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Airway inflammation following exposure to diesel exhaust: a study of time kinetics using induced sputum

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Airway inflammation following exposure to diesel exhaust: a study of time kinetics using induced sputum. C. Nordenhäll, J. Pourazar, A. Blomberg, J-O. Levin, T. Sandström, E. Ädelroth. ©ERS Journals Ltd 2000.
ABSTRACT: The adverse health effects of particulate matter pollution are of increasing concern. In a recent bronchoscopic study in healthy volunteers, pronounced airway inflammation was detected following exposure to diesel exhaust (DE). The present study was conducted in order to evaluate the time kinetics of the inflammatory response following exposure to DE using induced sputum from healthy volunteers.

Fifteen healthy nonsmoking volunteers were exposed to DE particles with a 50% cut-off aerodynamic diameter of 10 μm 300 $\mu g \cdot m^{-3}$ and air for 1 h on two separate occasions. Sputum induction with hypertonic saline was performed 6 and 24 h after each exposure. Analyses of sputum differential cell counts and soluble protein concentrations were performed.

Six hours after exposure to DE, a significant increase was found in the percentage of sputum neutrophils (37.7 *versus* 26.2% p=0.002) together with increases in the concentrations of interleukin-6 (12.0 *versus* 6.3 $pg \cdot mL^{-1}$, p=0.006) and methylhistamine (0.11 *versus* 0.12 $\mu g \cdot L^{-1}$, p=0.024). Irrespective of exposure, a significant increase was found in the percentage of sputum neutrophils at 24 as compared to 6 h, indicating that the procedure of sputum induction itself may change the composition of sputum.

This study demonstrates that exposure to diesel exhaust induces inflammatory response in healthy human airways, represented by an early increase in interleukin -6 and methylhistamine concentration and the percentage of neutrophils. Induced sputum provides a safe tool for the investigation of the inflammatory effects of diesel exhaust, but care must be taken when interpreting results from repeated sputum inductions.
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The adverse health effects of particulate matter (PM) pollution are of increasing concern as several epidemiological studies have revealed an association between elevations in ground level PM concentrations and increased morbidity and mortality from cardiovascular and respiratory causes [1–7].

Recently, attention has been directed towards the particulate air pollution emitted from diesel engines. The number of diesel-powered cars is rising and diesel engines emit up to 100 times more particles than petrol engines [8, 9]. Diesel exhaust (DE) particles contain a carbonaceous core, which adsorbs various metals and organic compounds on its surface. Consequently, the biological effects of DE may be explained by not only the gases and pure particles but also by these surface components.

The number of studies investigating the effects of DE has increased during recent years. In working environments, exposure to DE has been associated with various symptoms related to the eyes and airways [10–12]. In experimental chamber studies, DE has been shown to cause airway symptoms [13] and to induce an acute inflammatory response in human airways as reflected in bronchoalveolar lavage [14, 15] and bronchial biopsy [16] samples. Further, local nasal challenge with DE particles has been reported to enhance immunoglobulin E production in the nose [17] and to cause an increased cytokine response in the nose of allergic subjects [18].

The concentration of PM used in this study is comparable to high ambient levels in large European cities. From a global perspective, more than half of the world's megacities (cities with population >10 million) have annual mean PM concentrations ranging 200–600 $\mu g \cdot m^{-3}$ [7]. In addition, high concentrations of DE are found in various working environments such as mines, garages and other enclosed spaces containing running engines.

Fibreoptic bronchoscopy has become a central tool for the examination of air pollution effects in human airways. Bronchoscopy is an invasive and resource-demanding method and cannot be easily performed repeatedly in the same subject within a limited period of time. Thus there has been an increasing demand for a noninvasive and alternative method to be used in situations in which bronchoscopy is not practical. Sputum induction has been proposed as a safe, noninvasive and easily repeated method for monitoring inflammatory events in human airways, and the method of sputum induction has been validated in a number of

studies and reported to be safe and reproducible [19–23]. Analysis of induced sputum results has shown them to correlate well with data obtained from bronchial wash and bronchoalveolar lavage [24, 25]. In air pollution research, induced sputum has previously proved valuable in the evaluation of the airway effects of ozone [26] and nitrogen dioxide [27].

This study was conducted in order to elucidate the time kinetics of the inflammatory response following short-term exposure to DE in healthy human volunteers. In addition, the use of induced sputum as a noninvasive tool for the investigation of the inflammatory effects of DE was evaluated.

Methods

Subjects

The study population comprised fifteen healthy nonsmoking volunteers (two females and 13 males; mean age 25 yrs, range 22–33 yrs), with lung function within the normal range. None of the subjects had a history of allergy, asthma or any other pulmonary disease and none had experienced any respiratory tract infection for ≥4 weeks prior to or during the study period. As a part of the screening procedure for participation in the study, all subjects were tested as to their ability to produce sputum on induction with hypertonic saline. The study was approved by the local Ethics Committee of Umeå University and verbal and written informed consent was obtained from all individuals.

Study design

The study was performed using a single-blind crossover design with each subject acting as their own control. Each subject was exposed for 1 h on two different occasions, once to filtered air and once to DE at a concentration of particles with a 50% cut-off aerodynamic diameter of 10 µm (PM10) of 300 $\mu g \cdot m^{-3}$. In order to preclude any bias due to possible carry over effects, the exposures were conducted ≥2 weeks apart and in randomized sequence, with half of the study population being exposed to DE as their first exposure and the other half being exposed to air first.

Exposure

During each exposure, the subjects alternated between rest and moderate exercise on a bicycle ergometer (minute ventilation per square metre of body surface area 20 $L \cdot min^{-1} \cdot m^{-2}$) at 15-min intervals. All exposures were performed in an environmental chamber according to a previously described standard protocol [16, 28]. This exposure system has been carefully evaluated and validated [28]. DE was generated by an idling Volvo diesel engine (Volvo-BM TD45; Volvo, Gothenburg, Sweden; 4.5 L, 4 cylinders, 1991, 680 revolutions per minute (rpm)). Approximately 90% of the exhaust was shunted away and the remaining part diluted with air and fed into the chamber at a steady-state concentration. The concentrations of particles ($particles \cdot cm^{-3}$), NO_2, nitric oxide, carbon monoxide and hydrocarbons (HCs) were continuously recorded in the exposure chamber as previously described [13]. A gas analyser using infrared detection (Miran 1-A; Foxboro Co, East Bridgewater, MA, USA) was used for analysis of CO. NO, NO_2 and oxides of nitrogen (NO_x) were measured using an NO_x analyser with chemiluminescence detection (ECO-Physics CLD 700; Boo-Instruments, Stockholm, Sweden). HCs were measured using a hydrogen analyser with a flame ionization detector (model 3-300; (J.U.M. Engineering GmbH; Munich, Germany) with a heated prefilter (180°C) and calibrated with propane. The mass of the PM10 (in $\mu g \cdot m^{-3}$) was determined by weighing PM10-collecting filters. The air in the chamber was changed every 2–3 min, with the waste air being extracted through a tube in the ceiling. The DE entering the environmental chamber was standardized to give a PM10 concentration of 300 $\mu g \cdot m^{-3}$, associated with a median steady-state NO_2 concentration of 1.6 parts per million. The temperature and relative humidity in the chamber were kept at 20°C and 50% respectively.

Sputum induction

Sputum induction was performed 6 and 24 h after each exposure. The induction procedure was performed according to the method described by Pin *et al.* [23]. All subjects were pretreated with an inhaled β_2-agonist (0.5 mg terbutaline). Hypertonic saline was nebulized using an ultrasonic nebulizer (DeVilbiss 2000; DeVilbiss Co., Somerset, PA, USA), with an output of 1.5 $mL \cdot min^{-1}$. Inhalation was performed at intervals of 7 min with increasing concentrations (3, 4 and 5%) of saline solution. The forced expiratory volume in one second was monitored before and after every inhalation period. All subjects received all three concentrations of saline. Following each inhalation interval, subjects were advised to rinse their mouth with water and blow their nose before trying to cough sputum into a sterile plastic container. The samples obtained were kept on ice up to 1 h prior to processing.

Sputum processing

Sputum was processed according to the method described by Pizzichini *et al.* [19]. The expectorated samples were poured into a siliconized Petri dish and macroscopically examined, and portions that appeared more viscid and dense were selected with forceps and transferred to a 10-mL siliconized tube. After adding 0.1% diothiothreitol (DTT) at a volume equal to four times the selected sputum weight, the sputum was rocked for 15 min to dissolve the mucus and disperse the cells. The same volume of phosphate-buffered saline 0.01 M, pH 6.9 (PBS) was added and the rocking continued for 5 min. The mixture was then filtered through a 48-µm nylon filter into another 10-mL tube and centrifuged for 10 min at 300×*g* at 4°C. The supernatant was separated from the cell pellet, recentrifuged for 10 min at 1,000×*g* to further remove debris, aspirated and stored in Eppendorf tubes at -70°C for later analyses. The cell pellet was resuspended in 1,000 µL PBS. Total cell counts and cell viability were determined using a haemocytometer and trypan blue. The cell suspension was adjusted to $0{\cdot}5 \times 10^6$ $cells \cdot mL^{-1}$ and 50 µL placed in each cup of a Shandon 3 cytocentrifuge (Shandon Southern Instruments, Inc., Sewickley, PA, USA) and cytospins made on prewet slides. Six cytospin

slides per sputum sample were prepared (400 rpm, 5 min) and stained with May-Grünwald Giemsa. At least 400 nonsquamous cells were counted by two blinded observers and the results were averaged to yield the final differential cell counts reported. The differential cell counts were expressed as a percentage of the total nonsquamous cell count. The proportion of squamous cells was obtained by counting 400 additional cells and expressed as a percentage of the total cell count. Samples were considered suitable for analysis if squamous cell contamination was <20% and viability >50%. The total cell count is expressed as the number of cells per millilitre of the undiluted sputum on the assumption that 1g of sputum is equivalent to 1 mL.

Fluid phase mediators

The levels of seven different soluble proteins in the sputum supernatant samples were determined. Methylhistamine (M-hist), eosinophil cationic protein (ECP) and myeloperoxidase (MPO) concentrations were determined using sensitive commercial radioimmunoassay kits (Pharmacia & Upjohn AB, Uppsala, Sweden). Tumour necrosis factor-α (TNF-α), interleukin (IL)-6, IL-8 and growth-related oncogene-α (Gro-α) levels were measured using commercial enzyme-linked immunosorbent assay kits (R&D systems, Inc., Abingdon, UK). The concentrations presented have not been corrected for sputum dilution. All fluid-phase measurements were performed blind to the subject's exposure history.

Statistics

The Wilcoxon nonparametric signed-rank test for paired observations was used to compare data on cells and soluble markers after air and diesel exhaust exposures. Correlations between data were assessed using the Spearman rank correlation test. The primary end point was defined as a change in the percentage of neutrophils in induced sputum following exposure to diesel exhaust. A p-value of <0.05 was considered significant.

Results

Sputum induction was well tolerated by all subjects. The median (interquartile range) salivary contamination indicated by the percentage of squamous cells was 3.7% (1.6–9.0%) and the median cell viability was 76% (67–84%). The median weight of the selected sputum was 300 mg (158–410 mg). Only one sputum sample was discarded from the study due to insufficient volume.

Sputum cell counts

The results of the sputum differential cell count analysis are presented in table 1. Exposure to DE induced an increase in the percentage of neutrophils at 6 h (p=0.002) but not at 24 h, compared with the equivalent time points following air exposure (fig. 1). The median increase seen at 6 h was 49.8%. At 24 h after exposure, a minimal but significant increase (p=0.046) was found in the percentage of lymphocytes, whereas no change was seen at the earlier time point. No significant change was seen in the percentage of ciliated epithelial cells or squamous cells or total cell counts. Only very few samples contained eosinophils.

Irrespective of exposure, sputum induced at 6 h contained significantly lower percentages of neutrophils compared with sputum from the subsequent induction at 24 h. Correspondingly, the percentages of macrophages were higher at 6 h than at 24 h after exposure. The percentages of ciliated epithelial cells and lymphocytes were significantly lower at 24 h compared with 6 h following air exposure but not following diesel exposure.

Fluid phase mediators

Six hours after diesel exposure, a significant increase was seen in the fluid phase concentrations of IL-6 and M-hist compared with after exposure to air, whereas no significant changes were detected in the levels of ECP, Gro-α, IL-8, MPO or TNF-α. At 24 h after exposure, no significant changes were seen in the soluble protein concentrations (table 2).

Table 1. – Total and differential cell counts in sputum after air and diesel exposure

	Air	Diesel	p-value[#]
Total cell count 10^6 cells·mL^{-1}			
6 h	2.25 (1.30–2.78)	2.00 (0.56–3.10)	NS
24 h	1.90 (0.95–2.70)[+]	1.07 (0.36–2.70)	NS
Neutrophils %			
6 h	26.2 (13.5–37.9)	37.7 (22.2–46.3)	0.002
24 h	60.0 (55.6–65.25**,[+]	61.9 (45.9–76.9)*	NS
Macrophages %			
6 h	72.0 (60.0–74.0)	57.2 (40.4–72.1)	0.027
24 h	37.8 (32.1–47.5)**,[+]	37.3 (21.5–53.0*	NS
Lymphocytes %			
6 h	0.70 (0.55–1.10)	0.6 (0.35–1.20)	NS
24 h	0.47 (0.20–0.65)*,[+]	0.65 (0.40–0.80)	0.046
Ciliated epithelial cells%			
6 h	2.40 (0.70–10.8)	2.75 (0.70–4.20)	NS
24 h	1.15 (0.71–2.35)[+]	1.00 (0.30–2.15)§	NS

Data are presented as median (interquartile range) (n=15). The total cell count is expressed as the number of cells per millilitre of the undiluted sputum on the assumption that 1g of sputum is equivalent to 1ml. [+]: n=14; [#]: Wilcoxon nonparametric signed-rank test. [§]: p=0.10; *: p<0.05; ** p<0.01 *versus* 6 h.

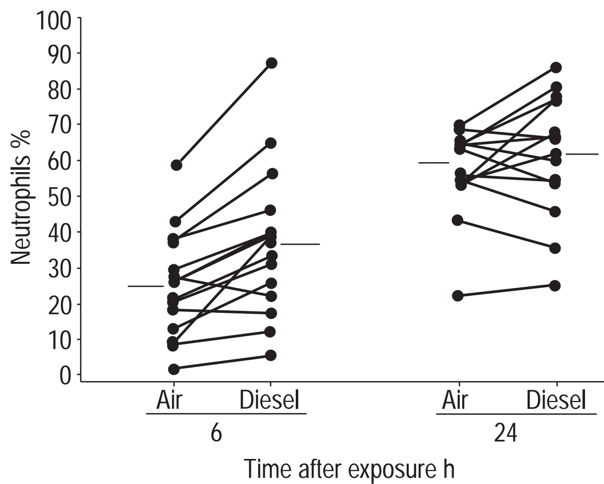

Fig. 1. – Percentages of neutrophils in induced sputum from healthy subjects at 6 (n=15) and 24 h (n=14) after exposure to air and diesel exhaust. The horizontal bars represent medians.

When comparing the levels of soluble proteins in sputum from the induction at 6 h with those from the subsequent induction at 24 h, there was a significant increase in ECP concentration at 24 h after air exposure. No other significant changes were seen in soluble protein concentrations from the two consecutive sputum inductions (table 2).

Discussion

Cellular and biochemical analysis of induced sputum revealed an inflammatory response in the airways following exposure to DE as compared to air. Inflammatory changes were found in differential cell counts as well as in fluid phase mediator concentrations. The inflammatory response to DE is likely to involve a sequence of events, with various cellular and protein effects suggested to appear at different time points following exposure. The levels of certain inflammatory substances are thus likely to depend on the time of sampling. In the present study, the time course of the inflammatory process following DE exposure was addressed by comparing levels of soluble proteins and cellular changes in sputum observed at 6 h after exposure to DE with those observed at 24 h.

The early increases in IL-6 and M-hist concentration probably represent an acute and transient response to DE and may result from direct effects of DE on airway cells. IL-6 is a pro-inflammatory cytokine that displays a diversity of functions, including regulation of immunological responses and induction of acute-phase reactions [29]. An increase in the release of IL-6 from human bronchial epithelial cells has recently been reported following *in vitro* exposure to DE particles [30], as well as to particles derived from residual oil fly ash [31]. Furthermore, air pollution particulates have been shown to induce IL-6 gene expression in bronchial epithelial cells *via* nuclear factor-κB activation [32]. Combined, these data suggest that epithelial cells, being the first cellular target of inhaled air pollutants, may release IL-6 upon exposure to DE. The early increase in M-hist most probably represents mast cell degranulation in the airway mucosa as indicated in a preceding bronchoscopic study [16].

The early neutrophilic response in sputum differential cell counts observed in the present study corresponds well with the previous data, in which an increase in neutrophil number was found in both bronchial biopsy and bronchial wash samples at 6 h after DE exposure [16]. The presence of a neutrophilic inflammation has further been reported in BAL at 18 h after DE exposure [14, 15]. Although the human data on the time course of the neutrophilic response to DE is limited, the results from these studies seem to suggest the peak response to occur sometime between 6 and 18 h following exposure.

Table 2. – Levels of soluble proteins in sputum after air and diesel exposure

	Subjects n	Air	Diesel	p-value+
ECP µg·L^{-1}				
6 h	15	9.2 (4.8–25.9)	15.1 (7.0–23.4)	NS
24 h	14	28.0 (11.4–40.2)*	18.0 (5.0–45.8)	NS
GRO-α pg·mL^{-1}				
6 h	15	552 (270–1050)	840 (252–1560)	NS
24 h	13	1020 (372–1500)	567 (321–795)	NS
IL-6 pg·mL^{-1}				
6 h	15	6.3 (4.5–14.4)	12.0 (6.9–22.2)	0.006
24 h	14	10.4 (5.6–19.2)	15.0 (10.8–40.8)	0.09
IL-8 pg·mL^{-1}				
6 h	15	990 (690–1650)	1290 (690–2550)	NS
24 h	13	1800 (1065–3188)	1560 (660–4200)	NS
M-hist µg·L^{-1}				
6 h	13	0.11 (0.00–0.14)	0.12 (0.05–0.18)	0.024
24 h	13	0.11 (0.00–15.5)	0.13 (0.11–0.14)	NS
MPO µg·L^{-1}				
6 h	15	162 (122–274)	252 (152–301)	NS
24 h	13	393 (172–543)	246 (152–1033)	NS
TNF-α pg·mL^{-1}				
6 h	15	0.00 (0.00–2.55)	1.50 (0.00–3.00)	NS
24 h	14	0.00 (0.00–3.19)	0.00 (0.00–3.19)	NS

Data are expressed as median (interquartile range). +: Wilcoxon nonparametric signed-rank test. ECP: eosinophil cationic protein; Gro-α: growth-related oncogene-α; IL: interleukin; M-hist: methylhistamine; MPO: myeloperoxidase; TNF-α: tumour necrosis factor-α. *: $p<0.05$ *versus* 6 h.

In spite of the increase in sputum neutrophil numbers, no significant change was seen in the fluid phase levels of IL-8 or Gro-α. Accordingly, SALVI *et al.* [16] did not find any increase in IL-8 levels in bronchial lavage fluid at 6 h after exposure to DE. These findings do not exclude an early transient increase in the levels of IL-8 and Gro-α. Six hours after exposure may be too late to detect any early change that might have occurred. As a marker for neutrophil activation, MPO levels were measured in the sputum supernatant, but no significant change was detected.

Exposure to DE was also associated with higher percentages of lymphocytes detected at 24 h, as compared to the equivalent time point following air. However, this change was small and only weakly statistically significant and should be regarded with caution. Nevertheless in support of the observation in this study, exposure to DE has recently been shown to cause lymphocytosis in bronchoalveolar lavage fluid and bronchial biopsy samples at 6 h after exposure [16].

The results from the present study indicate that the procedure of sputum induction may itself cause an inflammatory change in the composition of sputum. This is suggested by the significant changes in the cellular and biochemical components found between sputum obtained at 6 compared with sputum obtained at 24 h. Irrespective of exposure, there was a pronounced increase in the percentage of neutrophils at 24 compared with 6 h. Also, sputum induced at 24 h following air contained higher levels of ECP, compared with sputum induced at 6 h. Differences were further seen in the median percentage of ciliated epithelial cells between sputum obtained at 6 and 24 h, which is most probably explained by shedding of the ciliated epithelial cells during the first induction, thus resulting in lower percentages during the second induction.

Few studies have been conducted to compare sputum results between two consecutive inductions. In accordance with the present results, HOLZ *et al.* [33] recently reported a marked increase in the percentages of neutrophils and ECP levels in induced sputum 24 h after a previous sputum induction. Similar results were shown by NIGHTINGALE *et al.* [34]. In contrast, other studies have not revealed any significant change in sputum composition following sputum induction performed on consecutive days [35, 36]. The discrepancy seen between different studies most probably reflects methodological differences and different study populations, but further studies are needed to resolve this issue. Also, most studies using induced sputum have been performed in asthmatics, whereas the available data related to healthy subjects is more limited. In view of the results of the present study, it is suggested that possible inflammatory effects of the induction procedure should be taken into consideration when performing repeated inductions. However this issue might be of less importance if subjects are used as their own controls, as in the present study.

In conclusion, the results of the present study indicate that short-term exposure to diesel exhaust induces a time-dependent inflammatory response in the airways of healthy human subjects, involving an early increase in interleukin-6 and methylhistamine levels and neutrophil percentages possibly followed by a late increase in the percentage of lymphocytes. Sputum induction is a valuable and easily applicable method for the investigation of airway inflammation following exposure to diesel exhaust, but caution must be exercised as to the interpretation of results from repeated sputum inductions.

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