Use of Sputum Induction to Obtain Airway Lining Fluid After Ozone Exposure; A Pilot Study to Validate Sputum Induction as an Alternative to Bronchoscopy

Final Report

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ABSTRACT

Exposure of healthy subjects to ozone is associated with pulmonary inflammation as evidenced by increased cellular and biochemical markers of inflammation in bronchoalveolar lavage. Collection of bronchoalveolar lavage requires that subjects undergo bronchoscopy, an invasive procedure that requires willing subjects and skilled research personnel. Recently, sputum induction has been proposed as a non invasive method for sampling airway secretions for research purposes. Sputum induction relies on the fact that healthy subjects expectorate sputum when they inhale an aerosol of hypertonic saline. We and others have demonstrated that it is feasible to perform both cellular and biochemical measurements on this "induced sputum". To determine if analysis of induced sputum might reveal the pulmonary inflammatory effects of ozone exposure, we performed cellular and biochemical analysis of induced sputum collected 4 hours after air and ozone (0.4 ppm for 2 hours) exposures from 10 healthy subjects (age 30±5 years; 5 females) in a randomized crossover study where exposures were separated by 2 weeks. We found that the total number of non squamous cells were significantly higher after ozone exposure than after air exposure (7.4 vs 3.9 x 10^5/ml, $p<0.05$)) as were the percentage of the non squamous cells that were neutrophils (80±7.0% vs 51±20%, $p<0.05$) and the levels of myeploperoxidase in the sputum fluid phase (1622±635 ng vs 1273±578 ng, $p<0.05$). In addition, we found a trend for higher levels of IL-6 and IL-8 in induced sputum after ozone than after air exposures. Total protein levels and mucin-like glycoprotein levels were not signficantly different between exposures. We conclude that analysis of induced sputum reveals evidence of inflammation similar to that reported from analysis of bronchoalveolar lavage and that sputum induction thus represents a useful non-invasive method for studying the pulmonary response to ozone exposure in healthy subjects.

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DISCLAIMER

The statements and conclusions in this report are those of the contractor and not necessarily those of the California Air Resources Board. The mention of commercial products, their sources, or their use in connection with material reported herein is not to be construed as either an actual or implied endorsement of such products.

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SUMMARY

The project completed under this contract permits the following conclusions:

(1) Induced sputum collected after ozone exposure has significantly higher percentages of neutrophils and significantly higher levels of myeloperoxidase than induced sputum collected after filtered air exposure and trends for higher levels of interleukin-6 and interleukin-8.

(2) Sputum induction represents a non invasive method for evaluating the airway inflammatory response to ozone exposure in healthy subjects.

CONCLUSIONS AND SIGNIFICANCE

In this study we found that sputum induction represented a feasible method for collecting airway secretions after both ozone and filtered air exposures in healthy subjects. Analysis of the induced sputum samples revealed that the samples collected after ozone exposure had significantly higher percentages of neutrophils and significantly higher levels of myeloperoxidase than induced sputum collected after filtered air exposure. In addition, there were trends for higher levels of interleukin-6 and interleukin-8 in the induced sputum samples collected after ozone exposure. These ozone-associated changes in the composition of induced sputum are similar to those observed in bronchoalveolar lavage fluid collected after ozone and filtered air exposures. Thus, we conclude that sputum induction is a useful, noninvasive, and valid method for collecting airway secretions after air and ozone exposures, and therefore for evaluating the pulmonary response to ozone. In validating sputum induction as a noninvasive and easily repeated method for assessing the pulmonary response to ozone, we present the possibility that sputum induction might be used in

time course studies of the pulmonary response to ozone or in field studies of the pulmonary effects of ozone or of other environmental pollutants.

RECOMMENDATIONS

Our finding that markers of inflammation increase in induced sputum from healthy subjects after ozone exposure fits well with previous observations that markers of inflammation increase in bronchoalveolar lavage from healthy subjects after ozone exposure. Thus, analysis of induced sputum after ozone and filtered air exposures appears to reveal information qualitatively similar to that obtained by analysis of bronchoalveolar lavage.

Our recommendation for future studies would be to apply sputum induction in research protocols that would benefit from a noninvasive, easily repeatable, method for obtaining samples of airway secretions. As examples of such protocols we would foresee that sputum induction might be useful for protocols investigating time course responses to ozone since sputum induction could be used to obtain samples of airway secretions at multiple time points after an ozone exposure. In addition, we believe that it should be feasible to perform sputum induction in settings other than in hospital-based research laboratories. Specifically, it should be feasible to perform sputum induction in a mobile field laboratory, and thus to collect airway secretions from research subjects during field studies on the pulmonary effects of ozone or other airborne pollutants. Pilot studies to investigate this possibility should be undertaken before any large scale field study would include analysis of induced sputum as an outcome variable.

Introduction

Ozone (O_3), a product of photochemical reactions involving hydrocarbon vapors and nitrogen oxides, is a major component of urban smog. Clinical research into the effects of ozone exposure on lung inflammation has, to date, relied on the analysis of samples of lung secretions or lung tissue obtained by bronchoscopy (1-3). Thus, cellular and biochemical analysis of bronchoalveolar lavage and bronchial mucosal biopsies from healthy subjects exposed to ozone has demonstrated that ozone exposure is associated with inflammation in the lung. Although analysis of lavage and tissue samples obtained by bronchoscopy has contributed importantly to our current understanding of the pulmonary effects of ozone, bronchoscopy is an invasive procedure that requires research subjects willing to undergo it and research staff trained to perform it. Because of these factors, and because bronchoscopy is not easy to perform repeatedly in the same subject, bronchoscopy is not ideal for clinical studies of ozone's effects on inflammation in the lungs.

Cellular and biochemical analysis of induced sputum has recently been proposed as a non-invasive method for evaluating airway inflammation in asthma (4,5). We and others have shown that sputum can be induced from both healthy and asthmatic subjects by inhalation of aerosolized hypertonic saline, and that induced sputum can be analyzed semiquantitatively for cells and chemicals. To date, in asthmatic subjects, it has been shown that analysis of induced sputum reveals the inflammatory effects of aerosolized allergen challenge (6,7) and the antiinflammatory effects of prednisone treatment (8). Because analysis of induced sputum has been shown to be responsive to these interventions in asthmatic subjects, and because analysis of induced sputum reveals qualitatively similar inflammation to that revealed by analysis of bronchial lavage samples (9), we set out to determine if analysis of induced sputum from healthy subjects might reveal the inflammatory effects of ozone exposure in healthy subjects. We reasoned that if we could demonstrate the expected differences in cells and chemicals in induced sputum samples after ozone and control exposures that we could

then propose sputum induction as a noninvasive alternative to bronchoscopy for clinical research studies on the pulmonary effects of ozone or other air pollutants. A noninvasive alternative to bronchoscopy in this setting would have several advantages including the ability to repeatedly sample airway secretions and thereby more easily determine time course responses to ozone exposure as well as making possible the consideration of airway secretion sampling in field studies of ozone exposure.

Thus, in this study, we exposed healthy subjects to filtered air and to ozone (0.4 ppm) for two hours in an environmental chamber and collected induced sputum samples 4 hours later. We analyzed the induced sputum for total and differential cell counts and for the levels of myeloperoxidase, total protein, mucin-like glycoprotein, interleukin-6, and interleukin-8.

MATERIALS AND METHODS

Subjects

Ten healthy non-smoking subjects (age 30±5 years; 5 females) with no prior history of lung disease participated. All subjects signed consent forms approved by the Committee on Human Research at the University of California, San Francisco.

Protocol

Subjects participated in a three visit, single blind, randomized, cross-over study. Visit one was for obtaining of informed consent and for characterization (medical questionnaire and spirometry). Visit two was for exposure to ozone (0.4 ppm) for two hours or to filtered air for 2 hours followed by sputum induction four hours later. The airway response to the exposures was assessed by spirometry and by measurement of airway resistance. Visit three was for exposure to the gas not administered on visit 2 followed again by sputum induction four hours later.

Exposures

All exposures were carried out in a stainless steel and glass chamber (8X8X8 ft). Ozone was generated by passing 100% oxygen through an ozonator (Wellsbach no. T-408) and concentrations were maintained at approximately 0.4 ppm as measured by an ultraviolet ozone analyzer (Dasibi no.1003 AH). Air was passed through a filter, and chamber temperature and relative humidity were continuously monitored. The mean (±standard deviation) concentration of ozone during the filtered air exposures was 0.012±0.002 ppm and during the ozone exposures was 0.382±0.022 ppm. Subjects were exposed for 2 hours while wearing a Hans Rudolph Mouth Breathing Face Mask (Hans Rudolph, Inc, Kansas City, MO) fitted with a non rebreathing valve (Rudolph Valve #2600). During their exposures subjects performed intermittent exercise (15 of each 30 minutes) on a cycle ergometer (Corvial 400, Quinton Instruments Co., Seattle, WA) for 2 hours while wearing noseclips. The work level selected was based on the watts required to achieve and maintain a calculated minute ventilation of 25 L/min X

body surface area. During exercise a section of corrugated tubing was placed between the Rudolph valve on the facemask and a pneumotachograph (A. Fleisch , General Medical Corp, West Sacramento, CA) so that the subject's minute ventilation could be measured (flow signals recorded from the pneumotachograph were amplified [Validyne CD19 module, Validyne Engineering Corp., Northridge, CA], integrated [Validyne FV156 Intergrator] to yield volume signals, and then recorded on a visicorder [Honeywell 1858, Honeywell Test Instruments Division, Denver, CO]). The mean minute ventilation of the subjects during exercise during the filtered air and ozone exposures were similar (40.02±6.09 L vs 38.69 ±7.5 L). The mean temperature and relative humidities in the chamber during the filtered air and ozone exposures were also similar (19.45±0.66 Vs 19.8±0.31°C; 43.7±5.6 Vs 41.7±10.1 RH, respectively).

Pulmonary Function Tests

Spirometry was performed using a rolling seal Ohio 840 spirometer according to ATS criteria. Flow volume loops were generated by acquiring and digitizing the electrical volume signal versus time produced by the spirometer and then scaling these digitized computer counts to liters by application of a calibration factor. Flow was derived from the change in volume over the change in time (in real time) and flow versus volume was displayed on a graphic cathode ray screen as well as printed on hardcopy. All spirometric values were calculated from the raw data i.e. volume versus time and the values reported are the best of three separate maneuvers.

Specific airway resistance was calculated by measuring airway resistance and thoracic gas volume (Vtg) in a constant-volume variable pressure whole-body plethysmograph (Warren E. Collins Inc., Braintree, MA) every 30 seconds for 2.5 minutes.

Sputum Induction

All subjects were pre-treated with 180 µg albuterol administered by metered dose inhaler, and then inhaled nebulized sterile 3% saline for 20 minutes from a DeVilbiss

Ultra-Neb 99 (this nebulizer generates particles of a mean mass median diameter of 3.5 μm and has an output of 5.9 ml/min). Subjects were encouraged to cough throughout the procedure and interrupted inhalation of hypertonic saline every two minutes in order to expectorate all secretions, including both sputum and saliva, into a clean plastic container.

Sputum Processing

The volume of the induced sputum sample was determined and overlaid with an equal volume of dithiothrietol 0.1% (Sputalysin 10%, Behring Diagnostics Inc., Somerville, NJ). The sample was then mixed gently by vortex mixer and placed in a shaking water bath at 37°C for 15 minutes to ensure complete homogenization. The sample was removed from the water bath periodically for further brief gentle vortex mixing. Ten microliters of the homogenized sputum was used to determine the total cell count using a standard hemacytometer. 250μl of homogenized sputum (diluted in saline to prevent cell crowding on the slide) was spun in a cytocentrifuge (model 7 cytospin; Shandon Scientific, Sewickley, PA), and stained using Diff-Quik® stain (Baxter Scientific Products, Miami, FL). At least 200 non-squamous cells on each sputum slide were read by two investigators blinded to the subject classification. Averaged cell differentials of the two investigators are reported. The remainder of the homogenized sputum was centrifuged at 1037g for 5 minutes. The supernatant was aspirated and frozen at -70°C for later analysis.

Cell Count and Differential

Ten microliters of the pooled BAL was used to determine the total cell count of the BAL samples using a standard hemacytometer, and 250μL aliquots were spun in a cytocentrifuge (model 7 cytospin; Shandon Scientific, Sewickley, PA) onto glass slides that were then stained using the May Grunwald Giemsa stain. Two investigators, blinded to the subject's exposure history, each counted at least 200 cells and the cell counts of both investigators were averaged to yield the final percentages reported here.

Biochemical Assays

Myeloperoxidase levels in induced sputum supernatant samples were determined using a sensitive radioimmunoassay (Pharmacia Diagnostics Inc. Fairfield, NJ). Interleukin-8, and interleukin-6 levels were measured using specific and sensitive immunoassays (ELISA, Quantikine, R&D Systems, Minneapolis, MN). Mucin-like glycoprotein levels were determined by enzyme-linked immunosorbent assay (ELISA) as previously described (10). Total protein concentrations were determined using bicinchoninic acid (11).

STATISTICS

Data was entered on a computer spreadsheet (Microsoft Excel®, Microsoft, Redmond, WA) and exported to a statistics program (Statview®, Abacus Concepts Inc., Berkeley, CA) for descriptive and comparative statistics. The data is presented as the mean and standard deviation of the mean (mean±SD). The Wilcoxon signed rank test was used to compare induced sputum cell counts and differentials after air and ozone exposures, and the paired t test was used to compare chemical data in the induced sputum supernatants after air and ozone exposures. Correlations between data were made using the Spearman rank order correlation test. Differences or correlations with a p value < 0.05 were accepted as statistically significant.

RESULTS

As expected, there was evidence of mild bronchoconstriction, reflected by a rise in specific airway resistance and a fall in maximum expiratory flow, immediately after ozone exposures but not after filtered air exposures (Table 1).

Total and differential cell analysis of induced sputum after filtered air and ozone exposures revealed evidence of ozone-associated neutrophilic inflammation (Table 2). The total number of nonsquamous cells was significantly higher in the induced sputum samples collected after ozone exposure than after filtered air exposure and the number and percentage of these cells that were neutrophils was significantly higher. The nonsquamous cell percentage of macrophages was significantly lower after ozone exposure than after air exposure but the macrophage cell number in these samples was not significantly different. The percentage of squamous cells was lower in the induced sputum collected after ozone exposure but the total number of squamous cells was not significantly different.

Chemical analysis of the fluid phase of the induced sputum samples showed significantly higher levels of myeloperoxidase in the induced sputum collected after ozone exposure than after filtered air exposure (Table 3). We also found a trend for higher levels of IL-6 and IL-8 in the induced sputum collected after ozone exposures. The levels of total protein and of mucin-like glycoprotein were not significantly different in the induced sputum samples collected after the two exposures (Table 3).

Analysis of possible associations between neutrophil percentages, neutrophil cell numbers and fluid phase levels of IL-8 and myeloperoxidase revealed no statistically significant correlations. However, there was a trend toward a positive correlation between the ozone-associated increase in IL-8 and the ozone-associated increase in neutrophil cell numbers (r_S=0.5, p=0.1). In addition, there was a trend toward a positive

correlation between the ozone associated increase in IL-8 and the ozone-associated increase in myeloperoxidase (r_s=0.6, p=0.08).

DISCUSSION

The main finding of this study is that analysis of induced sputum from healthy subjects after air and ozone exposures reveals the known inflammatory effects of ozone on the lung. Specifically, cellular and biochemical analysis of induced sputum collected 4 hours after ozone exposure reveals significantly higher percentages and numbers of inflammatory cells (neutrophils) and higher levels of inflammatory mediators (significant increase in myeloperoxidase and a trend toward higher levels of IL-6 and IL-8) than induced sputum collected 4 hours after air exposure. These data demonstrate that sputum induction can be used to obtain airway secretions from healthy subjects after controlled exposures to ozone and that cellular and biochemical analysis of these secretions reveals the expected effects of ozone on markers of inflammation. Thus, sputum induction should prove useful as a noninvasive method for evaluating the pulmonary effects of ozone or other environmental air pollutants.

Cellular analysis of induced sputum after air and ozone exposures showed a significant increase in the neutrophil percentages and in the neutrophil cell counts in the induced sputum samples collected after ozone exposures (Table 2). The mean percentages of squamous cells and of macrophages were significantly reduced after ozone exposure but the absolute squamous cell and macrophage cell numbers were not. These findings are qualitatively similar to those described in published reports of cell counts and differentials in bronchoalveolar lavage fluid analyzed after air and ozone exposures (1-3). Even though the mean percentage of neutrophils in the induced sputum collected after filtered air exposures was high (Table 2), this mean percentage rose even higher after ozone exposure and the neutrophil cell numbers increased by over 200%. The relatively high percentage of neutrophils in induced sputum collected in baseline states has been noted previously (4) and most likely reflects the fact that sputum induction samples secretions mainly from large airways (9).

Chemical analysis of the fluid phase of the induced sputum revealed significantly higher levels of the neutrophil-derived inflammatory mediator, myeloperoxidase, in induced sputum collected after ozone exposure than after air exposure. Myeloperoxidase (MPO) is released by neutrophils when they are activated and combines with a variety of other inflammatory mediators (principally H_2O_2 and chloride ion [MPO-H_2O_2-Cl system]) to cause damage to other cells (12, 13). Surprisingly, in this study, the levels of MPO increased by only 27% despite an increase in neutrophil cell numbers of 227%, and there was no significant correlation between the increase in neutrophil cell number in induced sputum and the increase in myeloperoxidase. These findings suggest that the neutrophils that accumulate in the airways following ozone exposure are not highly activated.

There was a trend for higher levels of IL-6 and IL-8 in the induced sputum samples following ozone exposure (Table 3). IL-6 and IL-8 are members of a family of proteins called cytokines. Cytrokines function as mediators of cell-cell interactions and activity. IL-6 is produced by cells such as T lymphocytes, monocytes and fibroblasts. Ozone-associated increases in IL-6 have been described both for in *vitro* studies of ozone's effects on cultured airway epithelial cells (14) and for *in vivo* studies of ozone's effects on the lungs as reflected by changes in bronchoalveolar lavage (15). The implications of this finding are uncertain but available functional data on the effects of IL-6 in the lungs suggests that ozone-induced hypersecretion of IL-6 may have either proinflammatory or antiinflammatory consequences (16, 17).

IL-8 is a product of a number of cells including mononuclear phagocytes, fibroblasts, endothelial cells, and epithelial cells and one of its principal actions is to attract neutrophils into sites of inflammation (18, 19). Although cultured airway epithelial cells hypersecrete IL-8 in response to ozone exposure, IL-8 levels in nasal lavage fluid (20) or in bronchoalveolar lavage fluid (3) from healthy subjects do not increase significantly following ozone exposure (3). IL-8 levels in proximal airway lavage from healthy

subjects, which may sample a similar compartment to that sampled by sputum induction (9), do increase significantly after ozone exposure (3), as do IL-8 levels in bronchoalveolar lavage from asthmatic subjects (21). In this study, the mean ozone-associated increase in IL-8 levels in the induced sputum of 30% is surprisingly small given the mean increase in neutrophil cell numbers of 227%, and the correlation between ozone-induced changes in IL-8 levels in induced sputum and ozone-induced changes in neutrophil cell numbers was not statistically significant (r_S=0.5, p=0.1). These findings do not disprove involvement of IL-8 in ozone-induced airway neutrophilic inflammation in healthy subjects for it is possible that ozone-induced expression of IL-8 by airway epithelial cells is not fully reflected by airway luminal IL-8 levels even though this epithelial IL-8 expression may be a major stimulus to the accumulation of neutrophils in the airway lumen.

The levels of mucin-like glycoprotein or of total protein were not significantly different after ozone and air exposures (Table 3). Previous experiments in animals have shown that multiple expsoures to ozone are associated with airway submucosal gland hyperplasia and hypersecretion (22,23). It is possible that the effects of ozone on the tracheobronchial secretory apparatus only become manifest with multiple or continuous exposures and that this explains the discrepancy between this animal data and our findings in induced sputum.

In summary, cellular and biochemical analysis of induced sputum collected 4 hours after ozone exposure reveals significantly higher percentages and numbers of neutrophils and significantly higher levels of myeloperoxidase than induced sputum collected 4 hours after air exposure. There was also a trend for ozone-associated increases in IL-6 and IL-8 in induced sputum. We conclude that sputum induction is a feasible method for obtaining airway secretions from healthy subjects after controlled exposures to ozone, and that cellular and biochemical analysis of induced sputum reveals the expected inflammatory airway effects of ozone. Analysis of induced

sputum should therefore prove useful for investigation of the pulmonary effects of ozone or other environmental air pollutants.

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TABLE 1: PULMONARY FUNCTION BEFORE AND AFTER AIR AND OZONE EXPOSURES

	AIR		OZONE	
	Before	After	Before	After
SRAW (L[cmH2O/L/s])	6.2±1.5	5.9±0.9	5.9±1.1	7.0±1.4*
FEV1 (L)	4.1±0.8	4.2±0.8	4.0±0.8	3.8±0.6*
FVC (L)	5.1±1.2	5.1±1.1	5.0±1.2	4.8±1.1*
FEF25-75 (L/s)	4.1±0.9	4.4±1.0	3.9±0.8	3.5±0.7*

Definition of abbreviations: SRAW = specific airway resistance (airway resistance x thoracic gas volume); FEV1 = forced expired volume in one second; FVC = forced vital capacity; FEF25-75 = forced expired flow between 25 and 75% of the forced viatal capacity.
*Significantly different from the change found after air exposures, $p<0.05$.

TABLE 2: TOTAL AND DIFFERENTIAL CELL COUNTS IN INDUCED SPUTUM AFTER FILTERED AIR AND OZONE EXPOSURES

	AIR	OZONE	P Value
%Squamous Cells	59.7±17.8	41.7±20.4	0.04
Squamous Cells (x10^5/ml)	5.0±2.6	4.7±2.0	0.9
Total Cell Count (x10^5/ml)*	3.9±2.6	7.4±4.8	0.04
%Epithelial Cell*	4.3±3.6	3.8±4.7	0.6
Epithelial Cells (x10^5/ml)	0.2±0.2	0.2±0.2	0.9
%Macrophages*	43.7±19.9	14.8±6.4	0.009
Macrophages (x10^5/ml)	1.9±1.6	1.2±0.9	0.4
%Neutrophils*	51.0±19.9	80.4±7.3	0.009
Neutrophils (x10^5/ml)	1.8±1.3	5.9±4.1	0.007
%Eosinophils*	0.3±0.6	0.2±0.3	0.5
Eosinophils(x10^5/ml)	0.01±0.02	0.01±0.04	0.3
%Lymphocytes*	0.8±0.5	1.0±1.0	0.9
Lymphocytes (x10^5/ml)	0.04±0.04	0.07±0.08	0.2

*Total and differential cell counts of the non-squamous cells.

TABLE 3: FLUID PHASE CONSTITUENTS OF INDUCED SPUTUM AFTER FILTERED AIR AND OZONE EXPOSURES

	AIR	OZONE	P Value
Myeloperoxidase (μg/ml)	1273±578	1622±635	0.02
Interleukin-6 (pg/ml)	26.8±30.7	44.5±32.4	0.11
Interleukin-8 (pg/ml)	1153±635	1497±452	0.09
Mucin-like glycoprotein (μg/ml)	1347±1027	1600±964	0.26