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Differences in bronchoalveolar cell response to nitrogen dioxide exposure between smokers and nonsmokers

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Differences in bronchoalveolar cell response to nitrogen dioxide exposure between smokers and nonsmokers. R. Helleday, T. Sandström, N. Stjernberg. ©ERS Journals Ltd 1994.

ABSTRACT: We have previously reported on the bronchoalveolar inflammatory effects of the common air pollutant NO_2 in nonsmokers. In this study, we have investigated these effects in tobacco smokers.

Eight young nonbronchitic smokers and, as a reference group, eight healthy lifetime nonsmokers were exposed to 3.5 ppm NO_2 for 20 min. Bronchoalveolar lavage (BAL) was performed 3 weeks before and 24 h after exposure. The first recovered 20 ml was analysed separately and defined as the bronchial portion (BP), and the following fluid recovered as the bronchoalveolar portion (BAP).

Before exposure, the smokers had significantly less CD3+ cells and more alveolar macrophages (AMs) in the BP and the BAP, as well as reduced AM phagocytosis *in vitro* compared to nonsmokers. After NO_2 exposure, the smokers reacted with an increase of AMs and neutrophils in BAP. Nonsmokers reacted with an increase of neutrophils in BP, an increase of lymphocytes in BAP, and a tendency to reduced AM phagocytosis.

In summary, young smokers and nonsmokers differed to some extent in their reactions to NO_2 exposure. This is probably due to the pre- existing airway inflammation and compensatory mechanisms to oxidant stress in the smokers.
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Nitrogen dioxide (NO_2) is a common air pollutant, frequently found in ambient air. NO_2 is mainly produced by various combustion processes, especially in industrial locations and urbanized areas, but also in homes with gas stoves. The exposure limit for NO_2 for an 8 h workshift in Sweden and several other countries is 2.25 ppm (4 mg·m^{-3} air), and the peak exposure limit in the workplace is 5.5 ppm (10 mg·m^{-3} air). Concentrations up to the exposure limit for NO_2 frequently occur in the workplace in certain industries. In homes with gas stoves, indoor NO_2 may reach a maximum 1 h level of 0.25–1.0 ppm, and peak levels as high as 4 ppm [1]. During the combustion processes, a mixture of nitric oxide (NO) and NO_2 is generated, but much of the NO is rapidly oxidized and converted to NO_2 [2]. Earlier studies have demonstrated decreased lung function, increased morbidity in lung diseases [3, 4], and an increased risk of airway infections [5, 6] due to NO_2 exposure.

Since the mid 1980s bronchoalveolar lavage (BAL) has been used as a tool in the investigation of the effects of NO_2 in human airways [6–12]. In preceding studies we have described an inflammatory response in the lungs of healthy nonsmoking subjects after controlled chamber exposure to NO_2 evaluated with BAL. We found an increase in mast cells and lymphocytes after a single exposure [9, 10], but a different reaction after repeated exposures to NO_2, with decreased amounts of alveolar macrophages (AMs), B-cells and natural killer (NK)-cells in BAL, but no increase in lymphocytes or mast cells [11, 12].

It is known that tobacco smoking causes respiratory bronchiolitis [13], altered immune defence in the lung with increasing numbers and proliferation of AMs [14, 15], increasing numbers of neutrophils [16], risk of developing bronchiolar fibrosis [17], and pulmonary emphysema [18]. Smokers have also been found to have a different cell response to various situations and disorders compared to nonsmokers. Negative correlations between smoking and disease frequency have been reported for sarcoidosis [19], and extrinsic allergic alveolitis (hypersensitivity pneumonitis) [20]. Smoking patients with breast cancer respond with less irradiation induced pulmonary inflammation compared to nonsmoking patients [21]. Among the more than 4,000 individual components that have been identified, tobacco smoke contains NO and NO_2 [22]. It has not been clarified to what extent the low concentrations of nitrogen oxides reaching the lower airways may contribute to the acute and chronic changes in the lungs of smokers. Based on the above mentioned circumstances, we speculated whether smokers would respond with a more pronounced airway inflammation than nonsmokers when challenged with

NO_2, since they already have a state of augmented cell activation in the airways, or if they would have a protective effect of increased antioxidant levels [23–25], due to the repeated exposure of nitrogen oxides in the tobacco smoke.

The primary aim of this study was to evaluate the bronchoalveolar cell response in young smokers after exposure to NO_2, in a concentration which occurs in industrial indoor environments. The control group consisted of healthy nonsmoking subjects. The secondary aim was to investigate whether analysis of BAL fluid fractionated into proximal and peripheral airway portions would provide additional information regarding the inflammatory response to NO_2 exposure, when compared to conventional analysis of pooled BAL fluid.

Subjects and methods

Subjects

Sixteen subjects, medical students or manual workers, divided into two groups of eight each, participated in the experiment. One group consisted of smokers, six males and two females, with a smoking history ranging from 9–20 pack-years (median 14 pack yrs), currently smoking 3–35 cigarettes·day^{-1} (median 20 cig·day^{-1}), and aged 28–32 yrs (median 29 yrs). None gave a history of chronic bronchitis or other respiratory disorders. The control group consisted of healthy lifetime nonsmokers, all males, aged 24–35 yrs (median 26 yrs). Spirometry (forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC)) was normal in all subjects as a prerequisite for inclusion in the study. All were free of airway infection within at least six weeks prior to and during the study. They all gave informed consent to participate and the study was approved by the local Ethics Committee of the University of Umeå.

Design of the study

The subjects were exposed to 3.5 ppm (NO_2 6.7 mg·m^{-3}) in an environmental exposure chamber for 20 min, according to a protocol described previously [26]. The NO_2 concentration was very stable during the exposures. For more than 95% of the exposure time, 3.5 ppm was maintained. The maximum variability was ±0.05 ppm. During the last 15 min of the exposure, light work (75 W) on a bicycle ergometer was performed.

All subjects underwent bronchoscopy with BAL at least three weeks prior to the exposure, in order to obtain reference BAL fluid, and 24 h after exposure. Hence, they were their own controls in calculations of changes in bronchoalveolar lavage fluid (BALF) content comparing pre- and postexposure data.

The smokers were not allowed to smoke either during the night or in the morning before the NO_2 exposure, or in the morning before the second BAL performed 24 h after exposure.

Nitrogen dioxide exposure

The exposure chamber is built of aluminium with a volume approximately 14 m^3. Windows enable the operator to maintain visual contact with the subject inside the chamber, and conversation occurs *via* an intercommunication system. A lock must be passed to gain access to the chamber; thus, the climate inside can be kept very stable.

Air entering the chamber is pretreated in a climate aggregate placed on the floor above the exposure chamber. In summary, the aggregate consists of three refrigerating machines, an apparatus for steam-moistening in three steps, and a heater. In addition to cooling the air, the refrigerating machines can also dehumidify when lower humidity is required. The system has two ventilating fans, one to supply air and one to remove it. This makes it possible to create a lower pressure inside the chamber, which is necessary to prevent leakage from the chamber to the outer room. Air is supplied to the chamber through the perforated ceiling and removed near the floor at one of the walls. The desired climate is reached by setting the temperature, relative humidity and ventilation rate of the chamber to the required values.

During the exposures for nitrogen dioxide, the ventilation rate was 150 m^3·h^{-1}. The chamber air temperature (T) was kept at 21°C and the relative humidity (RH) was 45%, measured with an RH and T indicator HMI 14 (Vaisala, Finland). To generate the accurate concentration of gas (ppm) inside the chamber, a calculation was made based on the ventilation rate and the concentration of NO_2 in the gas tube supply. From the supply, gas with a content of 1% NO_2 passed through a Brooks mass flow controller 5850 TR series (Brooks Instrument B.V., Veenendaal, Holland) and the flow meter Rota L1.6/70-7437 (Rota Apparate- und Maschinenbau, Hannover, Germany) to obtain control of the supplied gas flow. From the flow meter, the gas entered the ventilating duct just before the duct reached the chamber. To measure the concentration of NO_2, a tube from the measuring equipment was inserted through the chamber wall and NO_2 was measured by a CSI 1600, oxides of nitrogen analyser (Columbia Scientific Industries, Austin, Texas, USA).

Bronchoalveolar lavage

Atropine was given subcutaneously as the only premedication, and lidocaine was used for topical anaesthesia. A flexible fibreoptic bronchoscope was used with the subjects in the supine position. The bronchoscope was inserted through the mouth and carefully wedged into the middle lobe bronchus. Four aliquots of 60 ml sterile phosphate buffered saline (PBS) pH 7.3 at 37°C was infused and gently suctioned back after each infusion to a siliconized container placed in iced water, to slow down the cell metabolism and keep the number of viable cells as high as possible. The initial 20 ml recovered from the first instilled aliquot was analysed separately and defined as the bronchial portion (BP), representing the proximal parts of the bronchi [27]. The subsequent

fluid recovered from further aliquots was pooled and defined as the bronchoalveolar portion (BAP), corresponding to the distal airways. The recovered BALF was immediately transported to the laboratory for analysis.

Lavage fluid analysis

The chilled BALF was filtered through a nylon filter (pore diameter 100 µm, Syntab Product AB, Malmö, Sweden) and centrifuged at 400 ×g for 15 min. The cell pellet was resuspended in balanced saline solution (PBS) to a concentration of 10^6 cells·ml^{-1}. The total number of cells in the lavage fluid was counted in a Bürker chamber. Cytocentrifuged specimens with 5×10^4 nonepithelial cells·slide^{-1} were prepared using a Cytospin 2® (Shandon Southern Intruments Inc., Sewikly, PA, USA) 1,000 rpm (96 G) for 5 min. Slides were stained according to May-Grünwald Giemsa for standard cell differential counts, and 400 cells per slide were counted. Mast cells were counted on at least 10 visual fields at 160× magnification on slides stained with acid toluidine blue and counterstained with Mayers' acid haematoxylin.

Cell differential counts, total protein and albumin concentrations were determined both in BP and BAP. Flow cytometry was performed only on the BAP, since the cell number of the BP was too small to allow for this processing. Lymphocyte subsets were determined with flow cytometry (Becton-Dickinson, Facscan, Stockholm, Sweden) using the means of double readings of each sample. The following antibodies were used: CD3+ (T-cells, Leu 4); CD4+ (T-helper cells, Leu 3a); CD8+ (T-cytotoxic, Leu 2a); CD16+ + CD56+ (natural killer (NK)-cells, Leu 11 and 19); and CD19+ (B-cells, Leu 16) (Becton-Dickinson, Stockholm, Sweden).

AM phagocytosis was measured as percentage of cells positive for yeast particle engulfment, using a glass surface adherence method described previously [28]. The method has been modified to enable the use of BAL cells. Briefly, 200,000 cells in 200 µl medium containing 10% pooled human AB+ serum were allowed to adhere to the glass surface for 30 min in cell culture conditions. After rinsing the nonadherent cells away, yeast cells labelled with fluorescein isothiocyanate (FITC) and opsonized with human serum were added to the slides (2.5×10^7 yeast cells in PBS buffer solution, pH 7.4). After 30 min, the phagocytosis was stopped by dipping the slides in ice-cold PBS containing 1 mM ethylenediamine tetraacetic acid (EDTA). The fluorescence of noningested yeast cells was quenched by dripping toluidine blue in saline (1 mg·ml^{-1}, pH 4.7) onto the slides. Yeast cell adherence (attachment) to an AM was defined as visible contact between a brown yeast cell and an AM. Engulfment was defined as the presence of fluorescent yeast cells within an AM.

Lysozyme positive macrophages were demonstrated with Lysozyme® antibody (Dakopatts A/S, Copenhagen, Denmark) with the use of an immunoperoxidase technique.

The albumin concentration was measured with a nephelometric method, Beckman array protein system (Beckman Instruments Inc., Brea, Ca, USA). The total intra- and interassay variability was 3.3% and the detection limit 6 mg·l^{-1}.

The total amount of protein in BAL was determined using the Bio-Rad protein assay dye reagent (Bio-Rad laboratories, Munich, Germany) based on the principle of protein binding to Comassie Brilliant Blue, G-250 (Bradford, 1975) giving a violet colour with highest absorption degree at 595 nm. Human albumin was used as standard.

Peripheral blood samples were drawn 3 weeks before and 24 h after exposure. Analyses of blood cells were performed with an autoanalyser at the Department of Clinical Chemistry, University Hospital of Umeå, Sweden, according to clinical routine.

Statistics

Wilcoxon's nonparametric signed rank test for paired and independent observations was used. A p-value <0.05 was considered significant.

Results

Results are presented in tables 1–4, with BAL data before and after 20 min single NO_2 exposure in smokers and nonsmokers. Statistical comparison of BAL data before exposure between the smoking and nonsmoking group can be seen at the bottom of each table.

Median BAL recovery before exposure was 120 ml in the smoking group (interquartile range 90–140 ml) and 140 ml in the nonsmoking group (interquartile range 115–165 ml); and after exposure 130 ml (55–140 ml) and 130 ml (95–160 ml), respectively. There were no significant differences between the groups, either before or after exposure.

Comparison of BAL data in smokers and nonsmokers before NO_2 exposure

Smokers had significantly higher numbers of total cells and alveolar macrophages compared to nonsmokers in the bronchial portion (BP) before exposure (both $p<0.02$). In contrast, the percentage of lysozyme positive alveolar macrophages was significantly lower ($p<0.001$) in smokers (table 1). In the bronchoalveolar portion (BAP), similar differences were found with significantly higher numbers of total cells and alveolar macrophages in smokers and lower percentage of lysozyme positive macrophages compared to nonsmokers (table 2). There were no significant differences in percentage of AMs, total numbers and percentages of neutrophils, lymphocytes, mast cells or eosinophils (data not shown) in either BP or BAP (tables 1 and 2).

Flow cytometry on BAP showed that smokers had fewer CD3+ cells and CD4+ cells in the BAP before exposure. No significant differences could be detected for the other lymphocyte subsets. The CD4+/CD8+ ratio

Table 1. – Cell characteristics for the bronchial portion (BP) in smokers and nonsmokers before and 24h after exposure to 3.5 ppm NO_2

	Total cells	Alveolar macrophages		Lysozyme positive	Neutrophils		Lymphocytes		Mast cells	
	$\times 10^7 \cdot l^{-1}$	%	$\times 10^7 \cdot l^{-1}$	% of AMs	%	$\times 10^7 \cdot l^{-1}$	%	$\times 10^7 \cdot l^{-1}$	%	$\times 10^7 \cdot l^{-1}$
Smokers										
Before exposure	25.2 (20.0–27.7)	93 (90–96)	24.0 (18.7–26.6)	0.6 (0.4–2.25)	2 (1–3)	0.6 (0.2–0.8)	6 (2–8)	0.6 (0.42–1.1)	0.22 (0.025–0.25)	0.045 (0.012–0.062)
After exposure	30.0 (16.6–32.6)	95 (93–97)	28.8 (11.6–31.0)	0.35 (0.2–2.05)	2 (1–3)	0.5 (0.3–0.7)	2 (2–4)	1.0 (0.4–1.7)	0.2 (0.05–0.2)	0.023 (0.0–0.060)
p*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Nonsmokers										
Before exposure	15 (11.6–17.5)	88 (77–91)	12.5 (8.5–16.0)	6.5 (6.5–9.0)	2 (1–3)	0.23 (0.15–0.35)	10 (8–19)	1.5 (0.8–2.7)	0.11 (0.04–0.14)	0.014 (0.004–0.021)
After exposure	20 (15.0–22.5)	86 (58–90)	13.0 (11.4–15.6)	5.5 (5.0–8.5)	4 (2–6)	0.78 (0.45–1.12)	8 (5–34)	1.4 (1.0–6.7)	0.01 (0.0–0.04)	0.002 (0.0–0.008)
p*	NS	NS	NS	NS	=0.08	<0.05	NS	NS	NS	NS
Smokers *versus* nonsmokers										
Before exposure										
p**	<0.02	NS	<0.02	<0.001	NS	NS	NS	NS	NS	NS

Data are presented as medians with the first-third quartile (Q_1–Q_3). *: Wilcoxon's rank sum test for paired observation; **: Wilcoxon's rank sum test for independent observations. NS: nonsignificant.

Table 2. – Cell characteristics for the bronchoalveolar portion (BAP) in smokers and nonsmokers before and 24h after exposure to 3.5 ppm NO_2

	Total cells	Alveolar macrophages		Lysozyme positive macrophages	Neutrophils		Lymphocytes		Mast cells	
	$\times 10^7 \cdot l^{-1}$	%	$\times 10^7 \cdot l^{-1}$	% of AMs	%	$\times 10^7 \cdot l^{-1}$	%	$\times 10^7 \cdot l^{-1}$	%	$\times 10^7 \cdot l^{-1}$
Smokers										
Before exposure	11.9 (10.0–15.1)	93 (92–93)	11.2 (8.4–14.0)	1.0 (0.35–2.6)	1 (0–1)	0.1 (0.0–0.1)	6.5 (5–8.5)	0.6 (0.4–1.1)	0.22 (0.15–0.28)	0.02 (0.02–0.03)
After exposure	14.2 (12.4–22.4)	92 (84–95)	12.0 (11.3–21.3)	0.6 (0.4–2.6)	3 (1–3)	0.3 (0.1–0.4)	8 (2–12)	1.0 (0.4–1.7)	0.18 (0.10–0.53)	0.02 (0.02–0.05)
p*	<0.05	NS	<0.05	NS	<0.05	<0.02	NS	NS	NS	NS
Nonsmokers										
Before exposure	8.5 (6.6–11.0)	86 (72–92)	7.7 (5.8–8.7)	8.0 (6.1–9.0)	1 (0–2)	0.1 (0.0–0.1)	13 (7–16)	1.0 (0.5–2.0)	0.14 (0.04–0.19)	0.01 (0.04–0.1)
After exposure	11.0 (9.5–16.6)	87 (60–90)	8.9 (8.0–10.4)	9.0 (6.5–10.6)	1 (0–2)	0.2 (0.0–0.2)	12 (8–38)	1.2 (0.8–6.6)	0.08 (0.01–0.12)	0.08 (0.03–0.2)
p*	NS	NS	NS	NS	NS	NS	NS	<0.05	NS	NS
Smokers *versus* nonsmokers										
Before exposure										
p**	<0.05	NS	<0.05	<0.001	NS	NS	NS	NS	NS	NS

Data are presented as medians with the first-third quartile (Q_1–Q_3). *: Wilcoxon's rank sum test for paired observation; **: Wilcoxon's rank sum test for independent observations. NS: nonsignificant.

had a very wide distribution in smokers (table 3), but without evident correlation to age and smoking history (smoking years/present smoking in cigarettes·day^{-1} *versus* CD4+/CD8+ ratio, data not shown).

Before exposure to NO_2, the *in vitro* phagocytosis by alveolar macrophages was significantly lower in smokers compared to nonsmokers, both in terms of percentage of phagocytosis positive AMs ($p<0.01$) and numbers of phagocytosed yeast particles ($p<0.01$) (table 4).

Before exposure, smokers had a median concentration of total protein and albumin in BP of 110 mg·l^{-1} (interquartile range 104–131 mg·l^{-1}) and 166 mg·l^{-1} (50–71 mg·l^{-1}), respectively, and in BAP 73 mg·l^{-1} (59–87 mg·l^{-1}) and 36 mg·l^{-1} (27–46 mg·l^{-1}), respectively. Before exposure,

Table 3. – Lymphocyte subpopulations in the bronchoalveolar portion (BAP) in smokers and nonsmokers before and after single exposure to 3.5 ppm NO_2

	CD3+ $\times 10^7 \cdot l^{-1}$	CD4+ %	CD8+ %	CD4+/CD8+ ratio ratio of %	CD19+ $\times 10^7 \cdot l^{-1}$	CD16+ + CD56+ % $\times 10^7 \cdot l^{-1}$
Smokers						
Before exposure	0.48 (0.10–0.67)	21 (8–35)	29 (10–52)	1.3 (0.3–2.1)	0.014 (0.040–0.031)	0.061 (0.026–0.065)
After exposure	0.66 (0.19–0.70)	34 (30–42)	27 (24–50)	1.4 (0.7–2.0)	0.090 (0.040–0.046)	0.063 (0.036–0.087)
p*	NS	NS	NS	NS	NS	NS
Nonsmokers						
Before exposure	0.78 (0.32–1.67)	48 (45–60)	30 (21–36)	1.53 (1.29–2.6)	0.017 (0.003–0.030)	0.037 (0.013–0.093)
After exposure	0.96 (0.60–5.40)	49 (46–56)	30 (24–32)	1.57 (1.52–7.3)	0.015 (0.006–0.038)	0.085 (0.063–0.200)
p*	<0.05	NS	NS	NS	NS	<0.05
Smokers *versus* nonsmokers						
Before exposure						
p**	<0.05	<0.05	NS	NS	NS	NS

Data are presented as medians with the first-third quartile (Q_1–Q_3). *: Wilcoxon's rank sum test for paired observation; **: Wilcoxon's rank sum test for independent observations. NS: nonsignificant.

Table 4. – *In vitro* phagocytosis by alveolar macrophages before and 24 h after exposure to 3.5 ppm NO_2 in smokers and nonsmokers evaluated in the bronchoalveolar portion (BAP)

	Phagocytosis positive %	Yeast adherence positive %	Phagocytosed yeast particles total number
Smokers			
Before exposure	79 (65–81)	11 (4–14)	117 (73–144)
After exposure	83 (72–85)	5 (3–7)	110 (104–120)
p*	NS	<0.05	NS
Nonsmokers			
Before exposure	90 (86–91)	3 (0–4)	415 (310–555)
After exposure	86 (84–88)	2 (1–4)	405 (286–498)
p*	=0.052	NS	NS
Smokers *versus* nonsmokers			
Before exposure			
p**	<0.01	<0.05	<0.01

Data are presented as medians with the first-third quartile (Q_1–Q_3). *: Wilcoxon's rank sum test for paired observation; **: Wilcoxon's rank sum test for independent observations. NS: nonsignificant.

nonsmokers had a median concentration of total protein and albumin in BP of 113 mg·l^{-1} (interquartile range 98–215 mg·l^{-1}) and 64 mg·l^{-1} (60–139 mg·l^{-1}), respectively, and in BAP 110 mg·l^{-1} (68–182 mg·l^{-1}) and 50 mg·l^{-1} (32–64 mg·l^{-1}), respectively. There were no significant differences between smokers and nonsmokers either before or after exposure.

Comparison of BAL data in smokers and nonsmokers after NO_2 exposure

All analysed cell parameters of smokers in the BP were unchanged after exposure to NO_2. In nonsmokers a small but significant increase in the total numbers of neutrophils ($p<0.05$), and a tendency towards increase in the percentage of neutrophils ($p=0.08$) were seen after the exposure (table 1). In the BAP of smokers there was a significant elevation in total cell numbers, total numbers of AMs (both $p<0.05$), total numbers and percentage of neutrophils ($p<0.02$ and $p<0.05$, respectively) after exposure. In the nonsmokers only the total number of lymphocytes showed a significant increase (table 2).

After exposure to NO_2, the lymphocyte subsets remained unchanged in smokers. In nonsmokers an increase in the total numbers of CD3+ and CD16+ + CD56+ cells was noted (both $p<0.05$), whilst the other lymphocyte subsets were not affected (table 3).

The *in vitro* phagocytosis assay showed a significant reduction in the percentage of yeast adherence positive AMs in the BAP of smokers after exposure. In nonsmokers there was a tendency towards a decrease in phagocytosis positive AMs ($p=0.052$) (table 4).

Comparison of peripheral blood cells in smokers and nonsmokers before and after exposure

Before exposure there were significantly ($p<0.05$) higher numbers of peripheral blood lymphocytes in the smoking group compared to the nonsmoking group, $28.4\times10^9 \cdot l^{-1}$ (interquartile range 25.2–32.2 cells·l^{-1}) and $21.6\times10^9 \cdot l^{-1}$ (18.3–23.6 cells·l^{-1}), respectively. There was a tendency ($p=0.059$) towards a decrease in blood lymphocytes in

nonsmokers after exposure, $19.2\times10^9\cdot l^{-1}$ (interquartile range 16.6–21.3 cells$\cdot l^{-1}$), but not in smokers, $24.0\times10^9\cdot l^{-1}$ (22.3–26.2 cells$\cdot l^{-1}$). All other cells, including neutrophils and monocytes, were unchanged (data not shown).

Discussion

This study demonstrated that young smokers and lifetime nonsmokers differed to some extent in their bronchoalveolar reaction to single exposure to NO_2, at a concentration occurring in certain industries. The pre-existing smoking-induced AM dominated airway inflammation in smokers tended to increase together with an increase in neutrophils in the peripheral airways, whilst the lifetime nonsmokers showed an increase in lymphocyte numbers and a mild increase in neutrophils in the proximal airways. The latter information was a new finding made possible by using a technique which separated the recovered BALF into a proximal and peripheral portion.

Before exposure we found significantly higher total cell numbers in the lavage fluid from both proximal and peripheral airspaces (BP and BAP) in smokers, due to a higher total number of AMs in comparison with nonsmokers. This is in agreement with earlier studies, both in BALF [15, 29] and in histopathological analyses of lung tissue from smokers [30]. To our knowledge, the low percentage of macrophages stained positive for lysozyme in smokers compared with nonsmokers is a previously unreported finding. This is probably an effect of the ingestion of particles from the cigarette smoke, which may also be the reason for reduced phagocytic capacity in smokers [31].

In accordance with some previous studies, we did not find any difference in the total number of lymphocytes in smokers and nonsmokers [29, 32]. In contrast to the more long-term smokers in a previous study [29], the young smokers investigated here showed lower numbers of CD3+ in BALF compared to the nonsmokers. Low T-cell numbers due to smoking has also been reported in an animal study [33]. The total numbers of CD4+ cells were reduced in our smokers as shown previously [29]. The reduction in some lymphocyte subsets due to tobacco smoke may be of importance for immune reactions, an issue that is of current interest to be explored in depth.

No significant difference between the smoking and nonsmoking group was detected concerning the neutrophil numbers before exposure, which in other studies have been shown to be more prominent in smokers [29, 34]. The explanation is probably differences in study populations; our smokers were relatively young with relatively few pack-years and a mild airway inflammation.

After exposure to NO_2, the smokers showed an increase in total BAP cell numbers, mainly due to a further increase in AM numbers. Thus, the pre- existing AM dominated inflammation in the young smokers appeared to be enhanced after NO_2 exposure. The exposure caused a small but significant decrease of the adherence of yeast particles to AMs *in vitro*. There was no evidence that this was of importance for the phagocytic process, since the ingestion of particles was unaffected. Interestingly, in the nonsmoking group there was a clear trend towards decreasing phagocytosis in terms of a reduced percentage of AMs that had ingested yeast particles ($p=0.052$) after exposure to NO_2. This is in agreement with earlier animal studies [35, 36], but previously undetected in man.

Both in previous and the present studies, nonsmokers responded to NO_2 exposure with an increase in lymphocyte numbers in the peripheral portion of BALF (BAP). The use of flow cytometry in this study enabled us to differentiate the lymphocyte subsets and to demonstrate that the increase in lymphocyte numbers in the peripheral BALF from nonsmokers after NO_2 exposure is due mainly to an increase in CD3+ cells (T-cells), and to a lesser degree in CD16+ + CD56+ cells (NK-cells). This increase was associated with a tendency to a reduction in the blood lymphocytes in nonsmokers and could be consistent with a migration of blood lymphocytes into the lung after the NO_2 challenge.

An interesting finding was the lack of lymphocytosis in the smokers after NO_2 exposure. Thus, the smokers differed in their response to NO_2 from the nonsmokers, both concerning effects on the macrophage and lymphocyte populations.

The induction of protective mechanisms against oxidant stress in the lungs of tobacco smokers is probably one reason for the difference in cell response to NO_2 between the groups. Smokers have been shown to have a higher antioxidant activity in AMs obtained from BALF [23]. This activity is due to enhanced concentrations of superoxide dismutase (SOD) and catalase (CAT). Glutathione (GSH), which is another molecule capable of protecting cells against oxidants is also increased in smokers, both in BALF [24] and epithelial lining fluid [25].

From recent studies with repeated exposures to NO_2, we have gained a positive experience with the use of fractionated analysis of recovered BALF divided into proximal and peripheral portions [11, 12]. This has enabled us to detect different inflammatory cell responses in the proximal and peripheral air spaces. The current study is the first in which we have analysed the BALF with this approach following a single exposure to NO_2. Interestingly, the data obtained revealed a mild neutrophilic inflammation in the proximal airways in nonsmokers which may not have been identified in previous single exposure studies in which the BALF was not analysed in different portions [9, 10]. When all fluid was pooled, cellular changes in the relatively small portion from the proximal airways (BP) may have been unidentified, due to the considerably larger contribution of cells from the periphery (BAP). The finding of a proximal inflammation in the airways following NO_2 exposure is also of interest in view of the previously demonstrated main deposition of this water insoluble gas in the terminal bronchioli. Hence, we propose an extended use of fractionated analysis of BALF in evaluating cell responses in airways after exposure to inhaled air pollutants, since this method yields increased information compared to pooled BALF.

The smokers also responded to NO_2 with an increase in neutrophils, but surprisingly at a different airway level,

compared to the nonsmokers. Smokers did not have an increase in neutrophil numbers in the proximal airways, where the nonsmokers had elevated numbers. Instead, the smokers developed a significant neutrophil increase in the BAP. The differences in response could be due to the pre-existing conditions in the airways of the smokers. The fact that neutrophils are relatively rare in healthy subjects, and that few cells were counted in small groups of subjects could potentially have influenced the results. Therefore, additional studies will be needed to verify these findings.

In this study, we were unable to identify an increase in mast cell numbers, which has been a distinct pattern in preceding series of NO_2 exposure [9, 10]. The reason for this is not clear at present, but mast cells are very few in numbers, and together with the small number of subjects and interindividual differences the possibility of variable results may increase. Furthermore, the handling of the BALF has changed to analysis of fractionated portions together with more rapid and careful handling in the laboratory. The total number of mast cells before exposure was also higher than in the earlier studies. To what extent each of these circumstances contributed to the outcome is under investigation.

In summary, there are several differences in the bronchoalveolar response to NO_2 exposure in smokers and nonsmokers. This may by due to the already existing airway inflammation and compensatory mechanisms to oxidant stress in the smokers.

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