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Bacterial-induced release of inflammatory mediators by bronchial epithelial cells

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Bacterial-induced release of inflammatory mediators by bronchial epithelial cells. O.A. Khair, R.J. Davies, J.L. Devalia. ©ERS Journals Ltd 1996.

ABSTRACT: This review focuses on bacterial induction and release of inflammatory cytokines and adhesion molecules by human bronchial epithelial cells, with special reference to *Haemophilus influenzae*, a pathogen commonly associated with chronic bronchitis. Studies investigating the mechanisms underlying bacterial colonization of the airways and bacterial-induced chronic airway inflammation have suggested that these are likely to involve localization of bacteria to the site(s) of infection in the respiratory tract and induction of a local airway inflammation resulting in the initiation of epithelial damage.

We have hypothesized that the gross airway epithelial damage observed in chronic infective lung disease is an indirect consequence of proteolytic enzymes and toxic oxygen radicals generated by large numbers of neutrophils infiltrating the airways. Furthermore, the infiltration and activation of the neutrophils is a consequence of increased release of proinflammatory mediators from the host respiratory epithelium, induced by bacterial products, such as endotoxin. This hypothesis is based on studies which have demonstrated that the concentrations of circulating cytokines, such as interleukin (IL)-8 and tumour necrosis factor-α (TNF-α), which have profound effects on neutrophil activity, are increased in endotoxaemia and that airway epithelial cells are a rich source of these cytokines.

Support for this hypothesis is provided by studies of cultured human bronchial epithelial cells incubated either in the absence or presence of purified endotoxin preparations from nontypable and type b *H. influenzae* strains which have demonstrated that these endotoxins lead to significantly increased expression and/or release of proinflammatory mediators, including IL-6, IL-8, TNF-α and intercellular adhesion molecule-1 (ICAM-1). Treatment of the cells with steroids can downregulate the expression and/or release of these inflammatory mediators. Additionally, these studies have demonstrated that culture medium collected from endotoxin-treated cultures, 24 h after treatment, significantly increases neutrophil chemotaxis and adhesion to human endothelial cells *in vitro*.

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Clinical studies of patients with chronic bronchitis have shown that many exacerbations of the disease in these patients are associated with bacterial infection, with nonencapsulated *Haemophilus influenzae* being one of the most commonly isolated organisms [1]. In contrast, studies of septicaemia, pneumonia and systemic *H. influenzae* infections in children have suggested that these are the most common clinical manifestations of *H. influenzae* type b [2, 3].

Several studies have established that nonencapsulated *H. influenzae* is present in the respiratory tract of the majority of patients with chronic bronchitis, and can be recovered occasionally from serial sputum cultures from virtually all chronic bronchitics [4]. Other studies have suggested that there is an association between purulence and the presence of nonencapsulated *H. influenzae* in sputum cultures, and that these bacteria can be isolated from the sputum more often during exacerbations than during symptom-free periods [5, 6]. However, since these bacteria can be isolated from the sputum of chronic bronchitics during remission [7] and have also been shown to be common inhabitants of the normal human upper respiratory tract of up to 80% of healthy adults [1], their role in the pathogenesis of chronic bronchitis is not entirely clear.

The role of bacterial infection in chronic airway inflammation

Studies of pulmonary infections have demonstrated that there is a close relationship between bacterial load and neutrophil recruitment [8], and have suggested that neutrophils play an important role in the pathogenesis of chronic lung diseases, due to their ability to release a variety of oxidants and proteolytic enzymes capable of causing acute and chronic lung injury [9]. Smallman *et al.* [10] have shown that the sol phase of purulent sputum, containing free elastolytic activity, caused gradual slowing of human ciliary beat frequency *in vitro*, and

that this attenuation in ciliary beat frequency was prevented by the prior addition of α-antiproteinase to the sputum.

Furthermore, some studies have demonstrated that bronchial secretions may also contain bacterial toxins, which can cause epithelial necrosis and disrupt ciliary ultrastructure [11]. Studies of bacterial lipopolysaccharide (LPS) have demonstrated that this is a major component of the outer membrane of Gram-negative bacteria, responsible for toxic manifestations of severe Gram-negative infections and generalized inflammation [12]. Animal studies have suggested that the effects of endotoxin may be mediated *via* proinflammatory cytokines such as interleukin (IL)-6, tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), since neutralizing antibodies to these cytokines protected against the lethality of *Escherichia coli* endotoxin injected into the animals [13, 14]. Indeed, studies in septic shock patients have demonstrated that the concentrations of circulating TNF-α were raised for several days in these patients and were associated with poor outcome, whilst stable or falling concentrations correlated with survival [15, 16]. Similarly, other studies have demonstrated that soluble receptors for TNF-α are also increased in clinical sepsis and experimental *E. coli* endotoxaemia, and suggest that this may be a natural host defence mechanism, in which the soluble receptors would bind the free circulating TNF-α and thereby prevent or attenuate the biological activity of this cytokine [17, 18]. More recently, VAN DEVENTER *et al* [19] have demonstrated that injection of human volunteers with low doses of *E. coli* endotoxin led to increased concentrations of IL-8 in the blood, 90 min after injection. Similarly, other studies of human volunteers challenged with purified endotoxin have shown that doses of 2–4 $ng \cdot kg^{-1}$ endotoxin led to maximal increase in plasma concentrations of TNF, 90–120 min after challenge [20]. More recently, SANDSTRÖM *et al.* [21] have demonstrated that administration, by aerosol, of 25 µg *E. coli* endotoxin into the lungs of healthy nonsmoking subjects led to nearly a 100 fold increase in neutrophils and a threefold increase in lymphocytes in bronchoalveolar lavage (BAL), collected from these individuals 3 h after endotoxin challenge [21]. Endotoxin treatment did not, however, affect the numbers of macrophages or mast cells in the BAL of these subjects (table 1). Collectively, these results suggest that the neutrophilic response observed in response to endotoxin challenge may be of greatest relevance in bacterial infections and possibly a consequence of endotoxin-induced increase in the concentrations of IL-8 and TNF-α.

The putative mechanisms of bacterial-induced chronic airway inflammation

Studies in healthy individuals, however, have demonstrated that the initial interaction of inhaled bacteria occurs with mucus within the airway mucosa, and that local defences, such as the mucociliary system and local antibodies, clear these out efficiently [22]. READ *et al.* [23] have hypothesized that, in individuals whose primary airway defences are compromised (*i.e.* impaired mucociliary clearance and increased mucus production), a "vicious circle of events" results in chronic airway inflammation and damage as a consequence of the host-mediated response to bacterial infections. These authors have speculated that in cigarette smokers, patients with chronic bronchitis or bronchiectasis and those with recent viral infections, bacteria such as *H. influenzae* remain attached to mucus in the respiratory tract for longer periods and, thus, replicate and make surface contact with damaged epithelium more readily at the nonciliated sites. This allows adherence of the bacteria to receptors, which are either inaccessible or unavailable on normal epithelium, and facilitates colonization of respiratory tract epithelial surfaces, where localized release of ciliotoxins, endotoxins, proteolytic enzymes, *etc.* may compromise the host defence system further; *i.e.* impair ciliary function, stimulate mucus production, break down local immunoglobulins and impair phagocytic function [24–26]. This consequently leads to an environment conducive to contiguous spread of the bacterium and a host-mediated, predominantly neutrophilic, counter effect, which results in further damage.

Although some studies have demonstrated that bacteria can generate and release specific neutrophil chemoattractants [27], others have demonstrated that bacteria such as *H. influenzae* can also release compounds which inhibit neutrophil chemotaxis [28], thereby suggesting that neutrophil infiltration in response to direct stimulation by bacterial products may be a self-limiting process and, therefore, unlikely on its own to account for the gross neutrophil infiltration which is observed in chronic infective lung diseases.

We hypothesize that chronic neutrophil infiltration seen in infective lung disease is an indirect consequence of bacterial-induced synthesis and release, from the host airway epithelium, of potent proinflammatory mediators which directly or indirectly influence the activity of neutrophils. These mediators result predominantly in large numbers of neutrophils, and to a lesser extent other inflammatory cell types, trafficking into the bronchial tree and releasing a variety of proteolytic enzymes and toxic oxygen

Table 1. – Data on cell content in BALF before and 3 h after LPS inhalation

	Total cells $\times 10^7 \cdot L^{-1}$	Total neutrophils $\times 10^7 \cdot L^{-1}$	Total lymphocytes $\times 10^7 \cdot L^{-1}$	Total macrophages $\times 10^7 \cdot L^{-1}$	Lysozyme +ve macrophages % of macrophages	Total mast cells $\times 10^4 \cdot L^{-1}$
Before exposure						
Median	6.8	0.06	0.49	5.9	7.5	0.06
Interquartile range	5.3–13.7	0.02–0.10	0.30–1.14	4.85–13.1	6.5–12.5	0.0–0.20
After LPS exposure						
Median	21.6	8.7	1.67	10.1	5.0	0.29
Interquartile range	15.5–28.9	2.5–14.9	0.10–2.46	8.1–14.1	4.0–5.5	0.10–1.42
p-value	<0.01	<0.001	<0.05	NS	<0.02	NS

BALF: bronchoalveolar lavage fluid; LPS: lipopolysaccharide; NS: nonsignificant. (From SANDSTRÖM *et al.* [21] with permission).

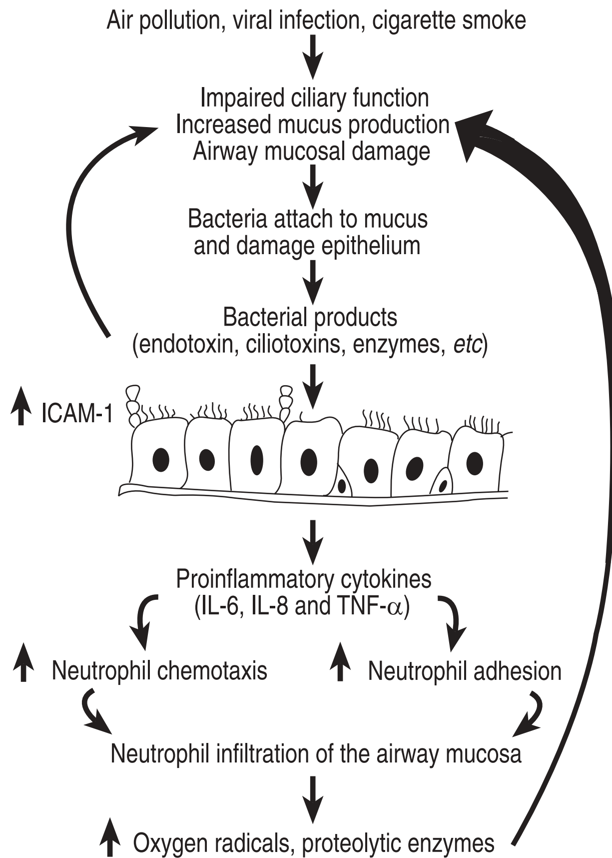

Fig. 1. – Putative interaction between airway epithelial cells and bacterial products in the amplification of neutrophil-induced airway epithelial damage. ICAM-1: intercellular adhesion molecule-1; IL: interleukin; TNF-α: tumour necrosis factor-α.

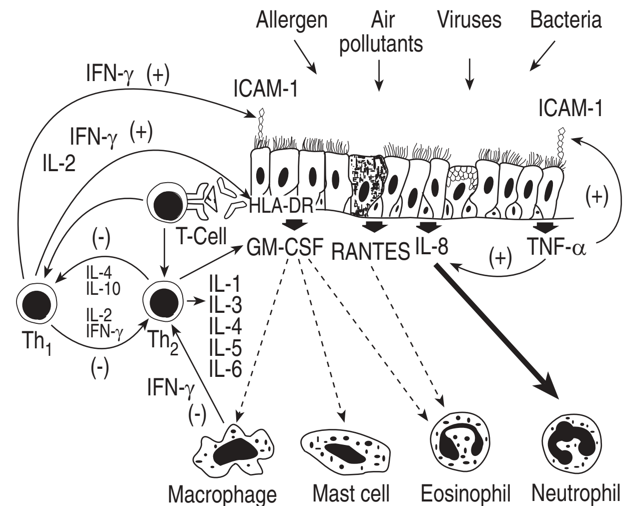

Fig. 2. – Schematic view of the role of epithelial cells in the modulation of airway inflammation. ICAM-1: intercellular adhesion molecule-1; IFN-γ: interferon-γ; IL: interleukin; GM-CSF: granulocyte/macrophage colony-stimulating factor; TNF-α: tumour necrosis factor-α; RANTES: factor regulated upon activation in normal T-cell, expressed and secreted; TH_1 and Th_2: type 1 and type 2 T-helper cells. (Modified from DEVALIA and DAVIES [30]).

radicals. In situations when the total load of these detrimental compounds is large enough to overwhelm the defence mechanisms which normally operate to limit the effect of these compounds, excessive mucus production and epithelial damage follows. Since activated neutrophils also have the capacity to synthesize and secrete IL-8 and other proinflammatory mediators, this becomes a self-perpetuating inflammatory process (fig. 1).

The role of airway epithelial cells in the aetiology of airway inflammation

The mechanisms responsible for the inflammatory cell influx in the central airways of patients with airway inflammation, such as those with chronic bronchitis, have not been well delineated. Although lung secretions, particularly in the presence of infection, have been shown to contain many neutrophil chemoattractants, the precise source of the factors initiating recruitment of neutrophils to the sites of infection is not well understood [29]. Whilst it is possible that bacteria themselves may generate some of these factors, there is increasing evidence to suggest that the airway epithelial cells may, indeed, be an important source and could provide a local mechanism to induce, amplify, or modulate on-going inflammation [30] (fig. 2).

Studies from our laboratory have demonstrated that human airway epithelial cells are capable of expressing and generating specific cytokines, including IL-6, IL-8 and TNF-α, which may play a role in activation and migration of neutrophils to sites of inflammation in the epithelium [31]. IL-6 is important in the induction of the acute phase response, augmentation of antibody production, and is released relatively early in the inflammatory response [32]. Studies of IL-8 have suggested that this cytokine has potent neutrophil activating properties, including neutrophil chemoattraction [33] and enhanced neutrophil binding at the site of inflammation [34], of which the latter may be a consequence of IL-8-induced expression of CD11b/CD18 (Mac-1) on the surface of neutrophils [34]. Recent studies by CROMWELL *et al* [35] have demonstrated that human bronchial epithelial cells are capable of generating IL-8 and that the expression of this cytokine may be upregulated by TNF-α, a multifunctional cytokine shown to be active in increasing epithelial permeability *in vitro* [36], and increasing the expression of intercellular adhesion molecule-1 (ICAM-1) [37]. Indeed, studies of human bronchial epithelial cells have demonstrated that these are also capable of expressing ICAM-1 [38], a member of the immunoglobulin supergene family, which is itself important due to its role in the recruitment and migration of neutrophils and eosinophils [39].

H. influenzae stimulates the release of proinflammatory cytokines from human bronchial epithelial cells

Whilst the majority of studies of bacterial endotoxins have concentrated on the effects of *E. coli* endotoxin, there are comparatively few studies with endotoxin from the nontypable strains of *H. influenzae*, which are the most important colonizing strains isolated in chronic bronchitis.

We have cultured human bronchial epithelial cells, as explant cultures on microporous membranes, and investigated the effect of purified endotoxins from a nontypable (NCTC 8143) and type b (NCTC 8467) *H. influenzae* strains, on epithelial permeability and expression and release of proinflammatory cytokines and ICAM-1 in these cell cultures.

Our studies demonstrated that the endotoxin from both strains of *H. influenzae* had no adverse effect on epithelial cell membrane integrity of the epithelial cultures, as indicated by a lack of difference between the permeability coefficients of control untreated cultures and cultures incubated for 24 h in the presence of 10–100 pg·mL^{-1} endotoxin. Indeed, both endotoxin preparations led to an initial increase in the electrical resistance of the cultures [40], suggesting that the permeability of the cultures was initially decreased rather than increased. This may be a consequence of an increase in cell volume, possibly resulting from chloride channel modulation [41], and/or changes in cell junctional complexes. Studies in our laboratory have demonstrated that incubation of epithelial cell cultures in the presence of histamine, a compound we have shown previously to be synthesized by *H. influenzae* and several other bacterial species [42, 43], leads to significant increase in the length of desmosomes present between adjacent cells [44]. Collectively, these studies suggest that the initial decrease in epithelial cell permeability observed in response to noxious compounds may be a naturally occurring protective mechanism, which serves primarily to preserve the integrity of the epithelium, but may be overcome by continuous exposure to these compounds.

Overall, these findings are in accordance with the findings of WIENER-KORNISH *et al.* [45], who have investigated the effect of *E. coli* endotoxin on sheep lung epithelium *in vivo*. These authors demonstrated that despite leading to a marked increase in interstitial pulmonary oedema and the number of neutrophils in the alveolar airspaces, *E. coli* endotoxin did not alter the alveolar epithelial permeability, as indicated by a lack of bidirectional movement of ^{125}I-labelled albumin across the alveolar epithelium. Furthermore, these authors demonstrated that treatment with *E. coli* endotoxin did not lead to any detrimental morphological changes in the alveolar epithelium.

Our studies have also demonstrated that *H. influenzae* endotoxin significantly increases the release of a number of proinflammatory cytokines, including IL-6, IL-8, TNF-α, from human bronchial epithelial cell cultures [40]. Release of IL-8 and TNF-α was increased twofold and threefold, respectively, 24 h after treatment of the cultures with 100 µg·mL^{-1} endotoxin from the nontypable *H. influenzae*, compared with control untreated cultures (fig. 3). Incubation of these cultures in the presence of 10^{-5} M hydrocortisone and 0.1–10 µg·mL^{-1} erythromycin significantly attenuated the endotoxin-induced release of these cytokines [40, 46]. Incubation of the epithelial cultures in the presence of endotoxin from the type b strain, however, led to a 65 fold increase in the release of IL-8 and a fivefold increase in the release of TNF-α (fig. 3). In contrast, analysis of the culture medium from *H. influenzae* endotoxin-treated cultures revealed that the release of granulocyte/macrophage colony-stimulating factor (GM-CSF) and the factor regulated on activation in normal T-cells expressed and secreted (RANTES),

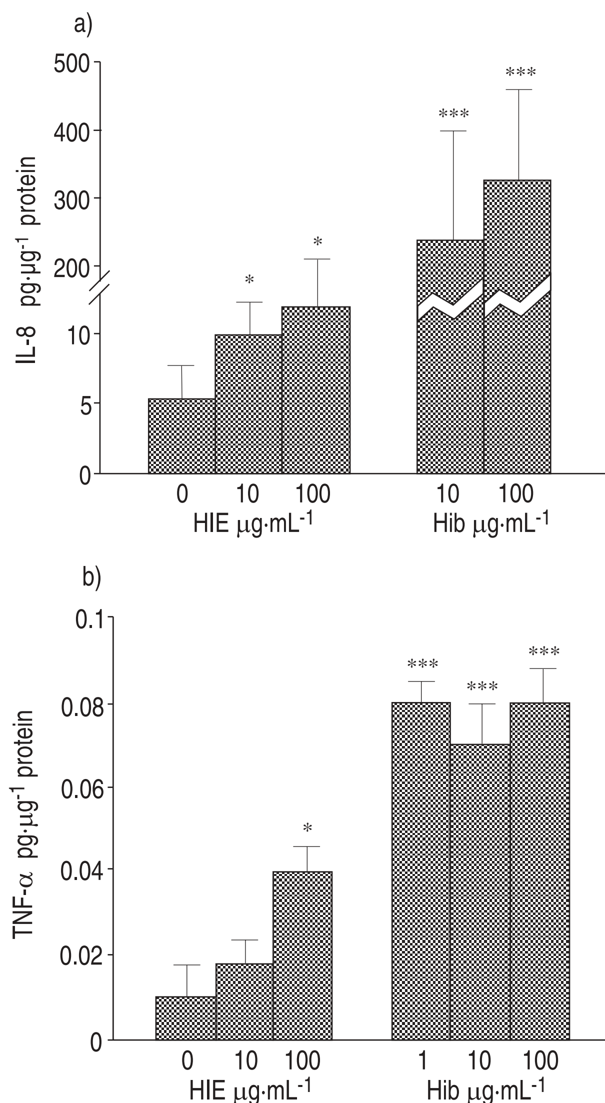

Fig. 3. – The effect of nontypable *H. influenzae* endotoxin (HIE) and type b *H. influenzae* (Hib) endotoxin on the release of: a) IL-8; and b) TNF-α by human bronchial epithelial cell cultures. Results are expressed as mean±SEM (n=6 at each concentration). *: p<0.05 *vs* untreated cells; ***: p<0.001 *vs* HIE-treated cells. For further definitions see legend to figure 1. (Modified from KHAIR *et al.* [40]).

cytokines known to have potent eosinophil chemotactic and activating properties, was not significantly increased. However, studies investigating the expression of ICAM-1 on epithelial cells demonstrated that the nontypable *H. influenza* endotoxin also upregulated the expression of ICAM-1 on these cells and that incubation of the cells in the presence of 10^{-5} M hydrocortisone significantly attenuated the expression of this cell adhesion molecule (fig. 4).

Our findings are in agreement with those of others, who have also demonstrated that steroids, at concentrations of 10^{-7} to 10^{-5} M, can decrease the release of cytokines and expression of ICAM-1 in cultured epithelial cells and bronchial epithelial cell lines *in vitro* [47, 48].

Numerous activities have been ascribed to IL-6, including its ability to contribute to the stimulation of humoral and cellular defence mechanisms [49]. Studies by HEREMANS *et al.* [50] have suggested that IL-6 may also

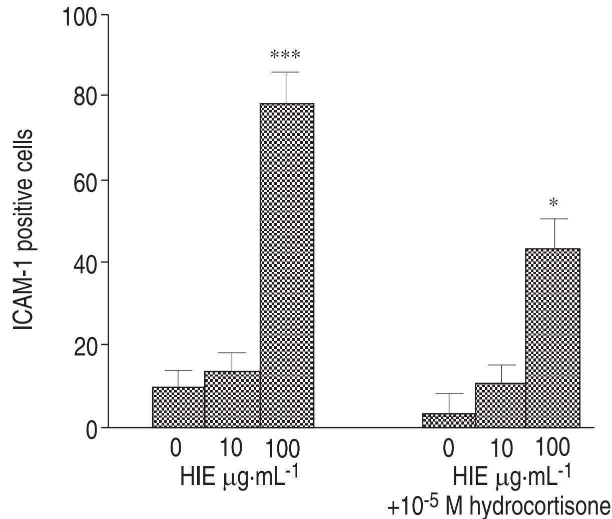

Fig. 4. – Effect of nontypable *H. influenzae* endotoxin (HIE) on the expression of surface ICAM-1 by human bronchial epithelial cell cultures and the influence of 10^{-5} M hydrocortisone. Results are expressed as mean±SEM (n=6 at each concentration). ***: $p<0.001$ *vs* untreated cells; *: $p<0.05$ *vs* cells treated with 100 $\mu g \cdot mL^{-1}$ HIE alone. ICAM-1: intercellular adhesion molecule-1. (Modified from KHAIR *et al.* [40]).

be involved in endotoxin-mediated reactions, since anti-IL-6 antibodies were found to protect mice against the generalized endotoxin-elicited Schwartzman reaction. DEHOUX *et al.* [51] have reported that during unilateral community-acquired pneumonia, the inflammatory response is localized within the human lung, and is limited to the site of infection, with locally enhanced production of TNF-α, IL-1β, and IL-6. Similarly, BOUTTEN *et al.* [52] have demonstrated that IL-8 levels in bronchoalveolar lavage fluid (BALF) from the involved lung of patients with unilateral community-acquired pneumonia are also increased and that locally increased levels of IL-8 are correlated with neutrophil counts and with total and free elastase concentrations. SMITH *et al.* [53] and DOHERTY *et al.* [54], have investigated the effects of *E. coli* endotoxin injected into rats and mice, respectively, and have demonstrated that this results in a significant increase in serum TNF-α activity. The studies of DOHERTY *et al.* [54] extended these observations by showing that specific neutralizing antibodies both to TNF-α and interferon-gamma (IFN-γ) protected the animals against the lethality of the *E. coli* endotoxin and suggested that TNF-α and IFN-γ may be acting synergistically to potentiate the toxicity of this endotoxin. Studies of TNF-α have shown that this is a proximal mediator in the cytokine cascade, which appears in the circulation of several species as a brief early peak after infusion of bacteria or bacterial lipopolysaccharides and induces secondary cytokines, such as IL-1, IL-6 and IL-8 [55, 56].

To investigate whether the cytokine profile induced by nontypable *H. influenzae* endotoxin was a direct effect or secondary to endotoxin-induced release of TNF-α, we investigated the effect of TNF-α monoclonal neutralizing antibody on endotoxin-associated release of IL-6, IL-8 and soluble ICAM-1 (sICAM-1). These studies demonstrated that although TNF-α neutralizing monoclonal antibody significantly reduced the release of IL-6 and sICAM-1, this did not block the release of IL-8, from bronchial epithelial cell cultures. These results are contrary to the findings of VAN ZEE *et al.* [57], who have shown that levels of IL-6 and IL-8 in baboons with septic shock and sublethal endotoxaemia parallel one another and peak after TNF-α, suggesting that TNF-α may induce the synthesis of both these cytokines. However, our results are in keeping with the findings of DEFORGE *et al.* [58], who have investigated the release of cytokines in LPS-stimulated human whole blood. These authors have demonstrated that the synthesis of IL-8 messenger ribonucleic acid (mRNA) and protein in the blood followed a biphasic pattern, and that this was not inhibited by addition of anti-TNF, anti-IL-1α and anti-IL-1β antibodies, either alone or in combination, in the primary phase, but was substantially reduced in the secondary phase (fig. 5). These results suggest that IL-8 release in the primary phase was a result of direct stimulation by LPS, and in the secondary phase a result of induction by TNF-α and/or IL-1.

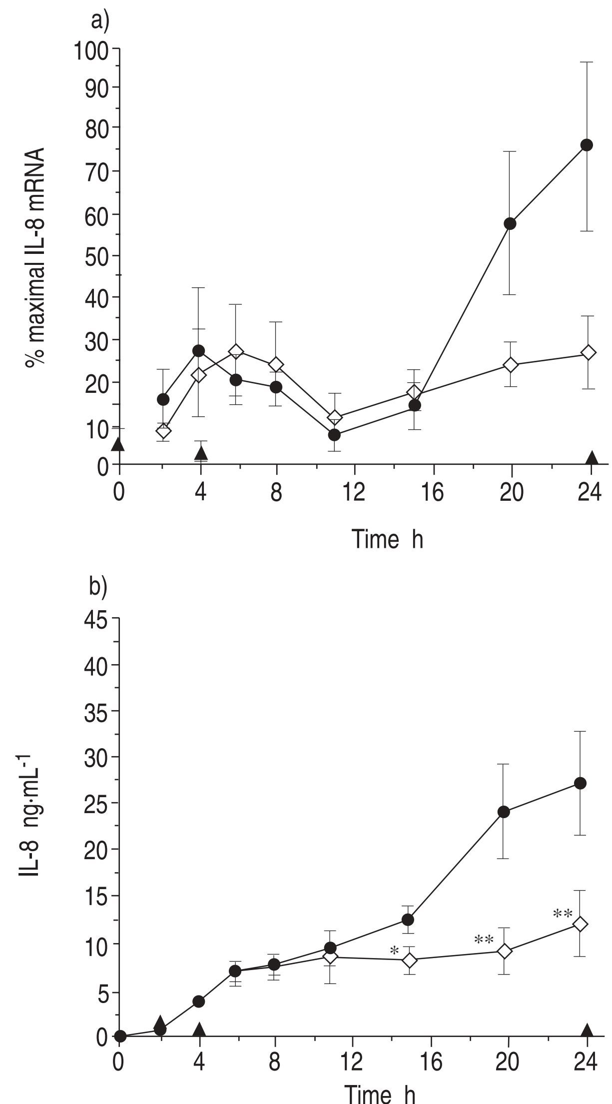

Fig. 5 – Inhibition of the second phase of: a) IL-8 mRNA expression; and b) IL-8 production in human whole blood, by anti-TNF and anti-IL-1 antibodies. (Modified from DEFORGE *et al.* [58]). —●— : LPS + control antibody; —◇— : LPS + anti-TNF-α + anti-IL-1; ▲ : control. mRNA: messenger ribonucleic acid; LPS: lipopolysaccharide; *: $p<0.007$ *vs* LPS + control antibody; **: $p<0.001$ *vs* LPS + control antibody. For further definitions see legend to figure 1.

Overall, the finding that *H. influenzae* endotoxin induced significant release of IL-6, IL-8 and TNF-α and increased the expression of ICAM-1 in human bronchial epithelial cell cultures may be of particular importance in bacterial-induced airway inflammation, since these mediators have been shown to exert a significant influence on neutrophil activity [32, 35, 37]. Additionally, since *H. influenzae* endotoxin was not shown to affect the synthesis and release of epithelial GM-CSF or RANTES, which influence primarily the activity of eosinophils [59, 60], these studies suggest that the effects of endotoxin are likely to be selective and directed towards synthesis and release of mediators that increase neutrophil recruitment and infiltration of the airways. Since endotoxin from type b *H. influenzae* was also shown to lead to a much greater release of IL-8 and TNF-α than endotoxin from the nontypable strain of *H. influenzae*, this may be of particular significance and could explain the differences in the pathogenicity of the two strains. Type b *H. influenzae* is a more virulent organism associated with more severe and invasive infections, such as meningitis, pneumonia, epiglottitis and septic arthritis, in which bacteraemia is a prominent feature [61]. In contrast, nontypable strains lead to less severe infections resulting from proliferation and contiguous spread of the bacteria within the respiratory tract, such as otitis media, sinusitis and lower respiratory tract infections, including infective exacerbations of chronic bronchitis [62].

Molecular mechanisms underlying endotoxin-induced proinflammatory cytokine synthesis

Studies with isolated monocytes and alveolar macrophages have demonstrated that endotoxin-induced synthesis and release of inflammatory cytokines including IL-6, IL-8 and TNF-α, from these cells is mediated *via* the cell membrane-bound CD14 receptor and that CD14 receptor activation may occur either in the absence or presence of specific LPS binding proteins (LBP) in the serum [63, 64]. Studies, particularly of LPS-induced TNF-α synthesis, have demonstrated that there is a threefold increase in the transcriptional activity and a 100 fold increase in cellular mRNA content [65].

Some studies, however, have demonstrated that although some cell types do not express CD14, they still have the ability to respond to endotoxin [66]. Studies by NAKAMURA *et al.* have demonstrated that LPS-induced Ca^{2+} increase in platelets and platelet aggregation can be blocked by selective platelet-activating factor (PAF) receptor antagonists, and suggest that there may be cross-reactivity between the LPS and the PAF receptors. Preliminary studies in our laboratory have also demonstrated that the bronchial epithelial cells do not express CD14, and suggest that the effects of *H. influenzae* endotoxin noted in our model may be mediated through a direct effect on epithelial cells or *via* an alternate cell membrane receptor, such as PAF receptors. Whilst some studies have demonstrated that PAF receptors are expressed on the airway epithelial cells in animals [68, 69], to our knowledge there is no report of the expression of PAF receptors on human airway epithelial cells.

Although LPS-induced signal transduction pathways leading to synthesis of inflammatory cytokines have not been fully elucidated, studies in monocytes and macrophages have suggested that these are likely to involve both cytoplasmic and nuclear components, including phospholipase C (PLC), protein kinase C (PKC) and transcription factors [70, 71]. It is thought that LPS-stimulated changes in cytosolic Ca^{2+}, resulting from activation of PLC, may play a role in the regulation of gene expression, *via* modulation of PKC activity [72], which itself influences the activation of specific transcription factors [73]. MUEGGE and DURUM [74] have reviewed the role of transcription factors and suggested that these are "third messengers", whose synthesis can be modulated by specific cytokines and which influence the expression of other cytokine genes by interacting with specific elements in the gene regulatory regions. Studies of transcriptional regulation of the TNF-α gene in monocytes have suggested that the promoter region of this gene contains binding sites for the transcription factors activating protein (AP)-1, AP-2 and nuclear factor-kappa B (NFκB) [75], and that LPS- induced transcription of this gene may be mediated by synthesis and activation of *c-jun* protein, a transcription factor that is essential for the transcriptional activation of the AP-1-responsive genes [76]. Indeed, BOYLE *et al.* [77] have suggested that increased binding of the AP-1 transcription factor complex to deoxyribonucleic acid (DNA) in human epithelial and fibroblast cell lines is a consequence of PCK-mediated dephosphorylation of the *c-jun* protein. Similarly, studies of the human IL-8 gene have demonstrated that this also contains potential binding sites for transcription factors AP-1, AP-2, NFκB, and NF-IL-6-like factor [78]. Whilst some studies have demonstrated that activation of NFκB and NF-IL-6-like factor is essential for induction of IL-8 by mediators such as IL-1, TNF-α and phorbol myristate acetate (PMA) [79], others have demonstrated that binding of the glucocorticoid-receptor complex to the AP-1 factor leads to inhibition of IL-8 synthesis [80–82].

To our knowledge, there are no studies of the mechanisms underlying *H. influenzae* endotoxin mediated regulation of the genes encoding the inflammatory cytokines. We have studied the effect of *H. influenzae* endotoxin on the expression of *c-fos*, *c-jun*, and NFκB in human bronchial epithelial cells by immunostaining, followed by quantification of the percentage of total positively stained cells in the cultures by colour image analysis. Our studies have demonstrated that the expression of all these transcription factors was significantly upregulated in cultures incubated in the presence of *H. influenzae* endotoxin, when compared with control untreated cultures. Moreover, preincubation of the cultures with hydrocortisone or erythromycin blocked the endotoxin-induced expression of these factors (fig. 6).

The biological relevance of *H. influenzae* endotoxin-induced release of proinflammatory cytokines

Despite increasing evidence for the role of airway epithelial cells in the generation of proinflammatory mediators, there is comparatively little information on the biological relevance of their release from epithelial cells,

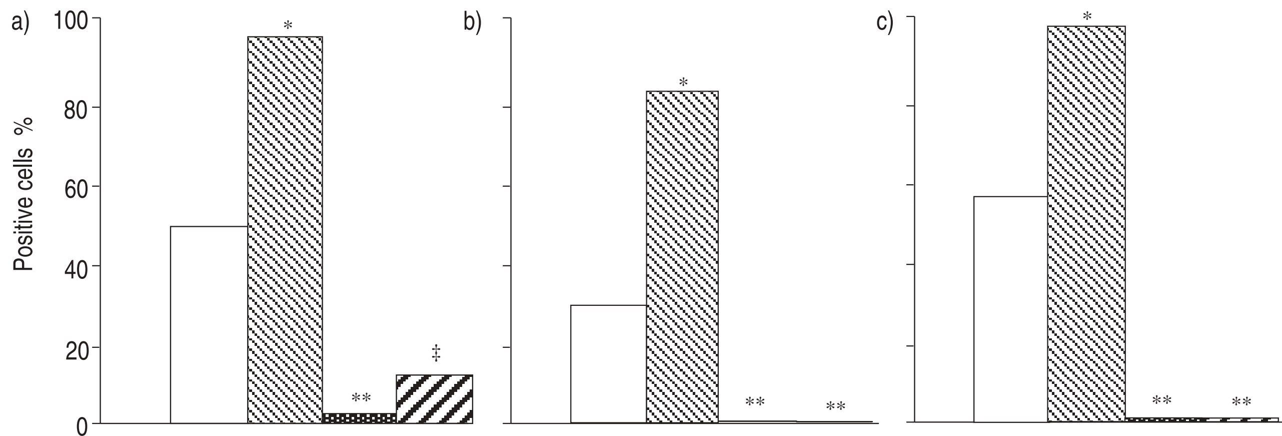

Fig. 6. – Effect of *H. influenzae* endotoxin (HIE) on the expression of: a) *c-fos*; b) *c-jun*; and c) nuclear factor-kappaB (NFκB) by human bronchial epithelial cell cultures and the influence of hydrocortisone (10^{-5} M) and erythromycin (1 µg·mL^{-1}). Results are expressed as mean±SEM (n=6 at each concentration). *: p<0.05 *vs* untreated control cells; **: p<0.001 *vs* cells treated with 100 µg·mL^{-1} HIE alone; ‡: p<0.05 *vs* cells treated with 100 µg·mL^{-1} HIE alone). ▭ : control; ▨ : HIE; ▦ : HIE + hydrocortisone; ▧ : HIE + erythromycin

either constitutively or following stimulation, in airway inflammation. We have investigated the effect of culture medium collected from confluent bronchial epithelial cell cultures incubated for 24 h (conditioned medium) in the presence or absence of 100 µg·mL^{-1} *H. influenzae* endotoxin, on neutrophil chemotaxis and adhesion to human endothelial cells *in vitro*.

Neutrophil chemotaxis in response to conditioned medium from *H. influenzae* endotoxin-treated and untreated cultures was assessed by a modification of the Boyden chamber technique [83]. These studies demonstrated that conditioned medium from the endotoxin-treated cultures increased the chemotaxis of neutrophils twofold, when compared with conditioned medium from untreated cultures, and that this increase in neutrophil chemotaxis was blocked by treatment of the epithelial cell cultures with 1 µg·mL^{-1} erythromycin [46]. More recent findings from our laboratory have indicated that the *H. influenzae* endotoxin-induced increase in neutrophil chemotaxis can also be blocked by the addition of IL-8 monoclonal neutralizing antibody to the conditioned medium, and suggest that IL-8 is most likely to be involved in this process. This is in agreement with the findings of others, who have also demonstrated that this cytokine primarily influences neutrophil activation [84]. Although others have also demonstrated that epithelial cell derived mediators are chemotactic towards neutrophils [85, 86], and indeed other cell types including lymphocytes [87, 88] and monocytes [86, 89], *in vitro*, the majority of these studies have employed animal models.

Similarly, the effect of conditioned medium on neutrophil adherence was investigated by estimating the number of neutrophils adhering to human endothelial cell cultures established from cell line ECV304 (European Collection of Animal Cell Cultures, Porton Down, UK) and preincubated in the presence of conditioned medium from *H. influenzae* endotoxin-treated and untreated cultures, for 6 h at 37°C. These studies demonstrated that adherence of neutrophils to endothelial cells preincubated with conditioned medium from endotoxin-treated epithelial cell cultures was significantly increased, when compared with neutrophil adherence to endothelial cells preincubated with conditioned medium from untreated cultures and, like neutrophil chemotaxis, was blocked by treatment of the epithelial cell cultures with 1 µg·mL^{-1} erythromycin [46]. Subsequent studies investigating the effect of addition of anti-TNF-α, anti-ICAM-1, and anti-E-selectin neutralizing monoclonal antibodies to the condition medium from the endotoxin-treated cultures have demonstrated that these antibodies blocked the endotoxin-induced adherence of neutrophils to endothelial cells, and suggest that neutrophil adhesion to endothelial cells is likely to be mediated *via* the expression of cell adhesion molecules such as ICAM-1 and E-selectin, on endothelial cells. Furthermore, the expression of these adhesion molecules was likely to be influenced by epithelial cell derived cytokines, such as TNF-α.

Our findings of the regulatory role of bronchial epithelial cell-derived TNF-α in the expression of endothelial cell adhesion molecules and their involvement in the adherence of neutrophils are in accordance with the findings of others. SCHLEIMER *et al.* [90] have investigated the role of different adhesion molecules expressed on human vascular endothelial cells, in adherence of eosinophils, basophils and neutrophils and demonstrated that increased expression of endothelial vascular cell adhesion molecule-1 (VCAM-1), but not ICAM-1 or E-selectin, was paralleled by an increased adherence of eosinophils and basophils, but not neutrophils. These results suggest that ICAM-1 and E-selectin, but not VCAM-1, are involved in the adherence of neutrophils to endothelial cells. BOCHNER *et al.* [91] have investigated the effect of IL-1β, a cytokine with several biological effects similar to TNF-α, on the adherence of neutrophils to human umbilical vein endothelial cells and have demonstrated that this cytokine significantly increases the adherence of neutrophils to endothelial cells.

In conclusion, our studies have demonstrated that human airway epithelial cells are capable of expressing and releasing potent proinflammatory cytokines and adhesion molecules, which play a prominent role in inflammation of the airways. Our finding that the expression and release from the epithelial cells of mediators which influence the activity of neutrophils is significantly enhanced by endotoxin from *H. influenzae*, and possibly other bacterial species, suggests that an interaction between bacterial products and airway epithelial cells is likely to play an important role in the development of bacterial-induced

respiratory disease. Indeed, we have demonstrated that *H. influenzae* endotoxin-induced release of the inflammatory mediators from the bronchial epithelial cells *in vitro*, parallels increased neutrophil activation (chemotaxis and adhesion) *in vitro*. This gives credence to the hypothesis that gross neutrophil infiltration of the airway epithelium observed in chronic infective lung conditions is likely to be an indirect consequence of bacterial compounds, such as endotoxin, which augment the synthesis and release of inflammatory mediators from the host airway tissue.

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