Reductions in lymphocyte subpopulations after repeated exposure to 1.5 ppm nitrogen dioxide

Thomas Sandström, Maj-Cari Ledin, Lissi Thomasson, Ragnberth Hellday, Nils Stjernberg

Abstract

In this investigation the effects of repeated exposure to 1.5 ppm NO₂ on immune competent cells in bronchoalveolar lavage (BAL) fluid was studied. Special attention was focused on effects on lymphocyte subpopulations. Eight healthy subjects were exposed to 1.5 ppm NO₂ every second day on six occasions. Bronchoalveolar lavage fluid was collected at least three weeks before the exposure series as reference and 24 hours after the last exposure. The results obtained were analysed using a non-parametric test for paired observations, with each subject as his own control. Significant reductions were found in the total number and percentage of T cytotoxic-suppressor cells in BAL fluid; this caused an increase in the ratio of T helper-inducer: cytotoxic-suppressor cells. The total number of natural killer cells in the BAL fluid was also reduced. The numbers of all other cell types were unchanged after exposure. No reduction of phagocytosis of opsonised yeast particles by alveolar macrophages in vitro was detected. It is concluded that repeated short term exposures to 1.5 ppm NO₂, a moderate occupational concentration, induces significant effects on immune competent bronchoalveolar lymphocytes. This indicates that previous findings of changes in the lymphoid immune system induced by NO₂ in animals may well be applicable to humans.

Nitrogen dioxide (NO₂) is one of the most common pollutants both in ambient air and in indoor air in some industrial workplaces. Environmental exposure to NO₂ has been associated with impairment of lung function and increases in respiratory illness as well as increased susceptibility to airway

infections.¹⁻⁵ During the last few years bronchoalveolar lavage (BAL) in combination with controlled chamber exposure has provided much information on the intrapulmonary inflammatory response of humans to NO₂⁶⁻⁹ as well as other air pollutants.¹⁰⁻¹³ The pulmonary inflammation reflected in BAL fluid from healthy subjects exposed to NO₂ has been found to deviate considerably from the results obtained in animal studies.⁸ As a consequence the significance of the data from animal studies has been questioned.

When the biological effects of exposure to NO₂ are considered, the indications of adverse effects on the immune system reported in animal studies attract interest.¹⁴⁻¹⁸ As the relevance of extrapolating from animal data to humans is ambiguous, it is important to clarify whether the effects on the immune defence in animals could be similar in humans. Currently only two studies have considered this question. Frampton and coworkers reported an impaired virus inactivation by human alveolar macrophages in vitro⁶ and Sandström and coworkers found effects on bronchoalveolar lymphocyte subpopulations after 20 minute exposure to 4 ppm NO₂ every second day for a total of six exposures.¹⁹

The primary objective in this study was to investigate whether repeated exposure to 1.5 ppm NO₂ would affect subsets of bronchoalveolar lymphocytes. The chosen exposure concentration is lower than in a preceding study with similar design¹⁹ and is frequently found in industrial indoor environments.

Subjects and methods

SUBJECTS

Eight healthy non-smoking male volunteers (mean age 24 (range 20-28)) gave their informed consent and the study was approved by the local ethics committee. All subjects were free of airway infection for at least six weeks before the study and none had a history of asthma. Pre-exposure spirometry was normal in all subjects.

DESIGN

Exposure to 1.5 ppm NO₂ (2.7 mg NO₂ m⁻³) in an environmental chamber was performed during 20 minutes according to a previously described protocol.^{8,9} Apart from the lower exposure concen-

National Institute of Occupational Health, Medical Division and the Department of Lung Medicine, University Hospital of Umeå, Umeå, Sweden
T Sandström, M-C Ledin, L Thomasson, R Hellday, N Stjernberg

tration in this study the design was identical with the preceding study upon which it was based.¹⁹ Light work was performed on a bicycle ergometer (75 W) during the last 15 minutes of each exposure. The exposures were repeated every second day on a total of six occasions for each subject. Flexible fiberoptic bronchoscopy with bronchoalveolar lavage (BAL) was performed three weeks or more before the exposure series in all subjects to obtain reference BAL fluid. The BAL after exposure was performed 24 hours after the last exposure to NO₂. Each subject was therefore used as his own reference in calculations of changes in the content of BAL fluid as a result of exposure.

METHODS

The exposure chamber²⁰ and the NO₂ exposure technique^{8,9} have been described elsewhere. The method of flexible fiberoptic bronchoscopy with BAL in the right middle lobe, the processing of the BAL fluid, and the staining of cells was performed as previously outlined¹¹ with a few additions. The first recovered 20 ml of the first instilled aliquot of 60 ml phosphate buffered solution (PBS-A) was analysed separately and defined as the bronchial portion (BP). The remaining recovered fluid of the total 4 × 60 ml instilled was defined as the bronchoalveolar portion (BAP).

Differential cell counts were determined in both BP and BAP. Flow cytometry was only performed on the BAP as the lymphocyte number in the BP had frequently been found to be insufficient to allow for accurate analysis with this technique.

Lymphocytesubpopulations were differentiated by flow cytometry (Facsan, Becton-Dickenson, Stockholm, Sweden). Means of blinded double readings of each sample were calculated. The selected monoclonal antibodies used to present the corresponding CD designation were; Leu 4 (CD3 +, T cells), Leu 3a (CD4 +, T helper-inducer cells), Leu 2a (CD8 +, T cytotoxic-suppressor cells), Leu 11 (CD 16 + Leu

19 (natural killer (NK) cells)), and Leu 16 (CD 20 +, B cells) (Becton-Dickenson, Stockholm, Sweden).

Phagocytosis by alveolar macrophages in vitro was measured by determining the proportion of alveolar macrophages that had engulfed one or several yeast particles. The applied glass surface adherence method described elsewhere²¹ was modified to enable the use of bronchoalveolar lavage cells. Briefly, 200 000 cells in 200 µl medium containing 10% pooled human AB positive serum were allowed to adhere to the glass surface for 30 minutes in cell culture conditions. After rinsing the non-adherent cells away, yeast cells labelled with fluorescein isothiocyanate and opsonised with human serum were added to the slides (2.5 × 10⁷ yeast cells in phosphate buffered saline (PBS), pH 7.4). After 30 minutes the phagocytosis was stopped by dipping the slides in ice cold PBS containing 1 mM ethylenediaminetetraacetic acid. The fluorescence of non-ingested yeast cells was quenched by dripping toluidine blue in saline (1 mg/ml, pH 4.7) on to the slides. Engulfment was defined as the presence of fluorescent yeast cells within an alveolar macrophage. Yeast cell adherence (attachment) to an alveolar macrophage was defined as visible contact between a brown yeast cell and an alveolar macrophage.

Peripheral blood samples were drawn before the first exposure and immediately after the last. Analyses of the total number of white blood cells were carried out at the Department 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

For the reference BAL, the recovery of the bronchoalveolar portion (BAP) was median 120 ml (inter-

Table 1 Cell characteristics for the bronchial portion (BP) and bronchoalveolar portion (BAP) of BAL fluid

	Total cell number		Alveolar macrophages		Neutrophils		Lymphocytes		Mast cells	
	BP (× 10 ⁷ /l)	BAP (× 10 ⁷ /l)	BP (%)	BAP (%)	BP (%)	BAP (%)	BP (%)	BAP (%)	BP (%)	BAP (%)
Before exposure to 1.5 ppm NO ₂ :										
Median	17.8	11.0	89	86	2	2	9	11	0.26	0.25
Q ₁ -Q ₃	11.5-30.5	9.4-12.2	84-91	82-92	1-4	1-2	7-13	7-14	0.18-0.39	0.18-0.31
After exposure to 1.5 ppm NO ₂ :										
Median	19.3	15.5	82	85	2	1	14	11	0.25	0.20
Q ₁ -Q ₃	12.8-25.0	10.3-23.8	74-89	72-91	1-5	0-4	7-19	6-14	0.19-0.34	0.15-0.36
Wilcoxon's paired rank sum test	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Q₁-Q₃ = first-third quartile.

Table 2 Lymphocyte subset characteristics for the bronchoalveolar portion (BAP) of BAL fluid

	T cells ($\times 10^7/l$)	T helper-inducer cells (%)	($\times 10^7/l$)	T cytotoxic-suppressor cells (%)	($\times 10^7/l$)	T helper-inducer/ cytotoxic-suppressor cells (ratio of %)	B cells ($\times 10^7/l$)	NK cells ($\times 10^7/l$)
Before exposure to 1.5 ppm NO ₂ :								
Median	0.8	47	0.40	29	0.24	1.9	0.01	0.05
Q ₁ -Q ₃	0.6-1.4	45-55	0.36	22-38	0.16-0.31	1.5-2.4	0.00-0.02	0.02-0.08
After exposure to 1.5 ppm NO ₂ :								
Median	1.2	51	0.40	20	0.14	2.4	0.01	0.02
Q ₁ -Q ₃	0.6-2.5	47-54	0.34-0.44	17-24	0.12-0.15	1.5-3.2	0.00-0.02	0.01-0.04
p Value*	NS	NS	NS	<0.02	<0.02	<0.02	NS	<0.05

*Wilcoxon's paired rank sum test. Q₁-Q₃ = first-third quartile.

quartile range 110-150 ml) and for the postexposure BAL median 130 ml (110-145 ml).

Table 1 presents the total cell number and the percentage of lymphocytes, alveolar macrophages, neutrophils, and mast cells before and after exposure for the BP and the BAP respectively. Eosinophil numbers were negligible and have been omitted. The total cell number and percentage of the mentioned cell types did not change significantly after exposure to NO₂ compared with before. The cell numbers in the BAP were likewise not significantly changed after exposure.

The flow cytometry data obtained with monoclonal antibodies for lymphocyte subsets in BAP did not show any significant change in the total number of Leu-4+ cells (T cells) (table 2). The proportion and total number of Leu-2a+ (T cytotoxic-suppressor) cells were significantly reduced after the exposure series ($p < 0.02$). The proportion and total number of Leu-3a+ (T helper-inducer cells) were not significantly effected. Depending on the change in the proportion of Leu-2a+ cells a significant increase in the ratio of Leu-3a+:Leu-2a+ (T helper-inducer:T-cytotoxic-suppressor) cells was found ($p < 0.02$). The total number of Leu 11+ and Leu 19+ cells (NK cells) was reduced ($p < 0.05$) but no significant effect on Leu 16+ cells (B cells) could be detected.

Table 3 Phagocytosis of opsonised yeast particles in vitro by alveolar macrophages from the bronchoalveolar portion of BAL fluid

	Phagocytosis positive macrophages (%)	Adherence positive macrophages (%)	Total no of engulfed yeast particles
Before exposure to 1.5 ppm NO ₂ :			
Median	83	8	204
Q ₁ -Q ₃	79-93	3-12	173-271
After exposure to 1.5 ppm NO ₂ :			
Median	89	5	307
Q ₁ -Q ₃	87-94	0-10	241-340
p Value*	NS	NS	<0.05

*Wilcoxon's paired rank sum test. Q₁-Q₃ = first-third quartile.

The total number of lymphocytes in the peripheral blood was also significantly reduced after the exposure series ($p < 0.05$). Before exposure there were 1.81×10^4 cells/l (median value) and 24 hours after the end of the exposure series 1.57×10^4 cells/l.

Concerning the phagocytosis of alveolar macrophages in vitro, the number of phagocytosis positive cells were unchanged even though the macrophages that had ingested yeast particles had engulfed a higher total number of particles after exposure ($p < 0.05$, table 3). The number of macrophages with adherent yeast particles was not affected.

Discussion

The present study confirms recent data indicating that repeated exposure to NO₂ in humans affects some subsets of BAL lymphocytes.¹⁹ The data from both studies imply that repeated exposure to NO₂ suppresses the number of T cytotoxic-suppressor cells, which causes an increase in the ratio T helper-inducer:cytotoxic-suppressor cells. Furthermore, NK cell numbers, normally low, appear to be further reduced by the exposure. The near to total absence of B cells in BAL fluid after repeated exposure to 4 ppm NO₂¹⁹ could not be confirmed at this lower exposure. A further indication of noxious effects of NO₂ on the lymphocyte population was the reduction in peripheral blood lymphocytes detected in both studies. The question as to whether the reduction in blood lymphocytes reflects similar changes in lymphocyte subsets as in BAL fluid has not been considered in this experiment, but will be an important issue in forthcoming studies.

The suppression of lymphocyte subsets in the BAP of BAL fluid is in agreement with the previous reports from animal studies by Richters and coworkers at the University of Southern California.^{14 15} According to their results inhalation of NO₂ causes adverse effects on the lymphocytic component of the immune system, not only within the lung, but also in the spleen, one of the major lymphoid organs. In agreement with our results, Richters and coworkers found the sub-

population of T cytotoxic-suppressor cells to be the most susceptible to exposure to NO₂.

T lymphocytes have a central modulating role in most inflammatory conditions and are important in regulating the defence against virus infections by causing lysis of virus infected and neoplastic cells. Furthermore, as suggested by the large number of immunological conditions that are associated with an altered ratio of T helper-inducer:cytotoxic-suppressor cells, it seems important to have a proper balance between these subpopulations to maintain appropriate immunological functions. The shift in the T cell subset ratio and reduction in cytotoxic suppressor cells found after repeated exposure to NO₂ may therefore have adverse consequences for the exposed populations and could partly explain the increased susceptibility to virus infections due to exposure to NO₂.³⁻⁵

To our knowledge, no experimental data from animals are currently available concerning direct measurements of NK cell activity or NK cell numbers in the lungs after exposure to NO₂. It is therefore not possible to directly compare our findings of reduced NK cell numbers with other sources. It should, however, be noted that Richters and coworkers found that exposure to NO₂ may facilitate tumour metastasis in animals.^{17,18} This could be explained by altered NK cell activity, as previously suggested.²² In a recent study by Selgrade and coworkers, rats exposed to NO₂ with a protocol similar to that of the present study developed suppression of NK cell activity in the spleen.²³ Lung cells were not assayed. Furthermore, ozone, an oxidant gas with chemical properties and biological effects that are close to those of NO₂, has been found to cause immunosuppression in terms of reduced NK cell activity.^{22,24} These circumstances inevitably lead to speculations as to whether exposure to NO₂ in humans may also lead to a diminished immunological defence against malignancies. Functional cell studies are now needed to answer this important question.

The question whether repeated exposure to NO₂ in humans may cause a reduced phagocytosis rate in alveolar macrophages, as in experiments with animals and animal cells,^{26,27} remains unsolved. In accordance with preceding studies in humans^{9,19} phagocytosis by alveolar macrophages *in vitro* remained undepressed after the exposure series. Regarding previous animal data, the studies were usually conducted with comparatively high doses of NO₂ which may not mimic the exposure in humans. Suppression of phagocytosis could, in those cases, represent a pronounced toxic effect on the macrophages. On the other hand, it may be that *in vitro* models, like the one used in the present study, may not completely reflect the capabilities of alveolar macrophages in the lungs. The processing of cells, even though careful, may still activate them, masking

a reduced *in situ* phagocytosis. Another possible explanation is that the suggested involvement of alveolar macrophages in the increased susceptibility to virus infections after exposure to NO₂,^{3-5,7} could be depending on impaired intracellular killing in humans rather than phagocytosis as such. It is suggested that the phagocytosis issue should be considered with complementary techniques on human cells in order to broaden the view.

It is now evident that repeated exposure to NO₂ induces compensatory mechanisms that are effective enough to reverse the acute bronchoalveolar inflammation induced by NO₂ involving lymphocytosis and mastocytosis.^{8,9} The standard BAL cell differential counts are not sensitive enough to detect effects caused by repeated exposure, but more specific cell analyses, such as flow cytometry, must be applied. This approach will advance further with use of more monoclonal antibodies for cell differentiation and cell surface markers for various degrees of activation. The technique may not only be applied on lymphocytes but by quenching the autofluorescence of alveolar macrophages the effects on this important cell population may also be better defined. Furthermore, there is a considerable need for further functional studies on cellular effects after exposure to NO₂.

It is concluded that repeated short term exposure to 1.5 ppm NO₂, which is a moderate occupational concentration, induces significant reductions in the proportion and total number of T cytotoxic-suppressor cells, changes the balance of T lymphocyte subpopulations, and reduces the total number of NK cells. This implies that previous findings of NO₂ induced changes in the lymphoid immune system in animals may well be applicable to humans.

We thank Jamshid Pourazar and Annika Hagenbjörk-Gustavsson for technical assistance. The work was supported by the Swedish Work Environment fund and the Swedish Heart and Lung Foundation.

Requests for reprints to: Dr Thomas Sandström MD, PhD, National Institute of Occupational Health, Medical Division, Box 7654, S-907 13 Umeå, Sweden.

- 1 Love GJ, Lan S, Shy CM, Riggan WB. Acute respiratory illness in families exposed to nitrogen dioxide ambient air pollution in Chattanooga, Tennessee. *Arch Environ Health* 1982;37:75-80.
- 2 Mostardi RA, Wobkenberg NR, Ely DL, Conlon M, Attwood G. The university of Akron study of air pollution and human health effects. II. Effects on acute respiratory illness. *Arch Environ Health* 1981;36:250-5.
- 3 Guidotti TL. The higher oxides of nitrogen: inhalation toxicology. *Environ Res* 1978;15:443-72.
- 4 Kulle TJ, Goings SAJ, Sauder LR, Green DJ, Clements ML. Susceptibility to virus infections with NO₂ exposure. *Am Rev Respir Dis* 1987;135(suppl):A58.
- 5 Morrow PE. Toxicological data on NO₂: An overview. *J Toxicol Environ Health* 1984;13:205-30.
- 6 Frampton MW, Finkelstein JN, Roberts NJ, Smeglin AM,

- Morrow PE, Utell MJ. Effects on nitrogen dioxide exposure on bronchoalveolar lavage proteins in humans. *Am J Respir Cell Mol Biol* 1989;1:499-505.
- 7 Frampton MW, Smeglin AM, Roberts NJ, Finkelstein JN, Morrow PE, Utell MJ. Nitrogen dioxide exposure in vivo and human alveolar macrophage inactivation of influenza virus in vitro. *Environ Res* 1989;48:179-92.
  - 8 Sandström T, Andersson M-C, Kolmodin-Hedman B, Stjernberg N, Ångström T. Bronchoalveolar mastocytosis and lymphocytosis after nitrogen dioxide exposure in man: a time-kinetic study. *Eur Respir J* 1990;3:138-43.
  - 9 Sandström T, Stjernberg N, Andersson M-C, *et al.* Inflammatory cell response in bronchoalveolar lavage fluid after nitrogen dioxide exposure of healthy subjects, A dose-response study. *Eur Respir J* 1991;3:332-9.
  - 10 Sandström T, Stjernberg N, Andersson M-C, Kolmodin-Hedman B, Lundgren R, Ångström T. Is the short term limit value for sulphur dioxide exposure safe? Effects of controlled chamber exposure investigated with bronchoalveolar lavage. *Br J Ind Med* 1989;46:200-3.
  - 11 Sandström T, Stjernberg N, Andersson M-C, Kolmodin-Hedman B, Lundgren R, Rosenhall L, Ångström T. Cell response in bronchoalveolar lavage fluid after exposure to sulfur dioxide. A Time-Response study. *Am Rev Respir Dis* 1989;140:1828-31.
  - 12 Rudell B, Sandström T, Stjernberg N, Kolmodin-Hedman B. Controlled diesel exhaust exposure in an exposure chamber: Pulmonary effects investigated with bronchoalveolar lavage. *Journal of Aerosol Science* 1990;21(suppl 1):411-14.
  - 13 Koren H, Devlin RB, Graham DE, *et al.* Ozone-induced inflammation in the lower airways in human subjects. *Am Rev Respir Dis* 1989;139:407-15.
  - 14 Damji KS, Richters A. Reduction in T lymphocyte populations following acute exposure to 4 ppm NO₂. *Environ Res* 1989;49:217-24.
  - 15 Kuraitis KV, Richters A. Spleen cellularity shifts from the inhalation of 0.25-0.35 ppm nitrogen dioxide. *J Environ Pathol Toxicol Oncol* 1989;36:114-19.
  - 16 Fujimaki H. Impairment of humoral immune responses in mice exposed to nitrogen dioxide and ozone mixtures. *Environ Res* 1989;48:211-17.
  - 17 Richters A, Damji KS. The relationship between inhalation of nitrogen dioxide, the immune system, and progression of spontaneously occurring lymphoma in AKR mice. *J Environ Pathol Toxicol Oncol* 1990;10:225-30.
  - 18 Richters A, Kuraitis K. A new relationship between air pollutant inhalation and cancer. *Arch Environ Health* 1983;10:69-75.
  - 19 Sandström T, Helleday R, Stjernberg N, Bjerner L. Effects on bronchoalveolar lymphocyte subsets and macrophages after repeated exposure to nitrogen dioxide. *Eur Respir J* (in press).
  - 20 Sandström T, Kolmodin-Hedman B, Stjernberg N, Andersson M-C, Löfvenius G. Challenge test to sulfur dioxide—symptom and lung function measurements. *Scand J Work Environ Health* 1988;14:(Suppl 1):77-9.
  - 21 Bjerner L, von Schoultz E, Norberg B, Henriksson R. Estramustine inhibits phagocytosis. *Prostate* 1988;13:49-55.
  - 22 Van Loveren H, Krajnc EL, Rombout PJA, Blommaert FA, Vos JG. Effects of ozone, hexachlorobenzene, and bis (tri-n-butyltin) oxide on natural killer activity in the rat lung. *Toxicol Appl Pharmacol* 1990;102:21-33.
  - 23 Selgrade MK, Daniels MJ, Grose EC. Evaluation of immunotoxicity of an urban profile of nitrogen dioxide: acute, subchronic, and chronic studies. *Inhalation Toxicology* 1991;3:357-7.
  - 24 Burleson Gr, Lynette LK, Stutzman JD. Immunosuppression of pulmonary natural killer activity by exposure to ozone. *Immunopharmacol Immunotoxicol* 1989;11:715-35.
  - 25 Acton JD, Myrvik Q. Nitrogen dioxide effects on alveolar macrophages. *J Bacteriol* 1972;24:48-52.
  - 26 Coffin L, Blommer JE. Time-dose response for nitrogen dioxide exposure in an infectivity model system. *Environ Health Perspect* 1976;13:11-5.
  - 27 Gardner DE. Influence of exposure patterns of nitrogen dioxide on susceptibility of infectious respiratory disease. In: Lee S, ed. Nitrogen oxides and their effects on health. Ann Arbor, Michigan: Ann Arbor Science 1980:267-88.

Accepted 17 February 1992