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Fine airborne urban particles ($PM_{2.5}$) sequester lung surfactant and amino acids from human lung lavage

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Kendall M. Fine airborne urban particles ($PM_{2.5}$) sequester lung surfactant and amino acids from human lung lavage. *Am J Physiol Lung Cell Mol Physiol* 293: L1053–L1058, 2007. First published July 6, 2007; doi:10.1152/ajplung.00131.2007.—Components of surfactant act as opsonins and enhance phagocytosis of bacteria; whether this process occurs with atmospheric fine particles has not been shown. We have studied the interactions of fine particles (urban $PM_{2.5}$) and surfactant removed from normal human lungs by lavage, using a surface analysis technique. The aim was to identify which of the chemical components of brochoalveolar lavage (BAL) deposit on the surfaces of urban $PM_{2.5}$. Deposition of surfactant components on urban $PM_{2.5}$ surfaces was reported in previous studies, but molecular identification and relative quantification was not possible using simple data analysis. In this study, we were able to identify adsorbed components by applying an appropriate statistical technique, factor analysis. In this study, the most strongly associated mass fragment on $PM_{2.5}$ surfaces exposed to BAL (and undetected on both untreated samples and saline controls) was di-palmitoyl-phosphatidylcholine, a component of lung surfactant. Amino acids were also strongly associated with BAL-exposed $PM_{2.5}$ surfaces and not other sample types. Thirteen mass fragments were identified, diagnostic of the amino acids alanine, arginine, asparagine, aspartic acid, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, serine, and valine. This study provides evidence that lung surfactant and amino acids related to opsonin proteins adsorb to nonbiological particle surfaces exposed to human lung lining fluid. Disruption of normal surfactant function, both physical and immunological, is possible but unproven. Further work on this PM-opsonin interaction is recommended.

bronchoalveolar lavage

EPIDEMIOLOGICAL STUDIES have shown that exposure to particulate air pollutants damages health (32). The exact mechanisms further underlying these air pollution effects remain unknown, although recent work (29, 39) has shown that cardiovascular system (acutely) and the developing respiratory system (chronic effects; Ref. 11) are most significantly affected. Effects on the cardiovascular system were seen on the day of exposure, indicating immediate short-term effects that were detectable within hours of exposures. It is likely that sensitivity to particles varies across the population with the young, the elderly, and those with preexisting cardio-respiratory disease being most at risk (31). Effects occur at ambient concentrations in industrial countries: These concentrations are low compared with concentrations recorded in earlier periods and with those common, now, in developing countries (8). Epidemiological and toxicological studies have demonstrated associations between specific PM characteristics and increased mortality and morbidity. Implicated characteristics include surface area, metals, acidic components, oxidative stress potential, elemental carbon, etc. (18a, 28, 30). The relative importance of these characteristics remains unknown.

The nature of ambient urban PM varies widely with location and time and is well studied: PM mass concentration, size range, surface area, bulk chemistry, and morphology have been studied at many locations (17). Using surface-sensitive techniques such as X-ray photon spectroscopy and time-of-flight secondary ion mass spectrometry (TOF-SIMS), it is now also possible to examine the surface chemistry of the ambient aerosol (21). With such inherent PM variability, it is perhaps surprising that PM is so consistently associated with specific health effects, both in terms of health outcome and concentration. But urban PM in cities around the world is similar in some key respects: combustion sources make an important contribution, mass concentrations exceed natural background levels, high concentrations of nano- or ultrafine size particles occur, and many particles comprise elemental carbon cores with hydrocarbon coatings and adsorbed trace species (17). These commonalities may be extremely important in causing the consistent associations found in epidemiological studies, and the effects may be dependent on interactions occurring at the site of first contact of particles with the body. PM depositing in airway and alveolar lining fluid represents the first contact with the body. Fine particles in the micron and submicron range are deposited in the deep lung in greater quantities than larger particles (38). In the alveolar spaces, these particles initially impact on the surfactant-rich alveolar lining layer (12). The alveolar lining layer has many functions, including maintenance of alveolar stability and immunological defence. The well characterised layer (~10% protein and 90% lipid by wet weight) supports defense reactions against foreign material such as biological particles (Reid KBM, personal communication; Ref. 40), but precise composition varies individually and temporally. Collectins [surfactant proteins (SP)-A and SP-D] attach to infectious particles to modify macrophage phagocytosis and host immunological responses (40). Dipalmitoylphosphatidycholine (DPPC) makes up ~50% of the phospholipid component of human surfactant and alone can account for the surface tension-lowering properties of surfactant (15). This occurs in an area-dependent way, thus reducing the force needed for lung inflation, preventing collapse and allowing alveoli of different sizes to exist while connected to each other. It also reduces fluid leakage into the alveolar spaces. DPPC is administered in respiratory distress syndrome to prevent alveoli collapse in newborns deficient in lung surfactant (Reid

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KBM, personal communication) and has been shown to be an effective treatment for lung inflammation after allergen challenge (1).

Understanding the initial interactions between PM and lung lining layer and assessing their downstream effects is likely to be important for explaining short-term health effects that occur within hours or days (30). It is known that surfaces of bacteria are opsonised by lung proteins immediately on deposition. Recent evidence has shown that polymer adsorbants modify $PM_{2.5}$ surfaces, and this affects their behaviour in lung fluid (19, 20, 22). These processes may be important for marking of particles as targets for phagocytosis. How sequestration of components of surfactant by deposited particles occurs is unmeasured. Surfactant sequestration by PM surfaces may be especially important where large surface areas are deposited, when PM deposition patterns are abnormal, or where these bio-molecules are in short supply (for example, in vulnerable individuals with respiratory infection, chronic disease or genetic predisposition; Ref. 7). Since the coating of deposited particulate by host proteins may modulate their biological activity, as they do for infectious particles, studies of such interactions may be expected to provide new information as to how inhaled pollutants may induce pulmonary and cardiovascular toxicity. Such studies, by necessity, are conducted on the nanoscale.

Toxicological studies have examined many of the effects of PM characteristics. However, in all but the animal/human exposure studies, the process of PM deposition in lung surfactant has been ignored. Animal studies may be a less than satisfactory model for effects in man because of the physiological differences between animal and human lungs and fundamental physico-chemical differences in the types, quantities, and activity of surfactant components (2, 18). Lung surfactant has been shown to be important in lung challenge by PM and allergen challenge (3, 13). However, the physico-chemical interactions involved are poorly understood.

The following key questions are addressed in this study: *1*) Which molecules adsorb from BAL to urban $PM_{2.5}$ surfaces? *2*) Of these, which are the most commonly adsorbed molecules? *3*) What patho-physiological consequences may be predicted as a result of adsorption?

Detailed statistical analyses of TOF-SIMS data identified which *m*/*z* values distinguish between BAL-exposed and untreated/control PM surfaces. This work aimed to identify which biological species adsorb to $PM_{2.5}$ samples exposed to BAL and presents a clear pattern of amino acid (AA) and DPPC adsorption. Examination of control samples was undertaken to confirm that these components did not occur on particles not treated with BAL.

METHODS

$PM_{2.5}$ samples were analysed by TOF-SIMS either untreated or treated with broncho-alveolar lavage (BAL) fluid (BALF) or a saline control solution for 4 h.

BAL preparation. Human BALF was collected in London during diagnostic fiber-optic bronchoscopy as described previously (22, 35). A sample of the supernatant from one subject was used per one or two $PM_{2.5}$ samples. Institutional Review Board approval to re-use these diagnostic samples was obtained from New York University as published by Kendall et al. (19). Concentrations of SP-A and SP-D in these BAL samples were measured using ELISAs (Yamasa, Japan), and surfactant DPPC was measured according to the methods used in Kendall et al. (20). These results are shown in Table 1.

Fine particle ($PM_{2.5}$) surface preparation. $PM_{2.5}$ samples were gathered as reported by Kendall et al. (19, 21). Twelve triplicate samples of $PM_{2.5}$ were collected on PTFE filters in New York City (n = 35; plus 1 lost sample). One sample from each triplicate was stored under nitrogen and analyzed untreated (*sample type 0*). One sample was placed in saline solution for 4 h (*sample type 1*), and one sample was placed in BAL for 4 h (*sample type 2*). After 4 h, the two liquid-treated samples were immediately rinsed with distilled water, dried under nitrogen, stored under nitrogen, and later analyzed. All samples were then analyzed by TOF-SIMS.

TOF-SIMS. TOF-SIMS (Univ. of Manchester) uses a pulsed high-energy ion beam to remove molecules or fragments of molecules from the surface of a sample. These fragment molecules removed from the surface (secondary ions) are accelerated into a "flight tube," and their mass is determined by measuring the exact time at which they reach a detector. Each mass fragment provides a fingerprint for specific molecules, which can be compared with a standard.

ToF-SIMS analysis was performed on a purpose-built BioToF-SIMS instrument, described elsewhere (6). A 15-keV Au/Ge primary ion source was used (Ionoptika). A primary ion pulser and mass-gate combination produced a 30-ns pulse of Au^+ ions with an equivalent continuous current of 1 nA. To improve mass resolution, the primary ion pulse was compressed to 5 ns using beam-bunching electronics. The ion beam was digitally scanned over a relatively large area of the exposed filter sample (1×1 mm^2) to minimize spot-to-spot variations in sample coverage or PM composition. Under these conditions, the primary ion dose for analysis was typically 2×10^{10} Au/cm^2, well within the static limit for the onset of observable damage ($\sim 1 \times 10^{13}$ Au/cm^2). Sample charge build-up during positive ion analysis was compensated with a low-energy (25 eV) electron flood gun (60-μs pulse width, DC equivalent current 20 nA). Under these conditions, it was not possible to stabilize the charge build-up during negative ion analysis of the PTFE filter samples, and consequently only positive ion spectra are reported.

Table 1. *Concentrations of SP-A, SP-D, and surfactant DPPC in BAL samples used to treat particle samples*

Treated PM Ref No:	Clinical BAL Ref. No:	SP-A, ng/ml	SP-D, ng/ml	Total Phospholipid, μg/ml
1298/1382	338	415	137	65.4*
1305	1302	388	129	
1310	378	303	69	74.8*
1315/1370	1402	991	90	
1320/1389	208	659	148	100*
1345/1375	1902	Not detected	61	
1365	802	367	88	
1394	380	124	165	42.5

*Average value from the measurement of two lavage aliquots. SP, surfactant protein; DPPC, dipalmitoylphosphatidycholine; BAL, bronchoalveolar lavage.

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Secondary ions were extracted by biasing the sample at 2.5 keV for 2 μs during the primary ion impact and mass analyzed in a dual-stage reflectron ToF-MS (Kore Technology, Cambridge, UK). Secondary ions were detected with a dual microchannel plate operated with 20 keV of postacceleration to increase the detection efficiency of high-mass molecules. Data were recorded with a 1-ns resolution time-to-digital converter over the mass range of 1–200 amu. For subsequent multivariate analysis, data were binned into 1-amu bins.

Standards of DPPC and lung proteins were analysed previously and can be found in studies by Wagner et al. (38, 39) and Kendall et al. (19).

Statistical analysis. The statistical method for the analysis of TOF-SIMS data was informed by the papers Lhoest et al. (24, 25) and Wagner et al. (38, 39). A statistical analysis was conducted to identify the molecules adsorbing from BALF to $PM_{2.5}$. No directly comparable method has been reported in the literature, and a new method for handling the data was developed.

The method adopted was *1*) data processing (normalization and mean-centring of mass data) and *2*) factor analysis (FA) in Matlab (version 6.5, The MathWorks, Natick, MA) of processed data. FA was used to identify inferred independent variables or "factors" that corresponded to sample treatment type. We then sought to determine the loadings of signature m/z values in each Factor to confirm which Factor represented which sample type. These indicator *m/z* values were selected based on prior works (19, 22), and FA was used to see whether they loaded as predicted to distinguish sample types. Once this was confirmed, we were able to establish which additional *m/z* values varied positively and negatively with each treatment to identify additional adsorbing species.

The 1- to 200-amu dataset was normalized first to the total intensity of each channel across all samples and then mean-centered to each sample mean. The samples were used as variables and the *m/z* values as observations. In the correlation matrix for these data, there were three factors with eigenvalues >1 according to the Kaiser criteria. We then generated the factor loadings matrix with varimax rotation to *1*) reduce the data to three variables to eliminate noise, *2*) explain the commonalities between samples of the same type, and *3*) explain systematic differences between samples of different types. This matrix of factor scores showed which *m/z* observations were positively or negatively associated with these three factors most strongly, thus discriminating between the factors. Using score plots, we looked at the three factors graphically. The plots for *factor 1* plotted against *factors 2* and *3* showed clear separation between the groups (Fig. 1). Plots of *factors 2* and *3* showed less separation, but a significant difference between most samples was clear.

To confirm the identity of different sample types, we examined the factor scores for each *m/z* in each factor. Factors with significantly positive or negative scores for certain *m/z* channels distinguished the different sample types. Each factor was allocated a sample type based on identified "fingerprint" *m/z* mass fragments known to be associated with each sample type (identified in Refs. 19, 21). Analysis identified *factor 1* as control/saline-treated samples (high in PTFE and hydrocarbons). *Factor 2* was identified as treated samples (high in hydrocarbons and DPPC). *Factor 3* was identified as untreated samples (high in trace metals and inorganic ions). To isolate the *m/z* values associated with BAL treated $PM_{2.5}$, we subtracted thus:

$$\text{BAL PM}_{2.5}\ m/z \text{ factor scores} > 1 - \text{control PM}_{2.5}\ m/z \text{ factor scores} > 1$$

$$\text{BAL PM}_{2.5}\ m/z \text{ factor scores} > 1 - \text{untreated PM}_{2.5}\ m/z \text{ factor scores} > 1$$

This subtraction determined which mass channels were positively associated with the BAL-treated $PM_{2.5}$. Each calculated *m/z* factor score therefore represents the relative strength of that association.

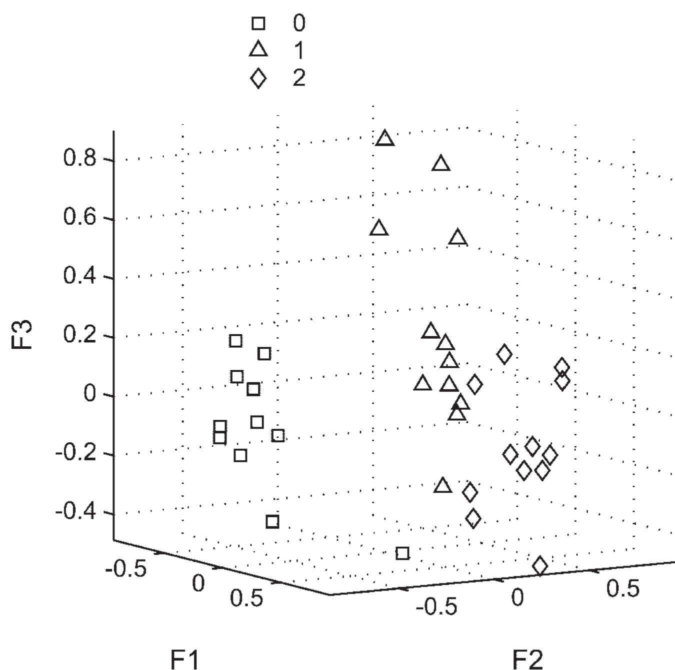

Fig. 1. A three-dimensional image of the 3 factors separated from the time-of-flight secondary ion mass spectrometry (TOF-SIMS) data by Matlab factor analysis: untreated (*sample type 0*), control (*sample type 1*), and human lung bronchoalvelolar lavage (BAL)-treated (*sample type 2*) sample types.

RESULTS

FA was able to establish three groups of samples from a blind dataset. Figure 1 shows the three-dimensional score plot of the three factors with eigenvalues of >1. Figure 1 represents graphically the statistical separation of the three sample types (marked 0, 1, and 2 in Fig. 1) using FA. The statistical grouping of the samples by TOF-SIMS *m/z* data is responsible for this statistical separation.

Untreated $PM_{2.5}$ factor (*sample type 0* in Fig. 1) separated more clearly because $PM_{2.5}$-associated trace species, such as inorganic species including metals, were present on these samples but were apparently removed by the liquids (saline and BALF) used in the treatment of *sample types 1* and *2*. *Sample types 1* and *2* were more difficult to distinguish because the process of removal of these $PM_{2.5}$ surface species exposed a large hydrocarbon surface and more PTFE, common to both sample types. However, the mass fragments representing molecules adsorbed from the lavage fluid onto the $PM_{2.5}$ surfaces in BAL-treated samples separated *sample types 1* and *2*.

A total of 17 mass fragments were identified only on BAL-treated samples. Where the sample $PM_{2.5}$ mass was low, the sample was only weakly linked to one *sample type 2* (BAL-treated $PM_{2.5}$), indicating that the adsorption of these bio-molecules was particle mass dependent, i.e., the bio-molecules did not adsorb to the PTFE (poly-tetra-fluoroethylene) filter substrate, which is hydrophobic.

Figure 2 shows the 17 mass fragments most strongly associated with $PM_{2.5}$ surfaces exposed to BAL. These molecules were absent on untreated and control $PM_{2.5}$ surfaces. Table 2 includes the identified biological molecules and the relative strengths of their associations (factor scores). An experienced TOF-SIMS operator identified 16 mass fragments as biological

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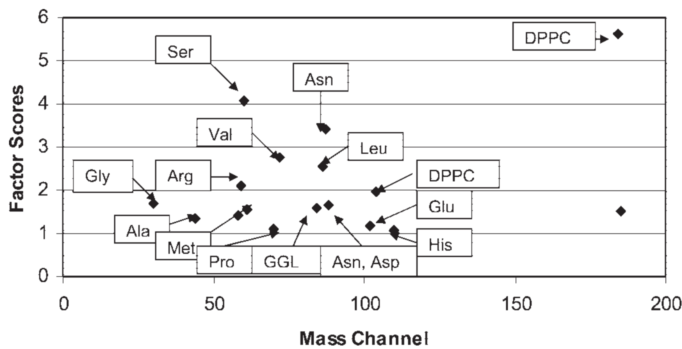

Fig. 2. Seventeen mass fragments detected by TOF-SIMS and only positively associated with BAL-exposed $PM_{2.5}$ surfaces. Factor scores represent the relative strength of the association of each mass fragment to the BAL-treated samples (<1 = no association; 5.63 is the strongest association). Labels indicate which fragments were identified by an experienced TOF-SIMS operator after comparison with data collected from the analysis of standards and should be read with Table 2.

in origin, after comparison with biological standards. The most strongly associated species at the BAL-treated $PM_{2.5}$ surface was identified as a DPPC fragment (*m/z*184). A number of other signals associated with BAL treatment in this study can be assigned to AA fragment arising from fragmentation of protein molecules (25, 34). Of these, the highest factor scores were associated with immonium ions of serine, asparagine, valine, leucine, and arginine.

DISCUSSION

The mass concentration and detailed surface chemistry of the New York City $PM_{2.5}$ samples have been described elsewhere (19). In summary, these samples were typical of mass concentrations measured in urban areas (17), and the surface chemistry was very similar to London $PM_{2.5}$ samples (21). The $PM_{2.5}$ samples used in this study may therefore be considered typical of "urban suspended fine particles," common to many urban areas due to their common sources and comparable urban atmospheric chemistry.

FA of nontreated, BAL-treated, and control urban $PM_{2.5}$ surface TOF-SIMS information was able to separate samples statistically by type, using only surface chemistry data as observations. Using the factor scores for mass fragments identified in earlier studies (19, 21), urban $PM_{2.5}$-associated salts and metals, biological molecules, and PTFE were the key differentiating mass fragments discriminating between the three sample types. The capacity of FA to separate sample types on the basis of TOF-SIMS surface chemistry data alone validated the methodology.

It was noted that immersion of urban particles in saline or BAL led to the removal of inorganic surface species, including metals. This is potentially important since these species have been seen as a source of free radical-generating reactions and thus of lung injury. This is an observation consistent with previous work and, we think, worthy of further study.

DPPC and AAs were the main adsorbants detectable on PM surfaces exposed to saline-diluted lung-lining fluid (BAL). After short periods in BAL, traces of DPPC and AAs can clearly be identified on the $PM_{2.5}$ surfaces but not on control or saline-treated surfaces. These results show that lung surfactant lipid and AAs adsorb to $PM_{2.5}$ surfaces. The source of the AAs is unknown, although it seems likely that SPs are adsorbed to the particle surfaces and act as a source of AAs released during analysis. Graham et al. (14) showed that *m/z* signals of AAs from proteins were related to their relative abundance in the original protein; in protein mixtures, serine and arginine were indicative of fibrinogen adsorption, and alanine, glutamine, and valine were indicative of albumin. The AAs that are important within the three-dimensional saccharide binding sites on the CRDs of SP-A and SP-D, are primarily glutamine, aspartic acid, asparagine, and arginine (Reid KBM, personal communication).

If $PM_{2.5}$ and lung surfactant interact in the lung, significant sequestration of these bio-molecules from the lung lining by extensive $PM_{2.5}$ surface might be expected. Certainly, this is widely observed for biological particles depositing in lung surfactant (40). Such a process may have significant conse-

Table 2. *m/z Values measured by time-of-flight secondary ion mass spectrometry on BAL-treated $PM_{2.5}$, not found on untreated or control samples*

Acronym	*m/z*	Full Name (s)	Factor Scores >1
Gly	30	Glycine	1.71
Ala, Arg, Asn	44	Alanine, arginine, asparagine	1.35
DPPC	58, 104, 184	DPPC	1.40, 1.96, 5.63
Arg	59	Asparagine	2.11
Ser	60	Serine	4.07
Met	61	Methionine	1.54
Pro, Asn	70	Proline, asparagine	1.10
Val	72	Valine	2.74
GGL	84	Glutamine, glutamic acid, lysine	1.60
Leu	86	Leucine	2.55
Asn	87	Asparagine	3.42
Asn, Asp	88	Asparagine, aspartic acid	1.67
Glu	102	Glutamic acid	1.18
His	110	Histidine	1.08
		Unidentified mass fragments in Fig. 2 with factor score >1	
	185		1.51

Factor scores represent the strength of association of each mass fragment with BAL-treated $PM_{2.5}$ surfaces. Sixteen of the 17 molecules have been identified as biological in origin (22, 34).

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quences where large surface areas are deposited in the lung or when deposition patterns are skewed toward certain areas (in vulnerable individuals with preexisting cardiac or respiratory disease) or where these biomolecules are in short supply (again in vulnerable individuals with certain genetic predispositions, respiratory infection, or chronic respiratory disease).

Significant quantities of data support the proposed hypothesis that surfactant interactions with PM are fundamentally important in PM health effects and require further study. Interactions of PM with surfactant lipids may lead to physical impairment of lung expansion, whereas interactions of PM with SPs may impair immunological processes. In Kendall et al.'s study (22), increased attractive and adhesive forces observed by AFM and SEM analysis showed clearly that the $PM_{2.5}$ aggregation occurs in BAL and suggested that this may be a role of lung opsonins. The level of aggregation or disaggregation of particles is generally related to the surface characteristics of the particles. Opsonization of inhaled solid particles by lung proteins, surfactant, or surfactant collectins SP-A and SP-D [which opsonise and aggregate biological particles (40)] may change the surface charge of solid particles in favor of aggregation and aid macrophage collection. This study and further data (Ref. 20; Kendall M, unpublished observations) show that particles adsorb DPPC and AAs in vitro, causing marked changes in surface characteristics and particle agglomeration rates. Since surfactant is already known to coat infectious particles and be involved in their clearance via macrophage recognition (40), based on these data here and other works, there is also now evidence to show that this process probably applies to urban fine particles (13, 19, 20, 22).

Deposition of large surface areas of foreign material, capable of removing DPPC and proteins involved in lung defense, into tiny quantities of lung lining fluid (estimated at 10 ml spread over $\sim$100 m^2 per person) may lead to significant health consequences. Surfactant is known to be necessary for normal lung function, and replacement has been shown to be helpful in the treatment of respiratory distress syndrome (33). Allergen challenge in asthmatics induces surfactant dysfunction, probably because of inhibiting proteins (3, 16). Chronic lower airway inflammation with mild or no clinical symptoms is also associated with impaired surfactant function (5). Babu et al. (1) showed that pulmonary surfactant dysfunction may also contribute to the very early asthmatic response to allergen and that exogenous surfactant administration could serve as a useful adjunct in controlling the early allergen-induced symptoms in patients with allergic asthma. Other studies raise concerns over the use of surfactant replacement materials sourced from animals, suggesting species differences in biochemistry and function of surfactant and highlighting a lack of understanding of the surfactant system (10). Inhibition or overload of the surfactant system by urban PM inhalation and surfactant sequestration may therefore be related to the observed health effects of PM exposure, such as increased infections in PM exposed rats (41) and PM exposed humans (9, 27). Damage to surfactant functioning may affect fluid balance at the alveolar surface and might play a part in the onset of pulmonary oedema in patients with incipient cardiac failure.

Conclusions. This paper identifies key biological molecules adsorbed to urban $PM_{2.5}$ surfaces following immersion in dilute human lavage fluid for short periods (4 h). DPPC (a major lipid in lung surfactant) was statistically the strongest associated molecule with BAL-treated $PM_{2.5}$ surfaces, but traces of AAs were also consistently associated, especially serine, asparagine, valine, and leucine. It is suggested that these indicate binding of lung SPs to particle surfaces.

It may be that one of the roles of these molecules is to agglomerate foreign material of nano dimensions so that they become "visible" to the scavenger macrophage cells (19, 22). But it is also possible that their sequestration onto the extensive particle surfaces presented by $PM_{2.5}$ will have patho-physiological effects. Toxicological cell culture assay studies, not including surfactant, and PM exposure studies using animals with different surfactant makeup should be interpreted with caution.

The adsorption of DPPC onto $PM_{2.5}$ could impair surfactant function, interfere with innate immunity, and deprive the host of critical lung defense molecules. Such a widespread challenge to the cardio-respiratory system could have serious implications for vulnerable individuals. Since surfactant components DPPC and collectins may well link innate and adaptive immunity (40), sequestration may have both short- and long-term health implications (immunological, respiratory, circulatory, and cardiac), depending on PM exposure and genetic variability. Since all these effects are observed in PM epidemiology, we should carefully examine interaction of surfactant with particles in the lung.

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