

Effects of TNF- α , IFN- γ and IL- β on normal human bronchial epithelial cells

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ABSTRACT: Several diseases affecting the airways such as asthma are associated with both epithelial damage and increased levels of pro-inflammatory cytokines. To investigate the possible relation between cytokines and epithelial damage, the effects of tumour necrosis factor- α (TNF- α), interferon gamma (IFN- γ) and interleukin-1 beta (IL-1 β) on normal human bronchial epithelial cells *in vitro* were studied.

The cells were exposed to these cytokines for 48 or 72 h, followed by morphological, immunohistochemical and metabolic studies.

Transmission and scanning electron microscopical analyses demonstrated damage to the mitochondria and an increase in cell processes induced by the cytokines. The use of antibodies against desmosomal cytokeratin showed a decrease in desmosome formation in IFN- γ -exposed cells. Decreased glucose oxidation rate and increased accumulation of nitric oxide were found in cytokine-exposed cells. N^o-monomethyl-L-arginine (L-NMMA) reduced nitrite production. X-ray microanalysis showed an increase in the intracellular sodium/potassium ratio of the cells after exposure to cytokines, which is an indication of cell damage. The cytokines induced both necrosis and apoptosis to varying degrees. IFN- γ and TNF- α generally potentiate each other's effects.

In conclusion tumour necrosis factor- α and interferon gamma, and to a lesser extent interleukin-1 β , can cause damage to epithelial cells, which may be a factor involved in epithelial shedding in airway diseases.

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Damage to the airway epithelium results in an imbalance of the chemical homeostasis and this plays an important role in the development of airway diseases [1]. Asthma and nasal allergy patients often lack smaller or sometimes even larger areas of the epithelium in their airways [2, 3]. In the airway walls of these patients, a significant accumulation of inflammatory cells, predominantly eosinophils and lymphocytes, is found. These cells in turn influence the pathogenesis of disease because they secrete or induce secretion of inflammatory mediators, *e.g.*, leukotriene C₄ and D₄ [4], interleukin (IL)-1, 3, 4, 5, 10 and 13, interferon-gamma (IFN- γ) and tumour necrosis factor- α (TNF- α) (reviewed in [5]).

Monocytes and macrophages, in asthma patients, have been shown to have an increased level of the inflammatory cytokine TNF- α [6, 7]. IFN- γ is secreted by T-lymphocytes and is known to inhibit cell proliferation [8, 9] and to enhance the cytotoxic effects of TNF- α [8]. IL-1 β is produced by macrophages, lymphocytes, and fibroblasts and elicits histamine release from mast cells at the site of inflammation (reviewed in [5]).

Release of inflammatory mediators from primary effector cells resident within the airways may in turn recruit and activate other mediators, thus amplifying the inflammation process. TNF- α , IFN- γ and IL-1 β are implicated in the pathogenesis of many diseases affecting the respiratory system such as asthma [10] and Sjögren's syndrome [8, 11].

TABIBZADEH *et al.* [12] have shown that TNF- α induces cell-cell dissociation of endometrial epithelial cells. If TNF- α can cause epithelial dissociation in the endometrium, it would appear possible that it does this by weakening the desmosomes, and that this in turn would cause epithelial shedding in the airway of patients suffering from asthma.

WU *et al.* [13] have reported that the destruction of salivary gland parenchyma in Sjögren's syndrome could be mediated by long-term exposure to elevated levels of cytokines such as IFN- γ and TNF- α *via* increased cell death processes. IFN- γ can also cooperate with apoptotic signalling pathways such as the Fas-APO-1 pathway to induce apoptosis of damaged epithelial cells [14].

The aim of this study therefore was to investigate whether, and by which mechanism, TNF- α , IFN- γ and IL-1 β can cause damage to airway epithelial cells. Possible mechanisms include necrosis, apoptosis and loss of cell-cell contacts. The effects of the cytokines on the structure of the cells were studied by transmission (TEM) and scanning electron microscopy (SEM), and desmosomes were, in addition, studied by immunocytochemistry at the light microscope level. Since the electron microscopical data indicated damage to the mitochondria, the functional aspects of the mitochondria were investigated through the use of glucose oxidation rate experiments. Nitrite measurements in the culture medium were performed to explore

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whether cytokines could induce an increase in nitric oxide production. Finally, X-ray microanalysis was used in order to reveal putative toxic effects of the cytokines.

Material and methods

Culture of epithelial cells

Normal human bronchial epithelial (NHBE) cells (Clonetics, San Diego, CA, USA) from a 16-yr-old female were used. Cell cultures were established at Clonetics Corporation's cell culture facility from normal human tissue. The cells were cultured in plastic culture flasks (Corning Costar Corporation, Cambridge, MA, USA) in bronchial epithelial basal medium (BEGM) (Clonetics), supplemented according to the manufacturer's instructions with recombinant human epidermal growth factor ($0.5 \mu\text{g}\cdot\text{mL}^{-1}$), insulin ($5 \text{ mg}\cdot\text{mL}^{-1}$), hydrocortisone ($0.5 \text{ mg}\cdot\text{mL}^{-1}$), transferrin ($10 \text{ mg}\cdot\text{mL}^{-1}$), adrenalin ($0.5 \text{ mg}\cdot\text{mL}^{-1}$), triiodothyronine ($6.5 \mu\text{g}\cdot\text{mL}^{-1}$), bovine pituitary extract ($13 \text{ mg}\cdot\text{mL}^{-1}$), retinoic acid ($0.1 \mu\text{g}\cdot\text{mL}^{-1}$), gentamicin ($50 \text{ mg}\cdot\text{mL}^{-1}$) and amphotericinB ($50 \text{ mg}\cdot\text{mL}^{-1}$) in a humidified atmosphere of 5% $\text{CO}_2/95\%$ air at 37°C . The culture medium was changed every 48 h. To achieve desmosome formation, confluent cells were cultured on Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY, USA) or in Petri dishes (Becton Dickinson, Plymouth, UK) in Dulbecco's modified Eagle's medium (DMEM): Ham's F12 (1:1) (Gibco BRL/Life Technologies, Paisley, UK) supplemented with 5% foetal bovine serum (Gibco BRL/Life Technologies), penicillin ($100 \text{ U}\cdot\text{mL}^{-1}$) (Sigma, St Louis, MO, USA), streptomycin ($100 \mu\text{g}\cdot\text{mL}^{-1}$) (Sigma) and 1% nonessential amino acids (Sigma).

Preparation of cultured cells for transmission electron microscopy

NHBE cells grown in Petri dishes in either BEGM or DMEM:F12 medium were fixed in 2.5% glutaraldehyde (Sigma) in 0.1 M cacodylate buffer (Agar Scientific, Stansted, UK) for 1 day. After being washed in 0.1 M cacodylate buffer, the cells were post-fixed in 1% OsO_4 in cacodylate buffer for 20 min. A second wash in buffer was followed by dehydration in graded series of ethanol, before the cells were finally embedded in Agar 100 Resin (Agar Scientific). Sections were cut and stained with 5% uranyl acetate/3% lead citrate and examined in a Hitachi H7100 transmission electron microscope (Hitachi, Tokyo, Japan) at 75 kV. The micrographs were judged by two observers.

Preparation of cultured cells for scanning electron microscopy

NHBE cells were grown and fixed as described above for TEM. After washing in 0.1 M cacodylate buffer, the cells were incubated with 2% guanidine hydrochloride and 2% tannic acid in distilled water for 8 h [15]. A second wash with distilled water was followed by a postfixation in 2% OsO_4 in distilled water for 15 h. The cells were again washed in distilled water and dehydrated in a graded series of ethanol. Subsequently, the cells were dried on coverslips by means of the critical point drying method. After drying, the coverslips were mounted on holders and examined in a LEO 1530 field emission scanning electron microscope (LEO, Cambridge, UK) at an accelerating voltage of 1 kV.

X-ray microanalysis

Cells were seeded out on 75-mesh titanium grids (Agar Scientific). The grids had been covered with a Formvar film (Merck, Darmstadt, Germany) and a thin carbon layer, and sterilized using ultraviolet light. The cells were allowed to grow in an incubator with a 5% $\text{CO}_2/95\%$ air atmosphere at 37°C [16].

After 2–3 days of culture, some of the grids were exposed to recombinant human cytokines, *i.e.* TNF- α ($5,000 \text{ U}\cdot\text{mL}^{-1}$), IL-1 β ($150 \text{ U}\cdot\text{mL}^{-1}$) or IFN- γ ($1,000 \text{ U}\cdot\text{mL}^{-1}$) (all from PeproTech, Rocky Hill, NJ, USA); unexposed cells served as controls. The concentrations of cytokines-used were based on previous experiments using other epithelial cells [17]. After 48 h of culture, the experiment was terminated *via* a quick rinse in distilled water (4°C) and the cells frozen in liquid nitrogen-cooled liquid propane (-180°C) and then freeze-dried under vacuum overnight at -130°C . Finally, the freeze-dried specimens were coated with a conductive carbon layer before analysis.

X-ray microanalysis was performed at 100 kV in the scanning-transmission electron microscopy mode of a Hitachi H7100 with an Oxford Instruments ISIS energy-dispersive spectrometer system (Oxford Instruments, Oxford, UK). Quantitative analysis was carried out based on the peak-to-continuum after correction for extraneous background [18] and by comparing the spectra from the cells with those from a standard. Spectra were acquired for 100 s and only one spectrum was obtained from each cell.

Immunohistochemistry

Confluent NHBE cells grown on glass slides in DMEM:F12 medium were fixed in methanol for 3 min at -20°C , and rinsed with tris-hydroxymethyl-amino methane (Tris)-buffered saline (TBS; 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.6) for 5 min. The cells were blocked using 10% normal human serum (NHS) (Sigma) in TBS for 30 min. After primary incubation with monoclonal anti-desmosomal cytokeratin (Sigma, catalogue No. C-1041) 1:1500 in TBS for 1 h at 37°C , the cells were rinsed twice with TBS. Thereafter, cells were blocked with 10% NHS for 30 min and incubated with a biotinylated secondary antibody (Dako, Copenhagen, Denmark) for 1 h. After rinsing with TBS, Oregon green-conjugated streptavidin (Molecular Probes, Eugene, OR, USA) was bound to the biotinylated secondary antibody. The specimens were mounted in Vectashield (Vector, Burlingame, CA, USA) mounting medium for fluorescence and examined using a Nikon eclipse E800 microscope (Nikon, Tokyo, Japan). Control slides were processed identically except that the primary antibody was omitted.

Glucose oxidation rate

The glucose oxidation rate was determined as previously described in detail [19]. Briefly, triplicate cell samples were placed in glass vials containing Krebs-Ringer's buffer with uniformly labelled D-U- ^{14}C -glucose (Amersham International, Amersham, UK) and 16.7 mM nonradioactive glucose. A rubber membrane was attached

to the shaft of the vial and the vial was placed into a 20-mL glass scintillation vial. The vials were gassed with CO₂:O₂ (5:95) and the membrane was sealed with a plastic lid. The cells were incubated for 90 min at 37°C. Glucose oxidation was terminated by the injection of 100 µL of 0.05 mM antimycin A (Sigma). By injection of 100 µL 0.4 M Na₂HPO₄ (pH 6.0), the ¹⁴CO₂, formed by cell metabolism, was released to the incubation medium and trapped in 250 µL Hyamine 10-X (Packard Instruments, Meriden, CT, USA) during a further 2 h incubation at 37°C. Finally, 5 mL of Ultima Gold (Packard Instruments) were added to the scintillation vials and the radioactivity measured in a liquid scintillator.

Deoxyribonucleic acid measurement

Sixty microlitres of cell suspension was diluted in 90 µL redistilled water and sonicated. Duplicate 50 µL aliquots/sample were placed in the wells of white plastic microtitre plates and prefrozen at -20°C. The samples on the microtitre plate were dried overnight at 56°C. Fifty microlitres of 17.8 mM 3,5-diaminobenzoic acid dihydrochloride solution, which binds the deoxyribose in the deoxyribonucleic acid (DNA), was added. The samples were incubated for 45 min at 60°C and, finally, 200 µL 1 M HCl was added to neutralize the pH. DNA was measured using a fluorophotometer (Perkin-Elmer LS-5B Luminescence Spectrometer; Perkin Elmer, Beaconsfield, UK) at 405 nm (excitation wavelength) and 520 nm (emission wavelength) [19, 20].

Cell viability

Cell viability was estimated by staining the cells with trypan blue (Sigma), and the fraction of necrotic cells (nuclei stained blue) was calculated using a light microscope.

Detection of DNA fragmentation was used as a marker of apoptosis. The terminal deoxyribonucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labelling (TUNEL) method [21] was used in conjunction with haematoxylin staining. Cells were grown to 90% confluence on eight-well sterile glass slides (Lab-Tek) and induced with cytokines (but not IL-1β), as above, for 72 h. The supernatant was then decanted and the remaining cells washed with ice-cold phosphate-buffered saline (PBS). Ice-cold methanol was used to fix the cells for 20 min. The cells were then rinsed again with PBS. To prepare the cells for the TUNEL assay, they were incubated with 20 µg·mL⁻¹ proteinase K for 10 min, and washed again. The cells were then labelled using the *in situ* Cell Death Detection Kit, POD (Roche Diagnostics Scandinavia, Bromma, Sweden), according to the manufacturer's suggested protocol. The cells were then briefly counterstained with haematoxylin and mounted using glycerol gelatin. For each experiment, the number of positive cells per 1,000 were determined.

Nitrite measurements

Measurements of NO₂⁻ in NHBE cell culture supernatants using the Griess reagent [22] were taken as an indicator of NO generation. To measure nitrite accumula-

tion, 10 µL of Griess reagent (equal volumes of 1% naphthylethylene diamine dihydrochloride in distilled water and a mixture of 10% sulphanilamide and 50% concentrated H₃PO₄) was added to triplicate aliquots of the cell culture medium (100 µL). The absorbance of the reaction product was measured at 546 nm. The final nitrite concentration was obtained by comparison with a standard curve obtained using sodium nitrite.

Statistical analysis

Data are presented as mean±SEM, and statistical analysis was performed using a paired or unpaired Student's t-test, or Wilcoxon's signed-rank test when the distribution of data was not normal. Significance was attributed to probability values <0.05. When experiments were performed in triplicate (glucose oxidation and nitrite measurements), the mean of the triplicate experiment was calculated and considered as one separate observation.

Results

Light microscopic morphology

NHBE cells grown as a monolayer in BEGM medium were exposed to 1,000 U·mL⁻¹ TNF-α, 150 U·mL⁻¹ IL-1β or 1000 U·mL⁻¹ IFN-γ for 3 days. Control cells had a polygonal shape and attained confluence in a few days (fig. 1a). NHBE cells stimulated with TNF-α had a rounder shape and did not always grow to confluence (fig. 1b). Some experiments were also carried out in DMEM:F12 medium. In this medium, the cells ceased spreading and started to grow in multilayers. When the cytokines were added to cells grown in DMEM:F12 medium, the cells were already more confluent than in BEGM medium, and their growth was not noticeably affected by TNF-α.

IFN-γ-exposed NHBE cells were capable of growing to confluence in BEGM, but showed marked morphological changes (fig. 1c). Many cells had lost their polygonal shape and appeared as drawn-out cells with long extensions. IL-1β-treated cells did not show any morphological changes, and no clear changes in growth rate and viability were seen in either of the culture media, compared to the controls. When the cells were exposed to the combination of IFN-γ and TNF-α the extensions were thinner and appeared more fragile compared to those observed with treatment with IFN-γ alone.

Transmission electron microscopy

The cells grown in BEGM were flat, with a small dome-shaped elevation containing the nucleus, and contained cytoplasmic vacuoles. Cells grown in DMEM:F12 medium were thinner and lacked vacuoles. The mitochondria of the control cells were normal and compact (fig. 1d). In all cytokine-stimulated cells, the mitochondria were swollen and had lost cristae (fig. 1e). The effect of IFN-γ was less pronounced than that of TNF-α, and the effect of IL-1β on the mitochondria was least pronounced.

In control cells, relatively few keratin filament bundles were present (fig. 1d). TNF-α, and to a lesser extent IFN-γ, caused an increase in the number of keratin filament

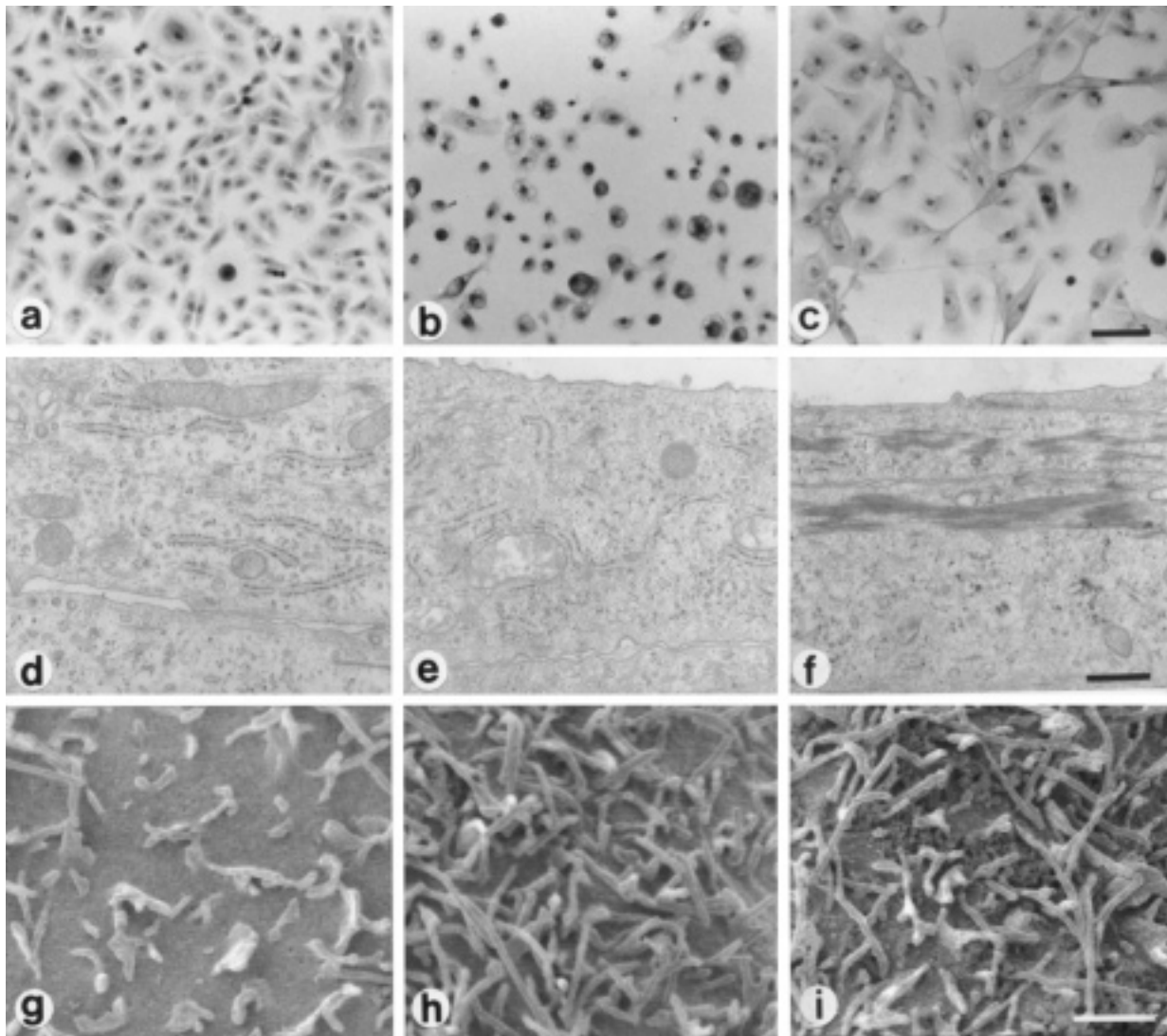


Fig. 1. – a–c: Light micrographs of normal human bronchial epithelial (NHBE) cells grown in bronchial epithelial basal medium (BEGM): a) control cells have a polygonal shape; b) tumour necrosis factor- α (TNF- α)-exposed cells have a rounder shape; and c) interferon gamma (IFN- γ)-exposed cells have a drawn-out shape with long extensions. d–f: Transmission electron micrographs of NHBE cells grown in Dulbecco's modified Eagle's medium (DMEM); Ham's F12 medium: d) intact and compact mitochondria in control cells; e) mitochondria in TNF- α -exposed cells are damaged and lack cristae; and f) increased keratin formation in TNF- α -exposed cells. g–i) Scanning electron micrographs of NHBE cells grown in BEGM medium: g) control cells with cell processes; h) TNF- α -exposed cells have numerous cell processes; and i) part of the population of IFN- γ -exposed cells has an increased number of cell processes. (Internal scale bars=100 μ m (a–c); 0.5 μ m (d–f); 2 μ m (g–i).)

bundles (fig. 1f). Bleb formation was found in IFN- γ -stimulated cells, but not in cells exposed to TNF- α or IL-1 β (not shown). The overall impression given by the cells exposed to TNF- α , and to some extent also to IFN- γ , was that a major change had taken place in the organization of the cells. There were no apparent morphological changes in the IL-1 β -stimulated cells compared with the control, except for the noted changes in the mitochondria. The effects of the cytokines were independent of the growth medium used.

Scanning electron microscopy

Control cells grown in BEGM were flat and they were sparsely but evenly covered with cell processes (fig. 1g). TNF- α -stimulated cells were round and showed more cell processes (fig. 1h). When cells were stimulated with IFN-

γ , two morphologically different populations of cells developed. One cell population had a long drawn-out shape with long extensions, and the other population had a round shape with no extensions. The drawn-out cells had a normal number of cell processes (not shown), but the round cells had an increased number of cell processes (fig. 1i). IL-1 β -treated cells had a morphology similar to that of the control cells.

Immunohistochemistry

Confluent monolayers of NHBE cells were established in BEGM with a low calcium concentration. In this medium, there was little or no cell/cell contact and hence no desmosomes. The DMEM:F12-medium has a high calcium concentration and this leads to rapid induction of cell/cell contact and desmosome formation [23].

In the absence of cell-cell contacts, immunocytochemical staining for desmosomal cytokeratin gave rise to spot-like staining in the cytoplasm, but no staining associated with the cell boundaries. When the serum-containing medium induced cell-cell contact, the spot-like staining shifted to the plasma membrane, within approximately 24 h.

In the control cells there was a continuous row of desmosomes lining the cell membrane (fig. 2a). IFN- γ ($1,000 \text{ U}\cdot\text{mL}^{-1}$) caused a marked reduction in the number of desmosomes. In some areas of the plasma membrane, a total loss of desmosomes was observed. The TNF- α and IL-1 β -treated cells did not show any major changes in the distribution of desmosomes. A combination of TNF- α ($5000 \text{ U}\cdot\text{mL}^{-1}$) and IFN- γ ($1,000 \text{ U}\cdot\text{mL}^{-1}$) reduced the number of desmosomes more than did IFN- γ alone (fig. 2b).

X-ray microanalysis

There was a significant increase in intracellular sodium and chlorine concentrations after TNF- α stimulation (fig. 3). The intracellular sulphur concentration was also increased, but the potassium concentration was not significantly affected. IFN- γ -exposed cells showed a significant decrease in magnesium, phosphorus and potassium concentration, whereas the chlorine concentration increased. IL-1 β -exposed cells showed a decrease in magnesium and phosphorus, whereas the chlorine concentration increased. The concentration of the other elements measured did not change significantly.

The mean elemental composition of the control cells (in $\text{mmol}\cdot\text{kg dry weight}^{-1}$) is given in table 1. Because the elemental content of the control cells varied somewhat between experiments, data on the cytokine effects are given as a percentage of the control value in each experiment. The (total) calcium concentration of the cultured cells was generally below the detection limit of the instrument.

Glucose oxidation rate

In NHBE cells cultured for 72 h in the presence of $1,000 \text{ U}\cdot\text{mL}^{-1}$ TNF- α , the glucose oxidation rate at 16.7 mM

glucose had decreased significantly compared to the control (fig. 4). There was also a significant decrease in the oxidation rate when the cells were treated with $1,000 \text{ U}\cdot\text{mL}^{-1}$ IFN- γ . When TNF- α was added together with IFN- γ , the suppressive effects of the two cytokines were potentiated. IL-1 β was not tested due to its minor effect on cell morphology.

Cell viability and deoxyribonucleic acid content

The viability of the cells, as determined by trypan blue exclusion, was not significantly affected by treatment with $1,000 \text{ U}\cdot\text{mL}^{-1}$ TNF- α or $1,000 \text{ U}\cdot\text{mL}^{-1}$ IFN- γ alone, but, when added in combination, the two cytokines increased the number of necrotic cells by $\sim 20\%$ (not shown). Induction of apoptosis by TNF- α and IFN- γ was determined by the TUNEL method. TNF- α or a combination of TNF- α and IFN- γ increased the number of apoptotic cells by 30%, whereas IFN- γ alone caused an increase of $\sim 15\%$. There was no significant difference between the DNA content of the surviving cells in any of the groups (not shown).

Nitrite measurements

In cells cultured for 72 h in the presence of $1,000 \text{ U}\cdot\text{mL}^{-1}$ TNF- α , nitrite production did not change significantly compared to control cells. There was a two-fold increase in nitrite production when the cells were exposed to $1,000 \text{ U}\cdot\text{mL}^{-1}$ IFN- γ . However, when the cells were exposed to both TNF- α and IFN- γ , there was a five-fold increase in medium nitrite as compared to control cells (fig. 5). Treatment with the nitric oxide synthase inhibitor N^{G} -monomethyl-L-arginine decreased nitrite production by 50% in IFN- γ -exposed cells and in IFN- γ and TNF- α -exposed cells (in both cases, $p < 0.05$). In all other experimental groups, the effect of the inhibitor was not significant.

Discussion

Morphological studies were undertaken to determine the cytotoxic effects of TNF- α , IFN- γ and IL-1 β on NHBE

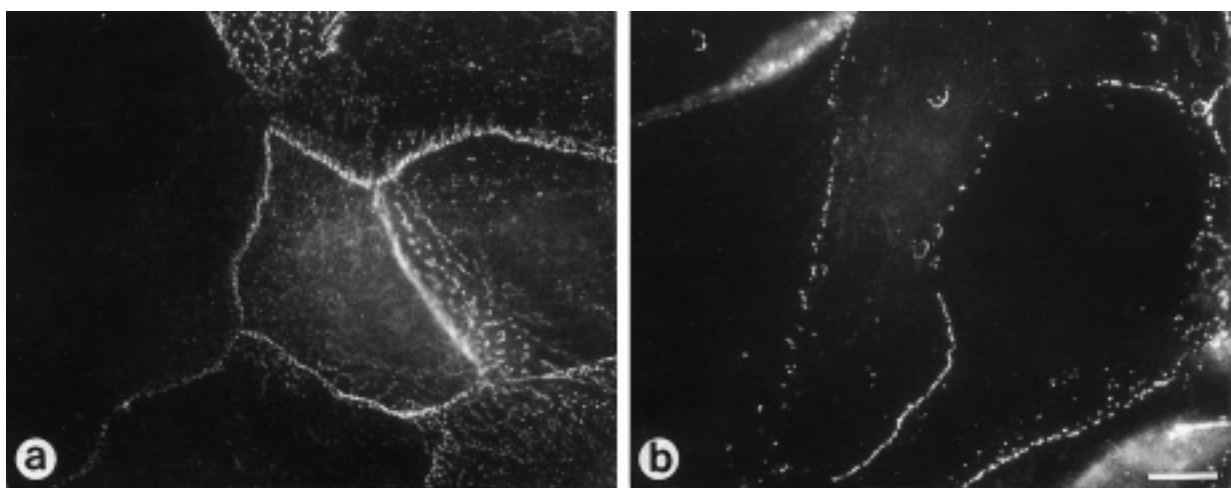


Fig. 2. – Desmosome formation in normal human bronchial epithelial cells grown in Dulbecco's modified Eagle's medium Ham's F12 medium: a) control cells with many desmosomes; and b) interferon gamma and tumour necrosis factor- α -exposed cells showing a decrease in the number of desmosomes. (Internal scale bar= $30 \mu\text{m}$.)

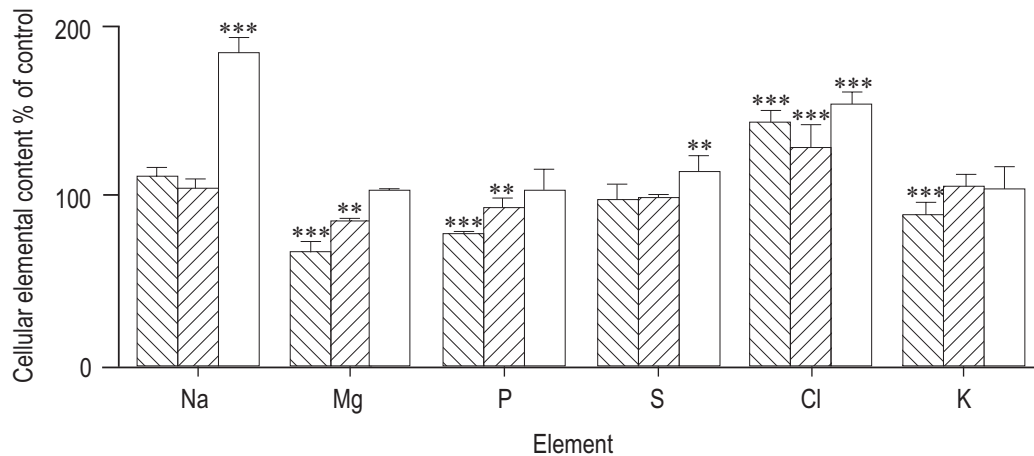


Fig. 3. – Effects of 1,000 U·mL⁻¹ interferon gamma (IFN- γ ; ▨); 150 U·mL⁻¹ interleukin-1 β (▧) and 5,000 U·mL⁻¹ tumour necrosis factor- α (TNF- α ; □) on the cellular elemental content of normal human bronchial epithelial cells. (Control values are shown in table 1.) Data are presented as mean \pm SEM and are based on four experiments. In each experiment, ~20 cells were analysed. **: p<0.01; ***: p<0.001, compared to the control.

cells. A common effect of the cytokines was a change in the structure of the mitochondria, namely swelling. Earlier studies have shown that TNF- α has an effect on mitochondrial structure, mainly swelling and disappearance of cristae in L929 cells [24] and in human thyrocytes [25]. The normal electron flow in the mitochondria of different cell lines, such as L929, PSC3H, C3HA, LM and WEHI 164 cells, is affected by TNF- α [24, 26, 27]. This may trigger the production of oxygen radicals, which might be implicated in the cytotoxic effects of TNF- α . The present data confirm that, in addition to the ultrastructure of the mitochondria being affected, their function is affected by TNF- α and IFN- γ . The significant decrease in glucose oxidation rate indicates a disturbance of mitochondrial glucose metabolism. The ability of NHBE cells to oxidize glucose decreases even more when TNF- α and IFN- γ are added together. Impairment of mitochondrial function may reduce production of adenosine triphosphate (ATP). This in turn might decrease ciliary activity in bronchial epithelial cells and impair transport of mucus.

The increased amount of keratin in TNF- α and IFN- γ -treated cells indicates that cytokines can affect the cytoskeleton. Since keratin filaments are coupled to desmosomes, this effect could be important for cell-cell contacts. The present data disagree, however, with those of ASAKAWA *et al.* [28] who found that neither the content nor the distribution pattern of keratin in mouse thyroid cells was affected by IFN- γ .

An increased number of cell processes as observed on SEM, and also on TEM, may point to an additional effect

Table 1. – The elemental content of cultured normal human bronchial epithelial cells (control).

Element	Concentration (mmol·kg dry weight ⁻¹)
Sodium	88 \pm 4
Magnesium	20 \pm 1
Phosphorous	416 \pm 8
Sulphur	232 \pm 7
Chlorine	132 \pm 4
Potassium	553 \pm 13

Data are presented as mean \pm SEM and are based on four independent experiments, in which a total of 140 cells were analysed.

of cytokines on the cytoskeleton, namely on actin filaments. ANDERSON *et al.* [29] have similarly reported that a microglial cell culture treated with IFN- γ and TNF- α showed an increased number of cell processes.

IFN- γ treatment induced a marked decrease in the number of desmosomes. This could be related to the loss of epithelial cells in the airways of patients with asthma or Sjögren's syndrome, in which IFN- γ levels are increased [30–32]. Although TNF- α has effects on cell structure, it does not seem to affect desmosome formation, and nor does IL-1 β . However, the combination of TNF- α and IFN- γ has a greater effect on desmosome formation than does IFN- γ alone. This potentiating effect of TNF- α may be explained by the fact that TNF- α can upregulate the number of IFN- γ receptors on the cell, which makes the cells more sensitive to IFN- γ [9, 13]. TNF- α has also been shown to enhance the recruitment of intracellular IFN- γ receptors to the cell surface [9], IFN- γ and TNF- α may also affect other cell contacts such as tight junctions [33].

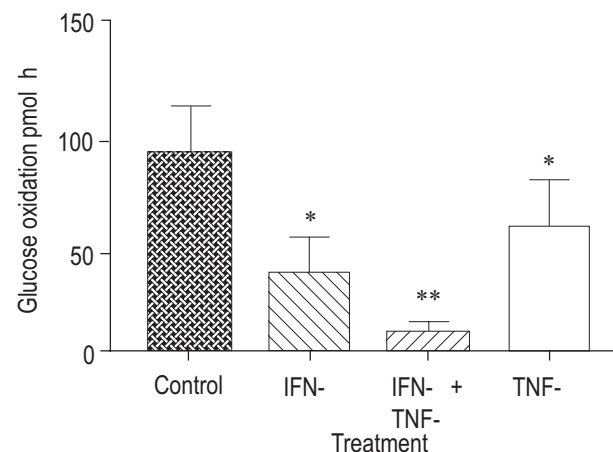


Fig. 4. – Glucose oxidation in normal human bronchial epithelial cells (10⁶) exposed to 1,000 U·mL⁻¹ interferon gamma (IFN- γ), 1,000 U·mL⁻¹ tumour necrosis factor- α (TNF- α) and a combination of 1,000 U·mL⁻¹ IFN- γ and 1,000 U·mL⁻¹ TNF- α for 90 min. Data are presented as mean \pm SEM of four–six experiments. *: p<0.05; **: p<0.01, compared to control.

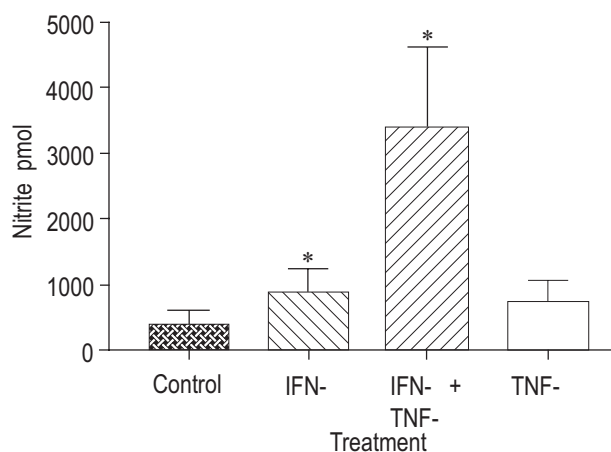


Fig. 5. – Nitrite accumulation in the medium of normal human bronchial epithelial cells (10^6) exposed to $1,000 \text{ U}\cdot\text{mL}^{-1}$ interferon gamma (IFN- γ), $1,000 \text{ U}\cdot\text{mL}^{-1}$ tumour necrosis factor- α (TNF- α) and a combination of $1,000 \text{ U}\cdot\text{mL}^{-1}$ IFN- γ and $1,000 \text{ U}\cdot\text{mL}^{-1}$ TNF- α for 72h. Data are presented as mean \pm SEM of five–eight experiments. *: $p < 0.05$; compared to control.

A possible method of determining whether TNF- α , IFN- γ or IL-1 β are toxic to the cells is to analyse the elemental content of the cells by means of X-ray microanalysis. Changes in the elemental concentrations may indicate a change in the physiological condition of the cells. Both IFN- γ and IL-1 β caused a decrease in the magnesium and phosphorous content. The decrease in phosphorous concentration points to a decrease in cellular phosphate, either bound (e.g. DNA or ribonucleic acid, phospholipids) or soluble (e.g. ATP). The present data show, however, that DNA content per cell is not affected by cytokine treatment. Damage to the mitochondria, as observed morphologically and *via* glucose oxidation rate measurements, may decrease production of ATP. Mg^{2+} is, together with K^+ , the preferred counterion for phosphate groups and a decrease in cellular phosphorous will also affect the concentrations of these ions. TNF- α caused an increase in the cellular sulphur content, which may be related to the increase in keratin. All cytokines caused a significant increase in the cellular chlorine concentration, and an increase in the intracellular sodium/potassium ratio, which is an indication of cell damage, in the order TNF- α >IFN- γ >IL-1 β . Despite the fact that a high activity of IL-1 β was used, the effects of this cytokine were less pronounced than those of the other two.

The cytokine-induced NO production may be a factor contributing to the observed inhibitory effects on cellular functions. Indeed, cytokine-induced NO may cause inhibition of glucose metabolism *via* inactivation of key mitochondrial enzymes in other cell types [34]. NO can also affect the epithelium by reacting with superoxide anions generated by inflammatory cells in the airways to form peroxynitrite ions. These ions may have toxic effects directly as well as through the generation of toxic hydroxyl radicals [35].

Necrotic and apoptotic cell death were investigated using trypan blue dye exclusion and TUNEL, respectively. The combination of TNF- α and IFN- γ caused a significant increase in necrotic cell death. This confirms again that TNF- α and IFN- γ potentiate each other's effects. The

TUNEL method showed a significant increase in apoptotic cell death in all three treatment groups used. These results show that cytokine-exposed cells undergo apoptosis to a greater extent than necrosis. TNF- α and IFN- γ are known inducers of apoptosis [14]. The combination of TNF- α and IFN- γ causes such a large insult that a sizeable proportion of the cells appears to undergo necrosis rather than apoptosis.

Epithelial damage and shedding of the airway epithelium are important features in a number of diseases such as asthma, nasal allergy and Sjögren's syndrome, and may in part be a consequence of increased levels of inflammatory mediators such as tumour necrosis factor- α and interferon gamma and, to a lesser extent, interleukin-1 β . This suggests that inhibition of the production of certain cytokines should be considered in new therapeutic attempts for the treatment of these diseases.

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