Inflammatory Cytokine Response to Ambient Particles Varies due to Field Collection Procedures

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***In vitro* assays of biological activity induced by particulate matter (PM) are a tool for investigating mechanisms of PM health effects. They have potential application to exposure assessment in chronic disease epidemiology. However, there has been little reporting of the impact of real-world PM collection techniques on assay results. Therefore, we examined the effect of sampling duration and post-sampling delays in freezing on PM-induced biological activity. Duplicate samples of respirable ambient Los Angeles PM were collected on polyurethane foam filters during 17 days and during three contemporaneous consecutive shorter periods. After collection, one duplicate was stored at ambient temperature for 24 hours before freezing; the other was frozen immediately. Cytokine response (IL-1β, IL-6, IL-8, and TNF-α) to PM aqueous extract was assessed in THP-1 cells, a model for evaluating monocyte/macrophage lineage cell responses. There was consistent 3- to 4-fold variation in PM-induced cytokine levels across the three collection intervals. Compared with levels induced by PM pooled across the three periods, continuously collected PM-induced levels were reduced by 25% (IL-6) to 39% (IL-8). The pattern of cytokine gene expression response was similar. Cytokine level variation by time to freezing was not statistically significant. PM-induced inflammatory response varied substantially over a weekly time scale. We conclude that long PM sampling interval induced less activity than the average of equivalent shorter consecutive sampling intervals. Time to freezing was less important. Implications for development of metrics of long-term spatial variation in biological exposure metrics for study of chronic disease merit further investigation.**

Keywords: air pollution; toxicology; exposure assessment; epidemiology

CLINICAL RELEVANCE

In vitro assays of biological activity induced by particulate matter (PM) are a tool for investigating mechanisms of PM health effects. They have potential application to exposure assessment in chronic disease epidemiology.

Toxicological, clinical and epidemiological study has demonstrated the health effects of ambient particulate matter (PM) less than 10 μm and 2.5 μm in aerodynamic diameter (PM_{10} and $PM_{2.5}$), respectively (1). However, PM composition is heterogeneous, and varies seasonally and geographically depending on local sources, meteorology, and atmospheric chemistry (2). Toxicological effects of PM of the same size vary by composition (3–5). Seasonal and geographic differences in acute cardiorespiratory health effects in time series studies likely reflect, at least in part, differences in composition (6–9). Unfortunately, the cost of measuring the health-relevant components of the PM mixture is high. It is not feasible to measure metals, the many toxicologically relevant species in organic carbon and elemental carbon, endotoxin, and other contributors to the aerosol, to name a few PM components, on the spatial and temporal scale necessary to evaluate chronic effects in large epidemiological studies.

A potential alternative to PM compositional analysis for exposure assessment in epidemiological studies is to evaluate biological effects of PM directly using *in vitro* assays, which have been widely used for many years to examine toxicological effects of PM. Assays of oxidative stress and inflammatory responses in PM-exposed cell systems, for example, could be used to provide an integrative assessment of biological activity of the PM mixture at modest cost. Assays reflecting different biological pathways of effect could be used to examine the mechanisms underlying epidemiological associations of PM with both acute and chronic disease. Associations in plausible pathways would also help reduce uncertainty in the causal interpretation of epidemiological findings.

An elegant series of studies in the Utah Valley with PM_{10} collected before, during, and after temporary closure of a steel mill demonstrated the relevance of this approach for human health. Particles collected during operation induced expression of inflammatory cytokines (IL-6 and IL-8) in a dose-dependent relationship when added to cultures of human respiratory epithelial cells (10). In rats, large lung lavage fluid inflammatory responses were observed only after intratracheal instillation of particles collected during mill operation (11). No response was observed to PM collected when the mill was not operating. A study of human volunteers showed larger inflammatory responses in lung lavage fluid after challenge with PM collected during periods of mill operation than during periods when the mill was not operating (12). These experiments suggested a biological mechanism for the results of several epidemiological studies that found associations of hospitalization and school absences due to respiratory illness with increases in the PM_{10} while the mill was operating (13–15). More recently, acute health effects have been associated with *in vitro* biological responses to PM (16).

There have been a few studies showing seasonal or broad spatial variability in PM proinflammatory or oxidant effects based on *in vitro* assessment of PM cellular response, or oxidative properties of PM (3, 5, 17, 18). However, to our knowledge, the use of toxicological assays to assess spatial variability in long-term PM biological activity would be a new paradigm in exposure assessment for chronic effects epidemiology.

In the southern California Children's Health Study, we are evaluating the long-term spatial variability in PM-induced proinflammatory

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activity in *in vitro* assays in model cell systems. We plan to evaluate the relationship of near-roadway PM-induced biological activity to respiratory health. However, we have identified several methodological challenges in the course of developing this study. For example, the impact of chemistry that might occur on filter-collected PM during prolonged collection periods has not been evaluated. If biological activity changes with duration of collection, for example, then the measured activity may not accurately reflect the activity that fresh airborne PM might produce in the lung. Two or more weeks of sampling in different seasons have been shown to be necessary to characterize average annual exposure to regional pollutants based on measurements from community monitoring stations (19). Characterizing smaller area variability in pollutant metrics reflecting near-roadway exposure requires temporally synchronized deployment at multiple locations, typically during multiple 2-week periods throughout the year (20).

In a monocytic THP-1 cell line reflecting alveolar macrophage response to PM (21), the impact of duration of particle collection on PM-induced cytokine response was evaluated. For a 17-day sampling duration that might be employed to characterize long-term exposure in an epidemiological study of chronic disease, we compared PM-induced cellular response to extract from a continuously collected sample to pooled extract from three shorter sampling intervals over the same period. We also compared the PM-induced cytokine response from filters frozen immediately after collection with the response from filters frozen after a 24-hour delay. A field study collecting PM from multiple locations over a large geographical area might result in a similar delay in recovering and freezing filters at the conclusion of sampling.

MATERIALS AND METHODS

Size-resolved PM was collected with a Harvard Cascade Impactor (22) modified for the Children's Health Study to include an additional cut-point of 0.2 and 0.5 μm in addition to standard-size cuts at 2.5 and 10 μm. Flow rate was set to 5.0 L/min, controlled by needle valve flow restriction, and measured before and after sampling with a Gilibrator bubble meter (Sensidyne LP, St. Petersburg, FL).

The collection substrate at each size cut was polyurethane foam (PUF), which allows larger PM mass accumulations before particle bounce or overloading occurs (22, 23). A Teflon backup filter was used to collect $PM_{0.2}$. Special cleaning procedures were used to remove endotoxin from the PUF. These included three repetitions of sonication in methanol; soaking in Milli-Q (MQ; EMD Millipore, Billerica, MA) highly purified water; sonication in mild HCl acid and MQ water solution; and soaking in MQ water. Filters were air dried at 50°C in polyethylene bags later used for shipping. All PUF handling was only with precleaned Teflon tweezers. Filters were stored in Petri dishes that underwent multiple HCl soakings, followed by MQ water soakings.

Eight samplers were deployed on a building rooftop at the Health Sciences Campus of the University of Southern California. Duplicate samplers collected PM during 17 days and during sequential Days 1–5, 6–10, and 11–17 over the same period (April 16 to May 3, 2010). From one of each duplicate sampler, the filters were removed at the conclusion of the corresponding sampling period and frozen immediately at −10°C. The other sampler remained outdoors for 24 hours before the filters were removed and frozen to simulate field procedures during large sampling campaigns. The PUF filters (including mass from PM_{10} to $PM_{0.2}$) were shipped on dry ice to the University of North Carolina, where they were stored at −20°C.

Before PM extraction, the PUF filters were equilibrated in a conditioning room at 22°C and at 33% relative humidity for 24 hours before weighing on a Mettler balance (Mettler Toledo, Columbus, OH) to within 0.002 mg. Particles were extracted from each filter by vortexing in 0.3 ml sterile distilled water for 10 minutes, followed by 30-second sonication. The filters were then lifted from the solution and mildly squeezed to expel excess water into the extraction solution. After re-equilibrating the filters for 24 hours in the conditioning room, postextraction weights were measured, and extracted mass was calculated based on the loss in mass during extraction.

The $PM_{10–0.2}$ extraction solutions were pooled from each sampler before cell challenge.

Cell Culture

The human monocytic leukemia cell line, THP-1, was purchased from ATCC (Rockville, MD). THP-1 cells have been used as a model system for cells of monocyte/macrophage lineage in the study of ozone effects (24). Cells were cultured in RPMI 1,640 medium (Invitrogen, Grand Island, NY) containing 10% FBS with penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in 5% CO_2; 1×10^6 cells in 0.5 ml medium were incubated in round-bottom polystyrene culture tubes with loosened caps for PM stimulation studies.

Cytokine Assays

One million THP-1 cells in 0.5 ml of RPMI 1,640 medium were incubated with PM at 37°C for 24 hours. The culture tubes were centrifuged at $500 \times g$ for 10 minutes. The supernatants were collected for measurement of inflammatory mediators, including IL-1β, IL-6, IL-8, and TNF-α, using the Meso Scale Discovery ELISA kit (Meso Scale Discovery, Gaithersburg, MD). Negative controls included the PBS solution and the extract from a field filter blank. Initially, THP-1 response to doses of 0, 25, 50, and 100 μg/ml of PM was used to determine optimum dose for subsequent assays.

Real-Time RT-PCR

THP-1 cells were exposed to PBS or PM for 24 hours. The cells were lysed with Trizol reagent (Invitrogen). Total RNA (100 ng), 0.5 mM NTP (Pharmacia, Piscataway, NJ), 5 μM random hexaoligonucleotide primers (Pharmacia), 10 U/μl RNase inhibitor (Promega, Madison, WI), and 10 U/μl Moloney murine leukemia virus RT (GIBCO BRL Life Technologies, Grand Island, NY) were incubated in a 40°C water bath for 1 hour in 50 μl 1× PCR buffer to synthesize first-strand cDNAs. The reverse transcription was inactivated by heating at 92°C for 5 minutes. Oligonucleotide primer pairs and fluorescent probes for IL-6, IL-8, IL-1β, TNF-α, and β-actin were designed and obtained from Integrated DNA Technologies (Coralville, IA). Quantitative fluorogenic amplification of cDNA was performed using the ABI Prism 7,500 sequence detection system (Perkin-Elmer, Palo Alto, CA). The relative abundance of IL-6, IL-8, IL-1β, or TNF-α mRNA levels was calculated using the difference between the cycle threshold of the cytokine mRNA sequence and the reference β-actin mRNA sequence.

Statistical Analysis

We used ANOVA of replicate assays on supernatant from each PM-challenged cell culture to evaluate the variability in the cytokine response due to period of collection, duration of collection, and time to freezing filters. For some analyses, equal extraction volumes were pooled across the three periods of PM collection and across time to freezing filters to provide sufficient PM mass to conduct a larger number of replicate assays. Because sample sizes were small, nonparametric ANOVA approaches were used to evaluate statistical significance. All hypotheses were two sided and assessed at a 5% level of significance. All analyses were conducted using the SAS Version 9.2 statistical package (SAS Institute, Cary, NC).

RESULTS

An appropriate concentration for the assay was determined using the 17-day continuous PM collection. Based on the pattern of four replicate assays of cytokine response to concentrations from 0 μg/ml (the PBS control) to 100 μg/ml (Figure 1), 50 μg/ml was selected for all subsequent assays. The variability of the replicate assays on the extract was low, except at the highest dose for each assay.

The replicate assays of cytokine response to a typical PM sample (at concentration 50 μg/ml) and to each of the negative controls are shown in Table 1. For cytokine responses, both the field filter blank extraction and the PBS negative controls were in agreement, indicating that the cleaning procedure for the filters removed all

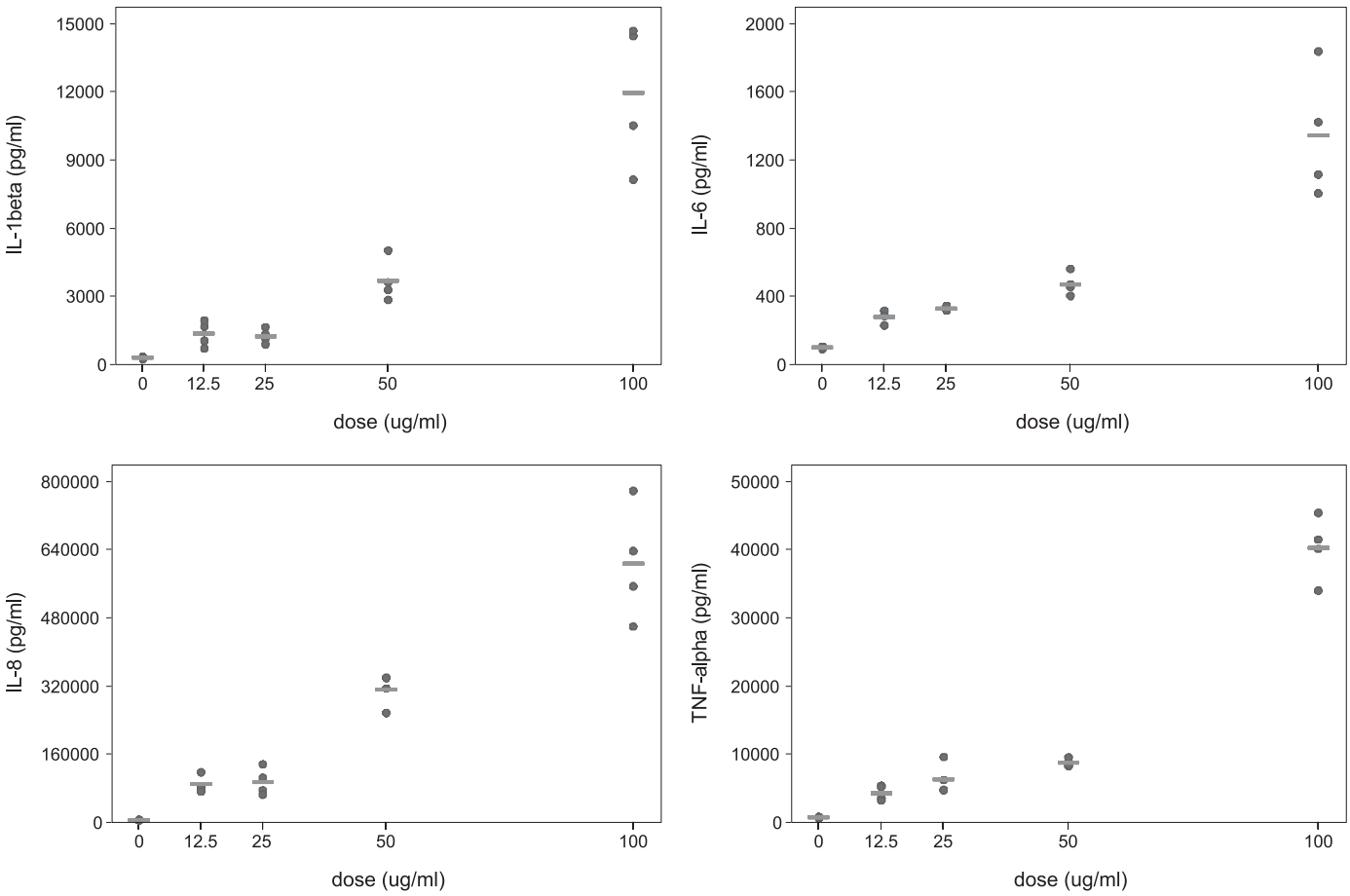

Figure 1. Cytokine response (IL-1β, IL-6, IL-8, TNF-α) to particulate matter at different doses from 0 to 100 μg/ml.

contaminants that are biologically active in this assay. The mean ambient PM-induced cytokine response varied from less than 10-fold (IL-6 and IL-8) to roughly 60-fold (IL-1β and TNF-α) larger than the mean of either negative control. In general, the replicate assays ($n = 3$) had low variability relative to the mean.

Each PM cytokine response was normalized to (divided by) the mean of the negative control filter extraction for the corresponding cytokine. There was 3- to 4-fold variability in this adjusted cytokine response across the three time periods ($P < 0.0001$ for all, except $P = 0.0006$ for IL-6; *see* Table 2). The second 5-day period PM induced the lowest, and the third (7-d) period induced the highest response for every cytokine.

To test the effect of freezing status, equal volumes of the three period-specific samples were then pooled by early and late freezing. A larger number of cytokine replicate assays ($n = 9$) were performed in these pooled samples to increase the power to identify differences in the response to PM frozen immediately and 24 hours after collection. There were small differences between the response to PM based on time to freezing (all less than 15%; *see* Table 3). Differences were not statistically significant for any cytokine.

Because there were no significant effects of delayed freezing, PM extract was pooled across time to freezing and the three collection periods. The final extract reflected the mass contribution of each period to the total mass collected during the continuous collection. PM-induced cytokine response was compared with that of continuously collected PM. The continuous collection during 17 days resulted in cytokine levels that were consistently reduced (by 25% for IL-6 to 39% for IL-8; Table 4). These differences were all statistically significant.

Cytokine RNA levels were also measured from the cells challenged with the continuously collected PM, and were found to be consistently reduced compared with levels from the pooled three-period collection (Table 5). The magnitude of the difference was similar to that observed for protein expression (except for TNF-α, which was reduced by only 15%). The differences were statistically significant for all cytokines.

TABLE 1. RESPONSE TO CONTROL AND AMBIENT PARTICULATE MATTER IN REPLICATE (FIRST THROUGH THIRD) CYTOKINE ASSAYS

Sample/Cytokines	Dose (*μg/ml*)	Protein (*pg/ml*) 1st	2nd	3rd	Mean
Field filter blank					
IL-1β	0	402	383	264	350
IL-6	0	167	102	77	115
IL-8	0	2,014	3,081	2,223	2,439
TNF-α	0	593	530	515	546
Laboratory blank					
IL-1β	0	369	293	267	310
IL-6	0	118	85	108	104
IL-8	0	1,762	2,165	1,775	1,901
TNFα	0	708	603	632	648
PM sample					
IL-1	50	17,992	17,860	25,311	20,388
IL-6	50	1,052	913	1,330	1,098
IL-8	50	188,891	162,512	193,733	181,712
TNF-α	50	27,260	30,599	34,713	30,857

Definition of abbreviation: PM, particulate matter.

TABLE 2. EFFECT OF PERIOD OF COLLECTION ON CYTOKINE RESPONSE

Cytokines/Period	Fold Increase*	*P* Value†
IL-1β		
1	60.0 (9.01)	<0.0001
2	35.4 (5.53)	
3	157 (13.5)	
IL-6		
1	15.2 (9.63)	0.0006
2	7.8 (3.06)	
3	22.3 (7.25)	
IL-8		
1	72.6 (5.08)	<0.0001
2	41.7 (6.01)	
3	148 (12.6)	
TNF-α		
1	55.6 (6.13)	<0.0001
2	29.5 (8.28)	
3	112 (13.6)	

* Mean (SD) fold increase over particulate matter–induced activity in field filter blank (based on $n = 6$ of 3 frozen immediately and 3 frozen after 24 h in each period).

† *P* value for heterogeneity by period, adjusted for whether frozen immediately or delayed.

DISCUSSION

We found marked variation in PM-induced THP-1 activity collected over consecutive 5- to 7-day periods. THP-1 reflects human proinflammatory macrophage response (25, 26), so this variation may have relevance for the variability in human response to ambient PM. The results are consistent with an emerging body of toxicological literature showing daily and seasonal variability in PM-induced inflammatory and oxidative stress responses, presumably due to changing composition of PM associated with meteorology and atmospheric chemistry (3, 4). We also showed that the biological activity of particles from the 17-day PM collection was reduced compared with the average of activity of the three shorter periods during the same 17-day interval. Gene and protein expression were consistently reduced across cytokines. The magnitude of reduction in cytokine levels induced by the single longer collection compared with the average of the three shorter intervals was substantial (from 25% for IL-6 to 39% for IL-8). There were no significant differences in activity induced by PM from filters frozen immediately after collection and PM stored at ambient temperature for 24 hours before freezing.

These results are relevant to the development of biological exposure indices for epidemiological studies of chronic disease. The optimum number of days of sampling to assess long-term (e.g., annual average) PM-induced biological activity that might be used in such studies has not, to our knowledge, been examined empirically. The large temporal variability in PM-induced activity indicates that, as for estimates of PM mass and other pollutants, sampling of several weeks across seasons is likely to be required. Studies of chronic effects of the small area spatial variation in near-roadway pollution have typically addressed this challenge by simultaneously measuring 2-week continuous integrated samples of a marker for the pollutant mixture. NO_2 and black carbon, for example, have been measured simultaneously at multiple locations in a community, and measurements repeated in different seasons (20, 27). Using these measurements, prediction models of average residential exposures at other locations have been developed based on nearby land use, such as traffic proximity, volume, and other characteristics, and average wind speed and direction. Our intention in the Children's Health Study is to use this approach to develop prediction models of average annual residential PM biological activity, using assays like the THP-1 cell system that we have used in this study. However, if the measurement of PM-induced biological activity decreases with duration of sampling, estimated exposure will be biased downward compared with the biological activity of the ambient PM to which the lung is exposed.

TABLE 3. EFFECT OF DELAYED FREEZING OF FILTERS ON SUBSEQUENT PARTICULATE MATTER–INDUCED CYTOKINE RESPONSE

	Freeze*		Difference†	
Cytokine	Immediately	Delayed	(%)	*P* Value†
IL-1β	46.2 (4.19)	50.0 (10.62)	8.5	0.52
IL-6	8.78 (1.64)	9.97 (1.59)	14	0.14
IL-8	34.6 (5.87)	39.4 (13.25)	14	0.58
TNF-α	19.5 (2.60)	20.5 (3.46)	5.0	0.52

* Mean (SD) fold change over blank control, based on nine replicate assays for immediate and for delayed.

† % Difference = ([delayed − immediate]/immediate) × 100; *P* value for difference.

Air pollution exposure assessment for chronic disease epidemiology based on PM-induced biological activity may still be a useful approach, even if long-term PM collection results in an underestimate of activity. As long as the measured biological activity is correlated with the true activity of inhaled ambient PM, the exposure index may provide a valid index of effect in a biologically relevant pathway. In this case, the measured activity could be adjusted upward to make it comparable to an ambient exposure experienced by the lung. Recent studies demonstrated differences in oxidative potential associated with PM collected in different European cities (17). Oxidative potential of PM also varied by traffic proximity within London and cities in The Netherlands (18, 28). To our knowledge, the impact of sampling duration on biological activity of ambient PM has not been studied previously by these or other investigators. Further investigation is required to assess the utility of these methods for epidemiological studies.

More generally, the impact of ambient PM collection and storage methods on biological activity has not been extensively studied by the toxicology research community. A recent study demonstrated that PM-induced biological activity varied depending on the collection method (29). Water extracts of PM collected on filters or impactors had lower oxidant activity than PM collected directly into a slurry. The investigators concluded that the difference was due to the loss of activity of water-insoluble particles from the extract of filters and impactors. Again, these limitations do not preclude use of filter-extracted PM for biological approaches to exposure assessment, as long as the measurement is correlated with the biological response produced in the lung, and further study is warranted to answer this question.

TABLE 4. EFFECT OF DURATION OF SAMPLING ON PARTICULATE MATTER–INDUCED CYTOKINE RESPONSE

	Collection Duration*		Difference†	
Cytokine	Pooled Three Period	Continuous	(%)	*P* Value†
IL-1β	42.5 (7.83)	26.8 (4.68)	−37	<0.0001
IL-6	9.44 (2.40)	7.04 (2.69)	−25	0.0006
IL-8	34.1 (8.26)	20.7 (3.91)	−39	<0.0001
TNF-α	16.8 (3.21)	11.4 (1.70)	−32	<0.0001

* Mean fold change over blank control (SD), based on 13 replicate assays for continuous and for pooled.

† Percent difference = ([continuous − pooled]/pooled) × 100; *P* value for difference.

TABLE 5. EFFECT OF DURATION OF SAMPLING ON PARTICULATE MATTER–INDUCED CYTOKINE GENE EXPRESSION

Cytokine	Collection Duration*		Difference† (%)	*P* Value†
	Pooled Three Period	Continuous		
IL-1β	18.7 (4.28)	12.5 (3.20)	−33	0.0005
IL-6	2.55 (0.41)	1.87 (0.77)	−27	0.03
IL-8	2.98 (0.44)	1.91 (0.31)	−36	<0.0001
TNF-α	2.11 (0.27)	1.79 (0.30)	−15	0.007

*Mean (SD) fold change over blank control, based on 13 replicate assays for continuous and for pooled.

† Percent difference = ([continuous − pooled]/pooled) × 100; *P* value for difference.

Other studies have found that the toxicity of PM in the ultrafine fraction (less than 0.1 μm in aerodynamic diameter) was greater than in the rest of PM_{10} (30–32). However, the water extraction efficiency of ultrafine particles from filters is less than that for larger particles, and decreases by size within the ultrafine fraction due to Van der Wall forces, becoming larger than the force available to remove the particle (33). For this reason, we did not include the $PM_{0.2}$ fraction in our assay of biological activity, because there are not good methods for extraction from Teflon filters without changing the biological activity of the extract.

PM in Los Angeles air is a complex mixture of crustal and combustion-derived metals, organic carbon containing polyaromatic hydrocarbons, and other reactive species in primary vehicular emissions that are the dominant PM source. If *in situ* chemistry of these components of PM is responsible for the reduced biological activity of the longer-duration collection PM that spent more time exposed on the surface of the filter, then these results may be generalizable to other cities with primary traffic sources. Primary emissions also undergo photochemistry in Los Angeles to produce secondary aerosol with relatively high nitrate concentrations, which differs from secondary aerosol high in sulfates in Eastern U.S. cities. It is also possible that bacterial growth on the filter occurring during the longer collection could induce an inflammatory cellular response, although this might be expected to increase rather than reduce activity. Bacterial growth could also change the chemistry of the PM on the filters, resulting in biological activity either increasing or decreasing, for example as a result of bacterial catabolism (and consumption) of polyaromatic hydrocarbons or other inflammatory components of organic carbon. Although filters from shorter- and longer-duration sampling periods were processed using standard procedures by the same technician, it is also possible that the differences were an artifact of handling procedures rather than duration of sampling.

For the spatial variability of PM-induced biological activity assays to be useful for predicting chronic disease in epidemiological studies, further investigation is warranted to see if the reduction of PM-induced response with longer duration of sampling is reproducible in THP-1 and other cellular systems. It is important to determine whether the PM-induced response varies over shorter sampling intervals than we examined. It would be useful to determine whether sampling duration–related variation in PM response is predictable, so as to allow adjustment of measurements made from long-duration collections to accurately estimate activity of ambient PM–induced exposure experienced by the lung. Finally, if sampling duration affects PM-induced biological activity, identifying the responsible PM component species may provide insight into the role of specific components in the etiology of human health effects of PM.

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