that the effect of tree pollens is largely independent of the effect of air pollution.

We examined also the association between air pollution and hospital admissions for chronic respiratory disease in three broad age ranges: 0-19 years, 20-64 years, and ages 65 and over. In the oldest individuals (65 and over), the hospital admissions for chronic respiratory disease are dominated by chronic bronchitis and emphysema. We saw no consistent associations between any of the air pollutants and hospital admissions in this group. Tree pollens were associated with admissions in this group with the strongest association seen with a lag of three days. Similarly, in the middle age group (ages 20-64), we saw no association with air pollution, but tree pollens were associated with admissions, particularly with lags of three and four days. The admissions in the youngest age group (ages 0-19) are dominated by asthma. In this group we saw strong associations between components of air pollution and admissions. Among the gases, CO was strongly, and SO_2 weakly, associated with admissions, with the coefficients for these two gases positive for all lags up to 4 days. The strongest CO association was seen with a 2 day lag (log RR

for a 1 ppm change = 0.078; 95% CI = 0.047 - 0.110). For SO_2 the strongest association was seen with a lag of 1 day (log RR for a 10 ppb change = 0.05; 95% CI = -0.01 - 0.11). Both PM_{10} and light scattering were associated with admissions with the PM_{10} association being the stronger of the two. For both PM_{10} and light scattering the strongest association was seen with a lag of 1 day (log RR for a $10\mu g/m^3$ change in PM_{10} = 0.03; 95% CI = 0.01 - 0.05). The association of $PM_{2.5}$ with admissions was inconsistent, with both positive and negative coefficients depending on the lag. When CO and PM_{10} or light scattering were considered in two pollutant models, the coefficient for CO remained stable, whereas that for the PM measure became unstable and statistically insignificant.

Discussion

Epidemiological studies of the association between air pollution and hospital admissions for respiratory disease are summarized in a review (Dockery & Pope, 1994). Most studies have reported positive associations between levels of particulate matter and hospital admissions with various lag times. Similar associations have been reported for ozone. The interpretation of many of these studies is limited, however, by the fact that generally other pollutants were not considered. In a previous study in Minneapolis-St. Paul and Birmingham, we reported inconsistent results (Moolgavkar et al., 1997). In Minneapolis, we found a strong and robust association of ozone with respiratory admissions. Although PM_{10} was associated with respiratory admissions in that city, this association was sensitive to other pollutants in the model and to the degree of smoothing of temporal trends. In Birmingham, we found no consistent associations between air pollution and respiratory admissions.

As reported by other investigators we found associations between various indices of air pollution and hospital admissions for chronic respiratory disease in King County. We also found associations between tree pollens and hospital admissions, but these associations appear to be largely independent of the effects of air pollution on admissions. A few published studies have considered the effects airborne allergens, including pollens, on hospital admissions and emergency room visits for respiratory causes. The results of these analyses have been mixed. Anderson et al. (1998) investigated daily admissions for asthma in London in the period 1987-1992. They reported associations with various components of air pollution, particularly ozone, but concluded that there was no confounding of air pollution effects by pollens, although there was some indication of a synergistic effect between SO_2 and grass pollens. In a study of emergency room visits for asthma in Israel, Garty et al. (1998) reported associations with NOx, SO_2, and high barometric pressure, but none with particulates or with airborne pollens or spores. In a study in Mexico City, Rosas et al. (1998) reported a significant association between aeroallergens and emergency admissions for asthma. They suggested that aeroallergens may be more strongly associated with admissions than pollutants and could be confounders in studies of air pollution. Neas et al. (1996) reported that particulate air pollution and aeroallergens appear to have independent effects on respiratory symptoms and peak expiratory flow rates in children.

In contrast to previous studies, we found only weak evidence of an association between ozone and hospital admissions for chronic respiratory disease. When we restricted analyses to the months April - October, which was the period during which data on ozone were available, there was little evidence of an association (figure 2). When we further restricted the analyses to the

months April – August in order to consider the tree pollens, however, there was some indication of an association with ozone: the coefficients for ozone with lags of from zero to three days were positive, with the strongest association seen with a lag of three days. This association was somewhat attenuated in a simultaneous analysis with tree pollens (see last panel of figure 4). Thus, the results for ozone were quite sensitive to the exact data set chosen for analyses. Among the gases, only CO was strongly and consistently associated with hospital admissions for chronic respiratory disease. Until recently, carbon monoxide has generally received short shrift in time series analyses of hospital admissions and mortality. Recent papers (Sheppard et al., 1999; Burnett et al., 1997a; Burnett et al., 1997b; Burnett et al., 1998) have reported associations between CO and hospital admissions for cardiovascular disease and between CO and daily mortality. Burnett et al. (1998) investigated daily mortality in Toronto and reported strong associations between CO and non-accidental deaths. They found, moreover, that CO was associated with both cardiac and non-cardiac causes of death. The association persisted in models incorporating multiple pollutants.

This paper adds to the growing body of literature on the association between CO and adverse health outcomes, both cardiac and respiratory. It is possible that, in these studies, CO is acting as a surrogate measure of mobile source pollution. A direct role of CO in cardiac and respiratory disease cannot be ruled out, however. Carbon monoxide decreases the ability of hemoglobin to carry oxygen to the peripheral tissues, and it is plausible that it could trigger a cardiovascular event in a compromised individual. There are plausible mechanisms by which CO could also affect respiratory admissions or deaths. Individuals with a severely compromised respiratory system could be pushed over the edge by the tissue anoxia resulting from exposure to CO. Second, recent laboratory research shows that CO may have physiological effects other than the formation of carboxyhemoglobin. For example, exposure to CO in concentrations up to 100 parts per million, which are in the range of concentrations for CO in urban environments, can trigger oxidative stress by the release of nitric oxide (NO) from platelets (Thom & Ischiropoulos, 1997). How such oxidative stress could trigger respiratory admissions or deaths is an open question; the finding indicates, however, that tissue anoxia is not the only mechanism by which the toxicological effects of CO could be mediated. The role of CO in the NO-cGMP (cyclic guanosine monophosphate) signaling pathway and its relevance to adverse health effects from air pollution is nicely summarized in a recent paper by Burnett et al. (1998).

Our analyses indicate that the association between air pollution and hospital admissions for chronic respiratory disease in the Seattle metropolitan area is largely attributable to admissions in the youngest age group. In a recent paper, Sheppard et al. (1999) examined hospital admissions for asthma in Seattle among individuals below the age of 65 and reported that PM and CO were jointly associated with these admissions. They reported an association with O_3 and no association with SO_2, whereas we found weak association with O_3 only in the summer months, and a modest association with SO_2. There could be a number of reasons for these discrepant findings. First, the two studies cover overlapping, but slightly different, time frames. Sheppard et al. examined admissions over the period 1987-1994, whereas we had one more year of data. Second, Sheppard et al. examined only asthma admissions (ICD – 9 code 493), which dominate chronic respiratory admissions in the younger ages; however, there is probably a significant number of chronic bronchitis and emphysema cases between the ages of 40 and 65, which would have been included in our analyses but excluded in theirs. There were other differences, as well, in the

analyses. For example, while both sets of analyses included only those cases with a primary diagnosis of the ICD code of interest, Sheppard et al. used a number of exclusion criteria, such as nonresidents, planned admits, and readmits within 14 days. Finally, and perhaps most importantly, we followed the usual procedure of eliminating days with missing pollution information in our analyses. Sheppard et al. filled in the missing information using a multiple imputation procedure. Both these procedures involve implicit assumptions. Our procedure of excluding the days with missing pollution information assumes that the data are missing completely at random.

With the general availability of powerful software packages, such as S-Plus, it is relatively straightforward to perform analyses that would have been unthinkable just a few years ago. The real challenge is in the interpretation of results. The results of most analyses are consistent in that some index (or indices) of air pollution is associated with hospital admissions for cardiac or respiratory causes and with non-accidental mortality. This association persists even after careful control of weather and temporal trends. Despite the preponderance of evidence that air pollution is associated with various respiratory end points, there are some puzzling exceptions. For example, a recent large study (Roemer, 1998) of children with respiratory symptoms in Europe found no association between indices of air pollution and exacerbation of symptoms or bronchodilator use. Carbon monoxide was not considered in this study. The more recent studies of air pollution also do not identify a single component as being the predominant predictor of health outcomes. Thus, for example, a recent large study in European cities (Zmirou et al., 1998) found that, in single pollutant models, SO_2, in addition to PM, was an important predictor of mortality. Moreover, although the coefficients of two-pollutant models were not reported in the paper, the authors state "The effect of particles when SO_2 was in the same model, however, was weakened and became unstable. This finding confirms that in the cities that were studied, sulfur dioxide was more consistently associated with daily mortality than were particles." These findings are contrary to the reported findings from many earlier analyses in the US. A recent study of cardiorespiratory admissions in Toronto (Burnett et al., 1997b) concluded that once the effect of gases was accounted for, indices of PM were not important predictors of admissions. These results indicate that there is considerably less consistency and coherence in the air pollution literature than some publications would have us believe.

Because NO_2 has been shown to have respiratory effects, we would have liked to investigate the association between NO_2 and hospital admissions for chronic respiratory disease in King County. Findings from epidemiological studies suggest that exposure to NO_2 is associated with alterations in lung function and with increased susceptibility to respiratory infection in children and adults (Speizer et al., 1980; Samet et al., 1987). Unfortunately we had no information on the oxides of nitrogen in King County because the levels were considered to be too low to warrant monitoring.

Human populations, particularly in urban areas, are exposed to a complex air pollution mix consisting perhaps of thousands of components. We probe this complex mixture by monitoring a half dozen so-called criteria pollutants. Chen et al. provide simulation studies of modeling bias that may be found in many studies of air pollution epidemiology. They find that the main results of underfit models (in which important covariates are omitted) are biased coefficient estimates and misguided confidence that the estimates are significant. The implications are that suggestive

but inconsistent results for any single pollutant must be considered in the context of the entire pollution mix, much of which is not accounted for in analytic models. With respect to the monitored components of air pollution, the most plausible interpretation of a positive association with adverse health effects is that the pollutant is simply an indicator of either a pollution source or, more generally, of the mixture of pollutants that is associated with adverse health effects. In view of the known toxicities of most criteria pollutants, a direct effect of the pollutant on human health cannot be ruled out, however. Driven largely by the regulatory imperative, research on air pollution has focussed on single components of the pollutant mix. Whatever the interpretation of regression analyses, it is clear that better insights into the effects of air pollution on human health will come only when the focus shifts from single components to the complex mixture.

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Table 1. Summary statistics for daily hospital admissions for chronic respiratory disease in King County over the period 1987 – 1995. The first column shows the statistics for total daily admissions. The second, third, and fourth columns show, respectively, the summary statistics for ages 0 – 19, 20 – 64, and 65 and over.

Summary Statistics

	copd	0 - 19	20 - 64	65 & up
Min:	0	0	0	0
1st Qu.:	5	1	1	1
Mean:	7.75	2.33	2.56	2.86
Median:	7	2	2	3
3rd Qu.:	10	3	4	4
Max:	23	14	10	12
Total:	3287	3287	3287	3287
Missing:	0	0	0	0

Table 2. Summary statistics for the average daily temperature and the average daily pollutant and tree pollen levels over the period 1987 – 1995. Note that the statistics for tree pollens are for the months March – August of each year. The statistics for $PM_{2.5}$ are for the monitor located in Duwamish; for the other pollutants, the statistics are for the average over all monitors (see text). The column nephavg refers to the average nephelometry readings. The PM measurements are reported in $\mu g/m^3$; the measurements for all gases are in parts per million.

Summary Statistics

	tmp	O3avg	SO2avg	COavg	PM25duw	PM10avg	nephavg	tree
Min:	15.000	0.000	0.000	0.520	3.000	7.000	0.093	
1st Qu.:	46.000	0.010	0.005	1.231	10.000	18.917	0.283	0.000
Mean:	53.378	0.016	0.008	1.779	18.114	30.006	0.580	118.711
Median:	53.000	0.016	0.007	1.610	15.000	26.333	0.427	5.000
3rd Qu.:	62.000	0.021	0.010	2.156	23.000	37.333	0.720	50.000
Max:	83.000	0.044	0.050	6.625	96.000	123.000	3.113	6588.000
Total:	3287	3287	3287	3287	3287	3287	3287	1656
Missing:	0	1338	153	0	2334	1263	1069	593

Table 3. Correlations among the pollutants. The $PM_{2.5}$ readings are from the Duwamish monitor. All other pollutant readings are averages over all the monitoring sites.

Correlations among pollutants

	PM25duw	O3avg	COavg	PM10avg	nephavg
PM25duw	1.00	-0.47	0.82	0.89	0.88
O3avg	-0.47	1.00	-0.55	-0.45	-0.55
COavg	0.82	-0.55	1.00	0.85	0.80
PM10avg	0.89	-0.45	0.85	1.00	0.88
Nephavg	0.88	-0.55	0.80	0.88	1.00

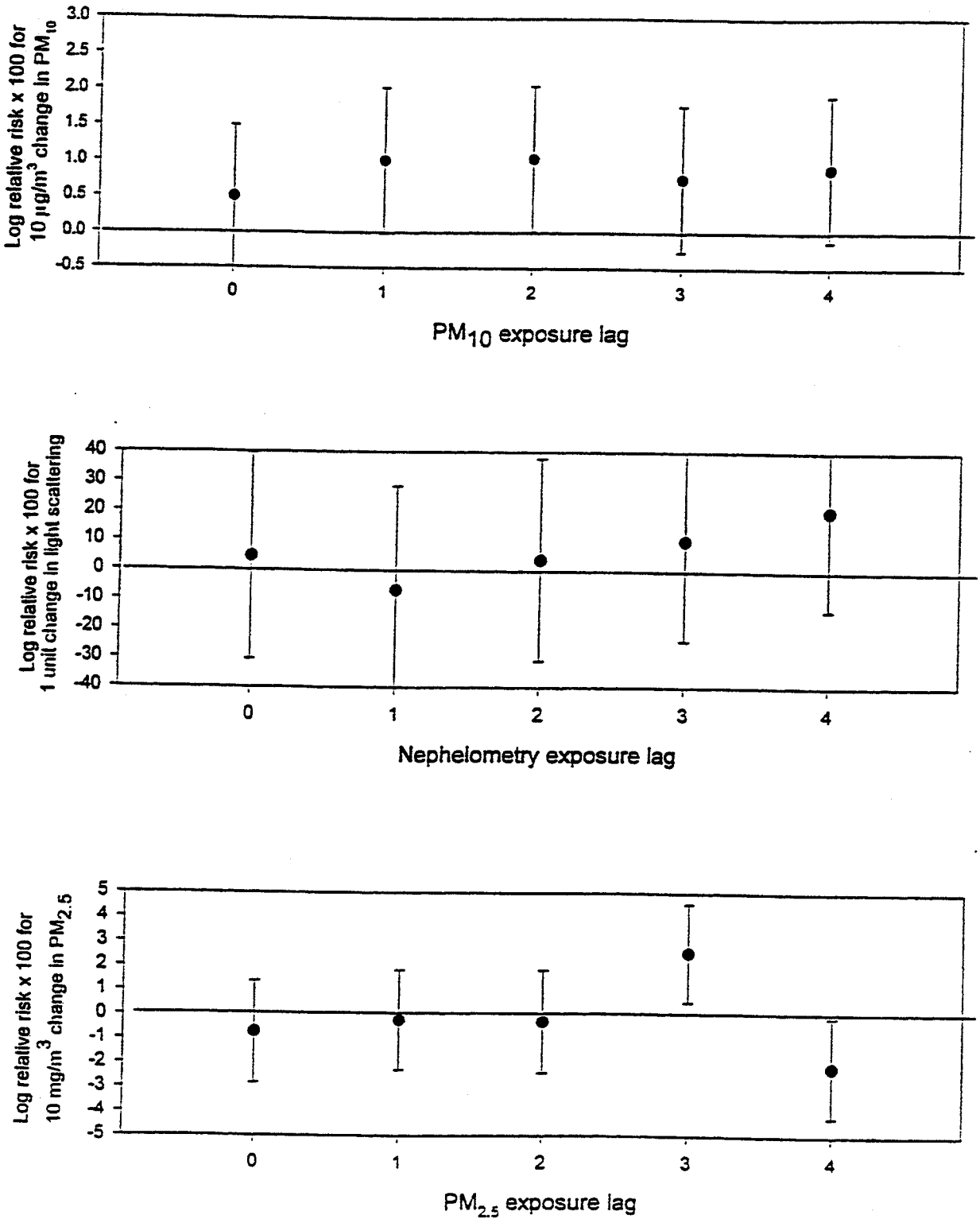
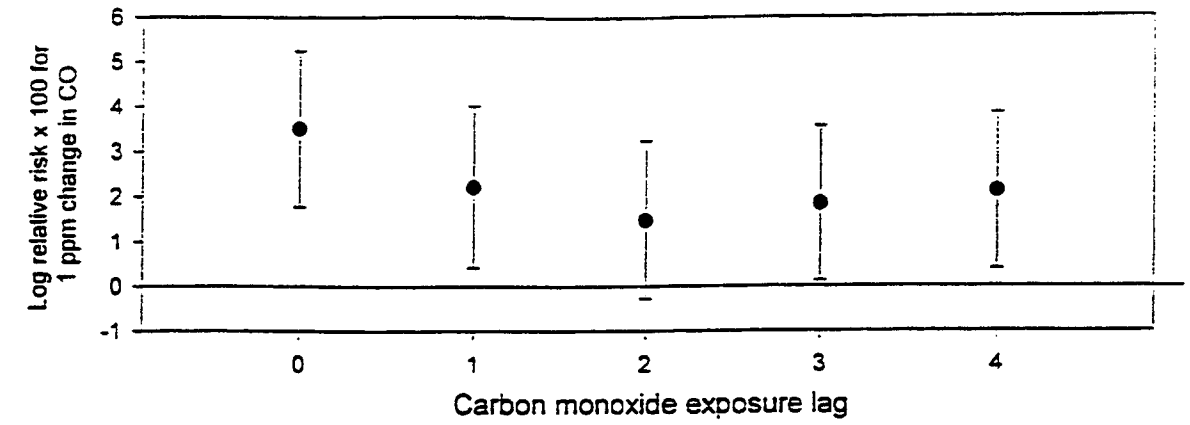
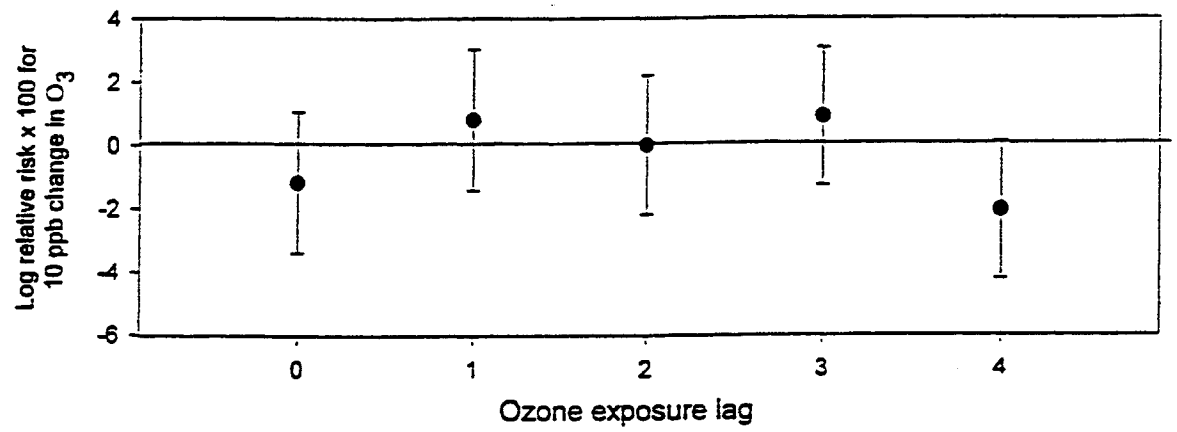


Figure 1. Chronic respiratory disease admissions associated with exposure to various indices of ambient particulate matter in single pollutant models. Point estimates along with their 95% confidence intervals are shown for lags between 0 and 4 days.





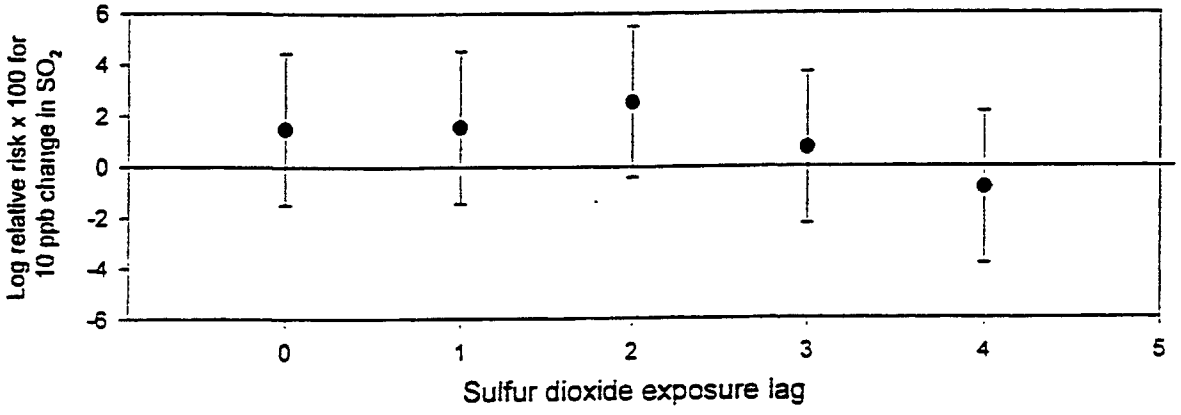


Figure 2. Chronic respiratory disease admissions associated with exposure to the gases in single pollutant models. Point estimates along with their 95% confidence intervals are shown for lags between 0 and 4 days.

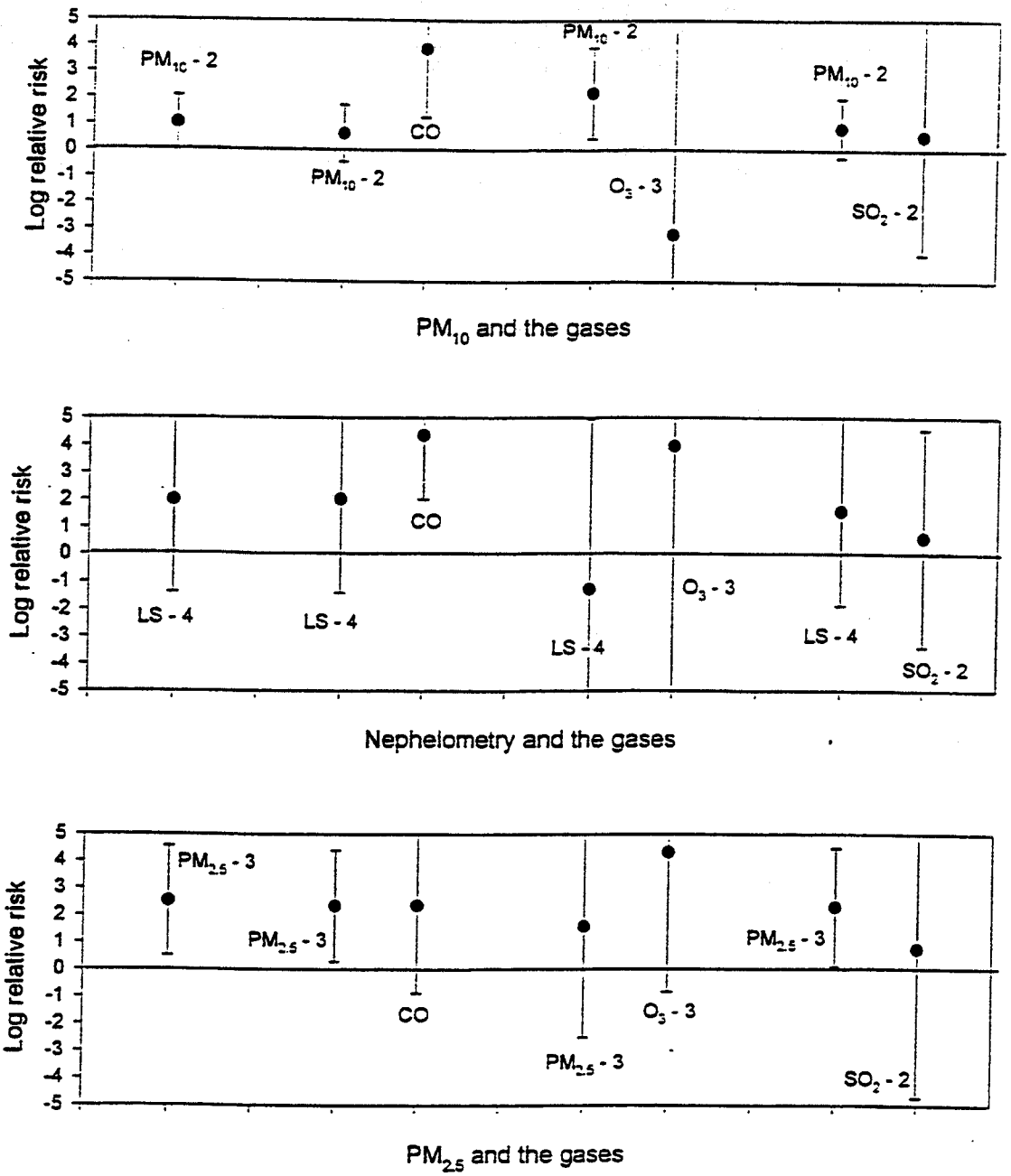


Figure 3. Results of analyses with two-pollutant models. In all panels, reading from left to right, the first line represents the results from a single pollutant analysis with the particulate matter metric being considered; the pairs of lines following the single pollutant analysis shows the results of two-pollutant models with the effect of the particulate matter index followed by the effect of the gas under consideration. The lag used for each of the pollutants is also shown. In all panels, for the gases, the coefficients are for a 100 ppm change in CO with a lag of zero days, a 1 ppm change in O_3 with a lag of three days, and a 1ppm change in SO_2 with a lag of two days. For all pollutants, the chosen lags are those that maximized the effect of the pollutant in single pollutant models. The top panel shows the point estimates along with their 95% confidence intervals for two-pollutant analyses with PM_{10} and the gases. The PM_{10} coefficient is for a $10\mu g/m^3$ change with a two day lag. The second panel shows the results of two-pollutant models with light scattering as the index of PM. The coefficient for light scattering is for a change of 100 nephelometry units with a lag of four days. The bottom panel shows the results of two-pollutant models with $PM_{2.5}$ as the PM index. The coefficient for PM is for a $10\mu g/m^3$ change with a three day lag.

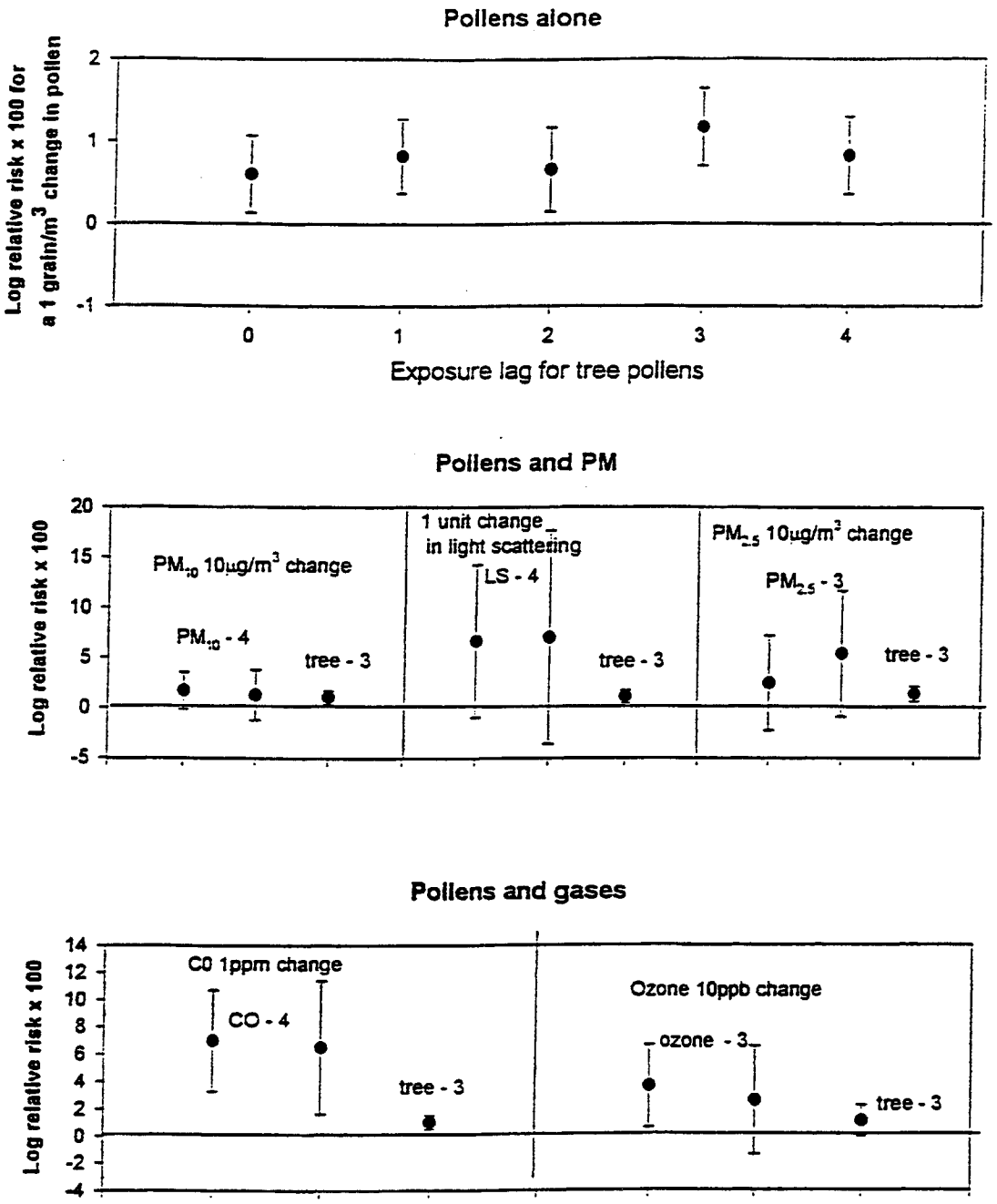


Figure 4. Results of analyses with tree pollens alone and jointly with PM and gases. All results in this figure are based on analyses restricted to the months March – August, which is the period over which pollen data are available. The top panel shows the results of analyses with tree pollens alone (coefficients with their 95% confidence intervals) with lags of from zero to four days. The second panel shows the results of single pollutant analyses with the various PM indices and also the joint analyses of these PM indices with tree pollens. As in figure 3, the results are presented for those lags that maximized the effect of that particular pollutant. In each box of the second panel, reading from left to right, the first entry shows the coefficient and 95% confidence interval for the PM index derived from the model with PM alone, the second entry shows the same for the PM index derived from the model with both PM and tree pollens. The last panel shows the results of analyses with single gases and also joint analyses with each of the gases and the tree pollens. Each of the boxes in this panel is to be interpreted as described for the second panel above. Note, in particular, the strong association between ozone with a three day lag and hospital admissions when analyses were restricted to the months March – August.

Chronic Inhalation Study in Rats Exposed to Car Exhaust Originating from Fuel with and without Ferrocene Additive

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Abstract

Gasoline engine exhaust deriving from the combustion of commercial fuel with 30 ppm ferrocene additive was compared to exhaust from the same fuel without ferrocene. Rats inhaled the exhaust after dilution of 1 : 20 and 1 : 40 for 18 hrs/day, 5 days/week for up to 24 months plus an additional exposure of 6 month to clean air. The high exposure concentration was the highest exhaust concentration technically feasible in an inhalation study as the limiting factor was the relative humidity of the exposure atmosphere. The exhaust was characterized by particle mass, size distribution and measurement of aliphatic hydrocarbons, polycyclic aromatic hydrocarbons, aldehydes and other components (phenols, ammonia, iron, platinum, NO_x, CO). At defined intervals, parameters of clinical chemistry, hematology, and broncho-alveolar lavage were measured as well as lung clearance and particle retention. Histopathological investigations were done in 20 animals per group after 12 months and in 200 animals per group after 30 months. In none of the investigations conducted, could differences in the toxic effects of the exhausts, deriving from fuel without or with ferrocene, be detected. The only exposure-related alterations which could be found were a loss in the iron concentration in the cell pellet of the BALF and slight alterations in the nasal cavity.

Introduction

Commercial use of metal compounds as additives for gasoline was stepwise restricted in Germany by the Gasoline Lead Act (Bundesgesetzblatt 1971), since the adverse toxicological effects of tetraethyllead as gasoline additive had become evident. However, in a test series with the organometallic iron compound ferrocene in the late 1980ies it was found that ferrocene added to gasoline in a concentration of 15 ppm was able to enhance octane, to reduce toxic exhaust compounds like CO and NO_x and to reduce fuel consumption (Schug et al. 1990). After combustion no unconverted ferrocene or other organometallic iron compounds could be detected, but only iron oxide (Fe_2O_3) which was emitted as particles. The only potential negative effect therefore seemed to be an enhancement in particle emissions. For this reason a test series to compare the exhausts from fuel without and with ferrocene was designed, including physical and chemical characterization of the exhausts, an inhalation study on chronic toxic and carcinogenic effects and in vitro studies on the mutagenic, cytotoxic and genotoxic potencies of the condensates, particle mass and gaseous phase of the exhausts. The hypothesis to be tested was that ferrocene used as gasoline additive may change the toxic properties of the exhaust e.g. by altering the size spectrum of particles or the chemical composition of the exhaust. Only the design and results of the inhalation study are reported here.

Study Design

The study was conducted according to EC / OECD guidelines with the exception that only two dose groups per exhaust type and only females were used. The high exposure concentration was defined as the highest exhaust concentration technically feasible and the low exposure concentration as twice the dilution. Two technically identical engines were used fed with commercial premium fuel without or with 30 ppm ferrocene respectively. A clean air group served as control. The engines chosen were the most common in Germany and technically representative for the traffic scene at study start and the following years.

Materials And Methods

Engines: VW Golf/ Passat 1.8 l, 66 KW, equipped with 3-way-catalysts, lambda sensors and automatic transmissions.

Driving cycle: To produce the exhausts a combined urban/freeway cycle was used, consisting of the US-72 cycle and a German freeway cycle. The average speed of the combined cycle was about 64 km/h (40 m.p.h.), the maximum speed 130 km/h (80 m.p.h.). The combined cycle could be repeated free.

Animals and housing conditions: 1440 female Wistar rats (Hsd/Cpb:WU) from Harlan CPB, Zeist, The Netherlands were used. The animals were randomly distributed into 4 exposure groups and the control group. They were kept pairwise in wire mesh cages Type III. Food and drinking water were given ad libitum. Lighting was in a 12 hrs light/dark cycle.

Exposure: The animals were exposed to the exhausts in 12m; stainless steel whole body inhalation chambers with horizontal air flow. Exposure was conducted for 18 hrs per day, 5 days per week for 12 months (chronic toxic effects) or for up to 24 months with additional 6 months in clean air (carcinogenic effects and special investigations). The exposure conditions are shown in Table 1 for the high dose groups. The exhausts were prediluted in dilution tunnels at the end of the exhaust pipes by a factor of 5 to avoid water condensation. Before entering the inhalation chambers they were diluted again to their final concentration. In comparison to older engines without catalysts, the emissions of the engines involved in this study were very low. For this reason the limiting factor for the high concentration was the relative humidity of the exhausts.

The average exhaust dilution for the high dose groups was about 1 : 20 and for the low dose groups 1 : 40. Already at dilutions of 1 : 18 condensation could occur which had to be avoided for hygienic reasons.

Investigations: The different investigations and the number of animals involved are shown in Table 2. *Body weight measurements* were conducted in 14 to 28 day intervals in all animals. *Food consumption* was measured during the first 13 weeks of the study, after this every 3 months in 10 pairs per group. 20 animals per group served for the *histopathological investigation* of chronic toxic effects after 12 months exposure. *Hematological, clinical*

Table 1: Exposure Concentrations (high dose groups, dilution 1 : 20)

Compound	Clean Air Control	Without Ferrocene	With Ferrocene
NO_x (ppm)	n. d.	16.0 ± 4.1	19.0 ± 6.9
NO_2 (ppm)	n. d.	0.8 ± 0.4	1.3 ± 0.6
CO (%)	n. d.	27.1 ±7.4	31.8 ± 7.9
CO_2 (%)	0.1 ±0.01	0.9 ±0.1	0.8 ± 0.2
CH_4 (ppm)	2.1 ± 0.2	3.0 ± 0.3	3.8 ± 0.5
CnHm – CH_4 (ppm)	0.9 ± 0.2	1.9 ± 0.4	3.7 ± 0.9
Particles ($\mu g/m^3$)	14.4 ± 5.5	85 ± 122	239 ± 335*
MMAD (µm) / δ_g	n. m.	0.90 / 5.39	0.63 / 2.17
Temperature (°C)	24.0 ± 0.3	25.1 ± 0.5	24.6 ± 0.3
Humidity (% r. h.)	61.7 ± 1.6	75.6 ± 3.5	75.9 ± 3.0

* enhanced by fallout of ammonium salts for a short time; n. d. = not detected; n.m. = not measured

chemical and urine analyses were conducted after 3, 6, 12, 18, and 24 months exposure in 10 animals per group and date. After 12 and 24 months exposure biochemical and cytological parameters of the *broncho-alveolar lavage* fluid (BALF) were determined in 10 animals per group. *Particle retention* measurements were conducted by determination of the iron content of the cell pellet of the BALF from the same animals. *Lung clearance* measurements of radiolabeled unsoluble test particles were done after 12 - 15 and 21 - 24 months exposure in 10 animals per group. Parameters of mechanical *lung function* were determined after 12 and 18 months exposure in 10 animals. 200 animals per group served for the investigation of *carcinogenic effects*; after 30 months experimental time, the complete organ spectrum of the animals of the high dose groups and the controls (600 animals) was investigated histopathologically. From the low dose groups, the respiratory tract and all macroscopically altered organs/tissues were investigated. 8 rats per group served as reserve for the different subgroups.

Results

The exposure concentrations in the inhalation chambers of the high dose groups are shown in Table 1. The greatest differences are seen with the average value for the particle mass. However, in this case the reason was not a high particle emission by the engine with ferrocene, but a temporary formation of ammonium salts in the exhaust tubes which connected the dilution tunnel with the inhalation chamber. For most of the exposure time the particle concentration in both inhalation chambers was in the range of about 50 $\mu g/m^3$, with

Table 2: Investigations And Number Of Animals

Investigation	Control	Without Ferrocene		With Ferrocene	
	clean air	low dose	high dose	low dose	high dose
Chronic toxic effects after 12 - 24 months exposure					
Pathology	20	20	20	20	20
Clinical chemistry*	20	20	20	20	20
Retention + BAL*	20	20	20	20	20
Lung clearance*	10	10	10	10	10
Lung function*	10	10	10	10	10
Carcinogenic effects after 24 months exposure and 6 months clean air					
Carcinogenicity	200	200	200	200	200
Reserve	8	8	8	8	8

* up to 24 months; BAL = broncho-alveolar lavage

no obvious differences between the different exhaust types. The particle concentration in the low dose chambers was only slightly above the background concentration (about 20 $\mu g/m^3$). Iron could not be determined in the exposure atmospheres. Ion chromatographic determinations of the particle mass resulted in about 60 - 90 % ammonium salts, mostly ammonium sulfate. The amount of toluene soluble compounds were determined in particles collected in the dilution tunnels. The values were in the range of about 24 % with no differences between the two exhaust types.

In the course of the study, no exhaust related clinical symptoms occurred. Body weight development of the high dose groups is shown in Figure 1. No differences occurred in body weight development between all five groups. After about 22 months, body weight decreased age-related.

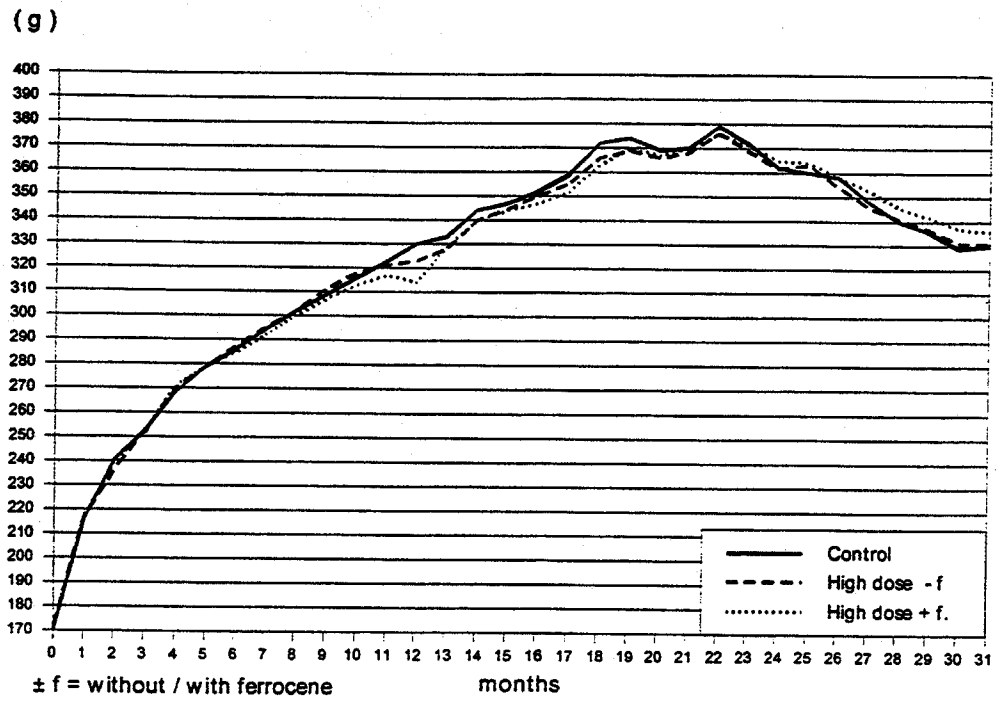
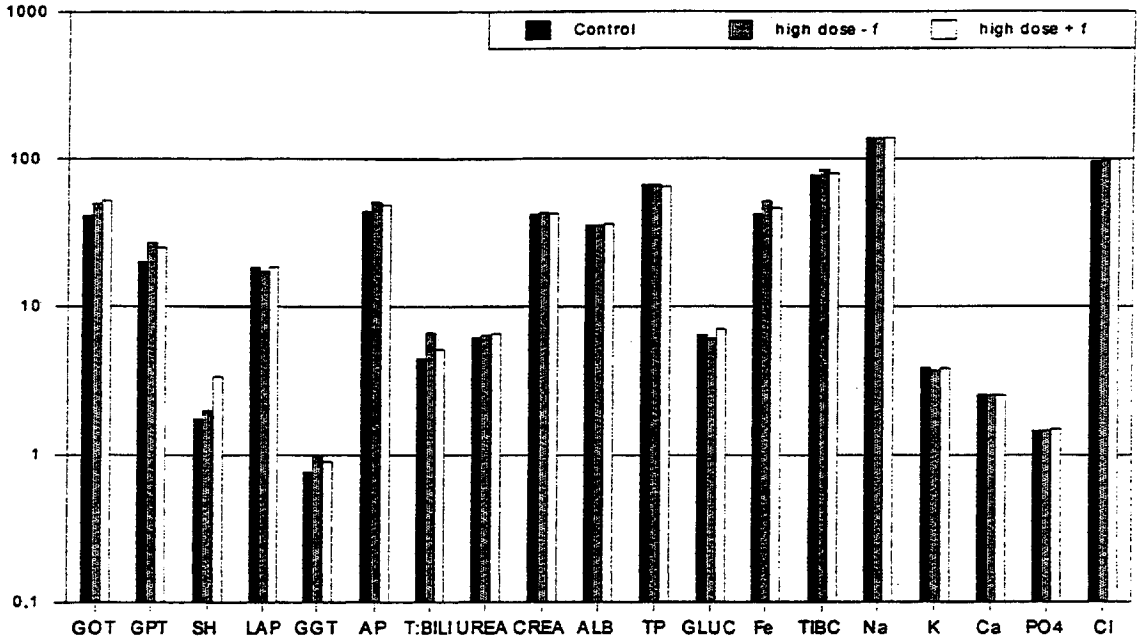


Figure 1. Body weight development (high dose groups and controls)

Mortality rates also were essentially equal in all groups. 50 % mortality was reached after about 104 - 116 weeks, 75 % mortality after about 122 - 126 weeks. At the end of the study after 30 months 9 - 12 % of the animals per group were still alive.

With none of the different special investigations like hematology, clinical chemistry, urinalysis, biochemistry and cytology in BALF, lung clearance and lung function measurements could any adverse effects of the exhaust inhalation nor any differences in the toxic potencies of the two exhaust types be detected at any time. Figures 2 - 3 show the results of clinical chemistry and biochemical and cytological determinations in BALF as an example. The biological half-time of radiolabeled particles in the lungs after 21 - 24 months exhaust inhalation was numerically but not significantly shortened compared to the control values, without differences between both exhaust types.

An exposure-related effect could only be found in the iron content of the cell fraction of the BALF. After 24 months exposure, compared to the control values, the iron content was significantly reduced in both groups without ferrocene and in the low dose group with ferrocene, but not in the high dose group with ferrocene.



Statistics: Anova + Dunnett's Test (two sided): no significant differences
± f = without / with ferrocene

GOT	Glutamate oxaloacetate transaminase (U/l)	GPT	Glutamate pyruvate transaminase (U/l)
SH	Sorbitol dehydrogenase (U/l)	LAP	Leucine aminopeptidase (U/l)
GGT	(γ-glutamyl transferase (U/l)	AP	Alkaline phosphatase (U/l)
T.BILI	Total bilirubin (µmol/l)	UREA	Urea (mmol/l)
CREA	Creatinine (µmol/l)	ALB	Albumin (g/l)
TP	Total protein(g/l)	GLUC	Glucose (mmol/l)
Fe	Iron (µmol/l)	TIBC	Total iron binding capacity (:mol/l)
Na	Sodium (mmol/l)	K	Potassium (mmol/l)
Ca	Calcium (mmol/l)	PO_4	Inorganic phosphate (mmol/l)
Cl	Chloride (mmol/l)		

Figure 2. Clinical chemistry after 24 months

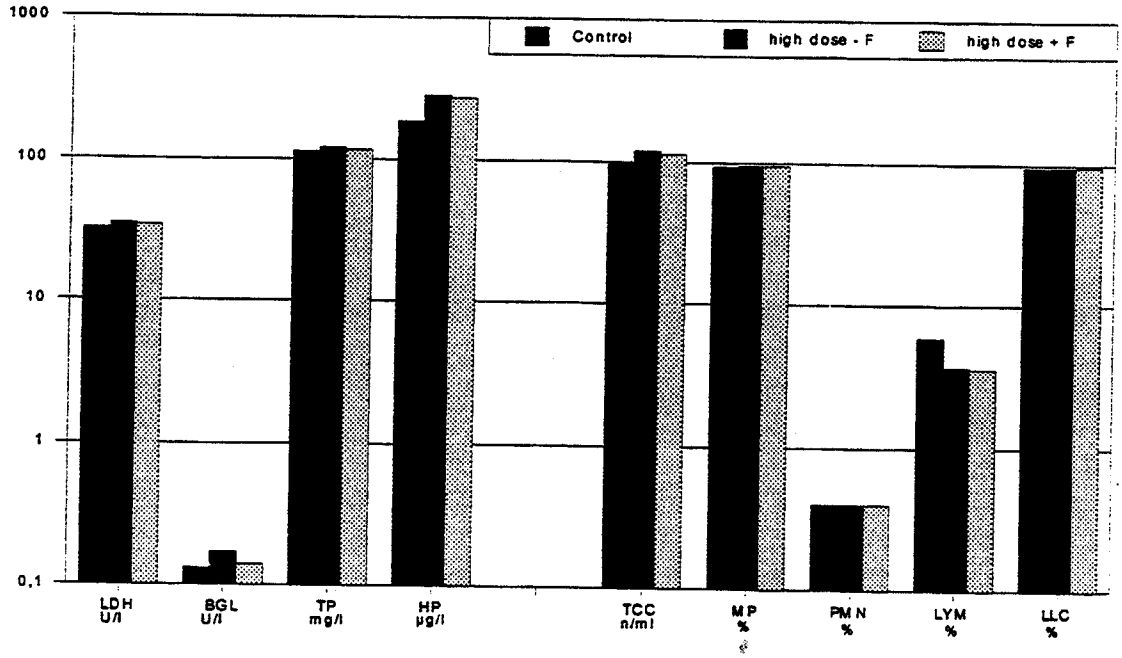
After 30 months experimental time only a few non neoplastic changes were found: in the nasal cavity hyaline eosinophilic cytoplasmatic inclusions and goblet cell hyperplasias could be detected. The inclusions showed dose-dependent increasing incidences compared to the control values which were statistically significant. The hyperplasias showed numerically but not significantly increased incidences. Both alterations thus indicated an exhaust-related effect, but again no differences existed between the two exhaust types without or with ferrocene.

In the target organs of the respiratory tract only a total of 5 tumours could be found: one adenocarcinoma in the nasal cavity in the low dose group with ferrocene, one bronchiolo-alveolar carcinoma each in the control group and the low dose group with ferrocene, and one bronchiolo-alveolar adenoma each in the low dose groups without and with ferrocene. In the

other organs also no statistically significant increase in any tumour type was observed, although more than 90% of the animals developed tumours. Table 3 shows the summarized tumour incidences for all organs.

Discussion And Conclusions

The inhalation of the highest feasible concentration of gasoline engine exhausts deriving from engines which were equipped with 3-way-catalysts and lambda sensors resulted in only very few exposure-related effects such as minor adaptive alterations in the nasal cavities and a loss in the iron concentration of the BALF. The use of 30 ppm ferrocene as gasoline additive did not detectably alter the toxicity of the exhaust. Ferric oxide (Fe_2O_3) as the only combustion product of ferrocene is classified to be of low toxicity. The inhalation of pure ferric oxide induces predominantly siderosis which is reversible after the end of exposure. Other adverse effects like lung cancer which was observed e.g. in ore miners were put down to combined exposure with carcinogenic compounds like radon, impurities like other metals, asbestos



Statistics: Anova + Dunnett's Test (two sided): no significant differences
± f = without / with ferrocene

LDH	Lactate dehydrogenase	TCC	Total cell count
BGL	β-glucuronidase	MP	Macrophages
TP	Total protein	PMN	Polymorphonuclear granulocytes
HP	Hydroxyprolin	LYM	Lymphocytes
LLC	Vital leucocytes		

Figure 3. Biochemical and cytological determinations in BALF after 24 months exposure

Table 3: Tumour Table - High Dose Groups - All Organs

	Clean Air	Without Ferrocene	With Ferrocene
Number of animals	200	200	200
% Animals with benign tumours	85	88	85
% Animals with malignant tumours	38	34	37
% Animals with metastasizing tumours	7	8	4

fibers or diesel exhaust. In animal experiments after intratracheal instillation or chronic inhalation of pure ferric oxide no lung tumour growth could be induced in Hamsters with cumulative doses up to 100 mg (i. tr.) or concentrations up to 40 mg/m³. As a result of these findings the maximum tolerable working place concentration for ferric oxide in Germany was defined as the maximum tolerable dust concentration of 6 mg/m³ (Elinder 1986, Greim1998). In our study, inside the inhalation chambers the iron concentration was below 0.1 µg Fe/m³ and thus far below toxic concentrations. The iron concentration deriving from 30 ppm ferrocene additive is calculated at about 700 µg Fe/m³ undiluted exhaust. However, measurements at the end of the exhaust pipes demonstrated that about 75% of the iron added with ferrocene is already lost in the exhaust pipe and catalyst. Only about 310 µg Fe/m³ deriving from ferrocene could be detected. After dilution at a ratio of about 1 : 20 the calculated Fe concentration in the high dose exposure with ferrocene was about 15 µg Fe/m³, a value close to urban background concentrations. The true value of 0.1 µg/m³ at the most was comparable to rural background values (Elinder 1986). As a result of these findings it must be concluded, that toxic effects of gasoline engine exhausts from modern engines equipped with catalysts and lambda sensors will no longer be detectable by means of inhalation studies, and that for this purpose other methods, like in vitro systems, will be the methods of choice.

As a result of the sum of investigations on potential effects of ferrocene on the toxicity of gasoline engine exhausts an extra license for the use of 15 ppm ferrocene in gasoline was given at the beginning of 1999 for a period of 5 years.

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A Chemical And Toxicological Comparison Of Urban Air PM_{10} Collected During Winter And Spring In Finland

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We have used a new high-volume low cut-off inertial impactor (HVLI) in a pilot study on chemical characterization and toxicity testing of ambient air PM_{10} in Helsinki, Finland. Ambient air PM_{10} was collected at 1100 l/min in two to four-day periods. Two different PM_{10} samples were selected to represent wintertime combustion-type and springtime resuspension-type PM pollution. The most abundant ions and elements were analyzed by ion chromatography and inductively coupled plasma mass spectrometry, respectively. The proinflammatory activation (NO and IL-6 production) and viability of cultured murine RAW 264.7 macrophages were tested in 24-hour incubations with increasing mass doses (30-2000 μg per 10^6 cells) from the collected PM_{10} samples. The winter sample had a higher assessed $PM_{2.5}$-fraction and sulphate content, and lower chloride, sodium, calcium, aluminium, copper, manganese, and especially iron contents than the spring sample. Both PM_{10} samples induced dose-dependent NO production in murine macrophages and the springtime PM_{10} produced also a strong, dose-dependent IL-6 production. In conclusion, the HVLI proved to be a suitable technique for short-term collection of relatively large ambient air PM masses enabling extensive chemical characterization and toxicity testing from the same samples.

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Introduction

The ambient air particulate matter (PM) pollution in Finnish cities has large contrasts in different seasons. In mid-winter, the 24-hour concentrations of $PM_{2.5}$ and PM_{10} are usually low and the PM originates largely from vehicle exhausts, whereas in springtime, the 24-hour concentrations of PM_{10} are relatively high and the PM originates mainly from resuspension of sand and other dusts (asphalt, tyres, studs etc.) from the road surfaces (Hämekoski & Salonen, 1996; Hosiokangas et al., 1999). The PM pollution during both seasons has been shown to be associated with respiratory health among asthmatic children (Salonen et al., 1996; Timonen & Pekkanen, 1997; Tiittanen et al., 1999).

A new high-volume, low cut-off inertial impactor (HVLI) has been developed by the Harvard School of Public Health for collection of large quantities of ambient air PM for chemical characterization and toxicity testing. The HVLI features a high flow rate, high collection capacity, sharp size cut-off curve, minimal particle bounce, and minimal amount of collection substrate material (Kavouras et al., 1999). We have installed the first full-size HVLI for a pilot study on chemical characterization and toxicity testing of ambient air PM_{10} in Helsinki, Finland.

Methods

The pilot study was conducted at a relatively busy traffic site in Helsinki, Finland, between March 5 and April 6, 1999. The average daily traffic in the nearest street (at 15 m distance) was about 13000 vehicles. Ambient air PM pollution was characterized by continuous monitoring of $PM_{2.5}$ and PM_{10} with the beta attenuation method (Eberline FH 62 I-R, Erlangen, Germany).

Construction of HVLI. The HVLI apparatus consists of three main parts: a PM_{10} inlet (Andersen G1200, Village of Cleves, OH), a slit impactor, and a blower (Y-Laite, Lahti, Finland). The 280-mm-long slit operates on an air flow of 1100 l/min and a pressure drop of 250 mbar, and it produces a lower PM size cut-off at approximately 0.12 μm. The HVLI apparatus was placed in a 9-m^3 extension cabin connected to the air quality monitoring station of the Helsinki Metropolitan Area Council (YTV).

PM collection and extraction. Ambient air PM_{10} was collected in two to four-day periods on a high-capacity polyurethane foam (PUF) (Merryweather Foam, Barbarton, OH), which was cut into a millimeter size of 320 (L) x 6.4 (W) x 6.4 (H). The PUF-strips were cleaned before use in four successive baths with 15-min sonications, two in water and two in ethanol. The collected PM samples were extracted from the PUF impaction substrate into 100% methanol by sonicating for 60 min. The PM_{10} suspension in methanol was divided into separate tubes for chemical analyses and toxicity testing, and then methanol was evaporated at +70 °C. Clean, methanol extracted PUF-strips and clean, non-methanol extracted PUF-strips were used as controls in chemical analyses and toxicity testing.

Chemical characterization of PM_{10}. Ions were extracted from the dry, methanol extracted PM_{10} samples with deionized water (Millipore Alpha-Q) and analyzed by ion chromatography (IC) (Pakkanen et al., 1996). Correspondingly, elements were extracted with 0.08 M HNO_3, and analyzed by inductively coupled plasma mass spectrometry (ICP-MS).

In-vitro toxicological testing of PM_{10}. The PM_{10} samples selected for chemical characterization were tested also in the murine macrophage cell line RAW 264.7, which was cultured at 37°C and 5% CO_2 in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum, 1% l-glutamine and 1% penicillin-streptomycin. The macrophages were exposed for 24 hours to five mass doses (30, 100, 300, 1000 and 2000 μg per 10^6 cells) prepared from each PM_{10} sample. Equal test volumes (6, 20, 60, 200 and 400 μl per 10^6 cells) from clean, methanol extracted PUF-strips and clean, non-methanol extracted PUF-strips were used in control experiments. In order to detect the proinflammatory activation of macrophages, the production of nitric oxide (NO) was analyzed spectrophotometrically as the stable metabolite, nitrite, according to the Griess-method (Green et al., 1982), and the production of interleukin 6 (IL-6) was analyzed by a "sandwich-type" enzyme immunoassay using a commercial ELISA kit (Pharmingen, San Diego, CA). The viability of the macrophages was assessed by using the spectrophotometric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) test to detect functioning mitochondria (Mosmann, 1983).

Statistical analysis. One-way analysis of variance and Fisher's test were used in the analysis of dose-relationships in the NO and IL-6 data with p-values <0.05 regarded as significant. The non-parametric Kruskal-Wallis test was used in the analysis of dose-relationships in the cell viability data.

Results

During the pilot study, the 24-hour average concentrations of continuously monitored ambient air $PM_{2.5}$ ranged from 4.8 to 37 $\mu g/m^3$ and the corresponding PM_{10} concentrations ranged from 8.2 to 76 $\mu g/m^3$. Two HVLI samples of PM_{10}, which represented contrasting PM pollution situations, were selected for chemical analyses and toxicity testing: during collection of Sample 10 (S10 - 79.9 mg) $PM_{2.5}$ constituted 83% of the total PM_{10} mass (little resuspension) and during collection of Sample 14 (S14 - 83.7 mg) $PM_{2.5}$ constituted only 45% of the total PM_{10} mass (much resuspension). As assessed by mass balance measurements, the PM_{10} extraction efficiencies with the present methanol procedure were 82% for S10 and 69% for S14.

In chemical analyses, S10 had a higher sulphate content and lower chloride, sodium, calcium, aluminium, copper, and manganese contents than S14. Moreover, the iron content in S10 was only about 10% of that in S14 (Table 1). Clean, methanol extracted PUF-strips and clean, non-methanol extracted PUF-strips contained less than 1% of the ions and elements in the ambient air PM samples.

Both PM_{10} samples S10 and S14 induced dose-dependent NO production in murine RAW 264.7 macrophages (Fig. 1A), whereas only S14 induced a clear-cut, dose-dependent IL-6 production (Table 2). Equal test volumes from clean, methanol extracted PUF-strips and clean, non-methanol extracted PUF-strips did not induce significant NO or IL-6 production. However, the largest test volumes from clean, methanol extracted PUF-strips were cytotoxic, and the largest doses from the PM_{10} sample S14 showed some additional cytotoxicity (Fig. 1B).

Table 1. The most abundant ions and elements (μg/mg PM_{10}) in the PM_{10} samples S10 and S14.

Ions /	S10		S14	
elements	IC	ICP-MS	IC	ICP-MS
NH_4^+	81.9		87.0	
Na^+ / Na	10.4	12.3	20.9	19.6
Ca^{2+}	4.2		7.9	
K^+	2.2		2.9	
Mg^{2+}/ Mg	1.8	1.7	2.0	1.9
NO_3^-	114.0		147.9	
SO_4^{2-}	108.5		66.7	
Cl^-	7.6		17.6	
Zn		0.95		0.94
Al		0.44		0.99
Fe		0.33		3.25
V		0.28		0.25
Pb		0.23		0.24
Mn		0.12		0.20
Cu		0.10		0.18
Ni		0.06		0.05

Discussion

The present HVLI technique proved to be a straightforward means to collect relatively large amounts of ambient air PM within a few days. There were no major problems in the installation or operation of the apparatus, although it was the first full-size setup of 1100 liters/min developed on the basis of a smaller prototype.

This model of HVLI with the lower PM size cut-off at approximately 0.12 μm was originally designed to be used in conjunction with a size selective inlet for $PM_{2.5}$ (Kavouras et al., 1999), but in our pilot study we wanted to use it with the inlet for PM_{10}. This was due to the contrasting features of urban air PM pollution in northern European countries like Finland, which have mainly combustion-type fine PM from automotive traffic in cold winter and resuspension-type coarse PM in spring (Hämekoski & Salonen, 1996; Hosiokangas et al., 1999). However, due to increased PM bounce-off the collection efficiency, especially in association with a high contribution of resuspension, was likely to be lower for PM_{10} than the estimate of 88-99% for $PM_{2.5}$ (Kavouras et al., 1999). We did not have any reference collection of PM_{10} in this pilot study, but the tendency towards a lower PM_{10} collection efficiency during high resuspension periods could be seen, when the collected HVLI masses were compared with the corresponding PM_{10} and $PM_{2.5}$ mass estimates derived from continuous beta attenuation measurements.

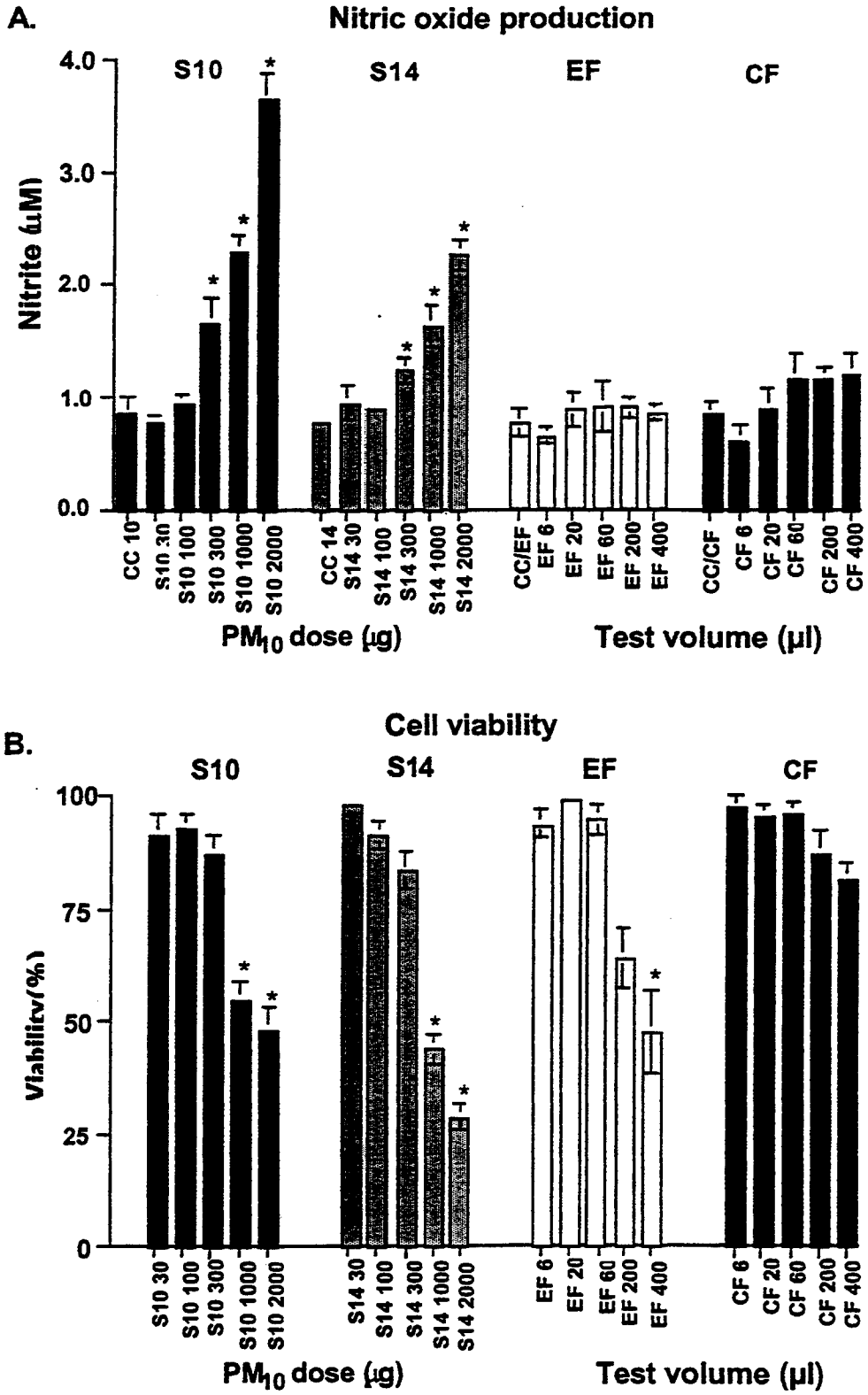

Figure 1. Nitric oxide (NO) production and cell viability in the murine macrophage cell line RAW 264.7 after a 24-hour incubation with increasing mass doses from PM_{10} samples S10 and S14 or with equal test volumes from clean, methanol extracted PUF-strips (EF) and clean, non-methanol extracted PUF-strips (CF). Means ± SEM usually from four experiments in duplicate are shown. *Significantly different from the respective cell control (CC) in the NO test (Fisher's test; $p<0.05$) or from the smallest test dose or volume in the cell viability test (Kruskal-Wallis test).

Table 2. Interleukin 6 (IL-6) production in murine RAW 264.7 macrophages by increasing mass doses from PM_{10} samples S10 and S14 or by equal test volumes from clean, methanol extracted PUF-strips (EF) and clean, non-methanol extracted PUF-strips (CF) after a 24-hour incubation.

Dose / volume per 10^6 cells	S10 (pg/ml)	S14 (pg/ml)	EF (pg/ml)	CF (pg/ml)
Control	0.28 ± 0.28	0.16 ± 0.16	0 ± 0	0 ± 0
30 µg / 6 µl	0.87 ± 0.56	28 ± 8.7*	0 ± 0	0.93 ± 0.93
100 µg / 20 µl	7.8 ± 1.7*	98 ± 18*	0.54 ± 0.54	0.05 ± 0.05
300 µg / 60 µl	14 ± 1.2*	150 ± 28*	0 ± 0	2.2 ± 2.2
1000 µg / 200 µl	22 ± 8.6*	350 ± 60*	5.0 ± 4.6	6.7 ± 4.7
2000 µg / 400 µl	12 ± 5.8*	810 ± 270*	0.59 ± 0.35	21 ± 19

Values are means ± SEM usually from four experiments in duplicate.
*Significantly different from the respective cell control (Fisher's test; $p<0.05$).

Despite the obvious selective losses in the coarse fraction of HVLI collected PM_{10}, we could demonstrate chemical differences between the winter and spring samples supporting our initial hypothesis of recognizing contrasting PM pollution situations on the basis of $PM_{2.5}/PM_{10}$ - ratio in continuous mass measurements. The winter sample S10 had a higher sulphate content than the spring sample S14 indicating a higher contribution of combustion sources. Moreover, S10 had lower contents of typical soil source ions and elements such as aluminium, calcium, manganese, copper, sodium, chloride, and most strikingly iron (USEPA 1996). The present results from IC and ICP-MS analyses were regarded to represent the watersoluble fraction of ions and elements in ambient air PM_{10}, as the overall levels measured from the methanol pre-extracted samples were roughly similar to the previously investigated, non-methanol extracted samples collected at the same traffic site (data not shown).

The present results showed that both the winter and spring samples of PM_{10} induced dose-dependent proinflammatory activation of the cultured murine macrophages, as assessed by NO production. The most striking difference in biological effects between the two PM_{10} samples was that only the spring sample S14 induced a clear-cut, dose-dependent IL-6 production. This finding may be associated with the 10 times higher content of soluble iron in S14 than in S10, as iron is one of the bioavailable transition metals in ambient air PM (Costa & Dreher, 1997) and has been involved in the acellular production of hydroxyl radicals from ambient air PM_{10} (Donaldson et al., 1997). Moreover, Ghio et al. (1992) have reported that surface complexed iron on silica dust has been involved in oxidant generation from murine alveolar macrophages and in lung inflammation of rats. Despite the present fascinating findings it is definitely too early to draw any conclusions from the chemical and biological differences between the two contrasting PM pollution periods on the basis of this pilot study. There may be also other characteristics in the springtime resuspension-type PM_{10}, which can potentially be involved in the activation of macrophages. Dong et al. (1996) and Becker et al. (1996) have shown that endotoxin in ambient air PM can be involved in the cytokine production (including IL-6) in murine and human alveolar macrophages. In our previous study, a commercial gram negative bacterial lipopolysaccharide induced NO and IL-6

production and cytotoxicity in the same murine macrophage cell line RAW 264.7 as used in the present study (Hälinen et al., 1999).

The PUF substrate cleaning with ethanol and water was necessary in order to guarantee low background levels both in chemical and toxicological tests, while methanol pre-extraction of PM_{10} from PUF-strips was necessary in order to guarantee a high extraction efficiency for the collected PM mass. These procedures were mostly successful and caused no interference in the IC and ICP-MS analyses or in the NO and IL-6 tests. However, there was some cytotoxicity due to either insufficient PUF cleaning and/or methanol impurities after pre-extraction and evaporation, which necessitates some changes in these procedures.

Conclusions

The HVLI proved to be a suitable technique for short-term collection of relatively large ambient air PM masses enabling extensive chemical characterization and toxicity testing from the same samples. The conversion of the present one-stage HVLI into a two-stage system allows simultaneous collections of the fine fraction ($PM_{2.5}$) and coarse fraction ($PM_{10-2.5}$) of ambient air PM_{10}, which will further improve the possibilities to analyze the chemical and toxic properties of contrasting ambient air PM pollution.

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(Submission to the proceedings of the 3rd colloquium on PM and human health)

An Exploratory Analysis of the Relationship between Mortality and the Chemical Composition of Airborne Particulate Matter

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ABSTRACT

We explored relationships between daily mortality and the major sources of airborne particulate matter (PM) using a newly developed approach, Factor Analysis and Poisson Regression (FA/PR). We hypothesized that by adding information on PM chemical speciation and source apportionment to typical PM epidemiological analysis, we could identify PM sources that cause adverse health effects. The FA/PR method was applied to a merged data set of mortality and extensive PM chemical speciation in New Jersey.

Statistically significant associations were found between mortality and several of the seven FA-derived PM sources, including oil burning, industry, sulfate aerosol, and motor vehicles. The FA/PR method provides new insight into potentially important PM sources related to mortality. For the data set we analyzed, the use of FA/PR to integrate multiple chemical species into source-related PM exposure metrics was found to be a more sensitive tool than the traditional approach using PM mass alone.

INTRODUCTION

Limited data are available on PM chemical speciation, thus traditional PM epidemiological studies generally use total PM mass or a single PM component (such as SO_4) as metrics of human exposure. This approach assumes that there is a linear dose-response relationship based on PM mass concentration. PM mass may only serve as a surrogate for the effects of individual PM constituents because PM is made up of different chemical components and sizes that come from various emissions sources.

Our study explored the relationship between daily mortality and PM by applying Factor Analysis and Poisson Regression (FA/PR), as first proposed by Ten Brinke *et al.* (1998), to a database with detailed PM chemical species measurements. Factor analysis was first used to convert chemical species measurements to vectors that can be used as PM source-related exposure metrics for mortality in the Poisson regression, adjusted for average temperature (T_{avg}). By adding information on chemical speciation and source apportionment to typical PM epidemiological analysis, we explored the relationship between multiple chemical species exposure and daily mortality. Risks associated with each significant PM source were estimated indirectly by incorporating FA, PR, and Multiple Regression (MR).

DATA

The environmental data were obtained from the Airborne Toxic Element and Organic Substances study (ATEOS, Lioy *et al.*, 1987) collected at three New Jersey sites: Newark, Elizabeth, and Camden. Chemical species measured were inhalable particulate matter (IPM: mass median aerodynamic diameter (d_{50}) $\leq$ 15 μm), fine particulate matter (FPM: $d_{50} \leq 2.5$ μm), and the trace metals and sulfate constituents of IPM. The sampling periods were two consecutive summers and winters with 39 sampling days in each period for a total number of 155 sampling days from 1981 to 1983. In addition to the pollutants measured by ATEOS, maximum 1-hour daily CO and average daily temperature were also included in the following analyses.

The mortality data were obtained from Public Use Data Tape Files: Mortality Detail for 1981-1983 (U.S. Department of Health and Human Service, National Center for Health Statistics). A subset of the New Jersey mortality data was extracted to match the ATEOS sampling sites. Accidental and homicide deaths (ICD codes > 800) were excluded. Two death categories were defined for statistical analyses in this study: total daily deaths (TDD) and cardiovascular and respiratory daily deaths (CRDD).

ANALYSIS METHODS

We used two approaches in the data analyses.

1. SIMPLE POISSON REGRESSION-- USING A SINGLE PM EXPOSURE METRIC

In the first approach, Poisson regression was applied to the merged environmental and mortality data to assess the association between single (and simpler) exposure metrics (FPM mass, IPM mass, and sulfate) and daily mortality count (TDD and CRDD, respectively) in each county, adjusted for the daily average temperature (T_{avg}) as a possible confounder. The Generalized Estimating Equation (GEE) (Liang *et al.*, 1986) was used to account for the possible auto-correlation of the dependent variable, mortality.

2. FACTOR ANALYSIS AND POISSON REGRESSION (FA/PR)-- IDENTIFICATION OF PM SOURCES SIGNIFICANTLY ASSOCIATED WITH MORTALITY

Factor analysis (FA) has been used extensively to identify important sources of ambient pollution (e.g., Morandi, 1985; Morandi *et al.*, 1987). FA converts multiple correlated environmental data (e.g., trace elements, sulfate, and CO in this study) into a reduced number of conceptually meaningful independent vectors, called factors. Sources can be identified by comparing the chemical species that have high factor loadings in each factor with the known source signatures or tracers from PM emissions sources. Examples of source tracers used are: geological sources -- Mn and Fe; oil burning sources -- V and Ni; motor vehicle emissions -- Pb. Factor scores, composite measures of factors resolved from FA, are PM source-related transformations of the original measurements of chemical species. In the second approach to the data analysis, these factor scores were used as exposure metrics to assess the relationship between daily mortality and individual PM sources.

Akaike Information Criterion (AIC, Sakamoto *et al*, 1986) was used to compare the simple Poisson models and the FA/PR models.

Due to the qualitative natures of FA, the FA/PR method cannot be directly used to quantify the risk associated with each source. Therefore, we incorporated multiple regression (MR) into FA/PR in order to estimate the risk of significant sources identified by FA/PR. Factor Analysis/Multiple Regression (FA/MR) was developed in previous studies to quantitatively apportion contributions of pollution sources. In FA/MR, source patterns and specific source tracers are first identified by FA, and total PM mass is regressed against a unique tracer of each PM source using stepwise multiple regression. [FA/MR]/PR was used to estimate relative risks (RR) of PM sources indirectly: total IPM mass was first apportioned to individual PM sources by FA/MR, and the individual source-

specific IPM masses for all significant sources were then regressed against the mortality using Poisson models. The relative risk (RR) estimation for Newark is shown as follows:

1) by FA/MR: $IPM_{total\ mass} = IPM_{oil\ burning} + IPM_{Zn\text{-}smelter} + IPM_{sulfate} + IPM_{dust} + IPM_{motor} + IPM_{ind\text{-}Cu}$

2) by PR: $Mortality = f(IPM_{oil\ burning}, IPM_{Zn\text{-}smelter}, IPM_{sulfate}, T_{avg})$

$RR = e^{(\beta * change\ of\ source\text{-}specific\ IPM\ concentration)}$ (β: regression coefficient from PR).

RESULTS

In the first approach, significant associations were found between three single exposure metrics - FPM, IPM, and sulfate - and TDD and CRDD in Newark. In Elizabeth, none of the three simpler exposure metrics was significantly associated with TDD or CRDD. In Camden, TDD was significantly associated with IPM and FPM; CRDD was significantly associated with three pollutants (FPM, IPM, and sulfate).

Statistically significant ($p \leq 0.10$) results of the second set of analyses are discussed in the following paragraphs by city.

In Newark, the factors for oil burning sources (tracers: V and Ni), industrial sources (tracers: Zn and Cd) and sulfate aerosol showed positive relationships with TDD. For CRDD, sulfate aerosol was identified as a significant source.

In Elizabeth, re-suspended dust (tracers: Fe and Mn) showed a negative association with TDD ($p = 0.08$). The industrial sources traced by Cd were a significant predictor for CRDD, and two other sources (soil dust and industrial sources traced by copper and lead) showed negative associations that may be statistical artifacts.

In Camden, oil burning sources and motor vehicle emissions (tracers: Pb and CO) were two important PM sources for TDD. Sources traced by copper showed a negative association with TDD. For CRDD, three PM sources were significant predicators for CRDD: oil burning, motor vehicles and sulfate aerosol.

The results of model comparison by AIC indicate that FA/PR is generally better than or equivalent to the conventional approach using PM total mass as an exposure metric. The relative risks of increasing 10 $\mu g/m^3$ IPM mass according to these two approaches, simple Poisson model and [FA/MR]/PR are presented in Table 1.

DISCUSSION

Consistent with previous PM studies, we found significant associations between single exposure metrics of PM (IPM, FPM, and sulfate) and daily mortality, although the ATEOS data set is very small. FA/PR appears to

provide new insight into the relationship between the individual PM sources and mortality. Not all identified PM sources found in the factor analysis contributed to the elevated daily mortality. The potentially important PM sources identified in our analyses are oil burning, sulfate aerosol, industry (Zn smelters or Cd), and motor vehicles. This study provides epidemiological evidence that differences in chemical composition may play a role in the health effects of PM. The detailed chemical measurement of PM constituents from the ATEOS study provided us an opportunity to investigate PM health effects by FA/PR. However, our small sample limits the statistical power to detect the small changes in daily mortality due to variations on exposures to different PM sources.

The observed positive relationships found in this study between ambient PM sources and increased mortality are supported by toxicological studies (e.g., Costa *et al.*, 1997; Dreher *et al.* 1997; Kodavanti *et al.* 1998; Holian *et al.*, 1998) which showed various chemical components of sources induce different biological reactions. For example, the adverse health effects of exposure to 10 $\mu g/m^3$ of residual oil fly ash was greater than those of exposure to the same concentration of volcanic ash, as suggested by Holian *et al.* (1998).

The associations between mortality and ambient pollution in Elizabeth did not show patterns similar to those in Newark and Camden, even though Elizabeth is in close proximity and similar environmental concentrations were observed. We hypothesize that the higher socioeconomic status of the population in Elizabeth may be the reason for the different pollution-mortality results in Elizabeth.

This exploratory study demonstrates that the FA/PR approach using PM chemical speciation provides a means to extract more useful information about relationships between daily mortality and various PM sources than does the conventional use of only total PM mass as an exposure metric. These results indicate that using source-specific exposure metrics composed of various chemical species is an improvement over the use of simpler mass-based PM exposure metrics, a finding that is supported by existing toxicological studies.

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Table 1. Relative risks of increasing 10 $\mu g/m^3$ by traditional approach using total PM mass and [FA/MR]/PR.

	Traditional approach	[FA/MR]/PR		
Newark	$IPM_{total\ mass}$	IPM_{oil}	$IPM_{Zn\text{-}ind}$	$IPM_{sulfate}$
TDD	1.01^{**}	NS	1.03^{**}	1.02^{**}
CRDD	1.02^{**}	NA	NA	1.04^{**}
Camden	$IPM_{total\ mass}$	IPM_{oil}	IPM_{motor}	$IPM_{sulfate}$
TDD	1.02^{*}	1.11^{**}	1.10^{*}	NS
CRDD	1.03^{**}	1.12^{*}	NS	1.02

*: $p < 0.05$ in [FA/MR]/PR.

**: $p < 0.01$ in [FA/MR]/PR.

NS: non-significant in [FA/MR]/PR at $p = 0.10$ level, but that source was significant in FA/PR.

NA: Non-significant sources in FA/PR at $p = 0.10$ level.

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Aerosol Synergism and Hydrate Formation – A Possible Connection

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Abstract

Earlier experiments using guinea pigs and other laboratory animals have shown that aerosols of sodium chloride potentiate the irritant effects of inhaled sulfur dioxide and formaldehyde. This potentiation was not seen with other vapors such as formic acid and acetic acid; nor was it observed when water insoluble aerosols were substituted for the sodium chloride. A simple explanation for these results would be that the potentiated vapors dissolved in the hydroscopic particulates and were thereby carried deeper into the respiratory tract. However such an explanation does not explain the observed difference between potentiated and nonpotentiated vapors. Additionally, the retention time for dissolved vapors is too short to permit the aerosol to transport the dissolved vapor for any appreciable distance. Here it is pointed out that both sulfur dioxide and formaldehyde are hydrated in water solutions to compounds (methylene hydroxide and sulfite, respectively) that have low vapor pressures and are known to decompose only very slowly in water solution. The physical chemistry associated with the formation of these compounds may provide the desired explanation for the observed potential effect of inhaled sodium chloride aerosols.

Aerosol Synergism and Hydrate Formation – A Possible Connection

Introduction

Soon after the highly publicized air pollution episodes in Donora, PA, in 1948 and in London in 1952, there was intense interest in determining how the various components of polluted air, some of which are present in only very low concentrations, interact to produce toxic effects. As both sulfur dioxide (SO_2) and particulate matter were present in these episodes, special importance was given to interactions between these components. A clear statement of this is found in a task force report for the National Institute of Environmental Health Sciences where it is stated "Sulfur dioxide is highly soluble in body fluids. Administered in particle-free air, it is taken up almost entirely by the upper airways (naso and oropharanyx). .... A large fraction of the accumulation mode of ambient aerosols is soluble and may exist as a solution droplet at low to medium relative humidities. These aerosols absorb sulfur dioxide. When inhaled, they probably carry sulfur dioxide and the products of its combustion with water, beyond the upper airways. These products include sulfite and bisulfite ions." (Nelson, N. *et al.*, 1977) Earlier this consideration had important consequences: the EPA Standards for the *Warning, Emergency, and Significant Harm Levels* of air contaminants included not only levels for SO_2 and particulates separately, but also included the product of their concentrations to account for possible interactions. (EPA, 1971)

Evidence from Animal Experiments

Of the many experiments to determine the existence of synergistic effects of inhaled gases and vapors, the most extensive, and perhaps the clearest, set of

experiments were developed by Amdur, in which she and her coworkers measured the pulmonary resistance and compliance of guinea pigs exposed to various combinations aerosols and vapors (Amdur and Mead, 1958; Amdur, 1957, 1960, 1966, 1969; Amdur and Underhill, 1968). Most of this work used well-defined components such as a sodium chloride aerosol of known particle size distribution (rather than "smoke" or lesser well defined components), giving reproducible experiments in which interactive effects between the aerosol and the inhaled vapor could be discovered and quantitated. Inhaled sodium chloride aerosols greatly enhanced the effect of sulfur dioxide on pulmonary resistance. Later a similar effect for sodium chloride aerosols was found with inhaled formaldehyde vapor. In this regard, see Figs. 1 and 2, modified from Amdur, 1957, and Amdur, 1960.

It is known that a sodium chloride aerosol is hydrated in the respiratory track, giving a solution in which the vapor might dissolve. A "simple" explanation for the observed synergistic effect between the inhaled vapor and particulates is that some of the vapor was absorbed on the particulates and transported deep into the laboratory animal's lungs. Additional evidence found to support this transport hypothesis includes:

1. Aerosols that are insoluble in water exhibited no synergistic effect with sulfur dioxide. See Fig. 3, from Amdur and Underhill, 1968.
2. Aerosols of KCl and NH_4SCN gave a greater synergistic effect with SO_2 than did NaCl. Compared to NaCl, KCl is 1.4 and NH_4SCN 2.1 times as effective in potentiating the guinea pigs' response to sulfur dioxide. 3N solutions of

KCl and NH_4SCN dissolve 1.35 and 1.97 times more SO_2 than a 3N solution of NaCl. (Amdur and Underhill, 1968)

Difficulties with the Simple Solution Hypothesis

There has been controversy regarding the ability of particulate matter to transport vapors in human airways. (Friedlander and Yeh, 1996; Wilson, 1995) In particular, difficulties arise in the concept that observed synergistic effects are solely from the solution of the vapor in the moistened particulates. I.e.:

1. The synergistic effect is not universal. See Fig. 4, which demonstrates that in the same guinea pig preparation as used by Amdur for the SO_2 and the HCHO experiments, inhaled sodium chloride had no measurable synergistic effect with inhaled formic acid vapor. Why then did formic acid, which has a lower vapor pressure in water than sulfur dioxide, show no synergistic effects?
2. The retention time in of sulfur dioxide in a droplet is too short to allow the aerosol to retain the dissolved sulfur dioxide or formaldehyde in solution. Assuming that diffusion from the particulate is the rate limiting step in mass transfer, then the fraction of SO_2 remaining at a time, t, will be (Crank, 1977):

$$\frac{M_t}{M_0} = \frac{6}{\pi^2} \sum_{n=1} \exp\left(-\frac{4\pi^2 n^2 Dt}{d^2} \right)$$

where:

M_0 = initial amount of absorbed SO_2

M_t = residual SO_2 at a time, t (same units as M_0)

t = time, s

d = particle diameter, cm

D = diffusion coefficient for dilute SO_2 in water at room temperature

Assuming d = 0.2 μm and D = 1.77×10^{-5} cm^2 s^{-1} (Leaist, 1987), then the time required for loss of half of the absorbed SO_2 is 7×10^{-7} s, which is too short to be important as a transport mechanism through the respiratory tract. This calculation shows that it diffusion is assumed to be the rate-limiting step for mass transfer of vapor from a particulate, then equilibrium is very rapid.

Wexler and Sarangapani (1998) give definitive calculations that if instantaneous equilibrium is assumed, then vapor transport is negligible. The same conclusion can be found through simpler calculations, as follows. Assume a vapor in an aerosol, for which:

D_m = diffusion coefficient of the vapor in air, cm^2 s^{-1}.

D_s = diffusion coefficient of the vapor in the aerosol particulate, cm^2 s^{-1}.

Pc = Air-mucus partition coefficient for the vapor, dimensionless

f = fraction of vapor on the liquid phase, dimensionless

Then the diffusion coefficient for the vapor in the aerosol will be:

$D_a = (1-f)D_m + fD_s$

And the partition coefficient (P_{c-a}) for the vapor where the two phases are aerosol vs. mucus is:

$$P_{c-a} = \frac{P_c}{1-f}.$$

With the values usually assumed for f (*i.e.*, <0.1), the partition coefficient and diffusion coefficient for the vapor in the aerosol are very close to those in air, leading to the conclusion that the dynamics of adsorption of the vapor will be

essentially the same with inhaled air as with inhaled aerosol. It is noted that this calculation assumes instantaneous equilibrium between the vapor phase and the particulates.

Methylene Hydroxide Formation as a Mechanism for the Retention of Formaldehyde on Aerosol Particulates

As a possible mechanism for the physical entrainment of formaldehyde as an aqueous solution by aerosols, we suggest that it is retained as a slowly decomposing hydrate (methylene hydroxide) and offer as evidence the following

1. Formaldehyde, in dilute water solution, exists primarily (*i.e.*, about 99.9%) as its hydrate. The reaction with water is: $CH_2O + H_2O \Leftrightarrow CH_2(OH)_2$
2. There are many measurements of the rate constants of chemical reactions of dissolved formaldehyde, in which the rate-controlling step is the dehydration of methylene glycol. These many experiments are in agreement that at room temperature the half-life for methylene glycol at room temperature in a dilute aqueous solution is about 2.4 minutes. (Le Hénaff, 1963)
3. The vapor pressure of formaldehyde over its dilute water solution is thought to be due to the small fraction of formaldehyde that remains unhydrated and unconverted to the glycol. This is not unreasonable, as the vapor pressure of glycols (as a result of the hydrogen bonding of their two "-OH" groups) in dilute aqueous solutions are very low.
4. There is experimental evidence that the dehydration of methylene hydroxide can be a rate-limiting factor in the volatilization of formaldehyde from aqueous solutions. Ledbury and Blair (1925), as cited by Walker (1963), were unable

to obtain "satisfactory partial pressure values for formaldehyde solutions at low temperatures with scrubbing rates that proved satisfactory at ordinary temperatures. At these temperatures the rate of dehydration [of methylene glycol] was too slow to allow the attainment of equilibrium and the scrubbing rate had to be lowered until equilibrium was the controlling factor". The effect of this rate limiting dehydration step could be far more pronounced in other situations - such as the volatilization from a submicrometer aerosol, where otherwise the rate of removal might be a matter of a hundredth or a thousandth of a second.

Sulfite Formation as a Mechanism for the Retention of Sulfur Dioxide on Aerosol Particulates

There is evidence for a similar rate limiting dehydration step for sulfur dioxide dissolved in aqueous solutions. Here the reactions are:

$$HSO_3^- + H^+ \xrightarrow{k_1} SO_2 + H_2O$$

$$HSO_3^- \xrightarrow{k_2} SO_2 + OH^-$$

In the above equations, the constants, k_1 and k_2 are the reaction constants for the formation of hydrated sulfur dioxide from dissolved sulfite. Note that both these reactions bypass the formation of sulfurous acid; there is, despite what many chemists still may believe, very strong spectrographic evidence that un-ionized H_2SO_3 is not present in aqueous solutions of SO_2 (Ley and König, 1938).

The question here is the speed of the conversion of sulfite ion to sulfur dioxide. Wang (1963) showed that this reaction rate is sufficiently slow to be measured in terms of minutes. His procedure was to inject a tracer in the form of

$NaH^{35}SO_3$ into a solution containing $^{32}SO_2$, H_2O, and $NaH^{32}SO_3$, which had been allowed to reach equilibrium under, controlled conditions of temperature, pH, and pressure. At intervals he flashed the SO_2 out of samples of this solution. The ratio of $^{35}SO_2$ to the total SO_2 gave the transfer of ^{35}S between the sulfite and the dissolved SO_2. At the time of initial transfer, none of the ^{35}S was present as sulfite; at equilibrium, there is equal distribution of ^{35}S and ^{32}S between the sulfite ion and SO_2. Fig. 5 shows the results from one such experiment. There the ratio of $^{35}S/^{32}S$, to the equilibrium ratio of $^{35}S/^{32}S$, as a function of time is plotted on a semi-logarithmic plot. The important factor to note in this plot, as well in his other data, is that the half-life for this transfer is nearly a minute. The relevance of this experiment is that the rate constant is measured in tens of seconds; whereas the time required to transport an aerosol from the ambient atmosphere to the alveoli is measured in seconds or a fraction of a second. The slow conversion of the sulfite ion to dissolved sulfur dioxide gives a plausible mechanism for the transport of SO_2 as sulfite on hygroscopic aerosols.

Formic Acid

Indirect evidence for the hydration hypothesis is that, as far as known, the rate constant, k, for the reaction between formate ion and the hydronium ion

$$COOH^- + H^+ \xrightarrow{k} HCOOH$$

is so rapid that a case cannot be made for this reaction being a rate-limiting step. In this regard, Delahay and Vielstich (1955), using classical polarographic techniques, found very rapid rate constants for both the association and dissociation of formic acid in aqueous solution.

Experimental Evidence for Aerosol Transport

Ichioka (1972) used a series of tubes 9 mm in diameter, 100 ml long, lined internally with moistened filter paper to model the respiratory tract. Through this system he passed dilute SO_2 alone and plus a NaCl aerosol. The SO_2 in the effluent from these wetted-wall tubes was collected in a bubbler. In reviewing these experiments as published, a major difficulty arises in that mass transfer calculations show that the total of SO_2 passing his system differs by factors between 3 and 5 from the amount of SO_2 absorbed on the wetted walls and in the bubbler. But even though this experiment can be severely criticized, the percent of the SO_2 passing through the wetted-wall tubes was less than 3% in the absence of a NaCl aerosol; adding a NaCl aerosol increased this fraction to 12%. This study, even though the mass transfer balance is flawed, seems to give credence to the hypothesis that the presence of a NaCl aerosol increases the transport of SO_2 through systems such as the respiratory tract.

Proposals for Future Research

The purpose of this note is to give plausible mechanisms for the transport of vapors by aerosols. Should the chemical reactions described here – the dehydration of methylene hydroxide and the neutralization and dehydration of sulfite ion – prove to be valid mechanisms for the retention of HCHO and SO_2 on hydroscopic particles, then tests with laboratory animals could be used to determine the importance of this means of transport. For example, the unattached HCHO or SO_2 could be stripped from the gaseous phase, and

the irritancy of the compounds attached to aerosol particulates measured directly in tests with laboratory animals or human subjects. The known rate of dehydration may also prove helpful in establishing whether the experiments carried out with guinea pigs, with their much smaller respiratory tract has much relevance to human exposures. In any case, the physical chemistry for the mechanisms proposed here has been in the literature for a number of years. The purpose of this note is to spur research to determine if aerosol physicists and pulmonary toxicologists will find this information useful in trying to understand aerosol transport of absorbed vapors and its relevance to the still important problem of air pollution.

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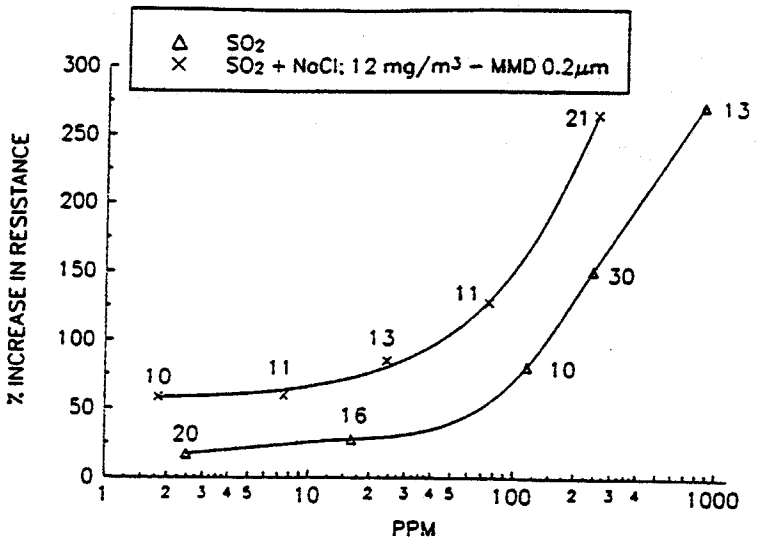

Figure 1: Dose-response curves for guinea pigs exposed to sulfur dioxide and to sulfur dioxide plus a sodium chloride aerosol. The number beside each point represents the number of animals exposed at each concentration (Results from Amdur, 1957)

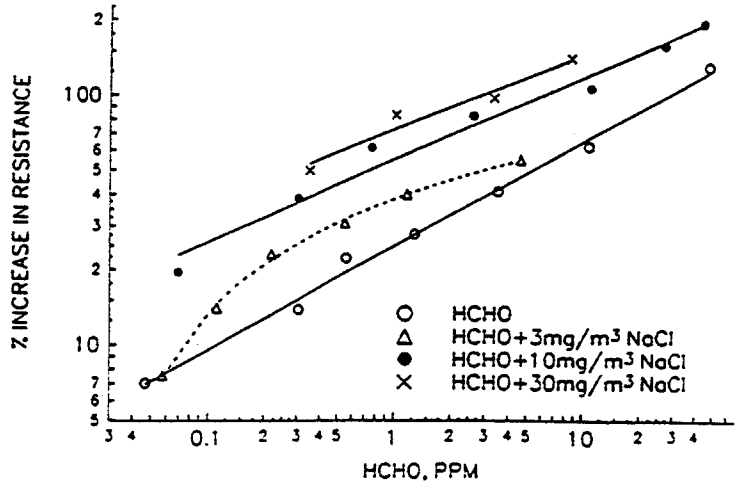

Figure 2: Dose response curve for guinea pigs exposed to formaldehyde and to a combination of formaldehyde and a sodium chloride aerosol (Results from Amdur, 1960)

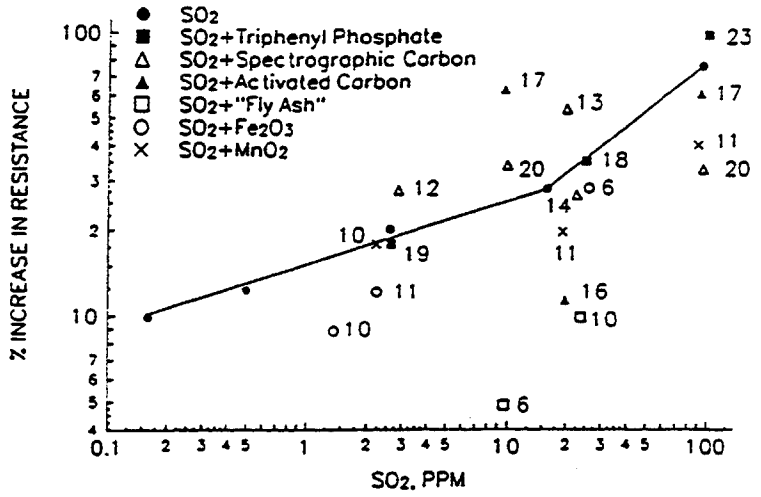

Figure 3. Response of guinea pigs to combination of insoluble aerosols and sulfur dioxide. Number beside each point indicates number of animals in each group. Results from Amdur and Underhill, 1968.

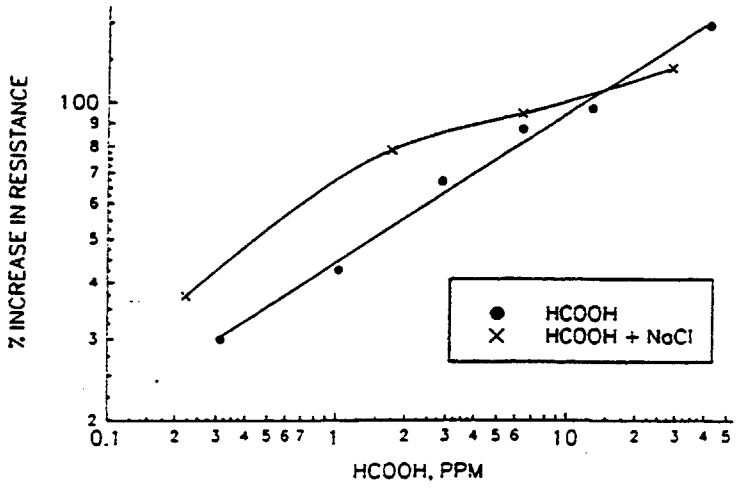

Figure 4. Dose-response curves for formic acid alone and plus 10 mg/m^2 sodium chloride. The percent increase in resistance at the end of 1-hour exposure is the criterion of response and the concentration is expressed as ppm. Results from Amdur, 1960

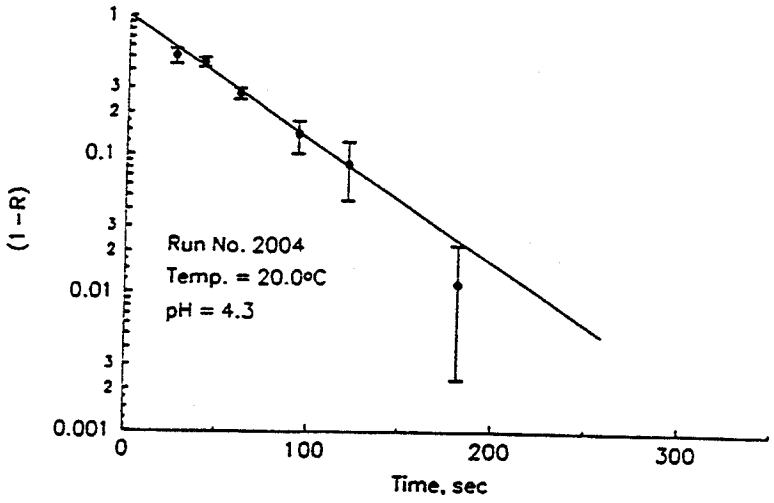

Figure 5. Equilibrium between labeled and stable sulfite ion in aqueous solution. . The X-axis gives equilibrium time in seconds. The factor, R, in the Y-axis gives the ratio of ^{35}S in the counting gas at time, t, to that at isotopic equilibrium. Results from Wang, 1963

Lack Of Concordance Between Reported Lung-Cancer Risk Levels And Occupation-Specific Diesel-Exhaust Exposure

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For several occupational groups, we compared information on the reported lung-cancer risk with estimated diesel-exhaust concentrations. Although none of the epidemiologic studies had concurrent measurements of diesel-exhaust concentrations, such data are available from more contemporary studies. Measurements of particle concentrations yield three, overlapping "order-of-magnitude" groups:

Truck drivers, dock workers, railroad workers (excl. shop workers, hostlers)	*5 – 100 $\mu g/m^3$*
Bus garage workers, railroad shop workers & hostlers	*50 – 700 $\mu g/m^3$*
Underground miners	*500 – 2,000 $\mu g/m^3$*

Bhatia et al. (1998) conducted a meta-analysis of lung-cancer risk with occupational exposure to diesel exhaust and found an overall meta-analysis relative risk (RR) value of 1.33, with a range of 1.11 to 1.49 in the subanalysis by occupation. If diesel exhaust were causally increasing lung-cancer risk by 50% for low exposure occupations (e.g. truck drivers, RR = 1.49), then the lung-cancer risk in a more heavily exposed population (e.g. railroad shop workers) should be much higher; however, the shop workers experienced a RR around 1.0 (Crump, 1999; HEI, 1999). Similarly, the added lung-cancer risk for bus garage workers (RR = 1.24) is half that of truck drivers, but diesel-exhaust concentrations are considerably higher in garage workers. There is an approximately two-orders-of-magnitude difference in potential diesel-exhaust exposure, yet, the epidemiologic relative risks cluster in a narrow range. Such a lack of concordance between reported lung-cancer risk and estimated exposure argues against a causal role for diesel exhaust in the epidemiologic associations.

Some epidemiologic studies of occupational groups show associations between surrogates of diesel-exhaust exposure and increased lung cancer, but causality has not been established. Causality would be supported by finding a linear relationship between diesel-exhaust exposure and reported lung-cancer risk. Thus, one can ask the question: Is there an exposure / response relationship across occupations between diesel-exhaust exposure and lung-cancer risk?

Methods of Analysis

We compiled information on the reported lung-cancer risk and on the estimated diesel-exhaust concentrations for various occupations.

Lung-Cancer Risk: For the meta-analysis results, we utilized Bhatia *et al.* (1998). For risk estimates from individual studies, we relied on the reviews by Bhatia *et al.* (1998), Cohen and Higgins (1995), California Environmental Protection Agency (CalEPA) (1997), Crump (1999),

and the Health Effects Institute (HEI) (1999). In those studies with subanalyses by age and/or duration of employment, the risk values for the highest category were usually given by the reviewers. For our analysis, we excluded studies published before 1980 and those studies in which diesel-exhaust exposure was ambiguous (for example, mixed or obscure job categories). Otherwise, we did not screen the studies for overall quality. Risk estimates were available for bus garage workers, dock workers, heavy equipment operators, railroad workers, truck drivers, and underground miners. For miners, we only used RR values for underground miners, because particle concentrations are so much higher underground than at the surface.

Diesel-Exhaust Concentrations: None of the epidemiologic studies had available concurrent measurements of diesel-exhaust concentrations. Rather, investigators used union records, interviews, questionnaires, and death-certificate information to assess indirectly the potential for diesel-exhaust exposure. However, data on diesel-exhaust concentrations in occupational settings are available for a later time period than when the actual exposures occurred for the worker populations in the epidemiologic studies. Thus, available information on diesel-exhaust concentrations for these occupations derives from more contemporary studies, but the relative ranking of the diesel-particle concentrations is likely to have remained constant. For data on diesel-particle concentrations we used Woskie *et al.* (1988), U.S. National Institute for Occupational Safety and Health (USNIOSH) (1990a, 1990b), Bagley *et al.*, (1990), Rubow *et al.*, (1990), Zaebst *et al.* (1991), Watts *et al.*, (1992), Watts (1995), and World Health Organization (WHO) (1996). In some studies, particle data were given as elemental carbon, which represents approximately 50% of the diesel-particulate mass. Thus, for our exposure/response analysis, we doubled those particle-concentration values given as elemental carbon.

Results

Table 1 summarizes the lung-cancer risk reported by various authors. The Bhatia *et al.* (1998) summary meta-analysis RR value is 1.33 for all occupations. In the subanalysis by occupation, the investigators reported a range of 1.11 to 1.49. Table 2 summarizes diesel-exhaust concentrations for various occupations. Particle-concentration measurements yield three, overlapping "order-of-magnitude" groups:

Truck drivers, dock workers, railroad workers (excl. shop workers, hostlers)	5 – 100 $\mu g/m^3$
Bus garage workers, railroad shop workers & hostlers	50 – 700 $\mu g/m^3$
Underground miners	500 – 2,000 $\mu g/m^3$

Figures 1 and 2 plot relative risk (+/- 95% CI) and diesel-exhaust concentrations (+/- range) for various occupational groups. Because of the large overall range of diesel-exhaust concentrations, we plotted these values on a logarithmic axis. A trend of increasing risk with increasing exposure to diesel exhaust is not apparent. For example, for a low-exposure occupation (*e.g.* truck drivers), the lung-cancer risk is increased 50% (RR = 1.49). The lung-cancer risk in a more heavily exposed population (*e.g.* railroad shop workers) should be much higher; however, the shop workers experienced a RR around 1.0 [RR = 1.08 from Crump (1999) and 0.8 from HEI (1999)]. Similarly, the added lung-cancer risk for bus garage workers (RR =

1.24) is half that of truck drivers, but diesel-exhaust concentrations are considerably higher for the garage workers.

Table 1. Reported Lung-Cancer Risk for Various Occupations

Occupation	Meta-Analysis Relative Risk [from Bhatia *et al.*, 1998] (*95% CI*)	Reported Relative Risk for Lung-Cancer [RR, SMR, OR] (*95% CI*)	Reference
Bus Garage Workers	1.24 (*0.93 – 1.64*)	0.90 (*0.77 – 1.04*) 1.01 (*0.82 – 1.22*) 1.22 (*0.71 – 1.96*) 2.43 (*1.32 – 4.47*)	Waller, 1981 Rushton *et al.*, 1983 Gustavsson *et al.*, 1990 Gustavsson *et al.*, 1990
Dock workers/Stevedores	not given	1.32 (*1.05 – 1.66*)	Gustafsson *et al.*, 1986
Heavy Equipment Operators	1.11 (*0.89 – 1.38*)	1.07 (*0.92 – 1.25*) 2.60 (*1.12 – 6.06*) 2.10 (*0.60 – 3.10*)	Wong *et al.*, 1985 Boffetta *et al.*, 1988 Hayes *et al.*, 1989
Railroad Workers (all)	1.44 (*1.30 – 1.60*)	1.35 (*1.20 – 1.52*) 1.41 (*1.06 – 1.88*) 1.72 (*1.27 – 2.33*) 1.59 (*0.94 – 2.69*) 2.46 (*1.24 – 4.87*)	Howe *et al.*, 1983 Garshick *et al.*, 1987 Garshick *et al.*, 1988 Boffetta *et al.*, 1988 Swanson *et al.*, 1993
Railroad Workers (excluding shop workers, hostlers)	not given	1.82 (*1.30 – 2.55*)	Garshick *et al.*, 1988 as used by CalEPA, 1997
Railroad Workers (excluding shop workers)	not given	0.7 (*0.6 - 0.9*)[a]	Garshick *et al.*, 1988 as calculated in HEI, 1999
Railroad Shop Workers	not given	1.08 0.8 (*0.6 - 1.1*) (a)	Garshick *et al.*, 1988 as given in Crump, 1999 Garshick *et al.*, 1988 as calculated in HEI, 1999
Truck Drivers	1.49 (*1.36 – 1.65*)	1.30 (*1.10 – 1.60*) 1.24 (*0.93 – 1.66*) 1.50 (*1.10 – 2.00*) 1.27 (*0.83 – 1.93*) 1.60 (*1.26 – 2.00*) 2.44 (*1.43 – 4.16*)	Ahlberg *et al.*, 1981 Boffetta *et al.*, 1988 Hayes *et al.*, 1989 Steenland *et al.*, 1990 Hansen, 1993 Swanson *et al.*, 1993
Underground Miners	not given	2.10 (*1.10 – 3.70*) 1.45 (*0.74 – 2.58*)	Lerchen *et al.*, 1987 Ahlman *et al.*, 1991

(a) Relative Risk per 10 years of exposure.

TABLE 2. Diesel-Exhaust Concentrations for Various Occupations

Occupation	Particle Concentration ($\mu g/m^3$)	Reference
Bus Garage Workers	14 – 326 [a] 220 – 370 [b] 10 – 730 [c]	USNIOSH, 1990a, as given in WHO (1996) Blome *et al.*, 1990, as given in WHO (1996) Gamble *et al.*, 1987, as given in WHO (1996)
Dock workers / Stevedores	13.8 [d] 24 [d]	Zaebst *et al.*, 1991 USNIOSH, 1990b
Heavy Equipment Operators	no occupation-specific exposure data are available	
Railroad Workers	engineer/firer 39 – 73 [e] braker/conductor 52 – 92 [e] shop worker, hostler 114 – 191 [e]	Woskie *et al.*, 1988
Truck Drivers	3.8 [d]	Zaebst *et al.*, 1991
Underground Miners	900 – 1,900 [f] 670 – 1,430 [g] 650 – 1,020 [h] 560 – 840 [i] 830 – 1,740 [j]	Bagley *et al.*, 1990 Watts *et al.*, 1992 Rubow *et al.*, 1990 Ambs *et al.*, 1991, as given in Watts (1995) Ambs *et al.*, 1994, as given in Watts (1995)

[a] Elemental carbon (actual diesel-particle concentration is two times this value)
[b] Incinerable fine dust
[c] Respirable dust
[d] Geometric mean elemental carbon (actual diesel-particle concentration is two times this value)
[e] Geometric mean of respirable particulate corrected for cigarette smoke but not for non-diesel particles. Range represents values for job sub-categories.
[f] Mean "diesel particulate matter" in haulageway. Range represents values from three different mines.
[g] Mean "diesel particulate matter" from five mines. Range represents values from three locations (haulageway, shuttle car, return).
[h] Mean " $< 0.8\ \mu m$ particle mass". Range represents values from three locations (haulageway, shuttle car, return) in two different mines.
[i] Mean "diesel particulate matter". Range represents values from two locations (haulageway and return).
[j] Mean "diesel particulate matter" in haulageway. Range represents values from two mines.

Our analysis was not intended to provide a quantitative risk estimate for diesel exhaust, because of uncertainties both in exposure and outcome. The currently available measurements of diesel-exhaust concentrations are valuable sources of data, but are problematic when applied retrospectively to estimate exposure. The HEI report (1999) discusses problems associated with

the various measures of diesel-exhaust exposure, specifically for the railroad workers and truckers. Likewise, our selection of epidemiologic studies should not be interpreted as an endorsement that these results reflect the effects of diesel-exhaust exposure. Several critical reviews (Muscat and Wydner, 1995; Stober and Abel, 1996; Cox, 1997; Morgan *et al.*, 1997; Stober *et al.*, 1998) have noted problems of bias, misclassification, residual confounding, and reliance on multiple comparisons.

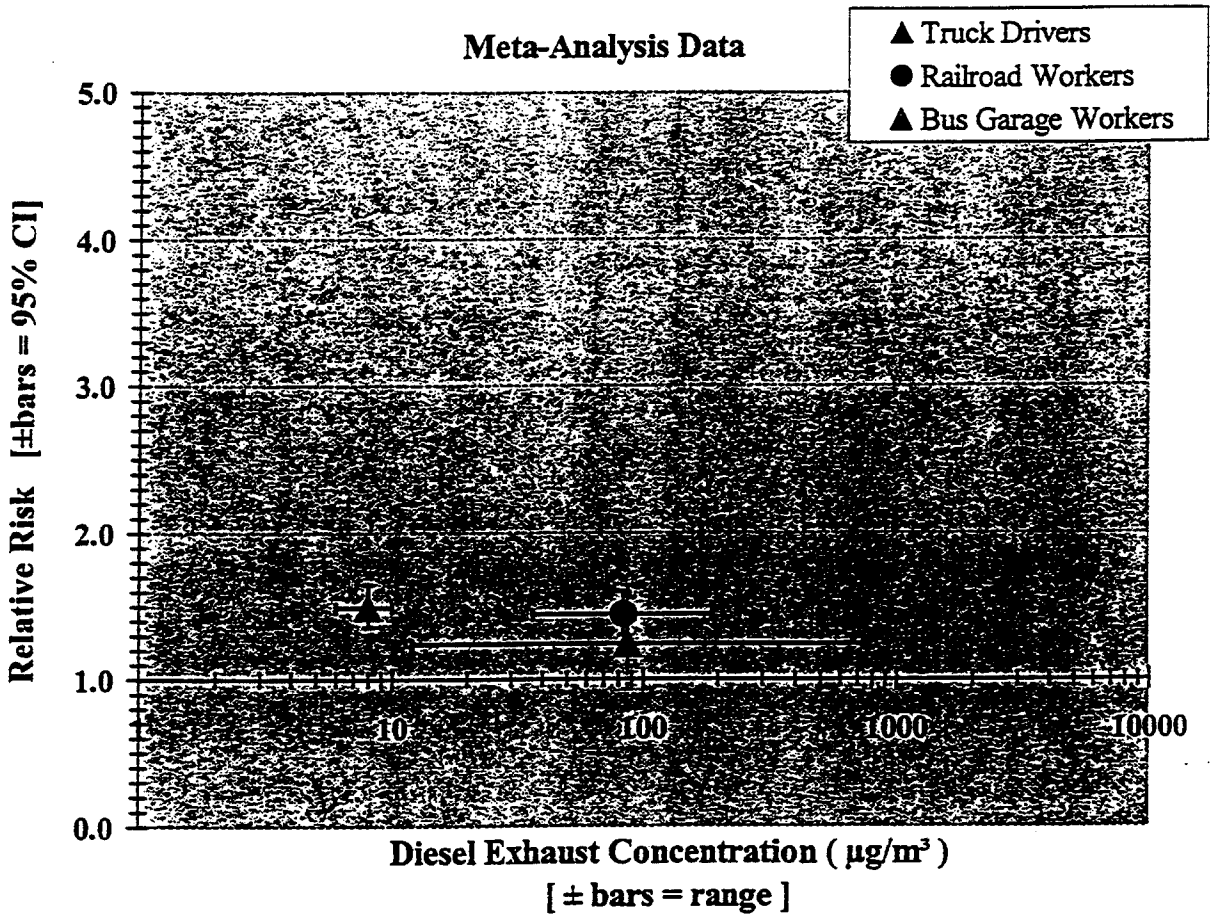

Figure 1: A plot of meta-analysis (Bhatia *et al.*, 1998) relative risk for lung cancer (RR from Table 1, +/ 95% CI) *versus* diesel-exhaust concentration (from Table 2, +/ range) for three occupational groups. Estimated diesel-exhaust concentrations is plotted on a logarithmic axis. Reported risk does not increase with increasing diesel exhaust exposure concentration.

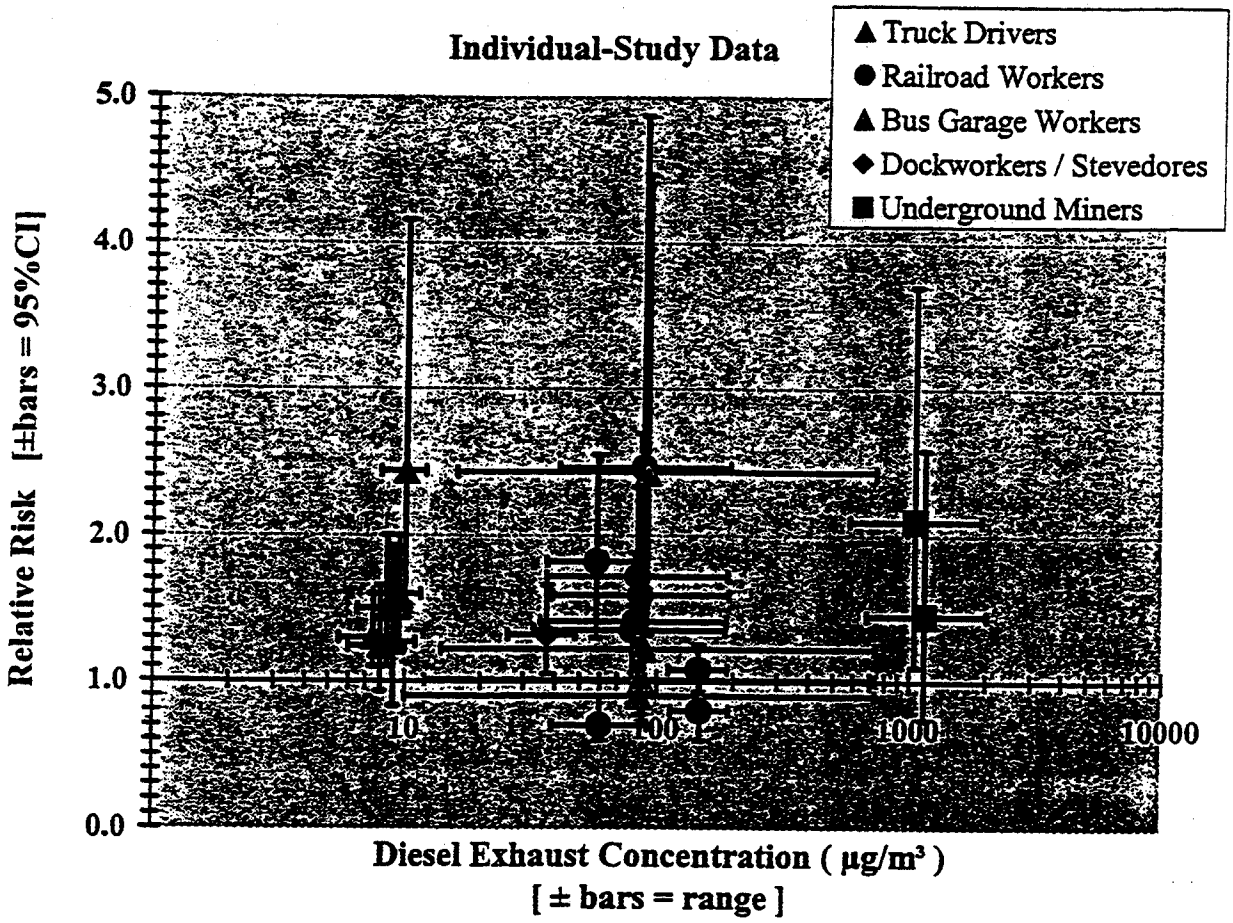

Figure 2: A plot of individual-study relative risk for lung cancer (RR from Table 1, +/ 95% CI) *versus* diesel-exhaust concentration (from Table 2, +/ range) for five occupational groups. Estimated diesel-exhaust concentrations is plotted on a logarithmic axis. Reported risk does not increase with increasing diesel exhaust exposure concentration.

Conclusions

Among different occupations, there is an approximately two-orders-of-magnitude difference in potential diesel-exhaust particle exposure, yet, the epidemiologic relative risks cluster in a narrow range. There is a lack of concordance between the level of reported lung-cancer risk and diesel-exhaust particle concentrations. Such a lack of concordance is not supportive of a cause-and-effect relationship. Although the data indicate that diesel-exhaust exposure by occupation span a far greater spectrum of values than do occupation-specific risk estimates, the interpretation of this lack of concordance remains uncertain because of the lack of concurrent exposure information.

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VI. MECHANISM-RELATED PAPERS (SESSION 3)

Polycyclic Aromatic Hydrocarbons And Mutagenic Activity In Bacteria And Human Cells In Culture Of Organic Extracts From Santiago, Chile, Respirable And Total Suspended Particulate Matter.

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The air in Santiago, Chile, is among the most highly polluted in the world. Due to the high levels of pollutants and the high incidence of respiratory diseases, specially in the most susceptible groups, Santiago has been declared a saturated zone for PM 10, 03, and CO. The levels of the 16 polycyclic aromatic hydrocarbons (PAHs) were determined by HPLC in organic extracts from respirable particles (OERP). Respirable particulate matter (fine and coarse) contains high levels of PAHs including six classified by IARC as carcinogenic, which represented at least 45 % of total PAH concentrations. A seasonal effect was observed with higher values in months with lower temperatures. Although a strong declining of PAH levels on OERP were observed in the last years, the levels of carcinogenic PAHs are still higher than those reported in cities of USA, Australia and Europe. OERP were highly mutagenic and contained direct and indirect mutagens which produce both frameshift and base substitution mutations in Salmonella thyphimurium. In addition, organic extracts from total suspended particles were also highly mutagenic at the tk locus in hlAlv2 human lymphoblasts in culture. In spite of the important decrease in PAHs in the period 1991-1996, direct mutagenic response has not changed significantly, suggesting that the levels of direct mutagenic pollutants (e.g nitroarenes) have not decreased considerably during the last years. These results suggest a high risk for Santiago's inhabitants since pollutants adsorbed in respirable particles are highly mutagenic and can damage DNA.

Santiago, Chile, with approximately 5.8 million inhabitants (almost 38% of the country's population), has spread out widely over a valley located 600 meters above sea level. It occupies an area of a hundred thousand hectars and is the city with the greatest atmospheric pollution levels in the country and one of the most air polluted cities of the Americas (Gil et al., 1993). Various factors contribute to the high atmospheric pollution levels that are reached. The city is built in a valley surrounded by high mountains, having poor ventilation that hinders the natural dispersion of the pollutants. Santiago is located in a zone of atmospheric stability, characterized by very little wind and rain (less than 300 mm a year), and a high content of suspended dust, particles and smog. In winter a thermal invertion layer is formed 600 and 900 meters above the city that can last for several days and contribute to diminish pollutant dispersion. Sometimes this layer can be as low as 150 m, between June and August (winter season), the levels of PM_{10} often surpass the Chilean 24 hour standard of 150 ug/m^3, reaching sometimes levels of 240-300 ug/m^3. There are over 3,200 fixed and 700,000 mobile sources. These include 9,000 buses and 37,000 trucks, running with diesel engines usually emitting high levels of particulates. Diesel fuel is usually of poor quality with a higher content of sulfur than similar fuel in developed countries. In addition, most of these engines are not well maintained.

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The above sources are responsible for the high levels of respirable particulate matter (PM_{10}), carbon monoxide (CO) and ozone(O_3), pollutants that surpass the corresponding air quality standards during many days of the year. In 1996 the Chilean Government declared Santiago as a saturated pollution zone for O_3, CO and PM_{10} and a latent zone for nitrogen dioxide. More recently (1997) a Plan to Prevent Air Pollution has been formulated. According to CONAMA (Chilean Environmental National Commission) its application in the next 14 years would avoid 10,994 premature deaths and more than 65 million hospital attentions for respiratory and cardiovascular diseases (CONAMA, 1997).

Air pollution in Santiago is a major public health problem and is the main cause of the high incidence of acute and chronic respiratory diseases, mainly affecting children and elderly people. Ostro et al., 1996, using data from Santiago, reported an association between PM_{10} and daily mortality from either respiratory or cardiovascular disease. They concluded that a 10 g/m^3 change in daily PM_{10} was associated with 1% increase in mortality. Faiz et al.,1995, have suggested that particles and O_3 may cause 1,546 premature deaths a year in Santiago.

Polycyclic Aromatic Hydrocarbons *(PAHs)* and their derivatives are air pollutants with a high risk to human health, as a susbtantial number of these compounds exhibit carcinogenic activity in experimental animals and therefore have a high probability of causing the same effect in humans (IARC,1983, IPCS,1998). PAHs are generated by the incomplete combustion of fuels such as petrol, diesel, oil, coal, and biomass. The principal sources of PAH emissions are vehicles, domestic heating, refuse burning, industrial activities and tobacco smoking. The presence of PAHs in the urban atmosphere represents a health risk due to the fact that there are millions of persons exposed and that exposure is permanent. The reactions of PAHs with nitrogen oxides are of great importance since both are emitted simultaneously from the same sources and these reactions can result in the conversion of inactive PAHs to nitroarenes, compounds of potent carcinogenic activity (Pitts et.al.,1985). Nitroarenes can also be formed by photochemical reactions under ambient conditions. Previous studies with Santiago's organic extracts from total suspended particles (TSP) have shown that these extracts contain high levels of PAHs and are highly mutagenic to Salmonella thyphimurium (Adonis and Gil,1993; Gil and Adonis, 1996).

The aim of this work was to investigate PAH levels and mutagenic activity of Santiago's fine and coarse fractions of respirable particles.

Methods

Respirable particulate matter PM_{10}. was collected daily in one of the stations of the monitoring net system stations (MACAM). A Dicotomic sampler (Sierra Andersen 244) placed at a height of 2 m above the ground, collected particles onto Teflon filters (37 mm) in Station B (Plaza Italia) in downtown Santiago. This station is representative of high vehicular emissions. The sampler allowed the collection of 2.5 m (fine fraction) and 2.5 – 10 m (coarse fraction). The volume collected was around 24 m^3 / day. Filter extractions, sampling preparation and cleaning as well as HPLC analytical procedure was performed as previously described (Adonis and Gil,1993; Gil and Adonis 1996).

Total suspended particles. were collected as described previously (Adonis and Gil. 1993).

Polycyclic Aromatic Hydrocarbons Analysis. PAHs were determined by ultraviolet detection at 254 nm and by fluorescence (260-300 nm excitation and 385-455 nm emission) with the specific wavelengths depending on the individual PAH. The identification of the PAHs contained in the samples was carried out by comparison of their retention times with those in a standard commercial mixture of PAHs as well as by the spectra of standards and samples, obtained using a Merck-Hitachi model L-4500 diode array detector equipped with a Chem Station DAD Manager 650 software. The PAHs identified and quantified were naphthalene, acenaphthene, acenaphthylene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene, benzo(g,h,i)perylene, and indeno(1,2,3-cd)pyrene. Total PAHs in the text refers to the determination of 16 PAHs. Standard compounds were purchased from Supelco, Inc.

Mutagenicity Assays. The Salmonella/microsome plate incorporation assay was performed using the Ames test (Ames et al., 1975) in the presence and absence of Aroclor 1254 induced Sprague-Dawley rat liver S9 mix, as described in Kado et al., 1986. All mutagenicity testing was accomplished using the frameshift tester strain TA98 of Salmonella thyphimurium.

Human Cell Mutagenicity Assay. Airborne particles were suspended in DMSO and mutagenicity was measured at the thymidine kinase (tk) locus in h1A1v2 cells (developed by GENTEST) after 72 hour exposure, as described previously (Hannigan et al.,1997).

The positive control was 1.0 ug/ml benzo(a)pyrene. The observed mutant fractions (MF) for the positive and negative controls were within the acceptable range in this assay (227 $\pm$14.7 positive control and 25.4 $\pm$7.15 historical negative control).

Results And Discussion

Polycyclic Aromatic Hydrocarbons adsorbed onto PM_{10}. Levels of total and carcinogenic PAHs adsorbed onto PM_{10} are shown in Figure 1. This figure shows that a seasonal effect predominated between April and July for both total and carcinogenic PAHs. Concentrations of the 16 PAHs determined over the cold season were higher than for the period of higher temperatures by a factor of at least 4. These approximate ratios of cold/hot season in urban air are similar to those reported in Europe, USA and Australia. The higher mean PAH concentrations in winter compared to spring and summer is explained by an increase in domestic heating, traffic congestion along with meteorological conditions which are less favourable for pollutant dispersion (thermal inversion layer). On the other hand, during summer physico-chemical and meteorological factors may significantly affect the atmospheric degradation of some of the reactive PAHs and lead to a greater loss rate. In addition, during January and February a considerable percentage of the total number of cars leave Santiago and industrial and commercial activity usually decreases. Thus, the relative proportions of individual PAHs differ significantly between the cold and hot season, possibly reflecting changes in PAH emission sources and atmospheric conditions.

The highest concentrations for total and carcinogenic PAHs were obtained in July: 85.3 ng/m^3 and 58.9 ng/m^3 respectively, and the levels of carcinogenic PAHs were 69% of the total 16 PAHs. The levels of 6 carcinogenic PAHs between March and August represented at least over 45% of total PAHs, and this includes: benzo(a)anthracene,

dibenzo(a,h)anthracene, benzo(b)fluoranthene, benzo(k)fluor-anthene, indeno(1,2,3-cd)pyrene and benzo(a)pyrene (BaP).

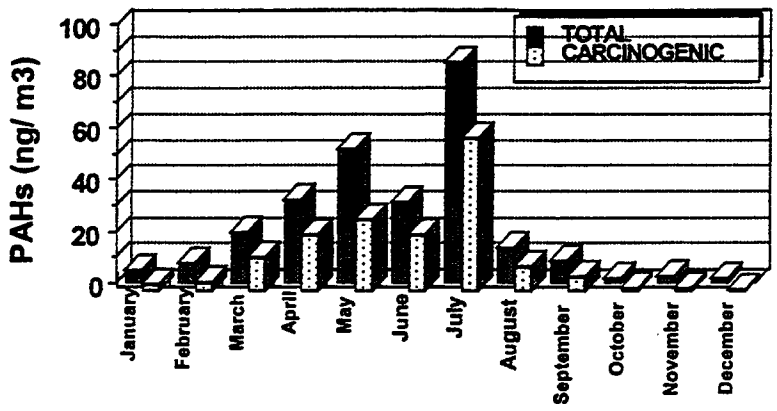

Figure 1. Levels of total and carcinogenic PAHs adsorbed onto PM_{10}. Coarse and fine fractions collected in the period January to December 1996 in downtown Santiago were separatly analysed once and added together to yield the above data. The values for each month represent the average of two organic extracts from 60-62 filters collected daily for coarse and fine fraction.

The annual average concentration in station B during 1996 was 22 ng/m^3 for total PAHs and 14 ng/m^3 for carcinogenic PAHs. The carcinogenic PAH levels in Santiago, specially BaP, are considerably higher than those reported for California, USA (Flessel et al., 1991), Brisbane, Australia (Müller et al., 1998), Pavia, Italy (Minoia et al., 1997), Birmingham, England (Smith and Harrison,1996) and Brescia, Italy (Monarca et al., 1997). Between May-August of 1996, the BaP average concentration for PM_{10} was 4.9 ng/m^3 whereas for other carcinogenic PAHs they were: 4.8 and 4.5 ng/m^3 for benzo(a)anthracene and dibenzo(a,h)anthracene, 4.3 ng/m^3 benzo(b)fluoranthene, benzo(k)fluoranthene and 6.7 ng/m^3 for indene(1,2,3-cd)pyrene. BaP has been regarded as the compound with the most important consequences for human health because it is a potent PAH carcinogen classified in group 2A (IARC, 1983). No Chilean or international standard exists for BaP or any other carcinogenic PAH since the World Health Organization has stated that there is no safe level for human exposure to carcinogenic compounds. Only recently Italy has stablished a standard for BaP of 1 ng/m^3 (Minoia et al.,1997). The levels of BaP in Santiago, although declining are still higher than 1 ng/m^3.

The distribution of total 16 PAHs in the fine and in the coarse fraction, in the period January-December 1996 is presented in Figure 2. It can be observed that the greater concentrations of total PAHs are found in the coarse fraction specially in the period March-August. The coarse fraction accounts for over 75% of the concentration of total and carcinogenic PAHs in respirable particles. The higher concentration of PAHs in the coarse fraction was rather surprising since previous reports have shown higher PAH concentrations are in the fine fraction (Monarca et al., 1997; Schnelle et al., 1996). Our results might suggest that in Santiago, high particle concentration and physico-chemical or atmospheric

conditions might favour fine particle condensation, specially in months with lower temperature and poor ventilation. Pistikopoulos et al., (1990) have described continuous particle size increase by condensation and redistribution of PAHs adsorbed on fine particles onto larger particles. The fact that this effect is not seen in month with higher temperatures might be due to the fact that the condensation mechanism depends on the fine particle concentration in the atmosphere and this is fairly low during the hot season.

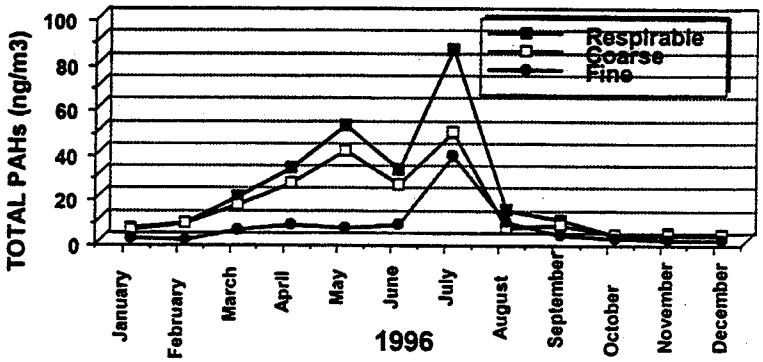

Figure 2. Distribution of total 16 PAHs in the fine and in the coarse fraction, in the period January-December 1996. Coarse and fine fraction (Plaza Italia station B) were separately analysed once and added together to yield the above data, constituting respirable particulate matter. Data for each month are the average of 2 organic extracts derived from 60-62 filters collected daily for coarse and fine fractions.

In spite of the high PAH levels found in this work, this might not be representative of the total levels of PAHs present in the air. PAHs of low molecular weight have a high vapour pressure and might be volatilised, specially in summer, or might pass through the filters in the vapour phase. In addition, since the samples were collected over 24 h, it cannot be excluded that reactions between PAHs and polar compounds such as nitrogen oxides, sulphur dioxide and with transient intermediates such as hydroxyl radicals and singlet molecular oxygen might decrease their concentrations. Such chemical or photochemical reactions occur mainly during atmospheric transport, but might also occur during sampling. Since gas samples were not collected in this study, underestimation of 2-3 ring PAHs is likely, but volatility is unlikely to significantly affect the measurement of 4 or greater ring PAHs, which are the compounds of greatest concern from the point of view of mutagenicity and carcinogenicity (IARC, 1986).

Seasonal variation and size distribution of mutagenic activity from respirable particles in Salmonella typhymurium. Seasonal variation for mutagenic activity of organic extracts from respirable particles (coarse fraction) in Salmonella thyphimurium TA98 strain is shown in Figure 3. Almost similarly to total and carcinogenic PAH levels, mutagenic activity was higher from April to August. The seasonal effect is clearly appreciated when the assay was done either in the presence or in the absence of activation fraction, suggesting that both direct and indirect (PAH) mutagens are present. During the months with higher mutagenic activity, only with the exception of July, direct mutagenic activity (in the absence of S9) was higher

than indirect mutagenicity indicating that in Santiago direct mutagenic pollutants might make an important contribution to the mutagenic activity of respirable particles.

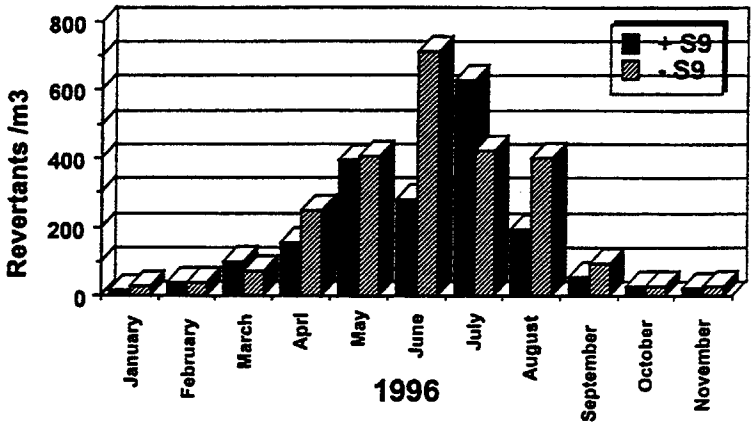

Figure 3. Mutagenicity of Coarse Particle Extracts collected in 1996. Mutagenic responses were examined in TA98 strain of S. typhimurium in the presence and absence of S9 by organic extracts from coarse fractions (2.5 - 10 m) of respirable particulate matter, Station B, Santiago, 1996. Data for each month correspond to 1 organic extract derived from 30-31 filters collected dairly for coarse fraction.

Although we have not identified the precise nature of direct acting mutagens in respirable particles, it is known that nitroarenes are ubiquitous direct acting environmental pollutants. In agreement with this finding, the contribution of mononitro and dinitroarenes to Santiago's TSP mutagenicity has been demonstrated by using strains TA98NR and TA98 1,8DNP6 (Adonis and Gil,1993). In addition, in results not shown here, we have been able to identify several nitroarene DNA adducts after incubation of Calf Thymus DNA with organic extracts from PM_{10} and TSP samples. High direct mutagenicity has been reported for samples collected in Oslo (Moller and Alfheim, 1980); Durham, North Carolina (Talcott et al., 1981) and Rio de Janeiro (Miguel et al., 1990), where it was suggested that nitroarenes might be involved. Nitroarenes are emitted by industrial process and heating and by diesel engines, however nitroarenes can also be formed in the atmosphere under field conditions, probably from the reaction of high concentrations of PAHs with atmospheric pollutants such as O_3 and NO_2 (De Flora et al., 1989). The mutagenic activity reported here for respirable particles is higher than samples collected for similar studies in other cities of the world such as Genoa (De Flora et al., 1989), Rome (Crebelli et al., 1988), Rio de Janeiro (Miguel et al., 1990) and Mexico city (Villalobos et. al., 1995).

Seasonal variation and size distribution of mutagenic activity of organic extracts from fine and coarse fractions in the presence of activation fraction is shown in Figure 4. As it was previously shown for total and carcinogenic PAHs, indirect mutagenicity was also higher in the coarse than in the fine fraction. Thus, as was explained for higher PAH levels in the coarse fraction, fine particle condensation might explain the high mutagenic activity of the coarse fraction during the cold season.

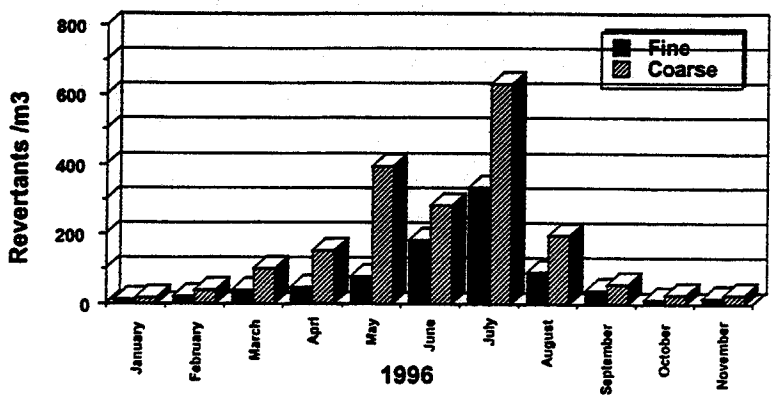

Figure 4. Seasonal variation and size distribution of mutagenic activity of organic extracts from fine and coarse fractions in the presence of activation system (S9). Mutagenic responses were examined in the TA98 strain of S. typhimurium by organic extracts from respirable particulate matter, Station B, 1996.

Human Cell Mutagenicity. In order to investigate whether the high mutagenic activity of Santiago's particulate matter to Salmonella thyphimurium can also be observed in human cells in culture, we investigated mutagenic activity at the tk locus in h1A1v2 human lymphoblast. Table 1 shows that organic extracts from two samples of total suspended particles, were clearly mutagenic at the tk locus in h1A1v2 cells under the conditions of this assay. The responses of cultures exposed to all test concentrations from both samples were statistically significant both in comparison to the historical negative controls and to the concurrent negative controls. The mutagenic potencies (420x $10^6/m^3$) of the organic extracts from TSP collected in Santiago were 400 fold higher than that reported for PM_{10} in Los Angeles, USA in 1993 (Hannigan et al.1997).

In conclusion, this investigation has shown that organic extracts from Santiago's respirable particulate matter contain high levels of PAHs, including six that have been classified by IARC as 2A and 2B carcinogens. Although substantially declining in recent years, the levels of carcinogenic PAHs in PM_{10} are still higher than those reported for other urban areas in USA, Australia and Europe. The organic extracts from respirable particles are also highly mutagenic to different strains of Salmonella typhimurium and those of TSP are mutagenic to human cells in culture, suggesting that particles contain both indirect-acting (PAHs) and direct mutagenic pollutants (nitroarenes). The observed higher PAH levels as well as direct and indirect mutagenicity in the coarse fraction in the cold seasons compared to the fine fraction, might be due to the high particle concentration, as well as physico-chemical and/or atmospheric conditions that favour fine particle condensation to enrich the coarse fraction with PAHs and nitroarenes.

In spite of a significant decrease in PAHs, in the last years the direct mutagenicity of respirable particles has not significantly changed in the period 1994 -1996. Thus, these results together with in vitro detection of nitro-PAHs-DNA-adducts with organic extracts from respirable particles, suggest the important contribution of nitro-PAHs and/or other compounds present in the direct mutagenic response. These pollutants probably represent the higher risk for human health, thus efforts might be improved to decrease the emissions of the main sources of these environmental pollutants.

Table 1. Dose-dependent mutagenicity of Santiago's airborne particles at the tk locus in human h1A1v2 cells.

Organic Extract (µg/ml)	Mutant Fraction (per millon)	
	13863 (% survival)	13903 (% survival)
3.13	50.4 ± 11.6 (94)	37.5 ± 6.83 (93)
B(a)P*	(7.2×10^{-4})	7.2×10^{-4}
6.30	45.1 ± 9.6 (82)	45.0 ± 11.30 (87)
B(a)P*	1.4×10^{-3}	1.5×10^{-3}
12.5	66.9 ± 14.6 (71)	47.1 ± 12.5 (76)
B(a)P*	2.7×10^{-3}	2.9×10^{-3}
25.0	66.2 ± 12.4 (66)	56.0 ± 6.17 (73)
B(a)P*	5.5×10^{-3}	5.8×10^{-3}
Benzo(a)pyrene (1 µg/ml)	227 ± 14.7 (31)	Positive Control
DMSO	15.6 ± 4.03 (100)	Concurrent Negative Control
DMSO	25.4 ± 7.15 (100)	Historical Negative Control

* BaP concentration in the organic extracts examined.
Both pollutant samples 13863 and 13903, were assayed for B(a)P content (ug/ml) by HPLC as described in Methods and are given in the table. Data are represented as the mean ± SEM for triplicate determinations at each concentration.

Acknowledgements

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Particulate Surface-Phospholipid Surfactant Interactions Affecting The Expression of Toxicity: *InVitro* Genotoxic Activities of Diesel Exhaust Particulate Soot and of Quartz Dust, National Institute for Occupational Safety and Health, Morgantown, WV, USA

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The National Research Council - National Institute for Occupational Safety and Health Postdoctoral Research Program supported the work of Dr. N. Gao. Address correspondence to W. E. Wallace, NIOSH, 1095 Willowdale Rd., Morgantown, WV 26505, USA. E-mail: wew2@cdc.gov

Diesel exhaust particulate (DEP) soot and respirable mineral quartz dust expressions of cytotoxic or genotoxic activities in vitro following particle pre-treatment with dipalmitoyl phosphatidylcholine (DPPC) are reviewed. Incubation of soots and dusts with DPPC, a major component of pulmonary surfactant, dispersed in saline is used to model the physical conditioning of particles depositing on the bronchio-alveolar surfaces of the lung. DEP soot generated by some engines under certain operating conditions can express genotoxic activity in vitro after incubation in DPPC-saline dispersion, measured as procaryotic cell mutation or eucaryotic cell chromosomal or DNA damage. The activity is expressed by the surfactant-solubilized (coated) non-dissolved soot particles rather than by a surfactant extract of the particles. Adsorption of DPPC on insoluble respirable mineral quartz particles inhibits otherwise prompt membranolysis and DNA damage of pulmonary macrophage and other cells in vitro. Cellular digestion can remove adsorbed prophylactic surfactant, restoring toxic dust surface activity.

We are investigating the expression of toxicity *in vitro* by respirable particles mixed into a dispersion of the phospholipid surfactant dipalmitoyl phosphatidyl-choline (DPPC) in normal physiological saline. This is to model the effects on the surface characteristics of particles deposited in the lung of the surfactant fluid hypophase lining of conducting airways and pulmonary alveoli. This addresses questions of the biological availability of deposited particles and their toxic constituents. In the case of diesel exhaust particulate (DEP) soots there is the concern that their genotoxicity as measured conventionally by using their organic solvent extracts is compromised for prediction of disease risk by the physiologically implausible mode of challenge. In the case of quartz dust and other mineral dusts there is the concern that direct and prompt cytotoxic effects of particles may confound the *in vitro* identification or measurement of genotoxic events. We here review our studies of a diesel soot extracted by organic solvent or by DPPC-saline surfactant, testing *in vitro* genotoxic activities of both the dissolved and non-dissolved phases of both extractions. Also, our studies of the suppression of *in vitro* cytotoxicity and DNA damage by a respirable quartz dust and of the digestive removal of surfactant and delayed expression of some toxic activities of the dust are reviewed.

Review of Studies

DEP soot can express *in vitro* genotoxic activity as (non-dissolved) particles dispersed in phospholipid surfactant.

In Figure 1, mutagenic response was measured in the Ames assay using *Salmonella typhimurium* TA98 without S9 activation of test materials. DEP soot was prepared by mixture into dichloromethane (DCM), or DPPC surfactant in saline (DPPC-saline). The DCM preparation subsequently was partitioned into dimethylsulfoxide (DMSO). Each total mixture (T) was then fractionated into a supernatant (Su) extract fraction and a sediment (Sd) non-dissolved fraction by centrifugation and filtration. Sd fractions were re-suspended for mutagenicity testing in the appropriate solvent (DMSO or DPPC-saline). The pre-incubation (90 min) assay was performed. Mutagenic activity was found in the Sd non-dissolved particulate fraction of the DPPC surfactant preparation, and in the Su dissolved fraction of the DCM/DMSO organic solvent preparation (Wallace at al., 1985; 1987; 1990). No activity was presented in the Sd sediment phase of DEP soot that survives DCM organic solvent extraction: that implies particles *per se* are not necessarily active.

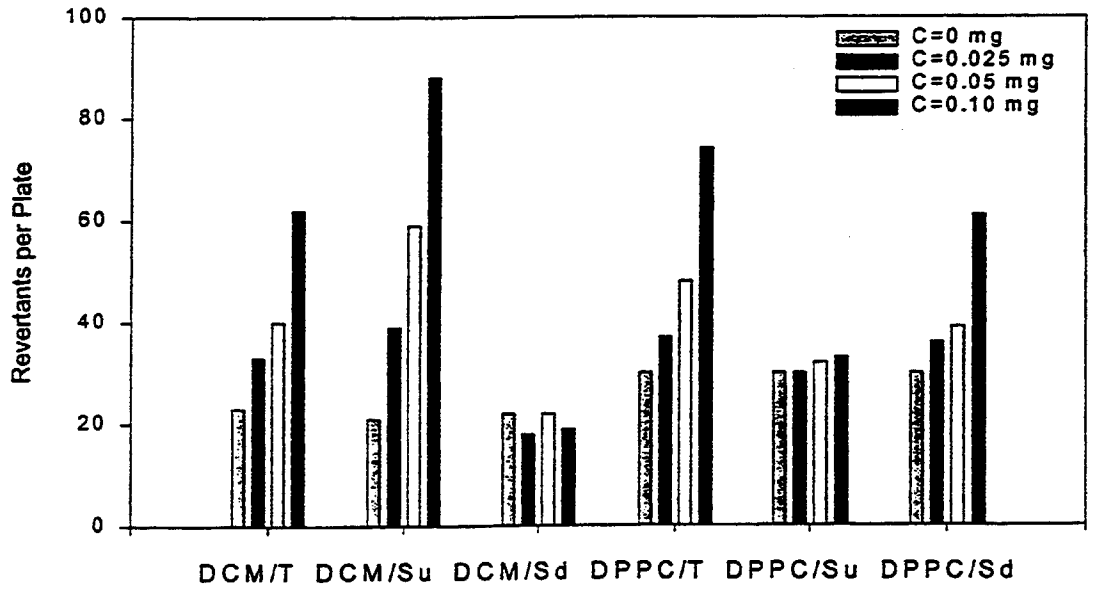

Figure 1. Mutagenic activity of DEP soot in *S. Typhimurium.* TA 98.

Figure 2 shows similar results for sister chromatid exchange (SCE) induction in Chinese hamster lung fibroblasts (V79 cells) in culture by fractions of the DEP soot. Cells were incubated for 5 h with DMSO or DPPC-saline T, Sd, or Su fractions and then incubated 39 h with bromodeoxyuridine. Increased SCE frequency was found in the DPPC Sd particulate fraction and in the DMSO solvent Su dissolved fraction (Keane et al., 1991).

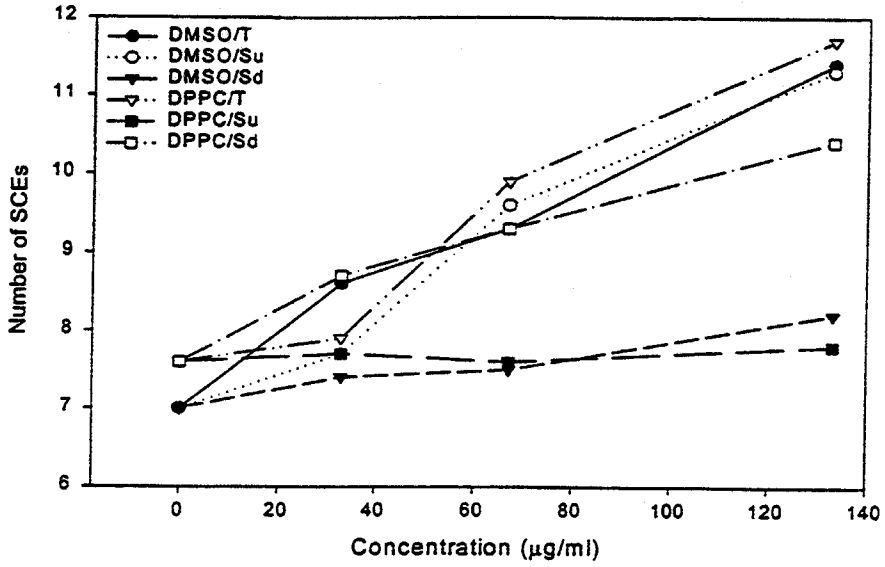

Figure 2. Sister chromatid exchange induced by DEP in V79 cells.

Figure 3 shows that micronucleated cells (MNC) in Chinese hamster ovary (CHO) cells *in vitro* is expressed by the DPPC Sd fraction of the DEP soot and the DMSO Su fraction. Cells were treated with the fractions for 24 h and then re-plated and incubated another 24 h. The MNC in 500 cells was determined for each fraction in each treatment. This MN response appears to be specific to particle composition and is not simply a response to uptake of any particle; that was indicated by microscopic counting of a greater number of DMSO sediment particles per cell compared to DPPC sediment particles per cell (Gu et al., 1992).

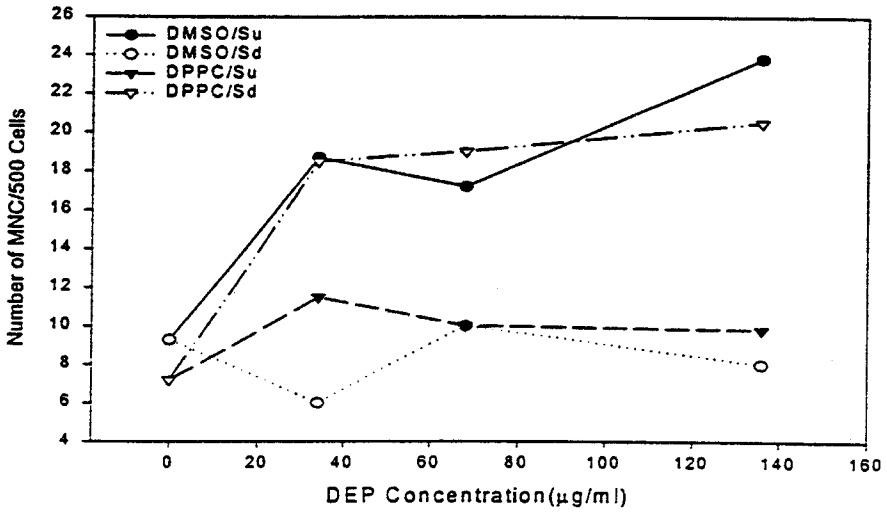

Figure 3. Micronucleus induction in CHO cells by DEP soot.

Figure 4 shows that unscheduled DNA synthesis (UDS) *in vitro* similarly was limited to the DPPC Sd fraction and the DMSO Su fraction of the DEP soot. The UDS assay was performed using cultured V79 cells incubated 16 h with the DEP fractions and with tritiated thymidine (Gu et al., 1994).

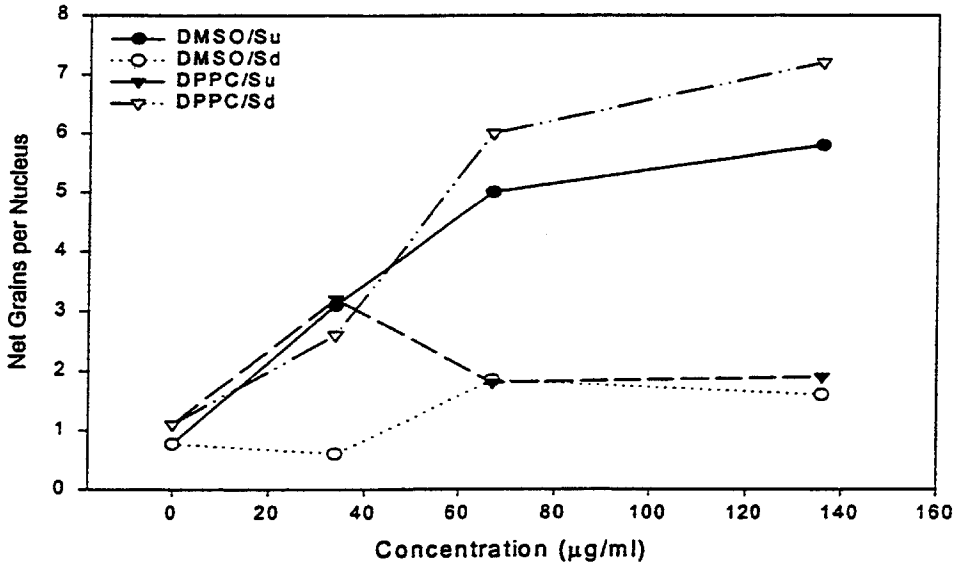

Figure 4. Unscheduled DNA synthesis induced by DEP soot in V79 cells.

DPPC adsorption suppresses otherwise prompt *in vitro* cytotoxic and genotoxic activities of quartz dust. Cellular digestion processes can remove DPPC and restore toxicities over several days.

In vitro cellular assays for release of β-glucuronidase (β-Gluc),β-N-acetyl glucosaminidase (β-NAG), and lactate dehydrogenase (LDH) from lavaged rat pulmonary macrophage show complete suppression of cytotoxicity after 2 h challenge with respirable quartz dust pre-treated by incubation in DPPC-saline dispersion (Wallace et al., 1985). Phospholipase A2 digestion removed DPPC from quartz dust in a cell-free biochemical assay system. Membranolytic activity was restored to original values when surfactant was removed. Kinetics are described by a model of enzymatic digestion of DPPC adsorbed as a bilayer on the quartz surface. (Wallace et al., 1992). In a cellular system, *in vitro* challenge of P388D1 cells by radiolabelled DPPC on quartz dust found approximately 60% of the DPPC was digested over a six day period (Hill et al., 1995). Surfactant loss also has been measured from quartz particles phagocytosed by lavaged rat alveolar macrophage: confocal fluorescence microscopy was used to follow the fluorescence intensity of a fluoroprobe-labeled analog of diacyl phosphatidylcholine. The fluorescence intensity diminished by one-half over a 5 to 7 day period, in parallel with the loss of viable cells (Liu et al., 1998). DNA damage induced *in vitro* by quartz dust was measured by the single cell gel electrophoresis assay for DNA migration as a measure of DNA single- and double-strand breaks. DNA damage was induced by native respirable quartz; but this activity was suppressed at 1 day after challenge by pre-incubation of quartz with DPPC-saline. After 5 days, DPPC-treated quartz expressed DNA damage activity which was on the order of one-half of

that of the native quartz (Gao et al., 1999). Micronucleus induction (MN) by quartz was observed in V79 cells; that activity similarly was suppressed at 1 day by DPPC-saline pre-treatment of the quartz. However, restoration of the MN activity of the DPPC-saline treated quartz was not seen over a 5 day incubation period with V79 cells (Liu et al., 1996).

Discussion

Expression of Genotoxic Activity by Surfactant-Treated Diesel Soot Particles. There is a current interest in the mechanisms of carcinogenicity of highly insoluble particulate materials which are considered to be not directly genotoxic. Diesel exhaust particulate soot is sometimes included in that set of materials because its genotoxic constituent organics are considered not to be biologically available *in vivo*. Genotoxic activity *in vitro* conventionally has been measured only on organic solvent extracts of soot, and lung lining fluids or surfactant surrogates for them have been found not to extract materials with mutagenic activity from DEP soot. However, Figures 1, 2, 3, and 4 show that DEP soot can express *in vitro* mutagenic, clastogenic, and DNA damage activity as a non-extracted dispersion of soot particles in DPPC-saline surfactant. Particles per se are not responsible for the activity. The genotoxic activity of DEP particles may be associated with the particle-borne organics; but DEP soot can express that activity as non-dissolved surfactant-solubilized particles under physical conditions representative of inhaled soot particles deposited in the lung. This questions a categorization of DEP with highly-insoluble non-genotoxic particulate matter whose potential for induction of neoplastic disease is limited to particle "overload" conditions of exposure.

Expression of Cytotoxic and Genotoxic Activities by Surfactant-Treated Quartz Dust Particles. In the case of quartz dust, the question of a direct physico-chemical mechanism for genotoxicity to target cells *in vivo* remains open; carcinogenicity frequently is postulated to be influenced by *in vivo* inflammatory response or other events involved in the pathogenesis of silicosis. Respirable quartz dust expresses both prompt cytotoxic and DNA damage activities *in vitro;* these are suppressed by adsorption of phospholipid surfactant. Longer term *in vitro* studies indicate that phagolysosomal digestion or extracellular phospholipase enzymatic digestion removes the prophylactic surfactant and restores toxicity. Current research is comparing this progression of events between quartz and other mineral dust of equally prompt *in vitro* toxicity but of significantly lower disease inducing potential *in vivo*, e.g., aluminosilicate clay dust.

Detailed information on methods, numerical data and statistics for the studies reviewed is given in the references below.

DEP soot used in these studies was graciously supplied by Lovelace ITRI.

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A Pilot Study Of Controlled Human Exposures To Concentrated Ambient Fine Particles In Metropolitan Los Angeles

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Abstract

We installed a Harvard particle concentrator with $PM_{2.5}$ size-selective inlet and 2 virtual impactor stages, in a movable human exposure laboratory. The exposure chamber, a modified body plethysmograph of ≈2000 liters volume, operated at flow near 200 l/min and pressure near 10 cm H_2O below atmospheric. Multiple pilot tests with an investigator (male, age 53, history of ectopic heartbeats but no known cardiopulmonary disease) as subject showed no untoward effects other than transient mild substernal irritation after exercise on one occasion, and mild ear discomfort from pressure changes upon opening or closing the chamber. Chamber $PM_{2.5}$ concentrations, measured by DataRAM nephelometer with allowance for relative humidity artifacts, ranged from 100 to 200 $\mu g/m^3$, typically 7-9 times outdoor concentrations. In-chamber concentrations were about 25% below inlet concentrations due to inhalation and wall losses; gradients within the chamber were small. In-chamber temperature rose modestly during exposures, but no special controls were required under the existing mild weather conditions. Subsequently, healthy adult volunteers (2 female, 2 male, age 19-41) completed 2-hr exposures to ambient $PM_{2.5}$ concentrated to 148-246 $\mu g/m^3$, at rest, and similar studies with HEPA-filtered air (control condition). Concentrator performance was generally as described above; occasionally the concentration factor decreased due to rapid fouling of impactor slits under humid and/or highly polluted ambient conditions. No medically significant changes in lung function, symptoms, arterial O_2 saturation, or Holter electrocardiograms (recorded during and for 22 hr after exposure in 3 subjects) were observed.

Background

Epidemiologic evidence associating ambient particulate pollution with adverse health effects in humans is extensive (American Thoracic Society, 1996; Environmental Protection Agency, 1996). Nevertheless, fundamental uncertainty and disagreement persist regarding what physical and chemical properties of particles (or unidentified confounding environmental influences) influence health risks, what patho-physiological mechanisms are operative, and therefore what air quality regulations should be adopted to deal with the health risks (Vedal, 1997). This lack of understanding reflects an inability of controlled laboratory investigations to demonstrate effects that support the epidemiologic findings. Ambient particle concentrations are generally too low to induce detectable effects in human laboratory volunteers, while artificially generated particles cannot realistically represent the range of potentially toxic components in the ambient mixture. The recent development of ambient fine particle concentrators has made it possible to perform laboratory exposures with concentrated ambient particles (CAP), i.e., "real-life" ambient aerosols at increased (but still realistic) concentrations. Preliminary findings from Harvard School of Public Health, USEPA Human Studies Division, and University of Toronto suggest increased toxic response to CAP (as compared to artificial particles) in animals, and potentially important cardiovascular responses in human volunteers. Accordingly, this type of exposure system may

provide a useful method for assessing the health effects of ambient particles and for identifying specific risk factors and the means of controlling them.

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Objectives

Objectives of this project are listed below. Numbers 1-3 are the subject of this paper; 4 and 5 will be addressed in future work.

1. Construct a concentrator at Rancho Los Amigos Medical Center (in metropolitan Los Angeles) suitable for controlled human exposures to CAP at "realistic worst case" concentrations (around 200 $\mu g/m^3$ for 2 hours with intermittent exercise).

2. Characterize the performance of the concentrator over the range of pollution and weather conditions experienced at the site.

3. Perform safety studies on healthy adult volunteers exposed to CAP as above for 2-hour periods at rest, and similarly to HEPA-filtered ambient air as a control condition.

4. Perform main experiment on healthy and asthmatic adult volunteers exposed to CAP as above for 2-hour periods with intermittent moderate exercise, and similarly to HEPA-filtered ambient air as a control condition.

5. Test statistically whether CAP exposures induce unfavorable cardiopulmonary responses relative to controls, and whether individual subjects' biological and/or exposure characteristics predict their responses.

Experimental Methodology

Facilities

The ambient particle concentrator essentially duplicates stage 1 (with 5 parallel virtual impactors) and stage 2 (with one similar impactor) of the 3-stage fine particle concentrator employed in animal studies at Harvard. Sioutas et al. (1995) have described the operating principles, design, and construction of the concentrator. A third stage of concentration is unnecessary owing to the generally high ambient fine particle concentrations in the Los Angeles area. A size-selective impactor inlet excludes ambient particles above 2.5 μm in aerodynamic diameter. In each stage, the major flow (80%), depleted of particles, is discarded, while the minor flow (20%), enriched in particles, is continuously drawn to the next stage, and ultimately through the exposure chamber. Total air flow is 5000 l/min; flow through the chamber is approximately 200 l/min; overall concentration factor is 10 to 12 theoretical, 7 to 9 typically observed. From 0% to 100% of the output air may be diverted through a HEPA filter to control

the exposure concentration. The exposure chamber is a whole-body plethysmograph modified by the addition of a foot well containing a pedal crank exercise device. Ports in the walls and ceiling of the chamber allow sampling of the exposure atmosphere and connection of electrocardiogram (ECG) or other cables to measure the subject's physiologic function. The chamber operates at a pressure usually between 8 and 16 cm H_2O below atmospheric, depending on the condition of the impactor slits.

Subjects

Volunteers (healthy only for this safety study, healthy and asthmatic for the planned main study) are recruited by word of mouth and newspaper advertisements, and compensated for participation. Subjects are screened by medical history, cardiopulmonary examination including a submaximal stress test, and methacholine challenge to evaluate airway reactivity. The experimental protocol has been reviewed and approved by the Rancho Los Amigos Institutional Review Board.

Biomedical Testing

Measures of response in this safety study included forced expiratory function tests, symptom evaluations by standardized questionnaire, measurements of arterial oxygen saturation by fingertip pulse oximetry, and 24-hour digital Holter electrocardiograms beginning shortly before exposure (in 3 subjects only). Subsequent studies will add venous blood sampling to evaluate hypercoagulability and leukocyte-endothelial activation, and analyses of sputum (induced 22 hr following the end of exposure) for cellular and biochemical indicators of airway inflammation.

Monitoring during Exposures

The following techniques are used to characterize exposure atmospheres:

Continuous real-time $PM_{2.5}$ monitoring in chamber by MIE DataRAM nephelometer.

Integrative $PM_{2.5}$ sampling in chamber and ambient air by MOUDI multi-stage impactor and by Harvard denuder (HDS) sampler, for the determination of total mass, nitrate, and sulfate, with size distribution.

Integrative $PM_{2.5}$ sampling in chamber for elemental and organic carbon analysis (main study only).

Continuous gas monitoring in ambient air for CO (by infrared), NO_2 (chemiluminescence), O_3 (ultraviolet photometer), andSO_2 (pulse fluorescence).

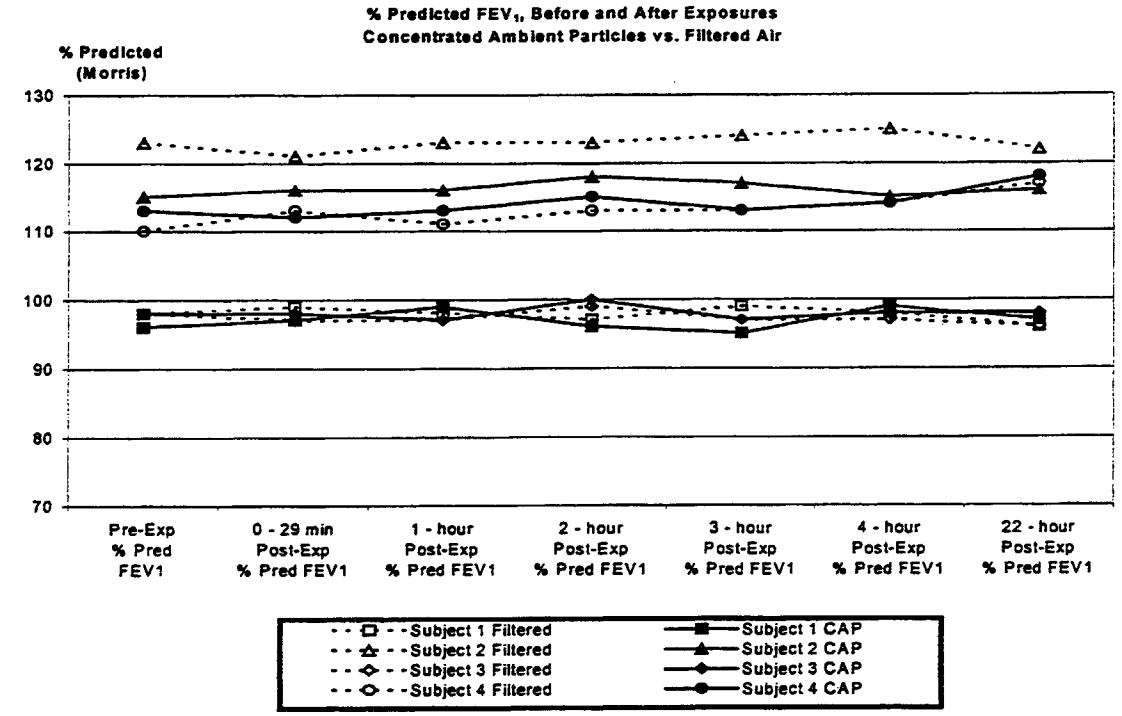
Results

Exposures

Table 1 summarizes results of monitoring inside the chamber during CAP exposure and filtered-air control experiments for the 4 safety-study subjects. HDS data, not shown, usually corroborated MOUDI data. Problems were encountered on occasion with monitoring as well as generation, but were resolved subsequently. Anomalously high mass readings in two filtered-air

studies were associated with large particles containing very little nitrate or sulfate. Their source was unclear; one possibility is debris from a HEPA filter which failed due to inadvertent overpressure.

Figure 1. Individual lung function and symptoms during and after exposures



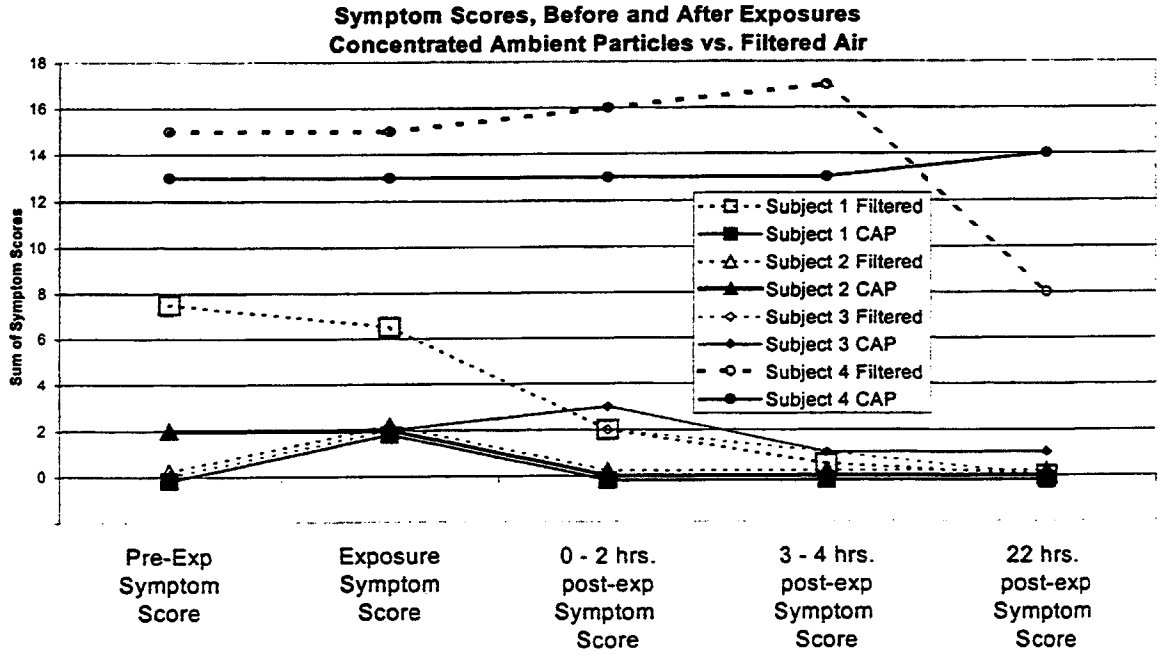


Table 1. Individual Exposure Data

Subject # (sex, age)	Exposure	DataRAM $\mu g / m^3$	MOUDI $\mu g / m^3$		
			Mass	SO_4	NO_3
#1	CAP	246*	269	9	95
(M, 29)	Filtered	51	246‡	0	1.6
#2	CAP	197*	216	26	66
(F, 41)	Filtered	7	19	0	0
#3	CAP	148	165	21.1	13.3
(F, 27)	Filtered	6	28	0.33	0.5
#4	CAP	153	143	17.7	22.2
(M, 19)	Filtered	10	102‡	0.2	0

*Adjusted post hoc to allow for absence of diffusion drying tube and for negative pressure effect (i.e., not returning exhaust to chamber).
‡Contamination suspected – most of the mass was >2.5 μm in aerodynamic diameter.

Responses

Pilot tests with an investigator showed little response, as indicated in the abstract. Figure 1 below illustrates the 4 safety study subjects' lung function (upper graph) and symptom scores (lower graph) during and after CAP and filtered-air exposures. No meaningful changes were seen in any subject. Table 2 summarizes findings from Holter electrocardiograms. No Holter records were obtained for Subject # 1. The remaining three subjects showed no meaningful changes in heart rate variability, S-T voltage levels, or incidence of ectopic beats between CAP and filtered-air exposures.

Table 2. Individual Holter ECG Results[a]

Subject (sex, age)	Exposure	SVE per hour[b]	VE per hour[c]	% RR >50[d]	Max. ST depress[e]	Max. ST elev[f]
#2 (F, 41)	CAP	3.01	ND	6	12	25
	Filtered	2.57	0.04	6	37	25
#3 (F, 27)	CAP	0.08	ND	4	62	12
	Filtered	0.08	ND	7	25	12
#4 (M, 19)	CAP	0.08	0.32	23	50	50
	Filtered	6.31	2.36	22	137	125

[a]Data collected during 2 hr exposure and for approximately 22 hr afterward (10 hr afterward for Subject #4 CAP study).
[b]Average number of supraventricular ectopic beats per hour of recording.
[c]Average number of ventricular ectopic beats per hour of recording. ND = none detected.
[d]Percentage of R-R intervals more than 50% different from adjacent interval.
[f]Maximum S-T voltage depression (in microvolts) seen in lead V1.
[g]Maximum S-T voltage elevation (in microvolts) seen in lead V1.

Conclusions

1. A 2-stage Harvard ambient particle concentrator can be operated successfully in the Los Angeles area under most atmospheric conditions, to generate exposure atmospheres equivalent to "worst-case" ambient conditions even when prevailing ambient pollution is light.

2. Fouling of virtual impactor slits, decreasing concentrator efficiency, may occur within a few hours under some Los Angeles ambient conditions.

3. A nephelometer with a diffusion drying tube on its inlet can monitor concentrated PM exposure concentrations in real time, agreeing reasonably well with gravimetric analyses of time-integrated filter samples. Thus, nephelometer readings provide adequate feedback for control of exposure concentrations (accomplished by diverting more or less of the concentrator airstream through a HEPA filter).

4. Healthy subjects at rest have not shown meaningful lung function, symptom, or ECG responses when exposed to concentrated ambient particles at approximately 200 $\mu g/m^3$. Thus, it is appropriate to proceed with exposures of healthy subjects at exercise in the main study.

5. Investigations of airway inflammation (by sputum induction), altered cardiac electrophysiology (by digital Holter monitoring), and blood hypercoagulability will be emphasized in the main study.

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LPS-Priming Amplifies Lung Macrophage TNF Production In Response To Air Particles

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We postulate that in the inflammatory milieu of diseased lungs, AMs may be 'primed' for enhanced responses to stimuli such as inhaled air particles. To test this hypothesis in vitro, we first cultured normal AMs with or without lipopolysaccharide (LPS). We then incubated the cells with particle suspensions (urban air particles (UAP, Washington, D.C.), residual oil fly ash (ROFA), concentrated respirable-size ($PM_{2.5}$) air particles (CAP), and inert TiO_2.) and compared rat and human AM production of the critical pro-inflammatory mediator, tumor necrosis factor (TNF). LPS priming caused a concentration-dependent increase in TNF production by rat AMs in response to UAP (e.g., control vs. LPS-primed: 0.9 ± 0.4, 8.6 ± 5.6 ng/ml),but not inert TiO_2. Priming also amplified responses of AMs to CAPs, although the potency of CAPs samples collected on different days varied (e.g., TNF, ng/ml control vs. LPS-primed human AMs: 0.9, 47.2 (CAPs #1); 1.5, 72.2 (CAPs #7). The soluble fraction of UAP and CAPs suspensions showed minimal potency for induction of AM TNF production, suggesting AM-particle interactions were essential for cytokine stimulation. The antioxidants NAC and DMTU blocked particle induction of TNF production (e.g., DMTU 20, 10, 2, mM in primed AMs + UAP: 97, 51, 9 % inhibition, respectively). The data suggest that AMs activated by extant pulmonary inflammation can promote further inflammation by an enhanced, oxidant-dependent, cytokine response to inhaled ambient air particles.

Significant associations between elevated levels of ambient air particles and increased mortality and morbidity have been identified. Particle health effects are primarily seen in people with pre-existing inflammatory lung disease (Dockery et al. , 1993; Schwartz, 1994). We hypothesize that in the inflammatory milieu of diseased lungs AMs may be 'primed' for heightened responses to air particles. To test this postulate, we compared the particle-induced TNF response of LPS-primed rat and human AMs with their control counterparts. The results show priming enhances TNF release by both rat and human AMs in response to UAP (SRM 1649 collected in Washington DC.) and concentrated ambient particles CAPs. We further report findings which identify the insoluble fraction as bioactive in AM particle responses in vitro.

Methods

Preparation of buffers and particle suspensions: A phosphate buffered saline was used for bronchoalveolar lavage in rats (PBS, BioWhittaker, Walkersville, MD). For priming and phagocytosis assays, we used RPMI-1640 (BioWhittaker) supplemented with 1% heat inactivated fetal bovine serum (Hyclone, Logan, UT), .075% BSA, penicillin, streptomycin and L-glutamine (RPMI 1%). The urban air sample SRM 1649 was collected in Washington D.C. (UAP) and was purchased from the National Bureau of Standards (Washington D.C.).) Titanium

dioxide (TiO_2) was provided by Dr. J. Brain. Concentrated ambient particles (CAPs) were collected from Boston air using the Harvard concentrator (Sioutas et al. , 1995). CAPs were released from filters into 0.9% saline through probe sonication of 0.9% saline. All buffers and supernatants from particle suspensions were tested for endotoxin using a Limulus assay kit (QCL-1000, BioWhittaker, Walkersville, MD). All reagents contained a final concentration of less than 0.1 endotoxin unit (EU) per ml with the exception of CAPs, which varied from 0 to 0.7 EU per 100 ug of particle suspension.

Macrophage isolation, priming and phagocytosis assay: Bronchoalveolar lavage (BAL) was performed on rats (female CD rats, 250 - 300g, VAF, Harlan Sprague Dawley Inc., Indianapolis, IN) using PBS as lavage buffer. Human subjects were lavaged under a protocol approved by the institutional review board. BAL fluid was centrifuged and cells were suspended in RPMI 1% at 1 x 10^6 cells per ml. All incubations were done in Ultra Low Cluster plates (Costar, Cambridge MA) which prevent cell adherence. AMs were first treated with bacterial lipopolysaccharide (LPS, E. coli serotype 0127:B8) at 10^6 cells per ml at 37 ^{0}C in humid 5% CO_2. Rat AMs were primed for 3 hours and human AMs were primed for 20 hours. After this priming period, AMs were washed 3 times and dispensed into wells containing particle suspensions or vehicle control. After 20 hours of particle incubation at 37 ^{0}C in humid 5% CO_2, supernatant was removed from each well for cytokine measurements. Cells were analyzed for relative particle load using flow cytometry. All reagents were obtained from Sigma (St. Louis, MO) unless otherwise specified.

Flow cytometric analysis: We used a Coulter ELITE flow cytometer (Coulter Corporation, Miami, FL) each equipped with a 15 mW 488 nm emitting air-cooled argon laser (Cyonics/Uniphase). Analysis of right angle light scattering (RAS) was used as a measure for relative particle uptake (cell associated particles deflect light and add to RAS signal, (Stringer et al. , 1995)). Data from 3000 AMs was collected using a linear scale ranging from 0-1023 relative intensity units.

Cytokine and nitrite assays: Cell supernatants from human and rat AMs were assayed for TNF bioactivity using the TNF-sensitive WEHI 164 clone 13 tumor cell line in a recently described micro-plate assay (Imrich et al. , 1998). Rat MIP-2 was measured with a sandwich ELISA using rabbit anti MIP-2 to coat the plates and goat anti MIP-2 as the detection antibody. This was followed by incubation with biotinylated horse anti goat IgG and avidin-biotin-peroxidase complex (Vector, Burlingame CA). Normal serums were used to block species cross-reactivity of reagents. The Griess method was employed for detection of nitrite.

Statistical Analysis: All data are reported as the mean value $\pm$ the standard error of the means. For most figures and tables, ANOVA analysis was performed with Fisher's PLSD adjustment for multiple comparisons using the Statview software package (Abacus Concepts, Berkley, CA). For dose-response analysis shown in Figure 1 and human data presented in Table 1, within-subject LPS-response and particle effects were estimated by fitting a mixed linear model with random subject effects to the square root of TNF using PROC MIXED in the SAS statistical software package (SAS Institute, Inc. NC). Differences between CAP samples were judged to be significant using the Bonferroni correction for multiple comparisons (significance criteria: $p \leq .0011$).

Results

LPS-priming amplifies UAP-mediated TNF production by rat and human Ams: Alveolar macrophages from rat were cultured +/- LPS ranging from 10-1000 ng/ml for 3 hours. Cells were then washed and treated with UAP at 25, 50 and 100 ug/ml. Each stimulus (LPS-priming or UAP incubation) caused concentration-dependent TNF release by AMs. When cells received both LPS-priming and UAP particle suspension, TNF release was greatly enhanced. The amplified TNF response was dependent on both LPS and UAP concentrations (Figure 1).

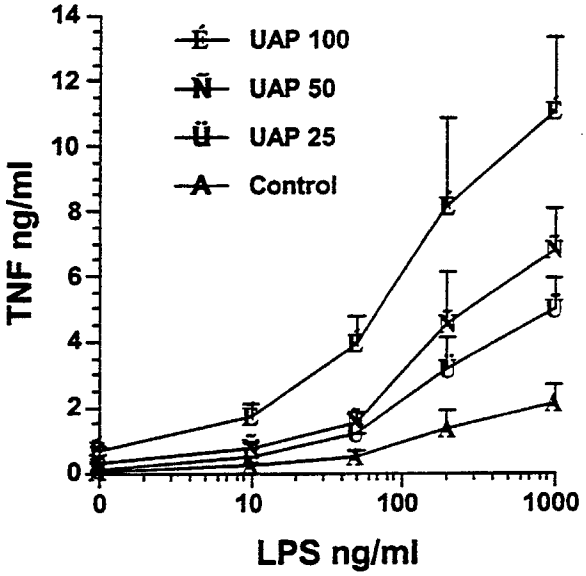

Figure 1. TNF production in response to UAP is enhanced by LPS priming and increases with UAP concentration. Rat AMs were pre-treated with LPS for 3 hours and incubated with UAP suspensions at 100 (diamonds), 50 (squares), and 25 (triangles) ug/ml or vehicle control (circles) for 20 hours (n=5). Statistical analysis (see methods) shows that both LPS and UAP have a concentration-dependent effect on TNF production (p = .0001), and that there is synergistic interaction between UAP and LPS on TNF production (p = .0186).

We next evaluated TNF release by primed rat and human AMs treated with UAP or TiO_2 suspensions for 20 hours. Results are summarized in Figure 2. Similar to findings in rat, UAP substantially augments TNF release by human AMs that have been pre-stimulated with LPS. The control particle TiO_2 does not stimulate or augment TNF release. We investigated whether observed changes in TNF release by primed > control AMs are due to changes in particle load. Right angle light scatter (RAS) was determined by flow cytometry and used as a measure of relative particle load (Stringeret al., 1995). In human, the RAS of control and primed AMs (incubated with particles) was similar while in rat, primed AMs were found to have less (~30%) relative particle load than their control counterparts. The cell clumping observed in primed rat AMs may partially explain this finding.

Effect of CAP incubation on TNF release by control and primed Ams

Human CAPs 50 ug	Control + particles	N	Primed control	Primed + particles	Primed increase over SUM
#1	.18 ± .14	4	.77 ± .3	2.08 ± .61	1.01 ± .35
#2	.4	1	.89	4.01	2.73
#3	.1	2	2.03	1.98	-.15
#4	.02	2	1.22	2.63	1.4
#5	.25 ± .15	5	1.25 ± .53	3.13 ± .81	1.63 ± .31
#6	.09 ± .06	3	.73 ± .42	3.16 ± 1.18	2.34 ± .81
#7	.26 ± .04	3	.73 ± .42	4.07 ± 1.22	3.08 ± .91
#8	.21 ± .03	3	.73 ± .42	2.45 ± 1.11	1.52 ± .67
#9	.04	2	1.03	1.62	.56
#10	.02	1	1.55	3.58	2.0
Rat CAPs 100 ug					
#1	.07	2	.69	2.29	1.52
#3	.02	2	.69	1.18	.47
#5	.05	2	.69	3.82	3.08
#10	.13 ± .01	5	.59 ± .18	2.79 ± .89	2.08 ± .73
#11	.08 ± .01	5	.59 ± .18	2.34 ± .7	1.67 ± .51
#12	.01 ± .01	5	.59 ± .18	1.58 ± .59	.98 ± .43
#13	.01 ± .01	5	.59 ± .18	.96 ± .34	.36 ± .21

Table 1. Priming augments TNF release by both rat and human AMs. Data presented in figure 4 as fold-increase is shown here as actual TNF production. In human cell assays, when considering all CAP samples together, there was significant evidence for synergy between priming and particles for TNF release ($p = .0001$). All particles tested except samples #3 and #9 were significantly synergistic (for details of statistical analysis, see methods). When comparing the potency of CAP samples to each other, we limited analysis to those samples tested in 3 or more subjects. For CAPs samples tested with human AMs, #7 is significantly different from #1 ($p = .0002$). In primed rat AMs, CAP samples #10 and #11 caused significantly enhanced TNF release ($p \leq .05$) while samples # 12 and #13 did not.

Concentrated ambient particles (CAPs) augment TNF release by primed Ams: We continued our investigations using concentrated ambient particles (CAPs). Control and LPS-primed AMs from human and rat were treated with one or more CAPs suspensions. Table 1 summarizes the TNF produced by AMs treated with CAPs at 50 ug/ml (human) and 100 ug/ml (rat). In primed AMs from both species, incubation with CAPs suspensions caused significantly enhanced TNF release. We observed differences in the biologic response of primed AMs to CAPs collected on different days (Table 1). Changes in RAS generated by rat AMs treated with 100 ug/ml CAPs varied as did the visible particle number contained in 100 ug/ml (observed by light microscopy).

Potency of CAPs correlates with relative particle load: We treated control and primed rat AMs with a panel of high, medium and low bio-active CAPs samples. We chose CAPs that were abundant enough for repeated experimentation (n=6) and measured 4 parameters in parallel. In supernatants from AMs incubated with CAPs suspensions, we measured nitrite, a footprint of iNOS activity, and the cytokines TNF and MIP-2. We also quantified the relative particle load (RAS) of AMs using flow cytometry. Results are shown in Figure 4. In control cells, particle suspensions stimulated moderate MIP-2 release while nitrite (not shown) and TNF production were only slightly elevated. After LPS-priming, particle-mediated TNF release was greatly enhanced while MIP-2 release was moderately enhanced above the expected sum of each stimulus alone. Nitrite production was increased in primed AMs and was generally unaffected by additional particle incubation (not shown). Increased RAS was detected in AMs incubated with particle suspensions. As previously observed, CAPs collected on different days varied in the particle load delivered to AMs. Samples which caused the greatest increase in RAS (i.e. UAP, CG35, CG36, E269) were also the strongest cytokine inducers. Statistical analysis showed a strong correlation of relative particle load (RAS increase) with TNF and MIP-2 release in primed AMs (r^2 = .96, 98, respectively). The correlation was weaker for control AMs (.67,.86, respectively), possibly reflecting additional mechanisms operative in control AMs (e.g., sensitivity to endotoxin adsorbed onto particles).

Most bioactivity was found in the insoluble fraction: Another approach was taken to analyze which component(s) of CAPs account for bioactivity. Particle suspensions were left unchanged or were separated by centrifugation into a pellet (re-suspended in saline) and a supernatant. Control and primed rat AMs were treated with these 3 preparations and TNF release was measured (Figure 3). For both control and primed AMs the majority of bioactivity is associated with the re-suspended pellet.

Discussion

The results show that pre-treatment of AMs with LPS markedly increases their TNF release in response to air particle suspensions (UAP and CAPs). Findings in rat were similar to findings in human. The relative magnitude of particle-enhanced TNF production was dependent on several factors including the duration and concentration of LPS-priming, the type or composition of particle and the load of particles delivered to the cell. We found strong correlation between particle load and cytokine release by primed cells. These findings support several statements. With respect to AM responses, most bioactivity of CAPs suspensions resides in the re-suspended pellet and can be attributed to insoluble mass. Particle suspensions made from CAPs collected on

different days vary in their proportion of soluble and insoluble mass. Because increased RAS (particle load) correlates so well with cytokine release by primed AMs, we postulate that most Boston air particles contain the component(s) necessary to augment TNF release in primed AMs and it is the proportion of insoluble mass that determines the potency of a given CAPs particle suspension.

In the overall picture of PM effects on lung health, extrapolation of these results to in vivo events is limited by a number of considerations. First, the physiologic significance of the changes observed in primed AMs remain to be established in vivo. Second, instillation studies in rat and mouse have shown both soluble and insoluble components of air particle suspensions are active in vivo (Dreher et al. , 1997; Li et al. , 1997). Most likely it is the combination of multiple lung cell responses driven by pre-existing health conditions that determine the physiologic outcome of PM exposure. The exposure of rats with pre-existing lung inflammation to inhaled CAPs may address some of these issues.

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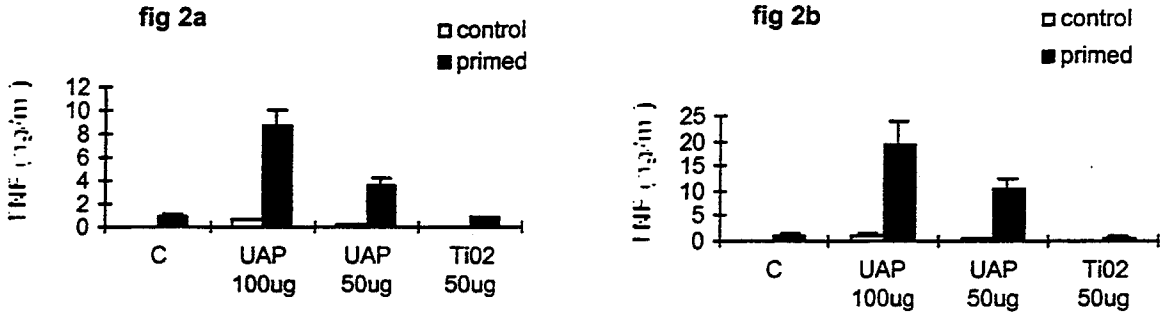


Figure 2. Particle-specific TNF release is seen in control (light bars) and primed (black bars) rat AMs after both 6 (n=14) and 20 (n=18) hours of particle incubation. At both time-points, UAP stimulated enhanced TNF release at 100 and 50 ug/ml (* = p value $\leq$ .05 when compared to the sum of TNF released from priming and particle incubation alone). The control particle TiO_2 did not stimulate or augment TNF release by control or primed AMs respectively.

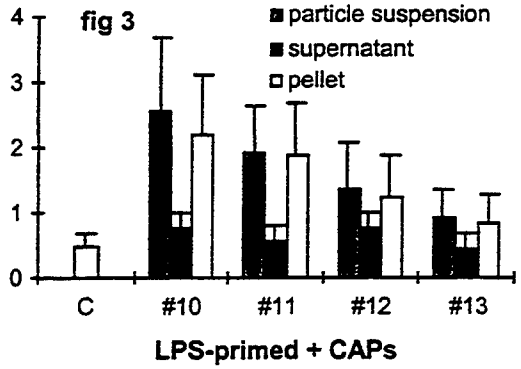
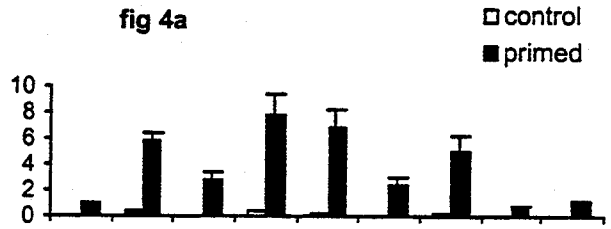
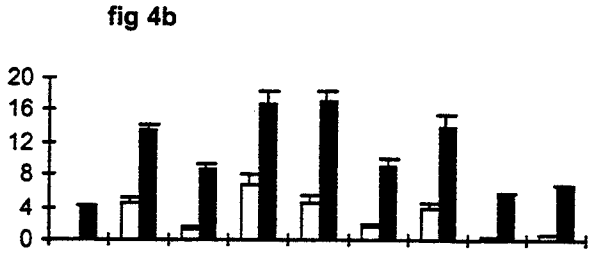


Figure 3. Particle suspensions that have been separated by centrifugation retain most of their TNF-inducing capacity in the re-suspended pellet while the supernatant has minimal bioactivity. Control and primed rat AMs (n = 4) were incubated with 4 CAP samples (5b) at 100 ug/ml. Cells were also incubated with particle suspension components separated by centrifugation (see methods). For a given particle type, there were no significant differences between TNF released by cells treated with particle suspensions (dark gray bars) and re-suspended pellets (light gray bars).





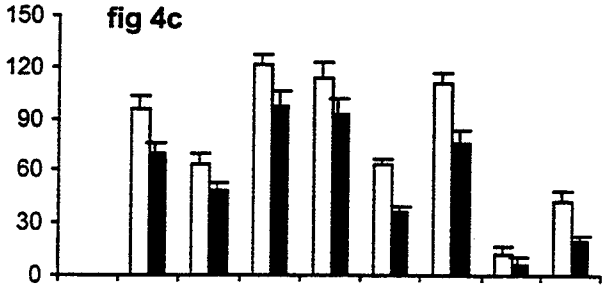


Figure 4. Analysis of cytokine release by primed AMs incubated with concentrated ambient particles (CAP) shows markedly amplified TNF release (A), expressed as fold increase over the expected sum of priming and particle incubation alone. The degree of cytokine release is strongly correlated to particle load as evaluated by flow cytometry for each sample (C). Similar results were seen with analysis of MIP-2 release in these same experiments (C).

PARTICLE-ANTIOXIDANT INTERACTIONS IN EPITHELIAL LINING FLUID

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As many of the absorbed materials on particles are recognised oxidants we hypothesised that oxidative stress may be a fundamental mode of injury associated with inspired particles. To test this hypothesis we examined the impact of three standard carbon black particles - CBP (M120, R250 or M880) on the respiratory tract epithelial lining fluid (ELF) antioxidant screen using a model exposure system. Initially, a dose response study was carried out. Antioxidant solutions (single and composite) were incubated with M120 (0, 50, 150 and 500 μg/ml) at 37°C for up to 6h under mixed conditions. The following observations were made: (1) Uric acid (UA) was not consumed in any of the conditions examined. (2) Ascorbic acid (AA) was consumed in a near-linear fashion with time, and in a CBP dose-dependent manner in both single and composite models. (3) Following an initial rapid loss of reduced glutathione (GSH) at 150 and 500 μg/ml CBP, a new steady state concentration, of approximately 80 and 50% of the starting GSH concentration, was established. In a second study, we compared the ability of M120, R250 or M880 (all at 150 μg/ml) to consume ELF antioxidants. The following observations were made: (1) UA was not consumed by any particle considered. (2) AA was consumed in a near-linear fashion, with time, however AA consumption rates varied markedly CBP type. (3) An initially high GSH consumption rate was noted with all CBPs. Where GSH consumption occurred it was always accompanied by the appearance of GSSG in solution. Together, these data demonstrate that particle size and surface area are important factors when considering particles/antioxidant interactions in the airways. Moreover, these data demonstrate that GSH and AA both represent significant substrates for CBP. Deposition of CBPs within the respiratory tract lining fluid may therefore compromise the airways antioxidant defensive screen and accelerate epithelial injury.

Carbonaceous material is a principal component of airborne particulate matter. It exists as either organic carbon or particulate elemental carbon, also known as carbon black particle (CBP). In London (UK), carbonaceous particles contribute up to 20% of the mass of PM10 while 87% of the elemental carbon is formed from diesel combustion. Diesel exhaust therefore represents an important source of urban air pollution, consisting of a complex of both oxidant gases and particulate matter. A wide variety of transition metals and hydrocarbons derived from the combustion process are absorbed onto the surface of these particles (Schuetzle et al., 1983; Draper, 1986). Whilst many of these gaseous components and surface absorbed materials are recognised oxidants, capable of causing oxidative stress in, and damage to the lung, little is presently understood about the toxicity of carbon black particles (CBPs) formed during diesel combustion. CBPs can contribute up to 20% of the total mass of PM10 in urban settings, and to date, have been regarded as toxic through

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their action as delivery vectors for materials absorbed on their surfaces.

In this study we sought to examine the potential toxicity of a range of CBPs through their ability to react with, and deplete, low molecular weight antioxidant defences present in ELF. We hypothesised the interaction of CBPs within this aqueous compartment represents the first physical interface encountered by inspired particulate matter. Given this, and the knowledge that the surface chemistry of particles is an important factor in determining the subsequent pulmonary response, we hypothesised that the oxidative capacity of particles could be demonstrated through their reaction with, and depletion of ELF antioxidants.

METHODS

Exposure system

To conduct these experiments we modified an exposure apparatus, previously used to examine the impact of gaseous oxidants on ELF antioxidants (Mudway and Kelly, 1998). In the model system, ELF was represented in two ways. First as single antioxidant solutions at physiological concentrations, ascorbic acid (200 μmol/L), uric acid (200 μmol/L) or GSH (400 μmol/L) and second, as a composite mixture of these three antioxidants. To standardise the system, a model carbon black particle (M120) was employed at three concentrations (50, 150 and 500 μg/ml). These values where chosen as they represent (a) the maximum UK hourly average value (b) the mean UK 8 hour running average and simultaneously the UK PM10 upper limit and (c) the maximum UK daily average (National Environmental Technology Centre, London 1995), respectively. These CBP doses were arrived at using the following assumptions (1) only 65% of the available CPB dose is deposited in the lung and (2) the "morphological volume" of the mucus and alveolar lining layers in humans is 12.6 ml (Hatch 1994). For comparison, we subsequently examined the impact of two other types of CBP (M880 and R250) on ELF antioxidants.

Exposures were carried in a 5.6l Perspex chamber. The chamber was maintained throughout at 37±2.8 °C, and the whole apparatus mounted on an orbital shaker to facilitate mixing of the aqueous phase. Antioxidant solutions were incubated with particles as 1ml aliquots in multi-well plates (Becton Dickinson UK Ltd., Oxford, UK.) fixed within the exposure chamber. Each well had a diameter of 1.5 cm, giving an exposed surface area of 1.78 cm^2. Prior to the addition of particles, solutions were allowed to equilibrate to 37°C. Concentrated particle solutions were then added to specific cells to give the desired final particle concentration. The chamber was closed and the orbital shaker switched on. At set intervals during the exposure: 30, 60, 120, 240, 360 min, the shaker was switched off, the chamber opened and 3 samples removed. These samples were centrifuged at 5000 rpm for 5 min (4°C) to separate the particles from the aqueous components and the supernatant decanted to a fresh tube and snap frozen in liquid nitrogen. At the end of the experiment all samples were transferred to - 80°C for longer term storage.

Characteristic of carbon black particles

The three different types of CBP used in this study were donated by the Cabot Corporation, Billerica, Ma, USA. Prior to utilizing the particles, their morphology and surface chemistry were examined. The diameter of single particles of carbon black type M120 was 50 nm, type M880 was 20 nm and R250 was 40nm. The surface area expressed as m^2/g was 32, 230 and 62, respectively for M120, M880 and R250 types of CBPs. Electron probe x-ray microanalysis (EPXMA) studies (Murphy et al. 1998) confirmed that CBP M120 was a pure carbon black, which contained only chloride and sulphur. Sulphur was presumably derived

from the fuel source during particle generation. M880 was found to contain sodium, silicon sulphur, chloride and potassium while R250 has silicon sulphur and iron.

Antioxidant solutions

Physiological ELF concentrations of AA, UA, and GSH/GSSG, were prepared: 200 µM AA (Hatch, 1994; Slade *et al.*,1993), 412 µM GSH and 17 µM GSSG (Cantin *et al.*, 1987), and 200µM UA (Hatch, 1994; Slade *et al.*,1993), either as pure antioxidant solutions, or as a composite mixture of the above moieties. Solutions were prepared in 0.9% (w/v) saline, both in the presence and absence of the chelating agents, ethylenediaminetetra-acetic acid (EDTA) and desferoxamine mesylate (DES) (0.1 mM final concentration). Solutions were also adjusted to pH 7.4, to reflect normal human airway secretions.

Preparation of CBP M120 stock solution

Three solutions of stock CBP M120 were prepared by suspending 10, 30 or 100 mg of dry CBP in 10 ml of 0.9% (w/v) saline adjusted to pH 7.4 at RT. These solutions were continuously mixed for two hours and then 50 μl of each was added to 1 ml aliquots of antioxidant solution in multi-well plates, giving the final CBP concentrations of: 50, 150 & 500 μg/ml, respectively. Control samples, which did not contain CBP, were run in parallel throughout. In a similar manner, stock solutions of CBP M880 and R250 were prepared.

Antioxidant determinations

UA, AA, GSH and GSSG were determined as previously described (Housley *et al.*, 1995).

Determination of antioxidant consumption/loss rates

Rates of antioxidant consumption, or formation of oxidised glutathione (GSSG) do not represent mol antioxidant verses unit mass reaction rates but rather antioxidant/GSSG concentration changes with time, or time per ug particulate. These rates therefore represent a composite of both direct consumption/formation by particles and that arising through secondary oxidation reactions. In addition, it is feasible that some loss of antioxidant occurs due to adsorption onto the particle surface. Rates have therefore been expressed either as the change in concentration with time (M s^{-1}), or change in concentration with time per particle concentration (M s^{-1} μg^{-1}). The former rate was determined from the gradient of a linear regression passed through a plot of antioxidant/oxidative damage marker concentrations against exposure time at each particle concentration used. In the majority of cases this relationship was linear with time. The 'goodness of fit' of the regression through each set of data points is indicted by the regression coefficient (r^2) and the probability that the regression line does not deviate significantly from the linear model (p). Gradients derived from linear regressions were only used where $p<0.05$. Where data could not be fitted by linear regression the 'best fit' polynomial regression was performed and the rate of consumption determined over the linear portion. The later rate expression, M s^{-1} μg^{-1}, was similarly derived by plotting the consumption rates observed under each particle concentration, derived as described above, against particle concentration.

Statistical analysis

Overall variations in data sets were determined using a two way, repeated measures analysis of variance. The factors employed for each analysis were as follows: for the '*rate of consumption (M s^{-1})*, with respect to the pure antioxidant and composite antioxidant data sets; particle concentration (0, 50, 150, 500 µg particle), and antioxidant (AA, UA, and GSH); for the '*overall consumption rate* (M s^{-1} μg^{-1}) *data set*' for each model system employed;

antioxidant (AA, UA and GSH) and exposure model (pure antioxidant and composite antioxidant solution). Pairwise comparisons of group means within each data set were conducted using the Student-Newman-Keul test, with corrections for multiple comparisons. Linearity of response was determined by linear regression analysis using the least squares method. Statistical significance for comparisons of means and linear regression analysis was accepted at $p < 0.05$.

RESULTS

M120/Antioxidant interactions in Pure and Composite Solutions

Consumption rates for AA, UA and GSH by M120 in pure and composite antioxidant solutions are given in Table 1. Significant increases in consumption rate were observed for AA at 50, 150 and 500 μg/ml CBP and 150 and 500 μg/ml CBP for GSH (initial rate) than under control (0 μg/ml) CBP exposure in pure antioxidant solutions. In comparison, in composite antioxidant solutions, GSH consumption rates were significantly increased only at 500 μg/ml CBP.

TABLE 1. Antioxidant Consumption Rates in Pure and Composite Antioxidant Solutions

MI20	Pure Antioxidant Solutions					
	Ascorbate		Urate		Reduced glutathione	
(μg/ml)	Consumption rate ($M\ s^{-1}$)	r^2	Consumption rate ($M\ s^{-1}$)	r^2	Consumption rate ($M\ s^{-1}$)	r^2
0	$4.5 \pm 3.3 \times 10^{-10}$	-0.69	$6.7 \pm 0.4 \times 10^{-10}$	-0.69	$8.5 \pm 2.3 \times 10^{-10}$	-0.32
50	$10.4 \pm 2.1 \times 10^{-10}$ *+	-0.92	$4.1 \pm 1.2 \times 10^{-10}$	-0.45	$8.2 \pm 0.8 \times 10^{-10}$ +	-0.59
150	$38.9 \pm 10.0 \times 10^{-10}$ *+	-0.99	$2.7 \pm 1.2 \times 10^{-10}$	-0.03	$53.4 \pm 2.3 \times 10^{-10}$ *+ •	-0.96
500	$90.5 \pm 8.5 \times 10^{-10}$ *+	-0.88	$9.7 \pm 1.2 \times 10^{-10}$ *	-0.67	$151.3 \pm 17.8 \times 10^{-10}$ *+ •	-0.90

M I20	Composite Antioxidant Solutions					
	Ascorbate		Urate		Reduced glutathione	
(μg/ml)	Consumption rate ($M\ s^{-1}$)	r^2	Consumption rate ($M\ s^{-1}$)	r^2	Consumption rate ($M\ s^{-1}$)	r^2
0	$9.9 \pm 1.3 \times 10^{-10}$	-0.98	$3.7 \pm 1.0 \times 10^{-10}$	-0.76	$4.7 \pm 8.6 \times 10^{-10}$	-0.01
50	$24.0 \pm 1.2 \times 10^{-10}$ *+	-0.98	$4.1 \pm 1.5 \times 10^{-10}$	-0.50	$3.2 \pm 1.5 \times 10^{-10}$	-0.08
150	$52.9 \pm 3.6 \times 10^{-10}$ *+	-0.97	$2.7 \pm 1.0 \times 10^{-10}$	-0.09	$68.4 \pm 8.5 \times 10^{-10}$ *+ •	-0.90
500	$81.2 \pm 3.7 \times 10^{-10}$ *+	-0.81	$2.5 \pm 0.4 \times 10^{-10}$	-0.19	$253.3 \pm 9.5 \times 10^{-10}$ *+ •	-0.81

Consumption rates are expressed as means ± SD (n=3). * Significant increase in the consumption rate of each antioxidant at a prescribed carbon black particles concentration compared with the 0 μg/ml carbon black particles control. For comparison of antioxidant consumption rates at a specific carbon black particle concentration: + Significant difference between consumption rate of AA and GSH vs. UA; and • Significant difference between consumption rate of AA vs. GSH. In all cases significance was assigned when $p < 0.05$.

To clarify the relationship between the antioxidant consumption rates and CBP concentration, plots of antioxidant consumption rate against CBP concentration were performed (plots not illustrated). In the case of AA and GSH, a near linear, positive relationship was observed: $r^2 = 0.98$, $p < 0.05$ (AA) and $r^2 = 0.98$, $p < 0.05$ (GSH). The gradients of the individual regression lines were then calculated, expressed as $M\ s^{-1}\ \mu g^{-1}$, broadly indicative of the substrate reactivity towards CBP. Comparison of these rates,

summarized in Table 2, revealed a significant difference in the overall consumption rates between GSH and AA versus UA and GSH versus AA, suggesting an overall reactivity hierarchy within the pure antioxidant model of GSH > AA >>> UA.

TABLE 2. Antioxidant depletion rates in pure and composite antioxidant solutions

	OVERALL ANTIOXIDANT CONSUMPTION RATES BY M120					
ANTIOXIDANT	Pure antioxidant solutions			Composite antioxidant solutions		
	($Ms^{-1}\mu g^{-1}$)	r	p	($Ms^{-1}\mu g^{-1}$)	r	p
AA	$17.3\pm1.5 \times 10^{-12}$	0.98	<0.05*	$13.3\pm 3.1\times10^{-12}$	0.90	<0.05*
GSH	$29.7\pm3.9 \times 10^{-12}$	0.98	<0.05*	$51.2\pm 2.2\times10^{-12}$	0.98	<0.05*+

Values represent means ± SD (n=3). * A significant difference between the overall rates of AA and GSH consumption vs. UA in each exposure model; + a significant difference between the overall rate of GSH consumption vs. that of AA in each exposure model. In all cases significance was assigned when p<0.05.

GSSG Formation

As with GSH consumption, complex formation kinetics where observed for GSSG. A rapid initial increase in GSSG at 150 and 500 μg/ml CBP, was followed by the establishment of a new steady state concentration after 2-4 hours of exposure. Because of these kinetics, GSSG formation rates were calculated only over the initial linear portion of the best fit polynomial regression. In all experiments, the rate of GSSG formation was significantly enhanced in comparison with the 0 μg/ml CBP control (data not shown).

Overall GSSG formation rates per unit CBP, calculated from the gradients of linear regressions were as follows: $14.3 \pm 3.2 \times 10^{-12}$ ($r^2 = 0.98$; $p < 0.05$) in the pure GSH model and, $25.3 \pm 0.9 \times 10^{-12}$ ($r^2 = 0.99$; $p < 0.05$) in the composite model, (n=3). These rates, approximately one half of the corresponding GSH overall consumption rates support a 2 : 1 GSH : GSSG reaction stochiometry.

TABLE 3. Particle characteristics with ascorbate and reduced glutathione depletion rates expressed per 150 μg of particle mass and corrected for available surface area

article	Dia (nm)	Surface Area (m^2/g)	AA Depletion rate ($\mu mol\ L^{-1}\ s^{-1}$)	AA Depletion rate (per m^2/g SA)	GSH Depletion rate ($\mu mol\ L^{-1}s^{-1}$)	GSH Depletion rate (per m^2/g SA)
120	50	32	$-5.3\pm0.4\times10^{-9}$	$-0.17\pm0.01\times10^{-9}$	$-7.5\pm1.2\times10^{-9}$	$-0.23\pm0.06\times10^{-9}$
880	20	230	$-25.8\pm0.01\times10^{-9}$*	$-0.11\pm0.004\times10^{-9}$*	$-22.0\pm7.4\times10^{-9}$*	$-0.10\pm0.01\times10^{-9}$*
250	40	62	$-12.9\pm0.2\times10^{-9}$*†	$-0.2\pm.003\times10^{-9}$*†	$-5.1\pm0.03\times10^{-9}$†	$-0.08\pm0.01\times10^{-9}$*

All rates are expressed as the mean ± standard deviation of three individual experiments. Statistical analysis was performed using a one-way ANOVA with pairways comparison performed using the Student-Newman-Kuels procedure. * indicates that the rate of antioxidant depletion for M880 and R250 are significantly different from M120. + indicates significant difference between that depletion rates observed for M880 and R250.

DISCUSSION

Concern about the health effects of particulate air pollution continues to grow on an international scale. As a consequence, increased effort is being made to understand the mechanisms underlying the biological toxicity of particulate matter. Whilst toxicity of pollutant gases, such as ozone and nitrogen dioxide, is widely acknowledged to involved oxidative stress, the mechanisms behind particulate toxicity are less well understood. As many of the absorbed materials on particles are recognised oxidants we hypothesised that oxidative stress may be also an important component of particle-induced lung injury. Moreover, as the surface of the lung is protected by a network of soluble antioxidant defences (Cantin et al., 1987; Slade et al., 1993; Kelly et al., 1996), we predicted that particles with oxidative capacity would interact with, and deplete, these antioxidants. In particular, we thought that the surface chemistry of the particles would play a major role in determining their oxidative capacity.

In our initial examination of this hypothesis we determined if a simple CBP, M120, would deplete AA, UA or GSH from a model system. The findings from these experiments support this theory, as time, and dose-related depletion kinetics were observed. Moreover, we observed that M120 preferentially depleted AA and GSH (with concomitant GSSG formation) as opposed to UA. This finding is in marked contrast to those observed for the pollutant gases ozone (Mudway and Kelly, 1998) and nitrogen dioxide (Kelly and Tetley, 1997) which react avidly with, and deplete, AA and UA but not GSH. From these findings, we conclude that deposition of CBPs within the respiratory tract lining fluid may compromise the airways antioxidant defensive screen and hence accelerate epithelial injury.

To investigate these findings further we next examined the reactivity of two further forms of CBPs, M880 and R250. The objective of this second study was to determine if particles, of variable morphology and surface properties, have different reactivities towards ELF antioxidants. The findings demonstrated that particle surface area, and surface chemistry, are both important determinants of particle reactivity. Whereas none of the particles examined had any reactivity with UA, both M880 and R250 had greater reactivities with AA and GSH than M120. However, when these reactivities were corrected for particle surface area the hierarchy changed to become, AA: R250 > M120 > M880; and GSH: M120 > R250 = M880, indicating the importance of available surface area in these reactions.

As lung epithelial lining fluid is the first interface encountered by inspired particles, the reactions between particles and antioxidants as they pass through this fluid layer will presumable modify the impact of these particles on the pulmonary epithelium. Indeed, it has been proposed that the antioxidants in this compartment help protect the lung from oxidative injury arising from inspired gaseous and particulate pollutants (Kelly et al, 1995). One possible interpretation of the present results is that as CBPs readily react with AA and GSH, their surface chemistry is changed as they pass through the ELF compartment and as a consequence they are less reactive when they reach the pulmonary epithelium. If this proves to be the case, the capacity of particles to react with ELF antioxidants represents an important process in detoxifying the particle and hence limiting their subsequent biological activity. If ELF antioxidants are acting in this way, this possibility requires further consideration, as individual variations in the concentrations of these moieties, may explain, in part, the differential susceptibility of subjects to PM10.

In conclusion, the finding of the present study contribute towards an improved understanding of particle-mediated lung injury. The finding that relatively chemically inert particles can result in a marked loss of airways antioxidants suggest that that particles, in combination with oxidant gases, will synergistically reduce the level of endogenous airway protection.

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A CARDIOPULMONARY RAT GENE ARRAY FOR SCREENING ALTERED EXPRESSION PROFILE IN AIR POLLUTANT-INDUCED LUNG INJURY

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ABSTRACT

Cardiopulmonary injury and repair processes involve complex and coordinated cellular events such as necrosis, inflammation, cell growth/differentiation, apoptosis and remodeling of extracellular matrix. These processes are regulated by expression of multiple mediator genes. Commercially available microarray blots and slides allow screening of hundreds to thousands of specific genes in a single sample preparation. However, often these blots do not contain cDNAs of specific interest and are expensive. In order to analyze the tissue expression profile of a large number of genes involved in cardiopulmonary injury and pathology, we developed a mini gene array filter using the gene array technology. This array consisted of 30 genes representing inflammatory and anti-inflammatory cytokines, growth factors, adhesion molecules, stress proteins, transcription factors and antioxidant enzymes. Using rat gene specific PCR primer pairs, cDNAs for these 30 genes were amplified and cloned into a TA vector. Plasmids with recombinant cDNA inserts were purified and blotted onto a nylon membrane. Total RNA was isolated from lung tissues recovered from rats exposed intratracheally (IT) to either saline (control) or residual oil fly ash (ROFA; 3.3 mg/kg). Lung RNA was also isolated from a separate group of rats instilled with metals found in one instillate of ROFA: nickel (Ni; $NiSO_4$; 1.3 μmol/kg) or vanadium (V; VSO_4; 2.2 μmol/kg). ^{32}P-labeled cDNA was generated from these RNA samples in a reverse transcriptase reaction and subsequently hybridized to the array blots. Densitometric scans of blots revealed a 2-3 fold induction of adhesion molecules and growth factor mRNAs such as VCAM-1, fibronectin (cFn-EIIIA) and PDGF-A chain. Increased expression of VCAM-1 following ROFA and metal exposure observed in the array blot was confirmed through hybridization of northern blots. Developing a customized gene array to study tissue specific markers provides a quick tool to screen the -gene expression profile of the target tissue.