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ORIGINAL ARTICLE

Ambient level of NO_2 augments the inflammatory response to inhaled allergen in asthmatics

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Abstract Air pollution constitutes an important factor for asthma aggravation, and there is increased concern about respiratory health effects of common air pollutants. The purpose of this study was to examine how exposure to a high ambient concentration nitrogen dioxide (NO_2) prior to a bronchial allergen challenge modulated the inflammatory response in the bronchi. Thirteen subjects with mild asthma and allergy were exposed at rest to either purified air or 500 $\mu g \times m^{-3}$ NO_2 for 30 min, followed 4 h later by an allergen inhalation challenge. The exposures (NO_2 or air) were performed in random order and at least 4 weeks apart. Lung function during NO_2/air exposure and allergen challenge was measured by plethysmography, and then hourly by portable spirometry after exposures. Subjective symptoms were recorded during and after exposure. Bronchoscopy with bronchial wash (BW) and bronchoalveolar lavage (BAL) was performed 19 h after allergen challenge. NO_2+allergen enhanced the percentage of neutrophils in both BW and BAL compared to air+allergen (BW 19 *vs.* 11, $P=0.05$; BAL 3 *vs.* 1, $P=0.02$ median values). The levels of eosinophil cationic protein (ECP) in BW was higher after NO_2+allergen compared to air+allergen (9.0 *vs.* 3.6 $\mu g/l$; $P=0.02$, median values). There was no NO_2-associated effect on symptoms or pulmonary function. These data suggest that ambient NO_2 can enhance allergic inflammatory reaction in the airways without causing symptoms or pulmonary dysfunction.

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Keywords asthma; allergy; bronchial responsiveness; air pollution; nitrogen dioxide (NO_2); lung function; inflammatory markers.

INTRODUCTION

Nitrogen dioxide (NO_2), a gas produced by combustion, is a common environmental air pollutant. The main outdoor sources are vehicular traffic, and indoor sources are gas appliances.

Individuals with asthma are more sensitive to NO_2 exposure than healthy subjects, according to results from controlled human-exposure studies. Short exposures to high ambient levels of NO_2 can increase exercise-induced bronchospasm (1) and the bronchial responsiveness to inhaled nonspecific agents in asthmatics (2). The main effect of NO_2 in these studies was on bronchial responsiveness, with pulmonary function not being affected at those NO_2 doses.

NO_2 can also enhance the asthmatic response to inhaled allergen (3,4). This amplifying effect on the asthmatic response has also been reported after repeated exposure to NO_2 in combination with low, non symptom-causing doses of allergen (5), suggesting that NO_2 may exert this effect commonly on asthma patients.

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The mechanisms for NO_2's enhancing effect on the asthmatic reaction to allergen appear to be related to an increased inflammatory reaction in the bronchi. The extent to which inflammatory cells and mediators are linked to this response is not well understood. Exposure to high doses of NO_2 alone (3000–7000 mg $\times$ m^{-3}), which occurs in certain industries and mines, has been shown to increase the number of neutrophils in bronchial lavage fluid in healthy subjects the first hours after exposure (6–8), while exposure to ambient levels of NO_2 ($<$1000 $\mu g\, m^{-3}$) does not seem to affect cell numbers in bronchial lavage fluid in humans (6).

Bronchial allergen challenge, on the other hand, gives an inflammatory response which is characterized in bronchoalvedar lavage (BAL) fluid by eosinophilia, which is noted as soon as 2–4 h after challenge and persisting up to 24 h post-challenge (9). Occasionally, neutrophilia occurs in the bronchi some hours after allergen challenge, but this usually subsides with normal values noted 24 h later (9).

These results suggest that the neutrophil and eosinophil constitute the prime effector cells in NO_2 and allergen-induced airway inflammatory reaction. However, to date there has been no experimental examination of the impact of a combination of NO_2 and allergen on the inflammatory response in the bronchi, though combination NO_2 and allergen exposure is likely to occur in asthma patients.

We hypothesized that NO_2 would enhance the airway inflammatory response of eosinophil and neutrophil granulocytes and mast cells to allergen inhalation.

We aimed to test this hypothesis in an exposure study, with short-term exposure to NO_2 and a subsequent allergen challenge.

METHODS

Subjects (Table I)

Thirteen adult subjects with mild asthma and allergy to birch or timothy pollen participated in the study. There were six men and seven women; the mean age was 29 years in the age range 23–39 years. All were nonsmokers.

At a pre-inclusion visit at least 4 weeks before the study started, a seasonal allergy to either birch or timothy pollen was confirmed by positive skin prick test ($>$3 mm). The diagnosis of extrinsic asthma was based on a typical history of attacks of dyspnoea during the pollen season and a positive bronchial challenge with the relevant allergen. Six subjects had a late phase reaction defined as =15% decline in forced expiratory volume

Table I. Anthropometric and clinical data

Subject	Sex	Age (year)	Smoking	Asthma duration (year)	Allergen inhaled	FEV_1 at inclusion (l)	FEV_1 at inclusion (% pred)	Skin prick test* (mm)	Histamine $PDSR_{aw}$ 100%† (SQ units)	Allergen $PDSR_{aw}$ 100%‡ (SQ units)	Allergen dose§ (SQ units)
1	M	39	E	25	B	3.75	86	6.5	129	6654	1980
2	F	23	N	6	T	3.16	85	7.5	172	1474	660
3	M	30	E	4	T	4.08	106	4.5	530	1097	440
4	F	23	E	3	B	3.61	119	7	478	2194	880
5	M	30	N	26	B	4.12	85	10.5	402	231	96
6	F	25	N	20	T	3.87	100	9	521	23	10
7	M	33	N	22	B	3.91	87	8	128	326	138
8	M	27	N	25	B	4.5	103	12	109	34	14
9	F	25	N	22	T	3.41	97	8.5	100	39	17
10	M	32	E	12	B	4.2	103	14	284	256	110
11	F	25	N	8	T	3.03	88	12	803	423	220
12	F	26	N	1	T	3.09	91	8	326	55	19
13	F	27	N	8	T	4	109	14	699	163	69
Mean		28		14		3.75	97				
Std		5		10		0.46	11				
Median								9	326	256	110
25th percentile									129	47	18
75th percentile									526	1286	550

Note: F=female, M=male; N=never smoker, E=ex smoker; T=timothy, B=birch. *Skin prick test $<$3 mm=positive reaction.
†Provocative dose of histamine causing a 100% increase in specific airway resistance.
‡Provocative dose of allergen causing a 100% increase in specific airway resistance.
§Inhaled allergen dose after NO_2/air.

in 1 s (FEV_1) 3–10 h after the allergen challenge. Airway hyperresponsiveness was also confirmed by a positive histamine inhalation test. Upon inclusion, the lung function expressed as FEV_1 was $97 \pm 11\%$ of predicted (mean $\pm$ SD; range 85–119%). All subjects were treated with inhaled β_2-agonist as needed, and 12 subjects were treated with inhaled steroids only during the pollen season, though none during the study period.

All subjects had a normal serum CRP-value on the first exposure day as an indication of no ongoing bacterial infection.

The study was approved by the Ethics Committee at Huddinge University Hospital.

Study design

All subjects were exposed for 30 min at rest in an exposure chamber to filtered air or to a concentration of $500\ \mu g \times m^{-3}$ (260 ppb) NO_2 on two separate occasions at least 4 weeks apart. The order of exposure was randomized (7 first NO_2, 6 first air). Four hours after exposure, a bronchial challenge with an individually fixed dose of birch or timothy pollen was performed. The time interval of 4 h was based on an established protocol from previous studies (4). The inhaled allergen dose was 40% of the dose estimated to cause 100% increase in specific airway resistance at the screening visit, based on a previous study with NO_2 and allergen from our laboratory (5). Bronchoscopy with bronchial wash (BW) and bronchoalveolar lavage (BAL) was performed 19 h after the allergen challenge.

Each experiment had the following design: after arrival to the laboratory in the morning, the subject rested for 15 min. Specific airway resistance (SR_{aw}) and thoracic gas volume (TGV) were measured with a whole-body plethysmograph, and FEV_1 with a portable spirometer. The subject then rested 10 min more before entering the exposure chamber. During the 30 min of exposure to air/NO_2, lung function (SR_{aw}, TGV) was measured after 4, 15, and 30 min of exposure, and an interview concerning symptoms was conducted after 3 and 26 min according to a standardized questionnaire.

After exposure, FEV_1 was measured hourly using a portable spirometer. Before and after the allergen inhalation, lung function was again measured by plethysmography. After the allergen inhalation, the subject went home and continued to measure FEV_1 hourly until 10 p.m.

The subjects were instructed to use an inhaled bronchodilator if necessary and to keep a record of symptoms and medication.

The exposure types were blinded to the subjects and the investigators analysing the bronchial lavage fluid and the results from the portable computerized spirometer.

Whole-body plethysmography

Airway resistance (SR_{aw}) and TGV were measured in a constant volume body plethysmograph (Model 2000 TB; Cardio-pulmonary Instruments, Houston, TX, U.S.A.) according to the method of Du Bois and colleagues (10,11).

The gas flow/box-pressure slopes were measured between gas flow +0.5 and $-0.5\ l \times s^{-1}$ (expiration–inspiration) as a mean of two or three measured slopes. The mouth/box-pressure slopes were measured between the endpoints, again as the mean of two to three curves.

Spirometry

The measurements of FEV_1 were made using a portable computerized spirometer (Diary Card spirometer, Micromedical Ltd. Chatham, Kent, U.K.) and the data later downloaded to a computer.

The maximal fall in FEV_1 from before allergen challenge to 3–10 h after allergen challenge, as well as the average fall in FEV_1 during this period, was used to measure the asthmatic reaction during the late phase.

Results for FEV_1 are provided in litres, and statistical evaluation of effects are normalized to each individual's best FEV_1.

Histamine bronchial challenge

Histamine provocation tests were performed by using an automatic inhalation-synchronized dosimeter jet nebulizer (Spira Elektro 2; Respiratory Care Center, Hameenlinna, Finland) with an adjustable aerosol delivery time according to a previously described method (5,12). SR_{aw} and TGV were measured 3 min after each dose. The provocative dose ($PDSR_{aw\ 100\%}$) was calculated by linear interpolation on a logarithmic scale.

Allergen bronchial challenge

The allergen challenge at the screening visit and during the study was performed with the same dosimeter jet nebulizer (Spira Elektro 2) as for histamine inhalation. Standardized and freeze-dried birch or timothy allergen extracts (Aquagen, Alk, Copenhagen, Denmark) were diluted and used at a maximum of four concentrations: 1000, 4000, 16 000 and 64 000 (Standardized Quality) SQ allergen units ml^{-1}. The nebulizer was set to nebulize for 0.5 s giving an output of 7.1 $\mu l\ breath^{-1}$. At each concentration, two and four breaths could be taken, and if needed even followed by eight and 16 breaths at the highest concentration providing doses from 14 to 7040 SQ allergen units. SR_{aw} and TGV were measured 15 min after each dose of allergen. The challenge proceeded until a 100% increase in SR_{aw} was reached. $PDSR_{aw\ 100\%}$ for

allergen was calculated by linear interpolation on a logarithmic scale.

The inhaled allergen dose in the study was set to 40% of the $PDSR_{aw\ 100\%}$ at the screening visit, or, when this was impossible to administer for practical reasons, the dose was as above (see Table 1). FEV_1 and SR_{aw} were recorded immediately before and 15 min after the single dose of allergen was inhaled.

Blood samples

Blood samples were obtained on two occasions; 1 day prior to exposure for CRP analyses and 1 day prior to BAL for analysis of albumin.

Questionnaire

After 3 and 26 min of exposure in the chamber, the subjects were asked 16 questions concerning respiratory symptoms and perceptions of discomfort (i.e. tight chest, cough, headache, odour), reporting symptoms on a scale of 0 (none)–7 (maximal).

Throughout the day, and then on the following morning, the subjects kept a self-administered daily record of bronchodilator medication and symptoms from bronchi, nose, and eyes.

Gas dilution and exposure system

NO_2 from a gas bottle (Alfax, approximately 8.000 mg $\times$ m^{-3} NO_2) was diluted in two steps to a final concentration of approximately 500 mg m^{-3} NO_2 and then was fed into the exposure chamber (volume 7 m^{-3}). The gas dilution and exposure system is presented in detail elsewhere (13).

Chemical analyses

NO_2 concentrations in the exposure chamber were measured with a chemiluminescence instrument (Model 8440 Nitrogen Oxides Analyser; Monitor Laboratories, Englewood, CO, U.S.A.). For calibration, an NO_2 permeation tube and NO calibration gas (Model 8500 Calibrator; Monitor Laboratories, Englewood, CO, U.S.A.; AGA Special gas, 100 ppm NO) was used. A calibration procedure was performed daily.

The subjects' individual exposure to NO_2 outside the chamber during the study days was measured with a personal passive (filter badge) sampler (Toyo Roshi Kaisha, Ltd, Tokyo, Japan) (14).

Exposure data

NO_2 concentrations in the exposure chamber were measured in the breathing zone of the subject. The concentration was 493 ± 10; 468–505 $\mu g \times m^{-3}$ (average of mean concentrations during 30 min $\pm$ SD; range). During exposure to filtered air, the NO_2 concentration was below 10 $\mu g \times m^{-3}$. The temperature in the exposure chamber was $24.0 \pm 0.8^{\circ}C$ (mean $\pm$ SD) during air and $24.4 \pm 0.8^{\circ}C$ during NO_2 exposure. The corresponding values for relative humidity were $39 \pm 12\%$ ($n=11$) and $36 \pm 8\%$ ($n=12$). The exposure to NO_2 in ambient air outside the chamber measured with the personal sampler was 18 ± 9 $\mu g \times m^{-3}$ (23 h mean $\pm$ SD, 9 a.m. day 1–8 a.m. day 2) during the NO_2 exposure and 25 ± 11 $\mu g \times m^{-3}$ during the air exposure, which was significantly different ($P=0.046$).

Bronchoscopy and bronchoalveolar lavage

All bronchoscopies were done between 8 and 9 a.m. by the same bronchoscopist (CB). VC and FEV_1 were measured before the procedure. Fibreoptic bronchoscopy was performed after premedication with morphine hydrochloride and scopolamine hydrobromide (Morfinscopolamin® Pharmacia, Uppsala, Sweden). Fifteen minutes before the bronchoscopy, all subjects inhaled 0.4 mg salbutamol. Local anaesthesia was administered before and during the procedure with lidocaine (Xylocain®, Astra, Södertälje, Sweden). The Olympus BI-IT 20 (Olympus Optical Company, Tokyo, Japan) bronchoscope was introduced through the nose or the mouth into the lung, and wedged in a subsegmental bronchus of the middle lobe. Lavage was performed using one aliquot of 20 ml, and three aliquots of 50 ml sterile warm 0.9% NaCl, instilled into the middle lobe subsegment. The fluid was gently aspirated after each aliquot and collected in propylene tubes. The first 20 ml that was instilled, the BW, was collected separately from the remaining 150 ml, the BAL.

Sample processing

The lavage fluid was filtered through a Dacron net (Millipore, Cork, Ireland). Cells were pelleted by centrifugation at 4°C, 400 g for 10 min, and the supernatants were stored at −70°C after an additional centrifugation at 4°C, 600 g for 10 min.

The cell pellets were resuspended in Hepes buffer (RPMI). The total number of cells was counted in a Bürker chamber, and their viability was tested by the exclusion of trypane blue. Slides for differential counts were prepared by cytocentrifugation at 500 rpm for 3 min (Cytospin 3 Shandon, Southern Products Ltd., and Runcorn, England). Slides were stained with May-Grünwald Giemsa, and 500 cells were counted. Mast cells were stained with acid toluidine blue and counterstained with Mayer's acid haematoxylin.

Analyses of soluble mediators

Analyses of soluble mediators were performed in cell free BW and BAL fluids .The fluids were distributed in portions and stored at $-70^{\circ}C$ until analysed.

ECP levels were measured with a fluoroimmunoassay, Pharmacia ECP Cap System FEIA, (Pharmacia & Upjohn, Uppsala, Sweden), with a detection limit $<2\,\mu g/l$.

MPO levels were measured with a competitive RIA (Pharmacia & Upjohn), which had a detection limit of $<8\,\mu g\,ml^{-1}$. In this assay, MPO competes with a fixed amount of ^{125}I labelled MPO for the binding sites of specific antibodies.

The levels of human IL-5, IL-8, eotaxin, and soluble-ICAM-1 were measured by ELISA technique using Quantikine immunoassays (R&D Systems, Inc, Minneapolis, MN, U.S.A.). According to the manufacturer, the minimum detectable concentrations for substances were IL-5 3.0 pg/ml, eotaxin 5.0 pg ml^{-1}, IL-8 10 pg ml^{-1}, and s-ICAM-1 0.35 ng ml^{-1}.

Statistics

Data obtained for NO_2 and filtered air were analysed for differences using the nonparametric Wilcoxon signed rank test.

The statistical program used was SPSS for Power Macintosh (SPSS Inc.Chicago, IL, U.S.A.). Probability values of $<5\%$ were considered significant.

Pair-wise correlations of delta values were calculated for r values (Pearsons correlation coefficient) with JMP® version 3.2.2 statistical software from SAS Institute Inc.

RESULTS

Specific airway resistance (SR_{aw}), and thoracic gas volume (TGV) (Table 2)

SR_{aw} was significantly higher at baseline on the NO_2 exposure day [4.0 $cmH_2O\,s^{-1}$ (3.04–6.02) NO_2 *vs*. 3.69 (2.88–4.89) air, $P=0.04$].

There were no significant differences in SR_{aw} or TGV between air and NO_2 during exposure in the chamber or immediately after bronchial allergen challenge.

Forced expiratory volume in 1 s (FEV_1) (Table 3)

FEV_1 in litres before NO_2 and control exposure were similar (3.68 ± 0.45 and 3.65 ± 0.48 mean ± SD, respectively) and before allergen provocation (3.67 ± 0.44 and 3.67 ± 0.48, respectively). The allergen provocation induced a small but (for group averages) statistically significant decrease in FEV_1 during the early phase of 4% (0.15 ± 0.19; $P=0.011$) in the NO_2 group and 5% (0.20 ± 0.32; $P=0.013$) in the control. FEV_1, averaged over the 3–10 h after allergen challenge, was 7% (0.26 ± 0.28; $P=0.006$) for the NO_2 group and 8% (0.28 ± 0.33; $P=0.009$) for the control group lower than immediately before allergen challenge. The maximal fall during late phase was 13% (0.49 ± 0.43; $P=0.002$) for the NO_2 group and 15% (0.55 ± 0.51; $P=0.004$) for the control group. There were no tendencies towards differences in effect between NO_2+allergen and control+allergen exposure during the early or late phase ($P>0.42$). Six in the air+allergen group and five in the NO_2+allergen group had a late FEV_1 reaction greater than 15%.

Subjective complaints

When the subjects were interviewed during exposure concerning subjective complaints, no difference between air and NO_2 exposure could be detected except for a slight increase in sense of anxiety and odour after 4 min of exposure to NO_2 [mean 1.1 *vs*. 2.1, $P=0.03$ (anxiety), and 1.4 *vs*. 2.1, $P=0.05$ (odour) on a scale in the range 1–7]. These effects had vanished after 28 min of exposure.

After exposures, subjective symptoms and medication did not differ significantly between air+allergen and NO_2+allergen.

Table 2. SR_{aw} and TGV

	Air	NO_2	*P*-value*
SR_{aw} ($cmH_2O\,s^{-1}$)			
Before expo	3.69 (2.88–4.89)	4.00 (3.04–6.02)	0.04
After expo	3.85 (2.83–5.82)	4.32 (2.89–5.52)	n.s.
Before allergen	5.33 (4.74–5.84)	4.92 (4.45–5.69)	n.s.
After allergen	6.50 (4.91–8.48)	6.23 (5.58–8.85)	n.s.
TGV (l)			
Before expo	3.28 (2.88–3.54)	3.52 (2.96–3.89)	n.s.
After expo	3.23 (2.87–3.71)	3.53 (2.91–4.09)	n.s.
Before allergen	3.30 (2.87–3.56)	3.36 (2.93–3.87)	n.s.
After allergen	3.59 (2.84–4.22)	3.36 (3.02–4.03)	n.s.

Data presented as median and interquartile range, $n=13$. *Wilcoxon's nonparametric signed rank test; n.s.=nonsignificant.

Table 3. Air/allergen *vs.* NO_2 /allergen (FEV_1*)

Subject	Air/allergen								NO_2/allergen							
	Pre-expo	Pre-allergen	Early† phase	Early phase % change	Late phase‡ (mean)	Late phase (mean) % change	Late phase (min)	Late phase (min) % change	Pre-expo	Pre-allergen	Early* phase	Early phase% change	Late phase† (mean)	Late phase (mean) % change	Late phase (min)	Late phase (min) % change
1	3.96	3.91	3.50	−10	3.97	2	3.91	0	3.82	3.68	3.85	5	3.86	5	3.71	1
2	3.10	3.21	3.03	−6	2.73	−15	2.37	−26	3.16	3.12	3.03	−3	3.00	−4	2.87	−8
3	4.11	4.15	3.82	−8	3.85	−7	3.75	−10	4.14	4.12	3.93	−5	3.71	−10	3.47	−16
4	3.54	3.64	2.46	−32	2.58	−29	2.11	−42	3.50	3.41	3.03	−11	2.73	−20	2.16	−37
5	4.13	4.12	4.15	1	3.62	−12	3.25	−21	3.91	3.95	3.91	−1	3.48	−12	2.95	−25
6	3.67	3.78	3.66	−3	3.67	−3	3.58	−5	3.87	3.92	3.71	−5	3.29	−16	3.12	−20
7	3.43	3.41	3.36	−1	2.82	−17	2.54	−26	3.77	3.85	3.66	−5	3.15	−18	2.66	−31
8	4.38	4.43	4.37	−1	4.28	−3	4.23	−5	4.41	4.40	4.43	1	4.38	−,45	4.34	−1
9	3.28	3.36	3.18	−5	3.22	−4	3.11	−7	3.31	3.28	3.18	−3	3.23	−2	3.14	−4
10	3.97	3.85	3.71	−4	3.67	−5	3.12	−19	3.98	3.92	3.76	−4	3.79	−3	3.53	−10
11	2.91	2.84	2.98	5	2.93	3	2.76	−3	2.91	2.98	2.90	−3	2.81	−6	2.71	−9
12	3.01	3.00	2.98	−1	3.10	3	3.09	3	3.07	3.12	2.50	−20	2.98	−4	2.92	−6
13	3.97	4.06	3.98	−2	3.66	−10	2.76	−32	3.99	3.98	3.95	−1	3.92	−2	3.79	−5
Mean	3.65	3.67	3.47	−5	3.39	−8	3.12	−15	3.68	3.67	3.53	−4	3.41	−7	3.18	−13
SD	0.48	0.48	0.54	9	0.53	9	0.62	14	0.45	0.44	0.55	6	0.49	8	0.57	12

*In litres. †FEV_1, during early phase immediately after allergen challenge. ‡FEV_1 during late phase 3–10 h after allergen challenge (mean value). §FEV_1 during late phase 3–10 h after allergen challenge (minimum value).

Cell parameters in BW and BAL fluid (Table 4 and Fig. 1)

There was a significant decrease in the recovered volumes of BAL fluid after NO_2+allergen ($P=0.03$). The median values of the recovered BW and BAL volumes after air+allergen exposure were 9 ml [(Interquartile range (IQR) 7–10 ml)] and 105 ml (IQR: 101–109 ml), respectively. After NO_2+allergen exposure, the corresponding figures were 8 ml (IQR: 8–10 ml) and 93 ml (IQR: 71–105 ml).

The cell viability after NO_2+allergen was unchanged in BAL, but decreased in the BW after NO_2+allergen exposure (75 *vs*. 47%; $P=0.01$, data not shown).

In the bronchoalveolar portion, both the total cell and macrophage concentrations decreased after NO_2+allergen (1420 *vs*. $1360 \times 10^6\ L^{-1}$, $P=0.002$; 1235 *vs*. $1170 \times 10^6\ L^{-1}$, $P=0.03$).

The percentage of neutrophils after NO_2+allergen exposure increased in BW and BAL (BW 11 *vs*. 19%, $P=0.05$; BAL 1 *vs*. 3%, $P=0.02$, median values).

The numbers of eosinophils and mast cells for the two exposures were not different.

Soluble mediators and albumin (Table 5)

ECP levels increased significantly in the bronchial portion (BW 3.6 *vs*. 9 μg/l; $P=0.02$) after NO_2+allergen, but not in the bronchoalveolar portion. There were no significant differences in IL-8, IL-5, and sICAM-1 levels between NO_2+allergen and air+allergen, neither in BW nor in BAL. Eotaxin and MPO concentrations in BW were consistently below detection levels. There were no significant differences between NO_2+allergen and air+allergen in albumin levels in lavage fluid (BW; 9 *vs*. 9; BAL; 27 *vs*. 28 mg l^{-1}, median values), or the ratio albumin in lavage fluid/albumin in serum (BW 0.21 *vs*. 0.21; BAL 0.63 *vs*. 0.67, median values).

Correlation between cell counts and ECP, IL-8, and IL-5

The neutrophil counts were associated with IL-8 levels ($r=0.59$, $P=0.04$) in BAL, but not in BW, and with ECP levels in both BAL and BW ($r=0.77$, $P=0.01$ and $r=0.60$, $P=0.03$, respectively).

The eosinophil counts in BW were associated with IL-8 levels ($r=0.68$, $P=0.01$). The eosinophil counts in BAL were correlated to the ECP levels ($r=0.57$, $P=0.04$) and ECP levels correlated with IL-5 levels ($r=0.79$, $P=0.001$) in BAL.

DISCUSSION

The novel finding in this study is that exposure to an ambient level of NO_2, in the dose tested, enhanced subsequent allergen-induced inflammatory reaction in the bronchi, as demonstrated by enhanced number of recruited neutrophils and levels of ECP. These inflammatory changes were not accompanied by any changes in pulmonary function or subjective symptoms.

Air pollution is generally recognized as a significant factor for asthma aggravation, and NO_2 exposure alone has been linked to increased asthmatic symptoms and enhanced bronchial responsiveness to both allergen and nonspecific agents (15). However, the understanding of mechanisms for potential synergistic actions between exposure to air pollution and allergen is incomplete.

NO_2+allergen caused decreased cell viability in BW in the current study. The cell viability in BW is typically not affected by allergen challenge (16,17) or NO_2 exposure (7,18). It is theoretically possible that the combined exposure to NO_2 and allergen leads to an increased release of cytotoxic substances in the bronchi. Since neither separate exposure to NO_2 nor allergen have been thought to cause such effect, and no previous study on combined exposure to NO_2 and allergen has been reported, these findings need to be corroborated before an effect of combined exposure can be determined with certainty.

The enhancement of neutrophils in the lower airways after exposure to NO_2 was an important finding in the current study. There are no published data on the cell response in BAL after exposure to NO_2 in asthmatic subjects at a comparable time after exposure. However, NO_2 has not earlier been found to increase neutrophils in BAL in asthmatics immediately after exposure (19). Normal subjects exposed to NO_2 did not show any change in neutrophil numbers in BW or BAL fluid at a comparable time (24 h after exposure), even though the concentrations were six- and 15-fold, respectively, higher than that used in the current study (20,21). The levels of neutrophils in BW and BAL after air+allergen in the present study were similar to those reported in untreated normal subjects (22) and after NO_2+allergen an about two-fold increase was seen. Allergen bronchial challenge *per se* caused no increase in neutrophils in asthmatics 24 h after challenge, although a transient increase of neutrophils in BAL fluid could be observed 2–4 h after allergen inhalation (9). This indicates that neither exposure to low levels of NO_2 alone nor allergen alone induces a late airway neutrophilia, which was observed in this study after combined exposure to NO_2 and a low dose of allergen. The potentiating effect of NO_2 on the neutrophilic response was relatively mild in this study. However, we cannot rule out the possibility that this potentiating effect of NO_2 could be even more pronounced when a higher allergen dose is chosen.

The quantity of MPO in both BAL and BW did not reach detectable levels by NO_2, suggesting that the neutrophils were not significantly activated, in terms of degranulation. An increase in MPO has been associated

Table 4. Differential cell counts in BW and BAL

	All cells	Neutrophils		Eosinophils		Macrophages		Lymphocytes		Mast cells	
	($\times 10^6\ l^{-1}$)	($\times 10^6\ l^{-1}$)	%	($\times 10^6\ l^{-1}$)	%	($\times 10^6\ l^{-1}$)	%	($\times 10^6\ l^{-1}$)	%	($\times 10^6\ l^{-1}$)	%
BW											
Air/allergen	100	7.0	11	1.8	2	81.0	77	8.3	5	2.0	2
	38–220	4.3–26.9	8–19	0.3–8.6	1–9	26.5–177.3	65–84	0.9–17.6	3–10	0.0–3.5	0–4
NO_2/allergen	160	24.0	19	1.2	3	104.4	72	7.2	4	1.0	1
	90–240	8.3–66.0	8–33	0.0–18.4	0–8	62.6–167.5	55–80	2.0–11.2	2–10	0.0–4.0	0–4
P–value*	n.s.	n.s.	0.05	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
BAL											
Air/allergen	1420	14.2	1	0.0	0	1235.4	89	120.0	10	3.0	3
	1250–1830	0.0–22.5	0–2	0.0–13.5	0–1	1045.6–1683.1	85–95	73.3–211.5	6–14	0.5–4.5	1–5
NO_2/allergen	1360	31.0	3	0.0	0	1169.6	88	122.1	8	4.0	4
	750–1590	15.1–61.2	1–7	0.0–24.2	0–2	650.5–1498.4	77–92	52.6–158.4	5–14	1.0–6.5	1–7
P–value*	0.002	0.02	0.02	n.s.	n.s.	0.03	n.s.	n.s.	n.s.	n.s.	n.s.

Data are given as medians and interquartile range. n=13.*Wilcoxon's paired rank sum test; n.s.=nonsignificant.

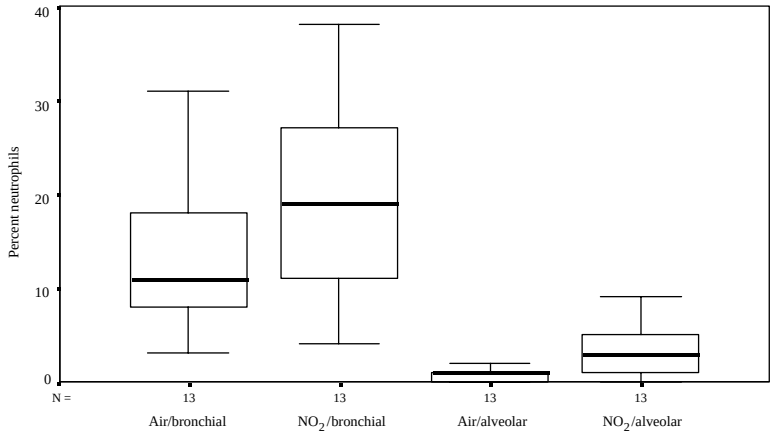

Fig. 1. Neutrophil percentage in lavage fluids after exposure to air+allergen compared to NO_2+allergen. The thick horizontal lines represent median values, boxes represent 25th–75th percentile range.

Table 5. Soluble mediators in BW and BAL

	Albumin(mg l^{-1})	ECP(μg l^{-1})	IL-5(pg ml^{-1})	Eotaxin(pg ml^{-1})	IL-8(pg ml^{-1})	MPO(μg ml^{-1})	sICAM-1(ng ml^{-1})
BW							
Air/allergen	9.00 8.00–14.50	3.60 0.00–11.50	0 0.00–9.00	0 0.00–0.00	56.80 21.05–84.65	n.d	18.70 14.10–26.55
NO_2/allergen	9.00 7.00–17.00	9.00 2.15–22.50	0 0.00–0.00	0 0.00–0.00	54.00 6.70–112.35	n.d	18.00 9.65–23.10
P-value	n.s.	0.017	n.s.	n.s	n.s.	n.s	n.s.
BAL							
Air/allergen	27.00 18.00–35.00	0 0.00–1.10	0 0.00–14.20	0 0.00–0.00	0 0.00–46.50	n.d	60.30 44.30–80.00
NO_2/Allergen	28.00 17.00–58.00	2 0.00–3.55	0 0.00–38.90	0 0.00–2.55	27.00 0.00–95.30	n.d	63.70 45.00–80.20
P-value	n.s	n.s 0.091	n.s.	n.s	n.s.	n.s	n.s.

Data are given as medians and interquartile range; n=13.*Wilcoxon's paired rank sum test; n.s.=nonsignificant; n.d.=non detectable.

with an increase of neutrophils in some NO_2 studies (23), but not in others (24). However, since MPO was not detected after the exposures in this study, the possibility that methodological differences exist between our experimental settings and those cited led to differences in observations which cannot be excluded.

There is increasing evidence that neutrophils play a role in asthma and especially in severe persistent asthma (25), during exacerbations of asthma (26), in status asthmaticus (27), and in fatal asthma of sudden onset (28). The fact that NO_2 exposure, even at a low concentration, promotes a neutrophilic type of inflammation in patients with allergic asthma might therefore be of potential clinical importance.

Although the mean levels of IL-8 were not affected in the current study, a significant overall correlation was seen between the number of neutrophils and IL-8 in BAL. This finding suggests that chemotactic signalling for further recruitment of neutrophils was still occurring 19 h after allergen challenge in this NO_2–allergen exposure model.

The levels of ECP after NO_2+allergen increased significantly in BW, and a tendency towards a similar effect was seen also in BAL. These findings support a previous observation that NO_2 increased the ECP levels in nasal lavage fluid after nasal allergen challenge (29). The ECP level in BW after air+allergen was about one-third of that seen 24 h after challenge with a three-fold greater allergen dose (16). After NO_2+ allergen, the ECP in BW increased three-fold and reached a level comparable to that reported after the higher allergen dose. It is noteworthy that in the current study, the levels of ECP increased although the numbers of eosinophils in BW were unchanged. One possible explanation of this difference in ECP and eosinophil response might be a selective effect on degranulation, but not on cell recruitment,

after NO_2 exposure. Another contributing explanation is that ECP in BAL fluid origins from both bronchial and submucosal eosinophils, though their relative contribution remains unknown. A less likely explanation is that ECP is produced by other cells, since this possible non-eosinophil ECP contribution to the overall ECP levels is not known (30).

NO_2 did not affect the asthmatic reaction to inhaled allergen measured as changes in FEV_1 in the current study, in contrast to what has been seen in several previous studies (3–5). The NO_2 exposure dose has been the same in two of these studies. However, two factors differ: the individual exposure to NO_2 outside the chamber and the dose of allergen. The personal exposure to NO_2 in ambient air (outside the chamber) during the study day happened to be higher after air than after NO_2 in the current study. This reduced the difference in NO_2 exposure between the NO_2 and the control experiment settings and might thereby have reduced the probability to detect the effect on lung function related to NO_2 exposure in the chamber. The allergen doses in the studies in which NO_2 affected lung function after allergen provocation were 10 and 100%, respectively, of $PDSR_{aw\ 100\%}$ (4,5). In the current study, the dose of allergen was intermediate, 40% of $PDSR_{aw\ 100\%}$. If this difference in allergen dose explains the different response in pulmonary function, it would imply that the NO_2-induced enhancement of the asthmatic reaction to inhaled allergen is not linearly related to the allergen dose.

To conclude, combined exposure to ambient levels of NO_2 and allergen was followed by a late increase in numbers of neutrophils, which differs from what has been reported after separate exposure to allergen or NO_2. Moreover, NO_2+allergen was followed by higher ECP levels in the bronchi than after air + allergen. Present data focus on subclinical inflammatory reactions that may occur due to the synergistic action of an air pollutant and allergen, a situation that may frequently occur in daily life. The clinical impact of the results needs to be evaluated in future studies to fully delineate the long-term effect of NO_2 exposure in asthma disease.

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