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Road tunnel air pollution induces bronchoalveolar inflammation in healthy subjects

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Abstract

Traffic related air pollution is associated with adverse respiratory effects.
The aim of the study was to investigate whether exposure to air pollution in a road tunnel cause airway inflammatory and blood coagulation responses. Sixteen healthy subjects underwent bronchoscopy with bronchial mucosal biopsies and BAL on two occasions, in random order; once at 14 hours after a 2 hour exposure to air pollution in a busy road tunnel, and once after a control day with subjects exposed to urban air during normal activities. Peripheral blood was sampled prior to bronchoscopies.

The road tunnel exposures included median concentrations of $PM_{2.5,}$ PM_{10} and NO_2 of 64, 176 and 230 μg/m^3, respectively. Significantly higher numbers of BAL fluid total cell number ($p<0.01$), lymphocytes ($p<0.01$) and alveolar macrophages ($p<0.05$) were present after road tunnel exposure vs. control. Significantly higher nuclear expression of the transcription factor component c-Jun was found in the bronchial epithelium after exposure. No upregulation of adhesion molecules or cellular infiltration were present and blood coagulation factors were unaffected.

In conclusion, exposure of healthy subjects to traffic related air pollution resulted in a lower airway inflammatory response with cell migration, together with signs of an initiated signal transduction in the bronchial epithelium.

Introduction

Ambient city air pollution has been shown to cause adverse health effects including respiratory and mucosal symptoms, worsening of asthma and COPD, but also increased mortality due to cardiovascular disease. The major contributor to these effects has been suggested to be particulate matter (PM) pollution ([1,2]). Much of the toxicity of PM appears to be mediated by ultrafine particles produced by combustion in gasoline and diesel engines, as well as other combustion sources ([3]). Additionally, road surface material, tire and break wear components and crustal matters contribute to the fine (1-2.5 μm) and coarse particles (2.5 -10 μm), which demonstrate clear toxicological properties ([4]). Particle size, chemical composition and bioavailability of chemical components have been found to be of importance for PM toxicity ([5]).

The airway epithelium is the primary target for environmental toxicants and substances, which can lead to inflammation and worsening of airway diseases. In exposure chamber studies diluted diesel engine exhaust has been shown to induce airway inflammation in human subjects. This response includes activation of signal transduction pathways in epithelial cells such as mitogen activated protein (MAP) kinases, nuclear factor kappa-B (NFκB) and activator protein-1 (AP-1), along with the subsequent up-regulation of a range of neutrophil chemoattractant cytokines, and endothelial adhesion molecule expression. This is followed by migration of neutrophils, T-cells and mast cells into the airway mucosa ([6,7,8,9,10]). In addition to this, oxidative stress and free radicals may contribute to diesel induced airway responses ([6, 7, 8, 9]).

So far, only one study has been published investigating health effects from exposure to road tunnel air pollution ([11]). This particulate traffic environment does not only include gases and PM from gasoline and diesel engines, but also resuspended road dust, components from wear of running vehicles and crustal materials ~~as well as irritant gases and nitric oxides~~. In the study by Svartengren and co-workers ([9]), allergic asthmatic subjects were shown to experience an increased hyperresponsiveness to inhaled allergen after a brief 30 minutes road tunnel exposure.

The present investigation was therefore directed to determine whether exposure of healthy subjects to road tunnel air pollution would result in airway inflammation, as reflected in bronchoalveolar lavage (BAL) fluid and bronchial mucosal biopsies.

Subjects and methods

Subjects

Sixteen healthy, non-smoking volunteers (6 female, 10 male) with a mean age of 28 (range 19-59) years participated in the study. All subjects underwent a physical examination including chest X-ray and blood routine examination two weeks before the study. None of the volunteers had a history of allergy, and airway infections were not allowed within six weeks before exposures. Chest X-ray and lung function tests were normal and *in vitro* screening for the presence of specific IgE antibodies against common inhaled allergens (Phadiatop®, Pharmacia-Upjohn, Uppsala, Sweden) were negative. No medication was allowed during the study. All participants gave their informed consent and the local ethics committee at the Karolinska Institutet approved the study.

Study design

All subjects underwent two bronchoscopies in random order with 3-10 weeks interval (figure 1). One was done 14 hours after a two hour road tunnel exposure and the other (serving as a control) was performed at the same time of day and was preceded by the subject's ordinary daily activities. This included work or educational activities and use of public transportations, but no transit through a road tunnel. The air pollution exposure on this occasion is considered as a low urban exposure, and contrasting to the road tunnel exposure.

Road tunnel exposure

The road tunnel exposures lasted for two hours, and took place the afternoon rush hours (4:00-6:00 pm) in the Söderleden road tunnel in the central part of Stockholm. The total length of the tunnel is nearly 1500 m and the traffic intensity is approximately 120 000 ~~35 000~~ vehicles/day. The subjects alternated 15 minutes intervals of light exercise on a bicycle ergometer (50 Watt), with 15 min of rest. Immediately before and every 20 min throughout the exposure, symptoms were recorded.

The exposure set up consisted of a small room (approximately 6 m^2) located between the two tunnel tubes, some 50-100 m from the tunnel exit. The room has doors on either side, which were open to the adjacent traffic roads during the exposure session.

Exposure measurements

Sampling of particles with a 50 % cut-off aerodynamic diameter of 2.5 and 10 μm, respectively ($PM_{2.5}$ and PM_{10}), was performed using Harvard impactors (Air Diagnostics and Engineering Inc., Maine,USA) at a flow rate of 10 L/min. The impactors were equipped with 37 mm teflon filters (Air Diagnostics and Engineering Inc.) with a pore size of 2 μm. Gravimetric analysis were performed after 24 hours of conditioning using a Mettler Toledo MT5 (Mettler, Greisensee, Switzerland). The concentration of airborne ultrafine particles (aerodynamic diameter 14 - 100 nm) was determined using a scanning mobility particle sizer (SMPS) system (Electrostatic classifier model 3071, TSI, USA) in combination with a condensation particle counter (CPC 3010, TSI). The total number of airborne particles in the range between 20 and 1000 nm in aerodynamic diameter were monitored with a P-Trak particle counter (TSI). Carbon monoxide (CO) levels were measured using an electrochemical sensor

(Dräger Pac III, Dräger, Germany). Nitric oxides levels were monitored during the exposure using a chemiluminescent instrument (AC 31M, Environnement, France).

Symptoms

Symptoms from eyes, upper and lower airways were recorded over the exposure session. The intensity was graded from 0 to 10, where 0 corresponded to no symptoms and 10 to severe symptoms, according to a modified Borg scale ([12]). Change in symptom reporting was calculated by subtracting the pre-exposure rating with the average of all ratings during the exposure.

Lung function tests

Lung function was measured immediately before bronchoscopies, including parameters for vital capacity (VC), forced vital capacity (FVC) and forced expiratory volume during the first second (FEV_1), using a spirometer (Jaeger Masterscope, Würzburg, Germany).

Peripheral blood

Peripheral blood was sampled before bronchoscopies and cell differential counts were performed using an autoanalyser (Advia 120 Hematology System, Bayer). Plasminogen activator inhibitor-1 (PAI-1) was analyzed in plasma samples using enzyme linked immunosorbent assay (ELISA) (Chromolize PAI-1, Biopool) and fibrinogen in plasma was analyzed by a kinetic fibrinogen assay.

Bronchoscopy, bronchoalveolar lavage and cell preparation

Bronchoscopy was performed with a flexible fiberoptic bronchoscope (Olympus F Type P30, Olympus Optical Co. Ltd, Tokyo, Japan) under local anesthesia (Lidocaine, Xylocain®, AstraZeneca, Södertälje, Sweden) after pre-medication with

morphine-hyoscine (Morfin-skopolamin, Meda, Solna, Sweden). Bronchial wash (BW) was performed by instilling two aliquots of 10 mL phosphate buffered sterile saline (PBS, 37 ˚C) in an upper lobe bronchus and then the fluid was gently aspirated. Thereafter five aliquots of 50 mL PBS was instilled in the middle lobe or lingual lobe for BAL, and the fluid was gently aspirated after each aliquot and collected in a siliconized plastic bottle kept on ice. The fluid was filtered through a single layer of Dacron net (Type AP32, Millipore, Cork, Ireland) and centrifuged at 400g for 10 minutes at 4^{o}C and cells and supernatant were separated. The cells were resuspended in RPMI medium and a total cell count was performed. Smears for differential counts were prepared by cyto-centrifugation (Cytospin 2, Runcorn, Cheshire,UK) at 22g for 3 min. After staining with May-Grünwald Giemsa, 500 cells were counted. The number of mast cells in 10 visual fields (16 x magnifications) was counted after staining with toluidine/haematoxylin. Bronchial mucosal biopsies were taken from proximal cristae. BAL was performed on one side and mucosal biopsies were obtained on the contra lateral side. At the second bronchoscopy, the sites were reversed.

Flow Cytometry

BAL cells were stained with monoclonal antibodies (mabs) against alveolar macrophage (AM) adhesion molecules, co-stimulatory molecules and activation markers (Table I, given in on-line available material). Isotype matched mouse immunoglobulin mabs were used as negative controls. For each analysis 5 x 10^5 cells were incubated with phycoerythrine (PE) conjugated antibodies (Becton Dickinson, Mountain View, Ca, USA) for 30 min, 4^{o}C, in the dark and thereafter washed twice with PBS. For identification of BAL cell lymphocytes, 2 x 10^5 cells were stained with

FITC (fluorescein isothiocyanate), PE and RPE-Cy-5 conjugated mabs against CD3, CD4 and CD8 (DAKO, Glostrup, Denmark). Natural killer (NK) cells and CD56+ T-cells were stained with CD3, CD45 and CD56/CD16 (Becton Dickinson). Cells were resuspended in Cellfix (Becton Dickinson) and analyzed by flow cytometry (FACSCalibur, Becton Dickinson) on the same day. Lymphocytes were gated by forward and side scatter properties and 10^4 cells were collected within the lymphocyte gate. The percentages of CD4+ and CD8+ T cell subsets were analyzed and the ratio was calculated. Natural killer (NK) cells (defined as CD56+/CD16+/CD3-) and CD56+ T-cells (defined as CD56+/CD16+/CD3+ cells) were calculated as percentage of all cells in the lymphocyte gate.

Soluble components in BAL fluid

Fibronectin was analyzed with a double-sandwich ELISA technique, as previously described ([13]). Total MMP (matrix metalloproteinase)-9 levels (active MMP-9 and pro-MMP-9) were measured in BAL using a commercially available ELISA (R&D Systems, Minneapolis, USA).

Bronchial mucosal biopsies

The staining and quantification procedures have previously been described in detail ([7, 8,14]). Briefly, endobronchial mucosal biopsy samples were fixed overnight in a solution containing chilled acetone and protease inhibitors before processed into glycol methacrylate (GMA) resins ([15]). The fixed biopsies were cut in two micron thin slices and placed on glass slides. Endogenous peroxidases were inhibited and non-specific antibody binding was blocked before primary mabs were applied and incubated over night. Mabs were directed against early signal transduction pathways (JNK, p38, c-Jun, p65), cytokine expression (interleukin (IL)-10, IL-8, Gro-α),

endothelial cells (EN4), endothelial adhesion molecule expression (p-selectin, ICAM-1) and inflammatory cell infiltration (neutrophils, mast cells, T cells) (See Table 1 – given in on-line available material). Finally, a biotinylated secondary antibody was added before the sections were developed and counterstained. In areas of intact epithelium and submucosa, positive staining was analyzed with the assistance of computerized image analysis and light microscopy (Leica Q500IW, Leica, Cambridge, UK), as previously described ([6, 17, 14]).

Statistical analysis

Statistical analysis was carried out with SPSS version 11.0 on a Windows based PC platform (SPSS, Inc., Chicago, IL, USA). To analyze the response to different exposures, the Wilcoxon´s non-parametric rank sum test for comparisons within the same individual was used. P-values from two-sided tests were considered significant if less than 0.05. Comparison of pre- and post-exposure lung function data were performed by Students t-test. The Pearson correlation test was used in order to determine significant correlation between exposure parameters.

Results

Road tunnel exposure

The median $PM_{2.5}$ and PM_{10} levels during the exposure occasions in the road tunnel throughout the afternoon rush hour were 64 (range 46-81) μg/m^3 and 176 (130-206) μg/m^3 respectively. The median NO respectively NO_2 levels were 874 (range 751-1032) μg/m^3 and 230 (180-269) μg/m^3 and the CO level was 5.8 (1.2-7.0) μg/m^3. The median number concentration of ambient airborne particles with an aerodynamic diameter between 20 and 1000 nm was 1.1 (range 1.0-1.3) x 10^5/ml measured by the P-trak instrument. The total number concentration of ultrafine airborne particles (particles less than 100 nm in diameter) was 0.85 (range 0.74-1.0) x 10^5/ml.

The NO, NO_2, CO, ultrafine particles (<100 nm), $PM_{2.5}$ and PM_{10} concentrations correlated as expected due to mainly engine combustion origin ($p<0.01$-0.05, $r=0.50$-0.74).

The 24-h average background levels of NO_2, $PM_{2.5}$ and PM_{10} measured at a busy street level in the central of Stockholm during the study period, were 46 μg/m^3, 13 μg/m^3 and 30 μg/m^3, respectively (The City of Stockholm Environment and Health Administration).

Cells in bronchoalveolar lavage

The total cell concentration in the BAL fluid increased significantly ($p<0.01$) after the exposure to road tunnel air vs. control day with normal activity. This was mainly due to increased concentrations of lymphocytes ($p<0.01$) but also of alveolar macrophages ($p<0.05$) (Table 2). The percentage of lymphocytes increased significantly from 9.4% to 17 % ($p<0.05$) with a reciprocal decrease of alveolar

macrophages from 89% to 82% after exposure ($p<0.05$). No statistical significant changes were seen for neutrophils, eosinophils, basophils or mast cells.

Flowcytometric analysis of BAL fluid

Analyses of lymphocyte subsets revealed a small but statistically significant decrease after exposure in the percentage of BAL fluid NK cells ($p<0.05$, Table 3). Also BAL fluid $CD56^+$ T-cells decreased significantly after exposure ($p<0.05$).

Soluble components in BAL fluid

Fibronectin concentrations at control were 50 ng/mL (40-129) and after tunnel exposure 71 (41-156) ng/mL, representing median and interquartile range, and MMP-9 concentration 0.54 (0.23-0.83) ng/mL and 0.32 (0.22-1.03) ng/mL, respectively without any significant difference.

Bronchial mucosal biopsies

Step-wise immunohistochemical analyses were carried out on the biopsy material based on the expected time course of the development of inflammation in the bronchial airway mucosa, as derived from earlier investigations ([7, 8, 16, 17]): 1/ early signal transduction pathways. If positive; 2/ cytokine and endothelial adhesion molecule expressions were determined, and finally 3/ inflammatory cell infiltration.

The evaluation revealed a significant increase in the nuclear expression of c-Jun in the bronchial epithelium after exposure to road tunnel pollution vs. control ($p = 0.034$), with no change in the expression of the signal transduction markers JNK, p38 or p65 (Table 4).

Since activation of an early signal transduction pathway was identified, we proceeded with readings of cytokine expression, adhesion molecule expression and

inflammatory cell counts, but could not identify any significant changes in these parameters (see Table 5 of on-line available material)

Symptoms

Symptom ratings increased significantly during the road tunnel exposure regarding symptoms from the eyes (mean ± standard deviation: 1.2±1.5, $p<0.01$), upper airways (1.4±1.2, $p<0.01$), and lower airways (0.76±1.2, $p<0.05$) as well as unpleasant smell (1.8±1.7, $p<0.01$).

Lung function

All subjects had a normal lung function at control and no significant changes occurred due to exposure.

Peripheral blood

Analyses of peripheral blood demonstrated that the total leukocyte numbers as well as plasma levels of fibrinogen and PAI-1 did not change over exposures.

Discussion

The present study demonstrated a two-hour exposure to air pollution in a busy road tunnel to cause an airway inflammatory response with elevations of T lymphocytes and alveolar macrophages in BAL, in comparison with a control day with the subjects exposed to urban air during normal activities. Additionally, an increased nuclear expression of the transcription factor component c-Jun was demonstrated in the bronchial epithelium after road tunnel exposure.

The air pollution exposure in the Stockholm road tunnel differed in several ways from the diluted diesel engine exhaust atmosphere investigated in a series of earlier studies ([5, 7, 8, 14, 17]). The $PM_{2.5}$ concentration in the present study constituted approximately 36 % of the PM_{10} concentration, indicating that a large proportion of the particles belonged to the coarse fraction, presumably originating from road surface and tire wear as well as resuspension of crustal material. In addition, the exposure situation in the road tunnel included exhaust fumes from vehicles run on different fuels, compared with the controlled chamber situation. Gasoline is the dominating fuel used in Sweden, and approximately 10% of the Swedish road fleet consists of diesel powered vehicles. The high levels of NOx and number concentration of ultrafine particles, demonstrates that an important source of exposure is fuel combustion from motor vehicles.

Of potential importance, when comparing the present study with the controlled diesel exposure studies, is the fact that the number concentration of PM_1 particles was considerably higher in the controlled diesel exhaust studies compared to the present study (4×10^6 particles/ml compared to 1×10^5 particles/ml). Additional aspects when considering the inhaled and subsequently deposited particle dose, are

workload and ventilation rate. During the exercise periods of the two-hour exposure the average minute ventilation increased to approximately 10 L/min/m^2, as based on ventilation measurements during step-wise exercise tests. This can be compared with approximately 20 L/min/m^2 during the mentioned diesel exhaust exposure experiments. Still, the present exercise levels would be more representative for most exposure situations encountered in the city environment. Interestingly, despite the lower minute ventilation rate and substantially lower concentration of ultra fine particles generated by the traffic, clear airway inflammatory responses were identified.

The present study confirms earlier human experimental data, as well as animal data, with controlled diesel exhaust challenges, suggesting T-cells to be important in the airway inflammatory response ([7, 10]). T-lymphocytes are important regulatory cells that interact directly with macrophages via receptors, and they also have the capacity to regulate inflammatory cell activity by cytokine release to either a Th:1 or Th:2 inflammatory response. Diesel exhaust particles have been demonstrated to interact with T-cell function and may also be a potent Th:2 adjuvant ([18]). We have recently confirmed that the T-cell dominated inflammation, induced by diesel exhaust exposure to 300 µg/m^3 PM_{10} for one hour, in healthy non-atopic subjects, leads to a Th:2 shift in the bronchial epithelium, with enhanced production of IL-13 ([17]). This cytokine, together with its companion IL-4, has the capacity to induce an allergic type inflammation ([17]). Worsening of asthma by traffic related pollution has found additional support in a diesel exhaust exposure study in asthmatic subjects treated with inhaled corticosteroids. These subjects experienced a doubling of their bronchial hyperresponsiveness, in terms of a doubling difference in metacholine PC_{20} after diesel exposure vs. air ([19]). Additionally, a preceding study by Svartengren and

colleagues, employing a short term road tunnel exposure of allergic asthmatics resulted in an enhanced airway responsiveness to allergen inhalation, which may likewise link to a state of enhanced Th:2 response by traffic induced air pollution.

In the present *in vivo* exposure study, the significant increase in BAL T cells after exposure was not accompanied by a change in the relation of CD4 or CD8 T cells, indicating an unspecific accumulation of these cells. There was however a significant reduction of relative numbers of NK cells and cytotoxic CD56+ T cells, both with important capacities to eliminate damaged or infected cells as well as to trigger the immune system by producing large quantities of key cytokines such as interferon (IFN)-γ and tumor necrosis factor alpha (TNF)-α. The decreased number of NK and cytotoxic T cells may indicate either a re-localization or that these cells are consumed in response to the road dust exposure as part of a first line defense against toxic substances.

Bronchoalveolar lavage analyses demonstrated an increase in the concentration of alveolar macrophages, in accordance with an earlier diesel engine exhaust exposure study investigating the late response at 18 hours post exposure ([6]). The concordant findings between road tunnel and diesel exposure highlight the importance of macrophages in the defense against traffic-related particulate air pollution. Alveolar macrophages have phagocytic, antigen presenting and immune modulating capacities, important for the immune defense against particulates and micro organisms. We have previously reported that the phagocytic ability of macrophages can be suppressed by ingestion of diesel particles during *in vivo* exposure of human subjects ([6]).

It is suggested that the alveolar macrophage increase may have been due to increased clearance after the tunnel exposure, while it remains to be demonstrated

whether these cells were secretively active. Fibronectin, which can be produced by many different cell types, often has its origin from activated alveolar macrophages, and has earlier been demonstrated to increase in BAL fluid after PM exposure, resulting in elevated numbers of alveolar macrophages ([7]). However, the results from the present study did not indicate any increased concentrations of fibronectin after tunnel exposure.

In the present study, bronchial mucosal biopsies were analyzed in a step-wise order to elucidate at what stage a potential inflammatory response in the bronchial mucosa would be. The analyses demonstrated an increase in nuclear translocation of c-Jun, which may indicate the presence of an early reactive epithelial response. Activation of the transcription factor AP-1, of which c-Jun is a sub-unit, may involve transcription of many down-stream inflammatory cytokines and chemokines, including TNF-α, IL-1β, IL-6, IL-8 and RANTES (regulated on activation normal T cell expressed and secreted) ([20]). The present findings therefore indicate potential for neutrophilic and lymphocytic cell recruitment during the forthcoming hours after biopsy sampling, through c-Jun enhanced transcription of IL-8 and RANTES. Further support for an assumption of a very early inflammatory state in the bronchial epithelium is that the vascular adhesion molecule expressions of p-selectin and ICAM-1 were not up-regulated. Importantly, it has been suggested from some earlier studies that inflammatory responses to air pollutants may develop slower after exposure to lower concentrations than higher. Controlled chamber exposure to diesel exhaust (DE) at PM_{10} levels of 300 $\mu g/m^3$ ([7, 8,14]) had already at 6 hours caused a fully established state of bronchial as well as bronchoalveolar inflammatory cell infiltration together with upregulated vascular adhesion molecules, cytokine expressions and signal transduction pathways. In contrast, at six hours after exposure to DE at PM_{10} levels

of 100μg/m^3 ([9]) there was only an early state of inflammation, which developed substantially after 18 hours ([21]). Consequently, it is expected that the early state of inflammation at 14 hours after exposure, may have developed further both in the bronchial and bronchoalveolar regions. Additional road tunnel exposure experiments with sampling at a later time point would be needed to more precisely determine the time course of the respiratory tract responses. The limited number of study subjects may have resulted in false negative results in some of the study parameters. Ideally, a large number of subjects should have been included, but exposure studies incorporating bronchoscopies and subsequent analyses of lung samples are very resource demanding. The current standard, based on a large number of studies suggests a similar number of subjects as used in this study, to be sufficient to demonstrate relevant inflammatory events induced by air pollution ([7-9,13,14,17]).

In the present study we addressed the hypothesis by Seaton and colleagues that exposure to ambient air pollution would impact on the coagulations system ([22]). We were unable to detect any significant change in systemic coagulation by road tunnel exposure, in similarity with a recent investigation in human subjects following diesel exhaust exposure ([23]). In contrast, the present investigators most recently demonstrated that diesel exhaust may locally reduce tissue plasminogen activator (tPA) release from the endothelium *in vivo* together with reduced vasomotor response, in healthy human subjects investigated with bilateral arm plethysmography at 6 hours after exposure ([23]).

It is concluded that a road tunnel exposure during light exercise results in an airway inflammation with cell migration reflected in BAL, together with indications of an initiated signal transduction in the bronchial epithelium as wells as increased symptoms from eyes and upper and lower airways. Forthcoming research may

determine whether further inflammatory development occurs in the subsequent hours beyond the sample time point in the present study.

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Figure 1. Study design. The subjects were randomized into alternative 1 or 2. Each subject served as their own control. Med exam = medical examination. Exposure 2 hours = road tunnel exposure. BR = bronchoscopy. postexp = post exposure,

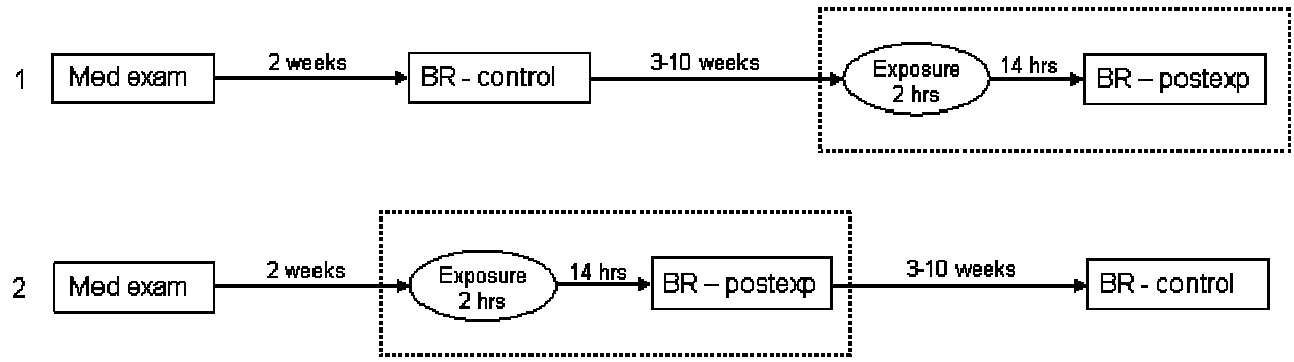

Table 1 (May be presented only as on-line material)

Characterization of antibodies used for staining of bronchoalveolar lavage cells before flow cytometry, and for immunohistochemical staining of bronchial mucosal biopsies.

Flow cytometry of bronchoalveolar lavage cells		
Antibody against	**Major function/marker of**	**Source**
Lymphocytes		
CD3	T-cell receptor	Dako, Glostrup, Denmark
CD4	T-helper cells	Dako, Glostrup, Denmark
CD8	Cytotoxic T-cells	Dako, Glostrup, Denmark
CD16	NK-cell marker	Becton Dickinson Pharmingen, CA, USA
CD45	Leucocyte	Becton Dickinson Pharmingen, CA, USA
CD56	NK-cell marker	Becton Dickinson Pharmingen, CA, USA
Bronchial mucosal biopsies		
Antibody against	**Major function/marker of**	**Source**
Signal transduction		
p-p38	p 38	Santa Cruz, CA, USA
p-JNK	c-jun N-terminal kinase	Santa Cruz, CA, USA
p-C-Jun	Activator protein-1	Santa Cruz, CA, USA
NFκB (p65)	Nuclear factor-κ-B	Becton Dickinson Pharmingen, CA, USA
Cytokines		
IL-8	Interleukin 8	Boehringer Ingelheim, Heidelberg, Germany
Gro-α	Gro-α	R&D, Abingdon, UK
IL-10	Interleukin 10	R&D, Abingdon, UK
Adhesion molecules		
CD62P	P-selectin, Microvasculature	Serotec Oxford UK
CD54	ICAM-1, Microvasculature	Dako, Glostrup, Denmark
EN4	Endothelium, Microvasculature	Monsan, Uden, the Netherlands
Inflammatory cells		
CD3	CD3, T lymphocytes	Dako, Glostrup, Denmark
NE	Elastase, Neutrophils	Dako, Glostrup, Denmark
AA1	Tryptase, Mast cells	Dako, Glostrup, Denmark

Table 2

Bronchoalveolar lavage cell numbers at control and after exposure to road tunnel air. Data are given as medians and interquartile ranges.

Cells x10^6/L	Control	Road tunnel exposure	p-value
Total cells	**80** (61-96)	**106** (88-133)	**<0.01**
Lymphocytes	**7.6** (5.1-11)	**13** (9.6-13)	**<0.01**
Alveolar macrophages	**67** (53-85)	**93** (62-103)	**<0.05**
Neutrophils	**1.2** (0.76-1.7)	**1.3** (0.66-2.0)	**ns**
Eosinophils	**0.04** (0-0.43)	**0.06** (0-0.34)	**ns**
Mast cells[a]	**3.5** (0.5-6.8)	**3** (1.2-6.8)	**ns**

[a]Number of cells in ten visual fields with 16x magnification

Table 3.

Bronchoalveolar lavage fluid lymphocyte subset data at control and after exposure in a road tunnel. Data are given as medians with interquartile ranges.

Marker	Control	Road tunnel exposure	p-value
%$CD3^+$ (T-cells)	**89** (85-93)	**90** (86-93)	**ns**
%$CD4^+$ (T helper-cells)	**69** (59-73)	**69** (62-74)	**ns**
%$CD8^+$ (Cytotoxic T-cells)	**24** (21-33)	**26** (21-31)	**ns**
% $CD56^+/CD16^+/CD3^-$ (NK-cells)	**5.7** (3.3-8.7)	**4.7** (3.9-8.1)	**<0.05**
% $CD56^+/CD16^+/CD3^+$ ($CD56^+$/T-cells)	**6.8** (4.5-9.0)	**6.2** (3.3-8.2)	**<0.05**

Table 4

Signal transduction markers in the bronchial epithelium of bronchial biopsies from healthy subjects, at control and after exposure to road tunnel air pollution. Data are given as medians with interquartile ranges.

Signal transduction marker positive nuclear staining/mm^2 epithelium	**Control**	**Road tunnel exposure**	**P-value**
JNK	**467** (299-610)	**454** (301-519)	**ns**
p38	**72** (34-175)	**124** (36-480)	**ns**
c-Jun	**1858** (689-2930)	**2257** (633-3857)	**<0.05**
p65	**1955** (768-2738)	**1015** (403-3130)	**ns**

Table 5 (May be published only as on-line material)

Cytokine expression, adhesion molecule expression and inflammatory cell numbers in bronchial biopsies from healthy subjects, at control and after exposure to road tunnel air pollution. Data are given as medians with interquartile ranges.

Marker	Control	Road tunnel exposure	P-value
Cytokines			
IL-10[a]	**0.34** (0.04-1.03)	**0.65** (0.06-2.92)	**ns**
IL-8[a]	**0.14** (0.05-0.59)	**0.21** (0.10-1.06)	**ns**
Gro-α[a]	**0.01** (0.00-0.04)	**0.00** (0.00-0.03)	**ns**
Adhesion molecules			
p-selectin [b]	**59.5** (46.9-66.0)	**52.0** (43.8-66.0)	**ns**
ICAM-1[b]	**61.3** (49.1-71.4)	**59.9** (49.5-65.5)	**ns**
Inflammatory cells			
Neutrophils submucosa [c]	**67.0** (51.9-103.1)	**86.7** (53.4-114.3)	**ns**
Mast cells submucosa [c]	**74.9** (47.8-83.9)	**74.7** (55.9-104.5)	**ns**
T cells ($CD3^+$) submucosa [c]	**164.2** (51.8-236.4)	**149.3** (98.4-232.1)	**ns**

a: % of epithelial surface with positive staining.
b: positively stained vessels in percent of pan-endothelial EN4 staining.
c: number of positively stained cells/mm^2 submucosa.

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