Effects of nitrogen oxides on natural killer cells in glass craftsmen and braziers

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Abstract

Objectives—To assess the effect of exposure to nitrogen oxides on peripheral blood natural killer cells.

Methods—Groups of glass craftsmen and braziers exposed to nitrogen oxides and non-exposed controls were studied. Air concentrations of nitrogen oxides were measured. Mononuclear cells isolated from peripheral blood samples were assayed for natural killer cell activity with K562 target cells in a ^{51}Cr release assay and the percentage of natural killer cells (CD16) was measured by flow cytometry. **Results**—Braziers were exposed to 1.2 ppm nitrogen dioxide and 8.6 ppm nitric oxide and glass craftsmen to 2.9 ppm nitrogen dioxide and 26.5 ppm nitric oxide. The natural killer cell activity of exposed workers was significantly lower than in non-exposed controls ($P < 0.05$ ANOVA Scheffe test). The percentage of natural killer cells in glass craftsmen was significantly greater than in controls ($P < 0.05$ ANOVA Scheffe test). Regression of natural killer cell activity against age, smoking habit, number of years worked and current exposure to nitrogen dioxide and nitric oxide gases was not significant. The percentage of natural killer cells was not significantly correlated with age, smoking habit, or numbers of years worked, but was significantly related to air concentrations of nitrogen dioxide ($P < 0.01$) and nitric oxide ($P < 0.001$).

Conclusion—Natural killer cell activity and the percentage of natural killer cells in peripheral blood cells were altered in workers exposed to nitrogen oxides.

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Chemicals such as polycyclic aromatic hydrocarbons, pesticides, aromatic amines, particles, drugs, alcohol, and oxidant gases (ozone and nitrogen dioxide) are capable of inducing immune responses in occupationally exposed workers.¹ Ozone and nitrogen dioxide are also common air pollutants. Nitrogen dioxide, at 0.25 ppm, has been shown to reduce the number of lymphocytes, natural killer (NK) cells, and the T-helper lymphocyte/T-suppressor lymphocyte ratio (CD4/CD8) in peripheral

blood of exposed rodents.² Immunological measures such as the ratio of CD4/CD8 cells in bronchoalveolar lavage and total lymphocyte count in peripheral blood were significantly decreased in human volunteers exposed to 1.5 ppm of nitrogen dioxide for one to two hours a day for seven days.³

Glass craftsmen who work with oxyacetylene burners are exposed to high concentrations of nitrogen oxides, which consist of nitrogen dioxide and nitric oxide formed by oxidation of atmospheric nitrogen. Glass craftsmen are not normally exposed to other potentially immunotoxic gases. Braziers are also exposed to nitrogen oxides but to a lesser extent, and to metal fumes. The occupational exposure limit (eight hour time weighted average) for nitrogen oxides is 3 ppm (5 mg m^{-3}) and for nitric oxide is 25 ppm (30 mg m^{-3}). Environmental air concentrations of nitrogen dioxide and nitric oxide have been reported as 25–50 ppb and 25–230 ppb, respectively. During episodes of high air pollution such as in London in December 1987, air concentrations were 130 ppb nitrogen dioxide and 900 ppb nitric oxide.⁴

Previous studies in glass workers exposed to nitrogen oxides have shown increased urinary excretion of nitrate and hydroxyproline compared with controls.⁵ The level of lipid peroxidation in the respiratory system was also increased, producing increased concentrations of pentane in expired air.⁶

Nitric oxide is produced endogenously by endothelial cells and immune cells in humans and laboratory animals. Nitrogen oxides have a role during inflammation and other physiological processes. Macrophages and several established macrophage cell lines produce nitrate and nitrite in vitro when stimulated with lymphokines, and the intermediate compound in the oxidation of L-arginine to nitrite and nitrate is nitric oxide.^{7,8}

This study was conducted in glass craftsmen, braziers, and controls to find the effects of exposure to nitrogen oxides on the immunotoxicity biomarkers, peripheral blood lymphocyte population, percentage, and activity of NK cells.

Methods

STUDY POPULATION

Eighteen glass craftsmen and 12 braziers were recruited from local industries. The glass craftsmen and braziers were exposed to naked oxyacetylene flames for eight hours a day, for five days during each working week. All the

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workers except one had been employed for at least 1.5 years in the same industry. The control population was recruited from university laboratory staff ($n = 10$) and office workers in the industry ($n = 11$). All workers gave written consent for the study, which had received ethical approval. The characteristics of the participants were determined from a set of questions. Controls were matched as closely as possible to the workers for sex (all male), age, and smoking habit (smoker or non-smoker).

AMBIENT MONITORING

Exposure of the glass craftsmen and braziers to nitrogen oxides was measured by personal monitoring. Air was sampled from the breathing zone at 20 ml/min for 200 minutes during the eight hour work shift with a personal pump. Nitrogen dioxide and nitric oxide were adsorbed on to a molecular sieve coated with triethanolamine. The nitrite formed was desorbed and measured at 540 nm after complexing with 1-naphthylethylenediamine by the method of Willey *et al.*⁹ Standards were prepared from sodium nitrite in the range 0–4.0 mg/ml. The sensitivity of the assay was 0.5 ppm nitrogen dioxide (940 $\mu\text{g}/\text{m}^3$) and 5.0 ppm (6250 $\mu\text{g}/\text{m}^3$) nitric oxide. Results were expressed as eight hour time weighted averages.

Air samples were collected at a different time from the blood sample because of the need for rapid processing of blood samples. Previous studies have indicated that, owing to the repetitive nature of the job, glass craftsmen were exposed to similar concentrations of nitrogen oxides at different times and on different days of the week. (Azari *et al.*, in preparation).

The exposure of eight of the control subjects to nitrogen oxides was measured as already described. Air concentrations of nitrogen dioxide and nitric oxide were below the sensitivity of the assay (0.5 ppm nitrogen dioxide and 5.0 ppm nitric oxide).

BLOOD SAMPLING

A 10 ml sample of peripheral blood was collected from all workers and controls into a lithium heparin tube on a midweek morning between 10.00 and 13.00. Blood samples were processed as soon as possible, and within three hours.

ASSAY FOR NATURAL KILLER CELL ACTIVITY

Mononuclear cells were isolated from heparinised blood by Ficoll metazoate density gradient centrifugation (400 g for 30 minutes), washed twice with RPMI 1640 medium and resuspended in RPMI 1640 medium supplemented with 10% foetal calf serum. The NK cell activity was measured by a modified ^{51}Cr

release assay,¹⁰ with the isolated mononuclear cells as effector cells and human chronic myeloid leukaemia derived cell line K562 as target cells. 200 μCi in 50 μl sodium chromate ($\text{Na}_2^{51}\text{CrO}_4$) was added to 0.8 ml aliquots of culture medium containing 5×10^6 washed target cells. The cells were incubated for 90 minutes at 37°C in humidified 5% CO_2 in air to allow maximum uptake of sodium chromate. The cells were then washed three times to remove excess sodium chromate and resuspended with 10 ml of medium and incubated again for 30 minutes to allow dying cells to disrupt. At this stage the labelled target cells were washed twice and resuspended to $5 \times 10^4/\text{ml}$. Aliquots (100 μl) of target cells were mixed with 100 μl of effector cell suspensions in round bottomed 96 well microculture plates to give effector/target (E/T) ratios of 100/1, 50/1, 25/1, and 12.5/1 in series of five wells at each ratio. The plates were centrifuged briefly (two minutes at 50 g) to bring effector and target cells in contact with each other. The cells were allowed to interact at 37°C in 5% CO_2 for four hours in an incubator. Release of ^{51}Cr was measured after centrifuging the plates at 50 g for 10 minutes and harvesting 100 μl of culture supernatant for counting in a gamma counter.

Spontaneous release (R_s) of ^{51}Cr was measured after incubation of labelled target cells in 200 ml of medium for four hours. The maximum release was estimated by counting the total ^{51}Cr in 5×10^3 labelled target cells: this value provided a convenient fixed upper reference point. The percentage ^{51}Cr release (NK cell activity) was measured from experimental (R_e), spontaneous (R_s), and maximum (R_m) release with the formula:

$$^{51}\text{Cr release} = \frac{R_e - R_s}{R_m - R_s} \times 100\%$$

Natural killer cell activity was expressed in lytic units. One lytic unit was defined as the number of effector cells required to achieve 20% lysis of target cells. The number of lytic units/ 10^7 effector cells was taken as the index of NK cell activity in separated blood mononuclear cells in this study. For checking assay reproducibility five blood samples from a single donor were measured on two occasions. The coefficients of variation were 6.5% and 4.3%.

MEASUREMENT OF PERCENTAGE OF NATURAL KILLER CELLS IN LYMPHOCYTES

For direct immunofluorescence staining, $10^5/50 \mu\text{l}$ mononuclear cells were incubated with 2 μl CD16 FITC antibody (Becton Dickinson) for 30 minutes, washed twice and resuspended in RPMI 1640 culture medium

Table 1 Characteristics and exposure of glass craftsmen, braziers, and control groups

		Concentration in inhaled air								
		NO ₂ (ppm)		NO (ppm)						
Population	n	Mean (SD)	Range	Mean (SD)	Range	Mean (SD)	Range	Mean (SD)	Range	Smokers n (%)
Glass craftsmen	18	2.9 (1.9)	1.7–7.6	26.5 (10.2)	14.8–48.9	29.4 (10.9)	16.0–58.0	10.1 (11.6)	0.1–43.0	8 (44.4)
Braziers	12	1.2 (0.9)	0.5–2.9	8.6 (2.7)	5.5–13.2	46.0 (11.0)	29.0–61.0	24.3 (8.8)	11.0–38.0	5 (41)
Controls	21	ND	ND	ND	ND	42.3 (10.9)	28.0–70.0	16.4 (12.2)	2.0–40.0	8 (38)

Figure 1 Natural killer cell activity in peripheral blood lymphocytes from workers exposed to nitrogen oxides (individual results and mean (SD)).

Figure 2 Percentage of NK cells in peripheral blood lymphocytes from workers exposed to nitrogen oxides (individual results and mean (SD)).

supplemented with 10% fetal calf serum. Samples were then analysed for immunofluorescence by flow cytometry with 10 000 events recorded.¹¹ The number of NK cells (CD16 stained) was expressed as a percentage of the total lymphocytes. The lymphocyte region was defined by backgating cells which had been stained with CD45FITC and CD14PE antibodies (Leucogate, Becton Dickinson).

STATISTICAL ANALYSIS OF THE RESULTS

Analysis of variance was used to test the differences between results in groups of workers and controls. Lymphocyte count in peripheral blood cells in glass craftsmen and controls were compared with unpaired *t* test. Multiple regres-

sion analysis was performed to find the influence of potential predictive variables: age, duration of employment, smoking habit (smoker or non-smoker), and exposure to nitrogen oxides.

Results

Table 1 shows the details of the workers and controls. The proportion of smokers was similar in each group. It was not possible to achieve individual matching for age: the mean age of the glass craftsmen was lower than those of controls and braziers.

Exposure of the glass craftsmen to nitrogen dioxide and nitric oxide (table 1) was higher than for the braziers. Most of the glass craftsmen were exposed to nitrogen dioxide above the occupational exposure standard (maximum exposure limit: 3 ppm as the eight hour time weighted average concentration). Concentrations in control air were below the detection limit of the analysis method used.

The mean NK cell activity in peripheral blood from the glass craftsmen was not significantly different from that of the braziers ($P > 0.05$, Scheffe test), but both were significantly lower than in controls ($P < 0.05$, Scheffe test, fig 1). The percentage of NK cells in blood from glass craftsmen was significantly greater than in controls ($P < 0.05$, ANOVA Scheffe test) but was not different from braziers (fig 2). Lymphocyte counts measured in peripheral blood of 12 glass craftsmen and 12 controls did not differ ($P > 0.05$, unpaired *t* test, table 2). Lymphocyte counts reported in this study ($1.5-3.1 \times 10^9/l$) were within the normal ranges reported by other investigators.^{3,10}

Regression of NK cell activity against age, smoking habit, number of years worked, and current exposure to nitrogen dioxide and nitric oxide gases showed no significant correlation ($P > 0.05$). The percentage of NK cells in peripheral blood lymphocytes also was not significantly correlated with age, smoking habit,

Table 2 Biomarkers of immunotoxicity in glass craftsmen and control population

Population	NK cell activity (lytic unit/ 10^7 effector cells)		% Natural killer cells (CD16) in lymphocytes		Lymphocyte count ($\times 10^9/L$)	
	Mean (SD)	n	Mean (SD)	n	Mean (SD)	n
Glass craftsmen	20.5 (11.6)*	18	17.5 (5.7)*	18	2.31 (0.44)	12
Braziers	20.4 (11.9)*	12	12.8 (4.5)	12	—	—
Control	36.1 (16.7)	21	13.6 (5.8)	21	2.11 (0.33)	12

* $P < 0.05$ v controls.

Figure 3 Percentage of NK cells in peripheral blood cells in relation to exposure to (A) nitrogen dioxide and (B) nitric oxide.

or number of years worked ($P > 0.05$). However there was a significant relation between the percentage of NK cells and both nitrogen dioxide ($P < 0.01$, $n = 29$) and nitric oxide concentrations ($P < 0.001$, $n = 27$) in inhaled air of the exposed workers (fig 3). Controls were not included in the analysis as inhaled air concentrations were below the lower limit of detection of the assay.

Discussion

Glass craftsmen in this study were exposed to nitrogen oxides in the inhaled air at concentrations of 1.7–7.6 ppm nitrogen dioxide and 14.8–49.0 ppm nitric oxide. Braziers were exposed at lower concentrations of 0.5–2.9 ppm nitrogen dioxide and 5.5–13.2 ppm nitric oxide. Exposure of some of the glass craftsmen exceeded the occupational exposure limit. The NK cell activity in peripheral blood from these workers was lowered in a similar manner to that previously reported after long term exposure to nitrogen dioxide at concentrations as low as 0.25 ppm.^{12,13} The percentage of NK cells in peripheral blood from the glass craftsmen was increased by exposure to nitrogen oxides. Depression of NK cell activity together with an increased percentage of NK cells in peripheral blood cells has also been reported by other workers in people exposed to benzidine, β -naphthylamine, or after physical exercise.^{11,14}

There was a wide variation in NK cell activity between people in the control group. Several factors influenced NK cell activity. There was also an increase with age. In future studies a base line should be established for people before exposure to nitrogen oxides, or blood samples taken before and after a break from exposure to nitrogen oxides, to study the recovery of the immune system.

Natural killer cells are a distinct population of non-B and non-T lymphocytes characterised by their ability to identify and lyse a variety of tumour cells, cells infected with viruses, and fungi.¹⁵ Previous studies have shown increased susceptibility to bacteria and viral infections among populations exposed to nitrogen dioxide.^{16,17} A lowered activity of NK cells, as found in this study, may have contributed to this susceptibility.

The role of nitrogen dioxide in immunotoxicity has been the subject of many studies, but few have considered the role of nitric oxide also present in the inhaled air. Nitrogen dioxide and nitric oxide are both free radicals and are biologically active gases. It is not possible to separate the effects of nitrogen dioxide and nitric oxide as they are produced together in combustion processes with nitric oxide as the dominant gas.⁴ Absorbed nitric oxide forms peroxynitrite, which dissociates to nitrogen dioxide endogenously.¹⁸

Previous studies have shown enhanced collagen breakdown and increased lipid peroxidation in the respiratory system in workers

exposed to similar concentrations of nitrogen dioxide and nitric oxide.^{5,6} The parallel lowering of activity of NK cells as the result of exposure to nitrogen oxides supports the suggestion of Kuraitis and Richters¹² that lipid peroxidation and collagen breakdown may be responsible for suppression of the immune system through production of altered antigenic determinants in the lung. The absence in this study of an effect of nitrogen oxides on lymphocyte count in peripheral blood contrasts with the results of other studies.^{3,19}

Measurements of NK cell activity and the percentage of NK cells in peripheral blood cells may have a part to play as biological markers of the immunotoxicity of exposure to nitrogen oxides. Samples of peripheral blood are easier to obtain in the work place than bronchoalveolar lavage fluid which has been used previously in volunteer studies for assessing the immunological effects of nitrogen oxides.

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