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Ozone-induced mediator release from human bronchial epithelial cells *in vitro* and the influence of nedocromil sodium

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Ozone-induced mediator release from human bronchial epithelial cells in vitro *and the influence of nedocromil sodium. C. Rusznak, J.L. Devalia, R.J. Sapsford, R.J. Davies. ©ERS Journals Ltd 1996.*

ABSTRACT: Although animal and human studies have demonstrated that ozone inhalation leads to airway epithelial inflammation and damage, the underlying mechanisms are not fully understood.

We cultured human bronchial epithelial cells as explant cultures and investigated the effect of 6 h of exposure to 0–500 parts per billion (ppb) O_3 with or without 10^{-5} M nedocromil sodium on: 1) epithelial cell membrane integrity; and 2) release of inflammatory cytokines and soluble intercellular adhesion molecule-1 (sICAM-1), as assessed by enzyme-linked immunosorbent assay (ELISA).

O_3 exposure led to significant epithelial cell damage at concentrations of 10–500 ppb O_3, as indicated by increased release of [^{51}Cr]-labelled sodium chromate. At concentrations of 10–100 ppb, O_3 induced maximal release of interleukin-8 (IL-8), granulocyte/macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor-α (TNF-α) and sICAM-1. The IL-8 and GM-CSF release increased significantly from 5.64±0.58 and 0.04±0.03 pg·µg^{-1} cellular protein, respectively, from control cells exposed to air, to 20.16±2.56 and 0.20±0.04 pg·µg^{-1} cellular protein, respectively, from cells exposed to 50 ppb O_3. 10^{-5} M nedocromil sodium significantly attenuated the O_3-induced release of both IL-8 and GM-CSF ($p<0.01$). The TNF-α and sICAM-1 increases after exposure to 10–50 ppb O_3, were also abrogated by treatment of the cells with 10^{-5} M nedocromil sodium ($p<0.05$). Similarly, the antioxidant, glutathione, at concentrations of 400–600 µM, significantly reduced the O_3-induced release of IL-8 ($p<0.05$).

In conclusion, these studies indicate that ambient concentrations of ozone may induce airway inflammation, through release of proinflammatory mediators from airway epithelial cells. This effect may be inhibited both by the anti-inflammatory drug, nedocromil sodium, and the naturally occurring antioxidant glutathione.
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Epidemiological studies have demonstrated that there is a clear association between episodes of air pollution and impaired lung function, cough and infections of the lower respiratory tract [1–3]. Increases in the ambient concentration of O_3, in particular, have been shown to decrease lung function and increase bronchial hyperresponsiveness, both in asthmatic and nonasthmatic children [4–6]. Studies investigating the pathophysiological effects resulting from inhalation of O_3 have demonstrated that this gas leads to marked histological change, including infiltration with inflammatory cells in the airways. Studies in Rhesus monkeys have demonstrated that, in addition to tracheal and bronchial epithelial damage, exposure for 8 h to 960 parts per billion (ppb) O_3 also led to neutrophil and eosinophil influx after 12–24 h [7]. Similarly, studies in humans have demonstrated that exposure of healthy volunteers for 4–7 h to 80–200 ppb O_3 induced epithelial damage and permeability and an increase in the number of neutrophils and several inflammatory mediators, in proximal airway lavage (PAL) and bronchoalveolar lavage (BAL) fluids collected 18–24 h after exposure [8, 9].

Although the precise mechanisms underlying O_3-induced inflammation of the airways are not clear, there is increasing evidence that airway epithelial cells may play a vital role, since they can express and synthesize a large variety of proinflammatory mediators, including eicosanoids, cytokines and cell adhesion molecules, which influence the growth, differentiation, proliferation and activation of inflammatory cells [10, 11]. More recently, we have demonstrated that exposure of human bronchial epithelial cells to nitrogen dioxide leads to significant release of interleukin-8 (IL-8), tumour necrosis factor-α (TNF-α), granulocyte/macrophage colony-stimulating factor (GM-CSF) and leukotriene C_4 (LTC_4) from these cells, *in vitro* [12, 13].

In view of the detrimental effects of air pollutants at the airway epithelium and the likely importance of the epithelial cells in modulating airway inflammation, we hypothesized that airway inflammation observed after

inhalation of O_3 is a result of increased synthesis and release of inflammatory mediators from airway epithelial cells and that anti-inflammatory agents inhibit the release of these mediators. To test this hypothesis, we investigated: 1) the effect of exposure for 6 h to 0–500 ppb O_3, on epithelial cell membrane integrity and the release of IL-8, GM-CSF, TNF-α and soluble intercellular adhesion molecule-1 (sICAM-1) from human bronchial epithelial cells *in vitro*; and 2) the effect of both nedocromil sodium (10^{-7}–10^{-4} M), a nonsteroidal anti-asthma drug, and glutathione (100–600 µM), a naturally occurring antioxidant compound, on any O_3-induced release of these inflammatory mediators. The mediators investigated were selected since they are known to play an important role in the initiation and maintenance of airway inflammation, which can lead to airway hyperresponsiveness.

Materials and methods

All chemicals and reagents were of tissue culture grade and, unless otherwise stated, were obtained from the Sigma Chemical Co. (Poole, UK).

Culture of bronchial epithelial cells

Bronchial tissue was obtained from five male and six female patients, who underwent lobectomy or pneumonectomy for lung cancer, at St Bartholomew's Hospital, London. All patients were smokers, not allergic to common allergens, and of mean (±SEM) age 59±7 yrs (range 27–69 yrs). Following resection, only tissue macroscopically free of tumour was further processed for culture.

Bronchial epithelial cells were cultured by an explant cell culture technique developed in our laboratory [14]. Briefly, the epithelium was dissected away from the underlying tissue and cut into smaller sections, approximately 1–2 mm^3 in size. All the sections were gently washed three times in sterile prewarmed Medium 199 containing 1% (v/v) antibiotic/antimycotic solution composed of penicillin, streptomycin and amphotericin B (Sigma, UK). Then 2–3 sections were explanted into untreated 60 mm diameter Falcon® "Primaria™" plastic culture dishes (Becton Dickinson Ltd, Oxford, UK). The explants were incubated for 2–3 weeks at 37°C in a 5% CO_2 in air atmosphere, in 2.5 mL aliquots of fresh sterile complete culture medium prepared by mixing 250 µg bovine pancreatic insulin, 250 µg human transferrin, 36 µg hydrocortisone, 3 mg L-glutamine, 1 mL of antibiotic/antimycotic solution and 2.5 mL Nu-serum IV (Flow Laboratories, Scotland, UK) in 100 mL Medium 199. Once the outgrowing epithelial cells had grown to confluence, the explants were removed from each culture dish and the epithelial cells allowed to grow over the areas previously occupied by the explants. Randomly chosen cultures were checked for purity as described previously [14].

Exposure of epithelial cell cultures to ozone

Exposure of cells to O_3 was carried out in an airtight polycarbonate aeration chamber, with a capacity of 5.5 L. O_3 in air was supplied, at a flow rate of 3 $L \cdot min^{-1}$, *via* Teflon tubing connected to an O_3 generator/monitor (Dasibi Model 1108, Quantitec Ltd, Milton Keynes, UK), and cultures were exposed for 6 h to 0–500 ppb O_3, at 37°C in a humidified tissue culture cabinet. The polycarbonate chamber was tilted gently at intervals of 2.5 s, to an angle of 10° from the horizontal in each quarter of the horizontal plane on a Luckham 4RT rocking table (Luckham Ltd, Burgess Hill, UK), thereby momentarily displacing approximately half the medium covering the surface of the culture plate during each tilt.

Effect of 0–500 ppb O_3 on epithelial cell membrane damage

Epithelial cell membrane damage was investigated by assessing the release of ^{51}Cr from cells radiolabelled with sodium chromate (Amersham International plc, Amersham, UK). Prior to exposure to O_3, explants were removed from 2–3 week old confluent cultures and the cells incubated overnight in the presence of 1.0 µCi [^{51}Cr]sodium chromate. Following this incubation, the culture medium was decanted and the cells were washed gently with Medium 199, to remove any radio-label not incorporated into the cells. The cultures were exposed to 0–500 ppb O_3 for 6 h, as described above. At the end of exposure, the medium was collected and the cells were gently washed with 1.0 mL fresh ice-cold medium. The culture medium and the wash were pooled and the adherent cells were scraped off into 3.0 mL Medium 199. Aliquots, 200 µL in volume, of all samples were mixed with 5.0 mL of Ready-Solv™ CP scintillation cocktail (Beckman-RIIC Ltd, High Wycombe, UK), and were assessed for ^{51}Cr radioactivity using a Beckman LS1800 scintillation counter (Beckman-RIIC Ltd, High Wycombe, UK). All radioactivity released into the medium was expressed as a percentage of total in the culture, according to the formula:

$$\frac{\text{Total cpm in medium}}{\text{Total cpm in medium + total cpm in cell pellet}} \times 100$$

where cpm=counts per minute.

Effect of 0–500 ppb O_3 on the release of IL-8, GM-CSF, TNF-α and sICAM-1 from human bronchial epithelial cells

Two to three week old confluent cultures were used for all experiments. Prior to exposure to O_3, cell cultures were equilibrated by incubation for 24 h in Medium 199 containing 1% serum-free supplement medium (SF-1 medium) (Northumbria Biologicals Ltd, Cramlington, UK). Following this initial incubation, the cultures were gently washed three times with prewarmed and preaerated SF-1 medium and sets of at least six cultures each from different individuals were exposed for 6 h to either 0 (air), 10, 50, 100 or 500 ppb O_3, as described above. At the end of exposure, the medium was collected from each culture and the cells were gently washed with 1.0 mL fresh ice-cold Medium 199. The

wash was pooled with the culture medium and stored at -70°C until analysis for the various mediators. The adherent cells were scraped off into l.0 mL Medium 199 and stored at -70°C until analysis for cellular protein, according to the method of LOWRY *et al.* [15]. Prior to analysis, the media samples were concentrated by freeze drying and, following reconstitution in 1.0 mL distilled H_2O, were analysed for IL-8, GM-CSF, TNF-α, and sICAM-1, using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Abingdon, UK). All results were expressed as pg mediator·μg^{-1} cellular protein.

Effect of 10^{-7}–10^{-4} M nedocromil sodium on the release of IL-8, GM-CSF, TNF-α and sICAM-1 from human bronchial epithelial cells

Sets of 2–3 week old confluent cultures equilibrated for 24 h in SF-1 medium, as described above, were incubated for 6 h in the presence of 10^{-4}–10^{-7} M nedocromil sodium. At the end of incubation, the culture medium and the cells were collected and treated as above.

Effect of nedocromil sodium on O_3-induced release of IL-8, GM-CSF, TNF-α and sICAM-1 from human bronchial epithelial cells

The first set of experiments indicated that O_3-induced mediator release was maximal at a concentration of 50 ppb O_3, and that IL-8 was the mediator which was released from the bronchial epithelial cells in greatest quantities. Subsequently, studies were undertaken to investigate the effect of 10^{-7}–10^{-4} M nedocromil sodium on 50 ppb O_3-induced release of IL-8. On the basis of these findings, further studies were undertaken to investigate the effect of the optimal concentration (10^{-5} M) of nedocromil sodium on 0–500 ppb O_3-induced release of all the different mediators.

Thus, in the first instance, sets of 2–3 week old confluent cultures, equilibrated in SF-1 medium, were exposed for 6 h to 50 ppb O_3 in the presence of 10^{-4}–10^{-7} M nedocromil sodium. At the end of incubation, the culture medium and the cells were collected and analysed for IL-8 and total cell protein, respectively, as described above.

In the second instance, sets of 2–3 week old confluent cultures, equilibrated in SF-1 medium, were exposed for 6 h to 0–500 ppb O_3, in the presence of 10^{-5} M nedocromil sodium and, at the end of incubation, the culture medium and the cells were collected and analysed for the presence of IL-8, GM-CSF, TNF-α, sICAM-1 and total cell protein, respectively, as described above.

Effect of 0–600 μM glutathione on 50 ppb O_3-induced release of IL-8 by human bronchial epithelial cells

Two to three week old cultures of bronchial epithelial cells were equilibrated in SF-1 medium for 24 h and then exposed for 6 h to 50 ppb O_3 in the presence of 0–600 μM glutathione. At the end of incubation, the medium and the cells were collected and analysed for IL-8 and total cell protein, respectively, as described above.

Statistical analysis

Results are expressed as mean±SEM. All results were tested for normality using a normal probability plot and the Shapiro-Wilk test, and then further tested by one-way analysis of variance (ANOVA) to test for significance of any differences across the O_3 exposed and treatment groups, followed by further analysis with Scheffé's multiple regression test to assess differences between individual treatment groups. In the case of ^{51}Cr release studies, results were analysed by the unpaired t-test and the Bonferroni correction applied. Probability values of less than 0.05 were considered to be significant.

Results

Studies of the effects of exposure for 6 h to O_3 demonstrated that this agent did not lead to any gross damage or detachment of the epithelial cells, as evidenced by light microscopy. More detailed studies investigating the effect of O_3 exposure on bronchial epithelial cell membrane integrity, however, demonstrated that this agent led to a dose-dependent release of ^{51}Cr, from cells "loaded" with ^{51}Cr overnight. Exposure of cells for 6 h to 10, 50, 100 and 500 ppb O_3 led to the release of 24±2, 28±1, 35±6 and 39±8% ^{51}Cr, respectively ($p<0.001$ for all comparisons with 5.2±0.3% ^{51}Cr in control cultures) (fig. 1).

Exposure of human bronchial epithelial cells for 6 h to O_3 significantly increased the release of IL-8, GM-CSF, TNF-α and sICAM from these cells, at concentrations of 10–100 ppb O_3.

Figure 2 shows the effect of O_3 exposure on the release of IL-8. These studies demonstrated that O_3 had a dose-dependent effect on the release of this cytokine, and was optimally active at a concentration of 50 ppb, significantly increasing the release to 20.16±2.56 pg·μg^{-1} cellular protein ($p<0.01$), compared with 5.64±0.58 pg·μg^{-1} cellular protein released from control cells exposed for 6 hours to air. Although significant release

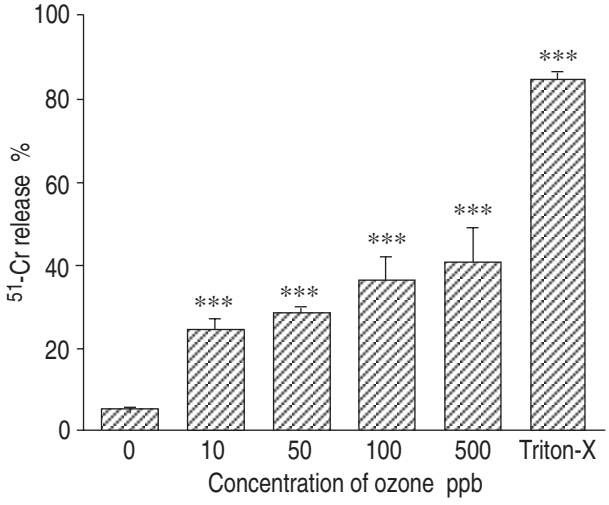

Fig. 1. – The effect of 6 h of exposure to 10–500 parts per billion (ppb) O_3, on the release of ^{51}Cr from human bronchial epithelial cells, *in vitro*. Triton-X (0.5%) served as a positive control. Results are expressed as mean±SEM; n=6 at each time-point. ***: $p<0.001$ *versus* 0 ppb O_3.

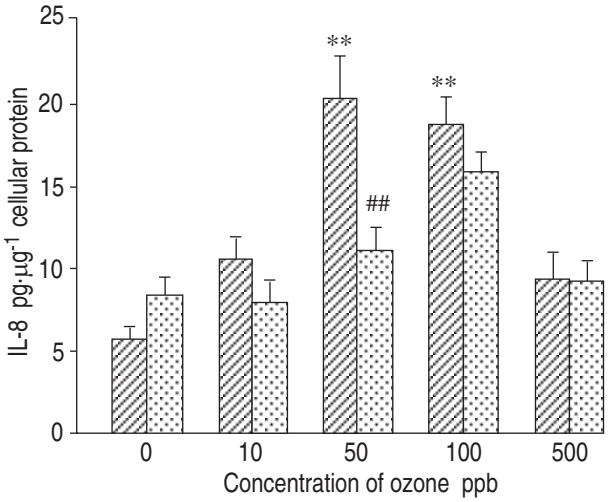

Fig. 2. – The effect of 10^{-5} M nedocromil sodium on ozone-induced release of interleukin-8 (IL-8) by human bronchial epithelial cells, *in vitro*. Results are expressed as mean±SEM; n=6 at each time-point. ▨ : without nedocromil sodium; ▭ : with nedocromil sodium. ppb: parts per billion. **: p<0.01 *versus* 0 ppb ozone; ##: p<0.01 *versus* 0 M nedocromil sodium

of IL-8 was also noted from cell cultures exposed to 100 ppb O_3 (18.67±1.50 pg·µg^{-1} cellular protein; p<0.001), this was not found to be the case for cultures exposed to either 10 or 500 ppb O_3 (10.38±1.31 and 9.08±1.66 pg·µg^{-1} cellular protein, respectively). Treatment of the cells with 10^{-4}–10^{-7} M nedocromil sodium attenuated the 50 ppb O_3-induced release of IL-8 at all concentrations after 6 h exposure and was maximally active at concentrations 10^{-4}–10^{-5} M, decreasing the release of IL-8 by nearly 50% (fig. 3). On the basis of this finding, the effect of only a single concentration of 10^{-5} M nedocromil on the release of other mediators was investigated in further experiments.

Studies investigating the effect on release of GM-CSF from human bronchial epithelial cells indicated that this was significant at 50 ppb O_3 (0.20±0.04 pg·µg^{-1} cellular protein; p<0.01), compared with cells exposed for 6 h to air (0.04±0.03 pg·µg^{-1} cellular protein). This increase

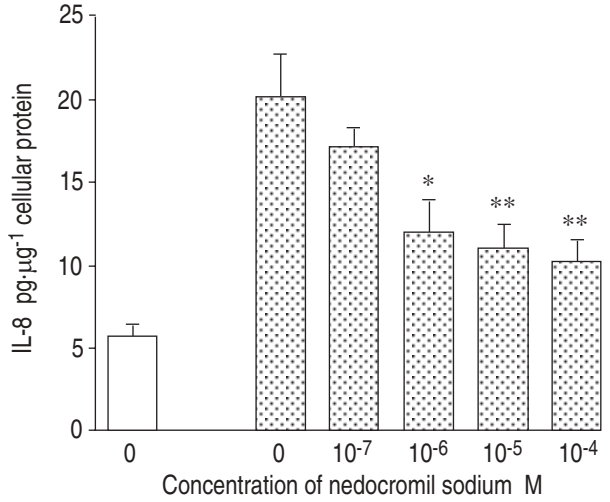

Fig. 3. – The effect of 10^{-7}–10^{-3} M nedocromil sodium on 50 parts per billion (ppb) O_3-induced release of interleukin-8 (IL-8) by human bronchial epithelial cells, *in vitro*. Results are expressed as mean± SEM; n=8 at each time-point. ▨ : 50 ppb O_3 exposure; ▭ : 0 ppb O_3 exposure. *: p<0.05; **: p<0.01 *versus* 0 M nedocromil sodium.

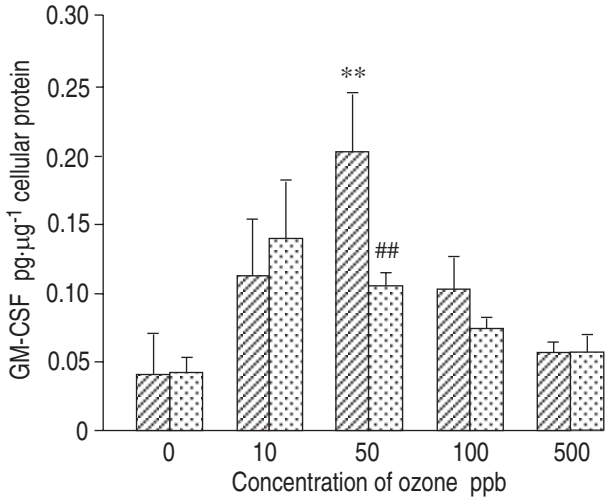

Fig. 4. – The effect of 10^{-5} M nedocromil sodium on ozone-induced release of granulocyte/macrophage colony-stimulating factor (GM-CSF) by human bronchial epithelial cells, *in vitro*. Results are expressed as mean±SEM; n=6 at each time-point. ▨ : without nedocromil sodium; ▭ : with nedocromil sodium. ppb: parts per billion. **: p<0.01 *versus* 0 ppb ozone; ##: p<0.01 *versus* 0 M nedocromil sodium.

in the release of GM-CSF at 50 ppb O_3 was significantly attenuated by treatment of the cells with 10^{-5} M nedocromil sodium (p<0.01). Exposure for 6 h to 10 and 100 ppb 0_3 also led to increased release of GM-CSF into the culture medium, although this was not found to be significant for either concentration of O_3 (fig. 4).

Analysis of TNF-α released from these cells into the culture medium demonstrated that this was also significantly increased from 0.06±0.05 pg·µg^{-1} cellular protein, in control cells exposed for 6 h to air, to 0.10± 0.01 pg·µg^{-1} cellular protein, in cultures exposed to either 10 or 50 ppb O_3 (p<0.01). Treatment of the cells with 10^{-5} M nedocromil sodium was shown to be effective in blocking both the 10 and 50 ppb O_3-induced release of TNF-α (p<0.05) (fig. 5).

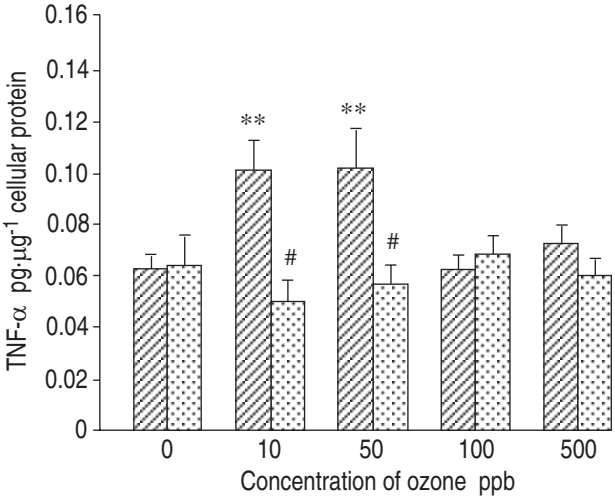

Fig. 5. – The effect of 10^{-5} M nedocromil sodium on ozone-induced release of tumour necrosis factor-α (TNF-α) by human bronchial epithelial cells, *in vitro*. Results are expressed as mean±SEM; n=6 at each time-point. ▨ : without nedocromil sodium; ▭ : with nedocromil sodium. ppb: parts per billion. **: p<0.01 *versus* 0 ppb ozone; #: p<0.05 *versus* 0 M nedocromil sodium.

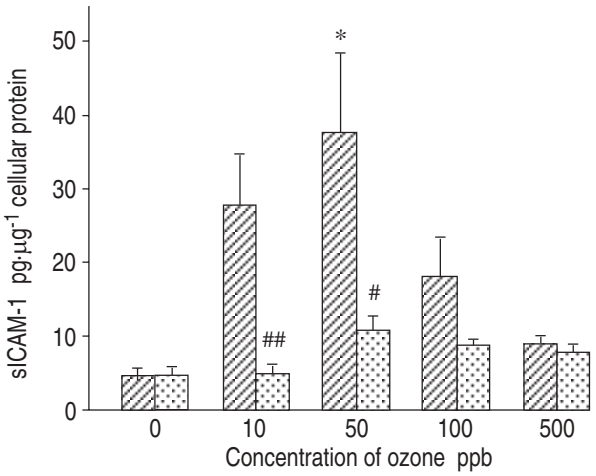

Fig. 6. – The effect of 10^{-5} M nedocromil sodium on ozone-induced release of soluble intercellular adhesion molecule-1 (sICAM-1) by human bronchial epithelial cells, *in vitro*. Results are expressed as mean±SEM; n=6 at each time-point. ▭ : without nedocromil sodium; ▭ : with nedocromil sodium. ppb: parts per billion. *: $p<0.05$ *versus* 0 ppb ozone; #: $p<0.05$, and ##: $p<0.01$ *versus* 0 M nedocromil sodium).

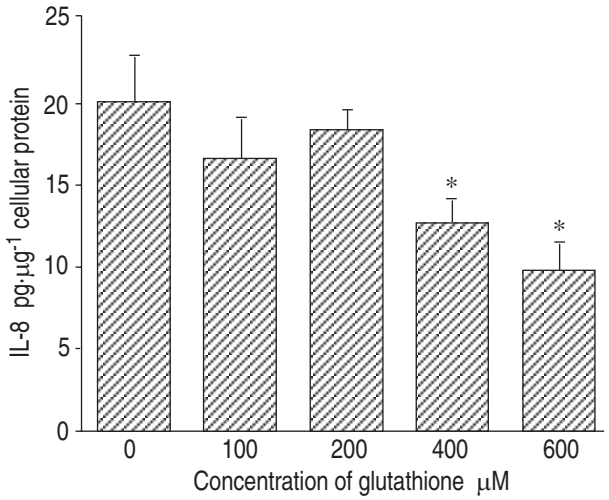

Fig. 8. – The effect of 100–600 µM glutathione on 50 ppb O_3 -induced release of interleukin-8 (IL-8) by human bronchial epithelial cells, *in vitro*. Results are expressed as mean±SEM; n=8 at each concentration. *: $p<0.05$ *versus* 0 µM glutathione.

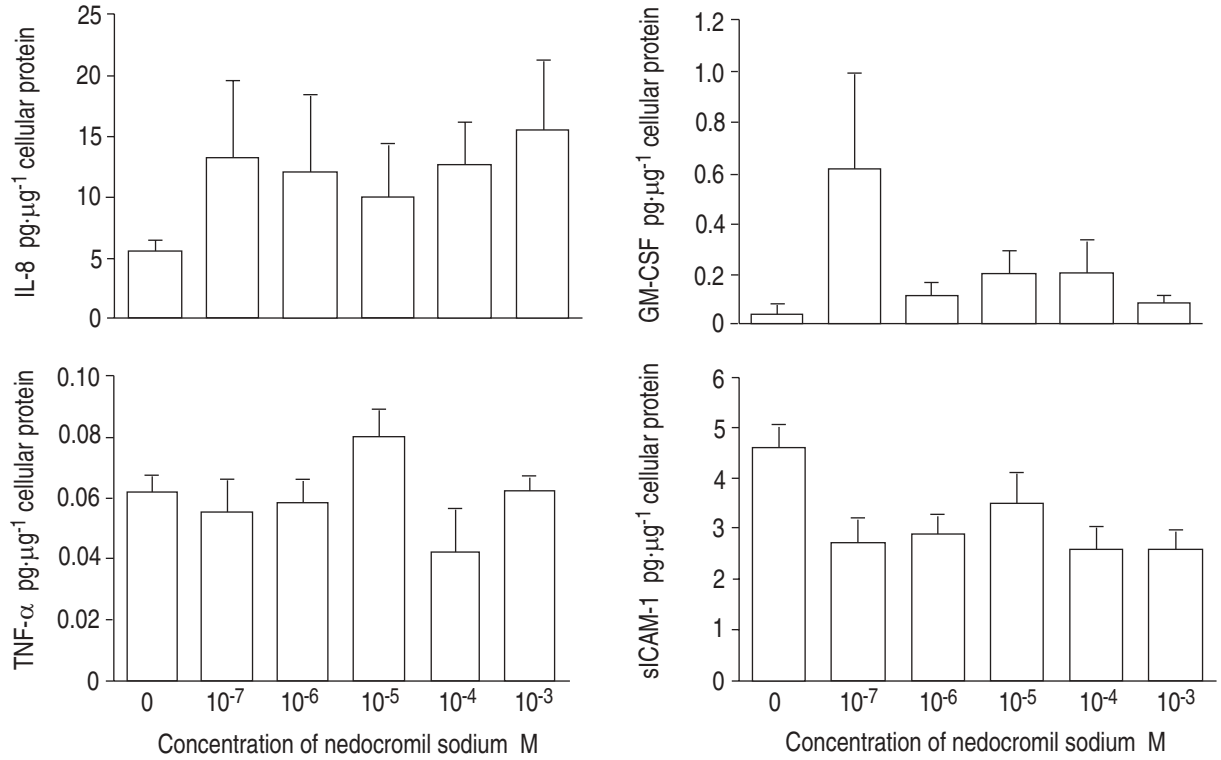

Fig. 7. – The effect of 10^{-7}–10^{-3} M nedocromil sodium on the constitutive release of IL-8, GM-CSF, TNF-α and sICAM-1 by human bronchial epithelial cells, *in vitro*. Results are expressed as mean±SEM; n=6 at each time-point. IL-8: interleukin-8; GM-CSF: granulocyte/macrophage colony-stimulating factor; TNF-α: tumour necrosis factor-α; sICAM: soluble intercellular adhesion molecule-1.

Analysis of the culture medium for the presence of sICAM-1 demonstrated that the release of this cell adhesion molecule was increased by exposure of human bronchial epithelial cells to 10–100 ppb O_3 and was maximal (increased eightfold) after exposure to 50 ppb O_3 (37.7±10.8 pg·µg^{-1} cellular protein; $p<0.05$), when compared to exposure to air for 6 h (4.53±0.45 pg·µg^{-1} cellular protein). 10^{-5} M nedocromil sodium was also shown to be effective in attenuating O_3-induced release of sICAM-1 from these cells (fig. 6).

Incubation of non-ozone-exposed human bronchial epithelial cells in the presence of 10^{-3}–10^{-7} M nedocromil sodium for 6 h did not significantly alter the constitutive release of IL-8, GM-CSF, TNF-α and sICAM-1 (fig. 7).

Incubation of human bronchial epithelial cells in the presence of glutathione, significantly decreased 50 ppb O_3-induced release of IL-8 from 20.16±2.56 pg·µg^{-1} cellular protein, in cells incubated in the absence of glutathione, to 12.95±1.25 ($p<0.05$) and 10.14±1.40 pg·µg^{-1}

cellular protein ($p<0.05$), in cultures incubated in the presence of 400 and 600 µM glutathione, respectively (fig. 8). Although incubation of cultures in the presence of 100 and 200 µM glutathione also led to decreased release of IL-8, this was not found to be significant (fig. 8).

Discussion

These studies have demonstrated that exposure of human bronchial epithelial cells for 6 h to 10–500 ppb O_3 leads to significant epithelial cell damage at all concentrations studied, and to significant release of IL-8, GM-CSF, TNF-α, and sICAM-1 at concentrations of 10–100 ppb O_3, *in vitro*. Additionally, these studies have demonstrated that mediator release from the cells can be significantly attenuated by treatment of the cells with 10^{-5} M nedocromil sodium and 400–600 µM glutathione. To our knowledge, this is the first report of the release of sICAM-1 by human bronchial epithelial cells and the inductive effect of O_3 on its release from these cells *in vitro*.

We have previously demonstrated that human bronchial epithelial cells cultured to confluence *in vitro* retain morphological and biochemical characteristics similar to those found *in vivo* [10, 14], and therefore render this model useful in the study of epithelial cell physiology. More recently, we have characterized the different epithelial phenotypes that constitute the cultures and have demonstrated that, of the total, approximately 0.5% are goblet cells, 40–45% are ciliated epithelial cells, 35–40% are glandular epithelial cells and 20% are basal epithelial cells [16].

Despite kinetic limitations and the lack of naturally occurring defence mechanisms, such as the presence of a "protective" epithelial lining fluid and endogenous "reducing" agents, which may limit the oxidant-induced cell damaging effect of O_3, cultured human airway epithelial cells offer a suitable *in vitro* model to study the effects of direct exposure to this agent and the mechanism(s) underlying these effects in epithelial cells.

Although a well-balanced human epithelial lining fluid would undoubtedly present the most optimal culture medium for use in such studies, such standardized preparations are not yet available commercially. Although a further limitation of the present study is that epithelial cells were cultured from surgical tissues of lung cancer patients with a history of smoking, and would possibly react differently to oxidant stress when compared to epithelial cells from healthy nonsmokers, the findings of these studies are nonetheless valuable since they give an indication of the mechanisms underlying O_3-induced airway inflammation and how anti-inflammatory agents, such as nedocromil sodium, may influence this inflammation.

The finding from the present studies that O_3 can lead to dose-dependent effects in human bronchial epithelial cells, however, is in agreement with the findings of others who have employed different epithelial cell models. Our studies have demonstrated that exposure to O_3 produces a bell-shaped response curve for the release of cytokines, and suggest that whilst O_3 may have stimulatory effects on cytokine release at lower concentrations, such effects become inhibitory at the higher concentrations. The demonstration that exposure to O_3 exhibited a linear dose-response curve for epithelial cell damage, as indicated by increased release of radiolabelled chromium from prelabelled cells, suggests that the inhibitory effect on cytokine release from these cells observed at the highest concentration of 500 ppb O_3 may be due to direct toxicity of this agent leading to: 1) detrimental effects at the cell membrane level; and 2) inhibition of the mechanisms upregulating the expression and release of the cytokines.

Similarly, DEVLIN *et al.* [17] have studied the effect of exposure to O_3 for 1 h, on BEAS 2B immortalized human airway epithelial cell line, and demonstrated that this led to a significant increase in the amount of interleukin-6 (IL-6), IL-8 and fibronectin (Fn) released by these cells. Furthermore, these authors demonstrated that all concentrations of O_3 (100–1,000 ppb) were significantly cytotoxic to these cells. DUMLER *et al.* [18], investigated the effects of exposure to O_3 using a primate epithelial cell model, and demonstrated that O_3 also produced dose-dependent cytotoxic effects in this model.

The present finding that O_3 is effective in inducing an inflammatory response, even at ambient concentrations of 50–100 ppb *in vitro*, also complements the findings of studies which have investigated the effect of exposure of human volunteers to O_3 at similar concentrations. DEVLIN and co-workers [8] have demonstrated that exposure of moderately exercising, healthy, nonsmoking individuals for 6.6 h to 80–100 ppb O_3 significantly increased the numbers of neutrophils, protein, IL-6, prostaglandin E_2 (PGE_2), fibronectin (Fn), and lactate dehydrogenase (LDH) in BAL fluid obtained from these individuals 18 h after exposure. Similarly, ARIS *et al.* [9] demonstrated that exposure of exercising, healthy subjects for 4 h to 200 ppb O_3 led to significant increases in neutrophils, protein, IL-8, GM-CSF, Fn, LDH and α_1-antitrypsin, in PAL and BAL fluids obtained 18 h after exposure. These authors observed that the number of neutrophils increased significantly in bronchial biopsy tissue obtained 18 h after exposure to O_3.

More recently, GWIZDALA *et al.* [19] demonstrated that, although there were no significant differences in spirometric values and symptom scores between asthmatics and healthy volunteers, after exposure for 6 h to 200 ppb O_3, there were significant increases in IL-6, IL-8 and polymorphonuclear neutrophil (PMN) numbers in BAL fluid obtained 24 h postexposure in asthmatics only. These studies suggest that O_3 may preferentially increase the production of inflammatory cytokines and inflammatory cell influx in asthmatics, possibly leading to an acute exacerbation at a later stage. Indeed, recent studies by MOLFINO *et al.* [20] have demonstrated that exposure of asthmatics to O_3 120 ppb for 1 h increased the airway response of these individuals to inhaled allergen, as indicated by a significant decrease (by approximately 50%) in the dose of allergen required to produce a 15% fall in forced expiratory volume in one second (FEV_1), when compared with exposure for 1 h to air.

Our finding that nedocromil sodium attenuates the release of inflammatory mediators from human bronchial epithelial cells *in vitro*, is in accordance with the

findings of others. MATTOLI and co-workers [21, 22] demonstrated that 10^{-5} M nedocromil sodium significantly decreased the release of GM-CSF and IL-8 from cultured human bronchial epithelial cells incubated with interleukin-1 (IL-1), but not from cells incubated in the absence of IL-1; in other words, constitutive release of this cytokine was not inhibited, as demonstrated in the present study.

Although several investigators have demonstrated that nedocromil sodium can inhibit the activation of different inflammatory cell types, including mast cells, eosinophils, monocytes, macrophages and platelets *in vitro* [23–27], the precise mode of action of this drug has only recently become clear. Studies have demonstrated that both sodium cromoglycate [28] and nedocromil sodium [29] block a chloride ion channel in rat basophil leukaemia cells, and have suggested that inhibition of this or a similar channel may inhibit mast cell activation, since influx of chloride ions into the mast cell is in part a prerequisite for calcium influx [30] and subsequent activation of this cell type. Furthermore, regulation of chloride channels, which are involved in cell volume change and thus inflammation [31], is also affected by nedocromil sodium [29, 32].

The findings of the present study suggest that nedocromil sodium may mediate its anti-inflammatory effects, at least in part, by affecting the synthesis and/or release of inflammatory cytokines, which directly or indirectly influence differentiation, growth and migration of inflammatory cells. It is possible that the inhibitory action of nedocromil sodium on the chloride ion channel may result in the abrogation of the initial stages of the signal transduction cascade essential for cytokine gene activation. Irrespective of what the specific mechanism is likely to be for the activity of nedocromil sodium, there is at present no information on the levels of nedocromil sodium *in vivo*. This lack of information has been due, in part, to technical difficulties in measurements resulting from the drug being applied topically into the upper and lower airways. Also, since the recommended daily dosage of nedocromil sodium for prophylaxis of asthma is 8–16 mg, the *in vivo* levels of this drug at the site of application are likely to be much higher than the concentration of 10^{-5} M nedocromil sodium investigated as the optimal concentration in these *in vitro* studies.

Our finding that glutathione is capable of reducing the O_3-induced release of IL-8 *in vitro* at concentrations of 400 and 600 µM, which are comparable to those found in the epithelial lining fluid *in vivo* [33], is both novel and important, since it suggests that the airway epithelial lining fluid is an important naturally occurring defence barrier, which acts to neutralize the oxidant stress from exposure to air pollutants, such as O_3 and NO_2, *in vivo*. Although it is possible that the susceptibility of the epithelial cells to O_3 *in vitro* may additionally be a consequence of natural degradation of glutathione under culture conditions, several studies have demonstrated that this is unlikely to be the case. CHEEK *et al.* [34] studied the stability of cellular anti-oxidant defence systems in rat alveolar type II cells cultured over a period of 4 days, and demonstrated that during culture the antioxidant enzyme activities and glutathione content of the cells were maintained at levels similar to those found in freshly isolated cells prior to culture. Similarly, SIMON *et al.* [35] have demonstrated that the glutathione-dependent antioxidant system is not altered in their rat alveolar epithelial cell culture system over a period of 7 days.

In summary, the results of these studies suggest that ambient concentrations of O_3, not normally found to be detrimental to health, may induce airway inflammation, through release of proinflammatory mediators from airway epithelial cells, an effect inhibited both by the anti-inflammatory drug, nedocromil sodium, and naturally occurring antioxidants, such as glutathione.

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