

## Effects of repeated exposure to 4 ppm nitrogen dioxide on bronchoalveolar lymphocyte subsets and macrophages in healthy men

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**ABSTRACT:** Following the basal descriptive studies of the bronchoalveolar inflammatory cell response induced by single exposure with nitrogen dioxide (NO<sub>2</sub>) in man, it was considered important to clarify the cell response to repeated exposure with NO<sub>2</sub>.

This investigation was, therefore, undertaken with bronchoalveolar lavage (BAL) 3 weeks before and 24 h after six repeated exposures with 4 ppm NO<sub>2</sub> (7 mg·m<sup>-3</sup>) in ten healthy volunteers. The exposures were performed during 20 min and every second day.

Analysis of the recovered BAL fluid demonstrated that repeated exposures to NO<sub>2</sub> caused a lung cell response different from that reported after a single exposure. Amounts of lavaged alveolar macrophages, B-cells, and natural killer (NK)-cells were decreased and the T-helper-inducer/cytotoxic-suppressor cell ratio was altered, but there was no lymphocytosis or mastocytosis as after single exposure. Lymphocyte numbers in peripheral blood were reduced after exposure.

These results suggest that repeated exposure with NO<sub>2</sub> adversely affects the immune defence. This could contribute to the increased susceptibility to airway infections reported to be associated with NO<sub>2</sub> exposure.

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The combination of bronchoalveolar lavage (BAL) and controlled exposure to nitrogen dioxide in healthy subjects has recently provided new insights into the response of the human lung caused by this common air pollutant. NO<sub>2</sub> exposure has been shown to cause an altered antiprotease activity, alpha<sub>2</sub>-macroglobulin increase in BAL fluid and impaired virus inactivation by human alveolar macrophages (AM) [1-3]. Significant acute inflammatory cell reactions, involving AM, lymphocytes and mast cells, have recently been reported following NO<sub>2</sub> exposure. These cell effects were observed in a dose-response study with 2.25-5.5 ppm NO<sub>2</sub> and a time-kinetic study with BAL 4-72 h after 4 ppm NO<sub>2</sub> [4, 5]. The cell data obtained in humans deviated considerably from animal studies, as recently reviewed [4], emphasizing the importance of obtaining data on the specific response in the human lung to air pollutants.

Following the basal descriptive studies on the bronchoalveolar inflammatory cell response after single exposure with NO<sub>2</sub>, we considered it important to determine the cell response in the human lung to repeated exposure to NO<sub>2</sub>. Since lymphocytes had previously been shown to be involved in the NO<sub>2</sub> induced acute inflammation and to play a central role in regulating

immune functions, we paid special attention to effects on these cells.

This investigation was, therefore, undertaken with BAL before and after six repeated exposures with 4 ppm NO<sub>2</sub> (7 mg·m<sup>-3</sup>) in healthy volunteers. The selected exposure concentration was based on indoor industrial environments, where workers may be exposed to peaks of this level a couple of times a week. We therefore chose to expose our subjects every second day. Six exposures were estimated to give a steady-state of inflammatory response, if any should be present.

### Subjects and methods

#### Subjects

Ten healthy, nonsmoking, male volunteers 23-37 yrs of age (mean 27 yrs) gave their informed consent to participate. All of them were free of airway infection within at least 6 weeks prior to the study and none had a history of asthma. Pre-exposure spirometry was normal in all subjects. The study was approved by the local Ethics Committee.



### Design of the study

Environmental chamber exposure with 4 ppm (7 mg NO<sub>2</sub>·m<sup>-3</sup>) for 20 min was performed according to a previously described protocol [4, 5]. Light work on a bicycle ergometer, 75 W, was performed during the last 15 min of the exposure. The exposure was repeated every second day up to a total of six exposures in each subject. Flexible fibreoptic bronchoscopy with BAL was performed 3 weeks or more before the exposure series in all subjects, in order to obtain reference BAL fluid. The post-exposure BAL was performed 24 h after the last NO<sub>2</sub> exposure. Each subject was, therefore, used as his own reference in calculations of changes in the BAL fluid content, 'after' exposure compared with 'before' exposure.

### Methods

The exposure chamber [6] and the nitrogen dioxide exposure technique [4, 5] have previously been described in detail. Flexible fibreoptic bronchoscopy with BAL was performed in the right middle lobe during local anaesthesia with lidocaine, according to a special scheme. The subjects received 0.5–0.75 mg atropine *s.c.*, but no sedative, before the investigation. The BAL and processing of the BAL fluid and the staining of cells was performed as outlined in detail previously [7], with a few additions. In this study the first recovered 20 ml of the first instilled aliquot of 60 ml phosphate-buffered saline (PBS) was analysed separately and defined as the bronchial portion (BP). The remaining recovered fluid of the totally instilled 4×60 ml was defined as the bronchoalveolar portion (BAP).

Cell differential counts, total protein, albumin and β<sub>2</sub>-microglobulin concentrations were determined in both BP and BAP. Flow cytometry was solely performed on the BAP since the volume of the BP was too small to fully allow for this processing.

Lymphocyte subsets were determined with flow cytometry (Becton-Dickenson, Facscan, Stockholm, Sweden) using the means of double readings of each sample. The following antibodies were used; T-cells (Leu 4), CD4+ T-helper/inducer cells (Leu 3a), CD8+ T-cytotoxic/suppressor cells (Leu 2a), natural killer (NK)-cells (Leu 11 and 19) and B-cells (Leu 16) (Becton-Dickenson, Stockholm, Sweden). Between 3–5,000 lymphocytes were sampled each time.

AM phagocytosis was measured as percentage of yeast particle engulfment positive cells, using a glass surface adherence method as described previously [8]. The method has been modified to enable the use of bronchoalveolar lavage 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 non-adherent cells away, yeast cells labelled with fluorescein isothiocyanate (FITC) and opsonized with human serum were added to the slides (2.5×10<sup>7</sup> 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 ethylenediaminetetraacetic acid (EDTA). The fluorescence of non-ingested yeast cells was quenched by dripping toluidine blue in saline (1 mg·ml<sup>-1</sup>, 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. At least 100 macrophages were counted.

Fibronectin was analysed with a double-sandwich enzyme-linked immunosorbent assay (ELISA) technique developed in our laboratory. Microtitre plates (Nunc, Denmark) were coated with rabbit-antihuman fibronectin antibodies (Dakopatts, Denmark), diluted 1:2,000 in carbonate buffer pH 9.6. After incubation at room temperature for 24 h, the titre plates were carefully washed. Phosphate-buffered saline (PBS) containing 0.05% albumin was added for 1 h and then washed away. BAL fluid sample was added in a diluted series together with horseradish peroxidase-labelled antihuman fibronectin 1:2,000 (Dakopatts) as second antibody and the plates were incubated for 90 min. The amount of bound peroxidase, which is proportional to the amount of fibronectin in the sample, was measured by analysing the enzymatic activity on orthophenylenediamine. Plasma fibronectin of nephelometric quality was provided from Sigma Chemicals and used as standard. The detection limit was 10 μg·l<sup>-1</sup>. Intra- and interassay variation was below 7%.

Albumin was measured with a nephelometric method, Beckman array protein system (Beckman Instruments Inc., Brea, Ca, USA) at the Dept of Clinical Chemistry, Umeå, Sweden. The total intra- and interassay variability was 3.3% and the detection limit 6 mg·l<sup>-1</sup>.

Peripheral blood samples were drawn before the first exposure and immediately after the last. Analyses of the total number of white blood cells were performed at the Dept of Clinical Chemistry, University Hospital of Umeå, according to standard hospital routine.

### Statistics

Wilcoxon's non-parametric signed rank test for paired observations was used. A *p* value <0.05 was considered significant.

### Results

The recovery of the bronchoalveolar portion (BAP) from the reference BAL was median 140 ml (interquartile range 115–150 ml) and in the post-exposure BAL median 150 ml (130–160 ml). Neutrophils were not significantly affected after exposure (mean and interquartile range: pre-exposure BP 1.5% (0.5–3.5%), BAP 1% (1–1%) and post-exposure BP 2% (1–5%), BAP 0% (0–1%). Eosinophil counts were negligible.



Table 1. - Cell counts in the bronchial and bronchoalveolar portions of lavage fluid before and after repeated exposure to NO<sub>2</sub>

	All cells ×10 <sup>7</sup> ·l <sup>-1</sup>	Macrophages ×10 <sup>7</sup> ·l <sup>-1</sup>	%	Lys+ AM % of AM	Lymphocytes ×10 <sup>7</sup> ·l <sup>-1</sup>	%	Mast cells ×10 <sup>7</sup> ·l <sup>-1</sup>	%
<b>Bronchial portion (BP)</b>								
Before	23.8	20.5	90	10	2.0	8	0.25	0.63
exposure	19.0-28.5	15.3-27.3	84-92	8-10	1.1-2.8	6-11	0.09-0.26	0.38-1.25
After	22.9	18.9	88	10	1.9	9	0.06	0.25
exposure	17.0-31.2	14.6-25.4	82-91	6-13	0.8-4.1	6-15	0.00-0.17	0.13-0.25
p*	NS	NS	NS	NS	NS	NS	<0.05	<0.05
<b>Bronchoalveolar portion (BAP)</b>								
Before	11.7	10.8	92	10	0.8	7	0.06	0.50
exposure	9.4-19.5	9.4-13.2	86-94	5-10	0.6-2.2	6-14	0.04-0.10	0.25-0.75
After	10.0	8.3	88	12	0.8	8	0.03	0.25
exposure	7.0-13.1	6.7-10.8	82-91	10-14	0.5-1.4	7-13	0.00-0.04	0.13-0.40
p*	<0.02	<0.02	NS	<0.05	NS	NS	NS	NS

The data are presented as median values with upper and lower quartiles. \*: Wilcoxon's paired rank sum test; ns: nonsignificant; Lys+ AM: lysozyme positive alveolar macrophages.

Table 2. - Lymphocyte subsets in the bronchoalveolar portion (BAP) of recovered lavage fluid

	All T-cells ×10 <sup>7</sup> ·l <sup>-1</sup>	T-helper %	T-suppressor %	T-helper/suppressor Ratio	B-cells ×10 <sup>7</sup> ·l <sup>-1</sup>	NK cells ×10 <sup>7</sup> ·l <sup>-1</sup>
Before	0.61	49	19	2.3	0.02	0.04
exposure	0.40-2.98	41-54	18-26	2.1-2.8	0.01-0.14	0.02-0.06
After	0.72	51	17	2.8	0.00	0.02
exposure	0.37-1.70	46-60	15-20	2.4-4.0	0.00-0.001	0.01-0.03
p*	NS	NS	<0.05	<0.01	<0.05	<0.05

The data presented are for 'before' and 'after' repeated exposure to NO<sub>2</sub> and given as median values with upper and lower quartiles. \*: Wilcoxon's paired rank sum test. ns: nonsignificant; NK cells: natural killer cells.

The total cell number in the BP was unaffected but the total cell number in the BAP was significantly reduced after exposure ( $p < 0.02$ ), mainly due to a decrease in AM ( $p < 0.02$ , table 1). The decrease in the subpopulation of AM in the BAP that stained positive for intracellular lysozyme was less pronounced than the decrease in the total number of AM. As a consequence, the percentage of lysozyme positive AM, of all AM, was elevated in the BAP after exposure ( $p < 0.05$ , table 1).

The total number and percentage of mast cells was significantly reduced in the BP ( $p < 0.05$ , table 1). A possible tendency to reduction in the total number of mast cells was found in the BAP ( $p = 0.08$ ).

The total number and percentage of lymphocytes and the total number of Leu 4+ cells (T-cells) were not significantly affected in the BAP (tables 1 and 2). The proportion of Leu 2a+ (T-cytotoxic-suppressor) cells was modestly reduced in all subjects after exposure ( $p < 0.05$ , table 2), whilst the proportion of Leu 3a+ (T-helper-inducer) cells was relatively unchanged. Consequently the ratio of the percentages of Leu 3a+/Leu 2a+ cells was significantly increased in all eight subjects ( $p < 0.01$ ).

The total number of Leu 16+ cells (B-cells) and the proportion of Leu 16+ cells of all lymphocytes, were both significantly reduced after exposure ( $p < 0.05$ , respectively). The total number of Leu 11+ and Leu 19+ cells (NK-cells) was similarly reduced ( $p < 0.05$ ) but the proportion was unaffected.

Table 3. - Phagocytosis of yeast particles *in vitro* by alveolar macrophage obtained from the bronchoalveolar portion (BAP) of recovered lavage fluid

	Total phagocytosis of yeast particles n	Engulfment positive cells %	Adherence positive cells %
Before	289	92	3
exposure	254-312	91-93	1-5
After	336	95	1
exposure	333-360	93-96	1-4
p*	<0.01	NS	NS

The data presented are for 'before' and 'after' repeated exposure to NO<sub>2</sub> and given as median values with upper and lower quartiles. \*: Wilcoxon's paired rank sum test. ns: nonsignificant.

The total number of lymphocytes in the peripheral blood was significantly reduced after the exposure series ( $p < 0.02$ ). Before exposure there were  $1.94 \times 10^4$  cells·ml<sup>-1</sup> (interquartile range  $1.90-2.24 \times 10^4$ ) and 24 h after end of the exposure series  $1.62 \times 10^4$  cells·ml<sup>-1</sup> ( $1.47-2.11 \times 10^4$ ).

The proportion of AM that had engulfed yeast particles *in vitro* was not significantly changed after exposure but the total number of engulfed yeast particles within the AMs was increased after exposure ( $p < 0.01$ ) (table 3).



The lavage concentrations of fibronectin,  $\beta_2$ -microglobulin and albumin were all unaffected in the BP and BAP after exposure.

### Discussion

This study describes the first data on bronchoalveolar cell effects in man caused by repeated exposure to NO<sub>2</sub>. The repeated exposures were found to cause bronchoalveolar cell reactions that were clearly different compared with single exposures [4, 5]. The previously described acute bronchoalveolar lymphocytosis and mastocytosis had totally disappeared after six repeated exposures conducted every second day. The apparent differences in inflammatory cell response in BAL fluid between single and repeated exposure to NO<sub>2</sub> most probably depend on compensatory mechanisms [9]. The fact that there is no persisting lymphocytosis and mastocytosis after repeated exposure cannot, however, be taken as a definite indication that repetitive exposure to NO<sub>2</sub> is harmless. The data implicate some small, but potentially important, effects of lymphocyte populations.

We found a significant reduction in the total number of lymphocytes in peripheral blood. This is in agreement with the adverse effects on lymphocytes of NO<sub>2</sub> exposure that have been demonstrated in several animal studies [10-14].

Of special interest are the small but statistically significant changes found in the bronchoalveolar lymphocyte subsets. The NO<sub>2</sub> exposure caused a decrease in the percentage of T-cytotoxic-suppressor (Leu 2a+) cells and a consequent shift of the ratio of T-helper-inducer/T-cytotoxic-suppressor (Leu 3a+/Leu 2a+) cells, which was elevated. These results may correspond with earlier animal studies, showing that T-cytotoxic-suppressor cells are the most sensitive cells to NO<sub>2</sub> exposure among the T-lymphocytes subsets [13, 15]. The findings should be considered with regard to the central role T-lymphocytes play in regulating immunological functions, e.g. mediating delayed hypersensitivity, regulating immunoglobulin production, and lysing virus-infected and neoplastic cells.

We also found a significant decrease in the number of B-cells which is in agreement with studies of NO<sub>2</sub> exposed mice, that showed humoral immunodepression [15] and depressed antibody response [16]. The reduction in NK cell (Leu 11+ and 19+) numbers does not appear to have been reported in the literature. To our knowledge, NK cell function after NO<sub>2</sub> exposure has not previously been studied. It should be stressed that the effects detected in the present material on B-cell and NK cell numbers should be considered together with the fact that these cells are relatively scarce in BAL fluid. This makes definite conclusions regarding altered numbers of those cells precarious and the results need to be confirmed in complementary studies.

The above mentioned effects on peripheral blood and BAL lymphocytes indicate that repeated exposure to NO<sub>2</sub> in humans causes adverse effects on the

immune system. These effects on the cellular and humoral immune system could well be connected with the previously described increased susceptibility to virus infections by NO<sub>2</sub> exposure [17, 18]. The susceptibility to virus infection, has also been postulated to be associated with effects on AM and phagocytosis [3, 19, 20]. In the present study, as well as in a study with single exposure with NO<sub>2</sub> [4, 5] we have been unable to confirm any decrease in AM phagocytosis *in vitro*. It should be emphasized that *in vitro* models may not completely represent the effects of AM *in situ*, since the adherence to the glass plate itself causes activation. We did detect some effects on AM by the repeated NO<sub>2</sub> exposure, in terms of reduced total number of recovered AM in the bronchoalveolar portion. This probably reflects the earlier activation of AM by the exposure, causing them to be more adhesive to the walls of the airspaces, but other explanations are also conceivable.

The cause of the reduction of mast cells in the bronchial portion of BAL fluid after the series of repeated exposures, compared with before exposure, is unclear. The implication of the mast cell in the acute pulmonary response to NO<sub>2</sub> has previously been established in a pharmacological study [21] and two BAL studies in man [4, 5], as well as in a morphological study in rodents [22]. In the present studies with repeated exposures, we have slightly enhanced the handling of mast cells, yielding somewhat enhanced mast cell numbers. This explains neither the absence of mast cell increase in this study, nor the significant decrease in mast cells that the data *de facto* indicate. This could be due to migration of mast cells into the interstitium or degranulation. Since mast cells are identified due to their metachromatic staining properties they appear "invisible" when degranulated [23]. The reason for the low mast cell numbers after exposure cannot be identified from present material. Since mast cells are relatively scarce, and few cells are counted, it should be kept in mind that there is a greater risk of chance effects.

It is concluded that repeated NO<sub>2</sub> exposure with 4 ppm for 20 min every second day, six times, in healthy volunteers causes small but potentially unfavourable effects on the bronchoalveolar lymphocyte population that may be of importance for the immune defence. AM phagocytosis *in vitro* was not found to be significantly affected, but the total number of AM recovered from the bronchoalveolar spaces with lavage was reduced. Complementary studies on the effects of NO<sub>2</sub> exposure on the immune defence in man are under progress.

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