

Effect of *Haemophilus influenzae* endotoxin on the synthesis of IL-6, IL-8, TNF- α and expression of ICAM-1 in cultured human bronchial epithelial cells

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ABSTRACT: Although studies of infective lung diseases have demonstrated that *Haemophilus influenzae* is a major pathogen, the mechanisms underlying pathogenesis by this organism are not clear.

We have cultured human bronchial epithelial cells (HBEC) to confluency and have investigated the effect of *H. influenzae* endotoxin (HIE) on: 1) epithelial permeability, by movement of ¹⁴C-bovine serum albumin (¹⁴C-BSA) across HBEC and measurement of electrical resistance of HBEC; 2) release of interleukin-6 (IL-6), interleukin-8 (IL-8) and tumour necrosis factor- α (TNF- α) into the supernatant, by enzyme-linked immunosorbent assay (ELISA); and 3) expression of intercellular adhesion molecule-1 (ICAM-1), by immunofluorescence staining.

HIE did not significantly increase the movement of ¹⁴C-BSA across HBEC. In contrast, HIE progressively increased the electrical resistance of HBEC, such that this was significant after 24 h. Compared with untreated cells, 10–100 $\mu\text{g}\cdot\text{ml}^{-1}$ HIE-treated cells released significantly greater amounts of IL-6, IL-8 and TNF- α , after 24 h, which was blocked by 10⁻⁵ M hydrocortisone. Similarly, incubation of HBEC with 10–100 $\mu\text{g}\cdot\text{ml}^{-1}$ HIE, significantly increased the total number of ICAM-1 positive cells, which were significantly decreased on incubation of the cells in the presence 10⁻⁵ M hydrocortisone. Conditioned medium from HIE-exposed HBEC lead to significant increase in neutrophil chemotaxis and adhesion to endothelial cells *in vitro*.

These results suggest that HIE may affect epithelial cell function and influence inflammation of the airway mucosa *via* induction of proinflammatory mediators.

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Studies of patients with chronic bronchitis have shown that exacerbation of disease in these patients is often associated with bacterial infection, with *Haemophilus influenzae* and *Streptococcus pneumoniae* being the organisms most commonly isolated [1]. These bacterial species have also been isolated from the sputum of such patients during remission, and consequently their role in the pathogenesis of chronic bronchitis is not entirely clear.

Studies of pulmonary infection have demonstrated that there is a close relationship between bacterial load and neutrophil recruitment [2], 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, which are capable of causing acute and chronic lung injury [3]. In addition, bacterial toxins can be detected in bronchial secretions, and have been shown to cause epithelial necrosis, disrupt ciliary ultrastructure, and produce cilio-inhibitory factors [4].

Although lung secretions, particularly in the presence of infection, have been shown to contain many neutrophil chemoattractants, the precise source(s) of the factors initiating recruitment of neutrophils to the sites of infection are not well understood [5]. Whilst it is possible that bacteria themselves may generate some of these compounds, there is increasing evidence to suggest that the airway epithelial cells may indeed be an important source of such compounds. Studies with human airway epithelial cells have demonstrated that they are capable of expressing and generating specific cytokines, including interleukin 6 (IL-6), interleukin-8 (IL-8) and tumour necrosis factor- α (TNF- α), which may play a role in activation and migration of neutrophils to sites of inflammation in the epithelium [6, 7]. IL-6 is important in the induction of the acute phase response and augmentation of antibody production, and is released relatively early in the inflammatory response [8].

Studies of IL-8 have suggested that this cytokine has potent neutrophil activating properties, including neutrophil

chemoattraction [9], and enhanced neutrophil binding at the site of inflammation [10], of which the latter may be a consequence of IL-8 induced increase in expression of CD11b/CD18 (Mac-1) on the surface of neutrophils [10]. Recent studies by CROMWELL *et al.* [7] have demonstrated that human bronchial epithelial cells (HBEC) are capable of generating IL-8, and that the expression of this cytokine may be upregulated by TNF- α [7], a multi-functional cytokine shown to be active in increasing epithelial permeability *in vitro* [11], and increasing the expression of intercellular adhesion molecule-1 (ICAM-1) [12]. Indeed, studies from our laboratory have demonstrated that HBEC are also capable of expressing ICAM-1 [13], a member of the immunoglobulin supergene family, which is itself important due to its role in the recruitment and migration of neutrophils and eosinophils [14].

In view of these findings, we have hypothesized that bacterial products may mediate inflammatory reactions in the airways by augmenting the synthesis of specific proinflammatory cytokines and adhesion molecules, and have investigated the effect of *H. influenzae* endotoxin (HIE) on epithelial permeability, the generation and release of IL-6, IL-8 and TNF- α and the expression of ICAM-1, in cultured HBEC *in vitro*. Additionally, we have studied the effect of hydrocortisone on the release of these cytokines and expression of ICAM-1, and assessed the biological relevance of cytokine release by investigating the effect of the conditioned medium from HIE-treated HBEC on neutrophil chemotaxis and adhesion to endothelial cells *in vitro*.

Material and methods

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

Culture of human bronchial epithelial cells

Bronchial tissue, which appeared macroscopically free of tumour, was obtained from 11 male and 5 female patients, who underwent lobectomy or pneumonectomy for lung cancer, at St Bartholomew's Hospital, London, UK. All these patients were smokers (mean 40 pack-years) with no history of allergy, and of mean age 63 yrs (range 43–80 yrs).

Pure cultures of HBEC were grown on microporous membranes by a modification of the technique developed in our laboratory and described fully previously [15]. Briefly, this involved incubation of approximately 2 mm³ pieces of bronchial epithelial tissue in 0.2 ml complete culture medium, in inserts placed into wells (insert wells) containing 0.4 ml culture medium at 37°C, in a 5% CO₂ in air atmosphere. On reaching confluency in 2–3 weeks, the explants were removed from the inserts and HBEC were washed in prewarmed medium, prior to further incubation to allow new cells to overgrow the areas of the insert membrane left barren by the removal of the explants. Such a system allowed simultaneous measurements of: 1) electrical resistance; 2) collection

of culture medium for analysis of the movement of radiolabelled markers across epithelial cell monolayers, and estimation of cytokines released into the culture medium; and 3) histochemical staining of the cells for ICAM-1.

Preparation of H. influenzae endotoxin

H. influenzae (strain NCTC 8143, Reference Laboratory, London, UK) were harvested from a pure broth culture, by centrifugation for 10 min at 6,000 \times g, and were lyophilized overnight in a Camlab SB6C freeze dryer (Camlab Ltd, Cambridge, UK). The endotoxin in the lyophilized bacterial preparation was then extracted, according to a modified technique of CAMPAGNARI *et al.* [16]. Ten grams of lyophilized bacteria were resuspended in 175 ml of water and, after sonication for 3 min, the suspension was incubated for 5 min at 65°C. On addition of an equal volume of 90% phenol (w/v), the suspension was incubated for a further 5 min at 65°C, spun, cooled rapidly on ice, and then centrifuged at 3,000 \times g for 30 min at 0°C. The aqueous phase was aspirated and dialysed against running water for 48 h to remove any phenol and low-molecular weight bacterial components. The dialysed suspension was centrifuged at 100,000 \times g for 4 h at 4°C, and the supernatant containing the crude endotoxin was digested with 100 μ g·ml⁻¹ ribonuclease A and 100 μ g·ml⁻¹ amylase, for 4 h at 37°C. At the end of this incubation period, the ribonuclease and amylase were inactivated by incubation for 1 h at 37°C, in the presence of 100 μ g·ml⁻¹ proteinase-K. This suspension was then subjected to a further cycle of phenol extraction, dialysis and ultracentrifugation as above, to obtain the "purified" endotoxin.

The endotoxin preparation was assessed for biological activity, by the Limulus amoebocyte lysate (LAL) test, according to the protocol outlined for use of E-Toxate® kits (Sigma Chemical Co., Poole, UK). The lipopolysaccharide in the preparation was assessed using a modification of the electrophoretic technique of TSAI and FRASCH [17]. Briefly, 20 μ l samples were electrophoresed on polyacrylamide gels containing 14% (w/v) acrylamide, 0.1% (w/v) sodium dodecyl sulphate (SDS), 4.0 M urea and 0.1 M Tris-HCl (pH 8.8) at 200 V, using Tris-glycine buffer, pH 8.3 (0.025 M Tris, 0.2 M glycine buffer and 0.1% SDS). Following electrophoresis, the gel was prefixed for 1 h in an aqueous solution containing 25% (v/v) 2-propanol and 10% (v/v) glacial acetic acid, and then stained using the Quicksilver stain kit (Amersham International plc, Little Chalfont, UK).

The preparation was also assessed for contaminating nucleic acids, by spectrophotometric absorbance at 280 and 260 nm, and for contaminating proteins, according to the technique of LOWRY *et al.* [18].

Effect of HIE on HBEC permeability, release of IL-6, IL-8 and TNF- α and expression of ICAM-1

Prior to exposure to endotoxin, fully confluent HBEC were washed with culture medium and incubated for 30

min in the presence of 0.025 μCi ^{14}C -bovine serum (^{14}C -BSA) (Amersham International plc, Amersham, UK). At the end of this incubation, the medium in each insert well was collected and analysed for total radioactivity, by liquid scintillation counting in a Beckman LS1800 scintillation counter (Beckman-RIIC Ltd, High Wycombe, UK). When the total radioactivity passing through the monolayer was found to be less than 0.5% of the total for ^{14}C -BSA added to the inserts at the beginning of the experiment, the experiment was continued.

HIE was added into inserts at concentrations of 10 or 100 $\mu\text{g}\cdot\text{ml}^{-1}$, and the electrical resistance of each HBEC was measured immediately and again after 30 min, 1, 2, 4, 6 and 24 h, with an EVOMTM microvolt-ohmmeter (World Precision Instruments, Owslebury, UK). Immediately after measurement of the electrical resistance at each specific time-point during incubation, the medium from each insert well was collected and replaced with fresh medium. The culture was further incubated, at 37°C in a 5% CO_2 in air atmosphere, and 25 μl aliquots of each sample were analysed for total radioactivity, prior to storage of the remaining sample, by freezing at -70°C, for analysis of IL-6, IL-8 and TNF- α . At the end of incubation, the medium in the insert well was collected and 25 μl were analysed for total radioactivity. The remaining medium was pooled with the medium collected from inside the insert and stored for analysis as above.

The cell culture insert membrane was then detached from the insert barrel and accurately cut into four quarters. Three quarters of the membrane were immersed in 0.5 ml 1 M NaOH and spun for 10 min to detach the adherent cells on the membrane. The cell suspension was incubated at 56°C for 1 h and stored by freezing at 70°C, prior to analysis for total cellular protein [18].

The remaining quarter of the membrane was stained by a double staining technique, incorporating an indirect immunoperoxidase staining procedure for cytokeratin and a direct fluorescein isothiocyanate (FITC) staining procedure, for ICAM-1. HBEC were fixed in fresh acetone for 10 min and then methanol for a further 10 min. The cells were rinsed with Tris/HCl buffered saline (TBS), pH 7.6, and incubated in the presence of a mouse anti-human anti-cytokeratin monoclonal antibody preparation, CAM 5.2 (Becton Dickinson Ltd, Oxford, UK), for 1 h at room temperature. The cells were then washed three times with TBS and incubated further in the presence of immunoperoxidase-conjugated rabbit anti-mouse antibody preparation P260 (Dako Ltd, High Wycombe, UK), for 30 min at room temperature. The cells were washed again with TBS and treated with a solution of 0.06% 3,3 diaminobenzidine tetrahydrochloride (DAB) and 0.03% hydrogen peroxide in 0.05 M Tris buffer, pH 7.6, for 20 min before counterstaining with Mayer's haematoxylin stain for 5 min. The immunoperoxidase and haematoxylin-stained cells were then washed in running tap water for 5 min, and incubated with FITC-labelled anti-human ICAM-1 monoclonal antibody preparation MCA675F (Serotec, Oxford, UK), for 1 h at room temperature.

The stained HBEC were mounted with glycerol and

examined by phase contrast and epifluorescent microscopy, by two independent observers blinded to the treatment regimens, using an Olympus BH-2 microscope with interchangeable phase contrast and epifluorescence optics. FITC fluorescence was visualized using the BP-490 and EY-455 exciter filters and DM-500 and O-515 barrier filters (Olympus Optical Co. Ltd, London, UK). The total number of cytokeratin-stained cells and fluorescein-stained cells were counted in six random fields, and the number of fluorescein-stained cells (ICAM-1 positive cells) in each field was expressed as a percentage of the total number of cytokeratin-stained cells in that field.

Prior to final analysis, the electrical resistance values were corrected for baseline resistance of the culture insert membrane and the appropriate incubation medium in the absence of HBEC, and the total radioactivity found in the insert well at each time-point was expressed as a cumulative percentage of the total added into the insert at the beginning of the experiment. IL-6, IL-8 and TNF- α were analysed in all frozen samples using commercially available enzyme-linked immunosorbent assay (ELISA) kits (British Biotechnology Ltd, Abingdon, UK) and expressed as $\text{pg}\cdot\mu\text{g}^{-1}$ cellular protein.

Isolation and purification of human neutrophils

Neutrophils were isolated and purified from human blood by dextran sedimentation and centrifugation on discontinuous density Percol gradients, according to the method of KLOPPROGGE *et al.* [19], and only preparations of >95% viability and >95% purity were used in further investigations.

Assay for neutrophil chemotaxis

Neutrophil chemotaxis was studied using the modified Boyden chamber technique [20]. Neutrophil migration was assessed over 90 min at 37°C in response to conditioned medium from untreated HBEC, HIE-treated HBEC, medium 199 \pm HIE and 10^{-9} M N-formyl-methionyl-leucyl-phenylalanine (FMLP). At the end of incubation, the membrane was removed and, after fixation in absolute alcohol for 5 min, was washed and then stained in Harris's haematoxylin stain (BDH Laboratory Supplies, Lutterworth, UK). The stained membrane was cleared in CNP 30 reagent (BDH Laboratory Supplies, Lutterworth, UK) and after mounting was immediately examined microscopically. Neutrophils were counted in 10 random high power fields, and neutrophil chemotactic activity was expressed as the mean number of neutrophils per high power field (hpf). All slides were read by two independent observers blinded to the experimental conditions.

Assay for neutrophil adherence

Neutrophil adherence was investigated by estimating the number of neutrophils adhering to human endothelial

cell cultures (HEC) established from cell line ECV304 (European Collection of Animal Cell Cultures, Porton Down, UK). Confluent HEC were incubated for 6 h at 37°C in: 1) conditioned medium from HIE-treated HBEC; 2) conditioned medium from untreated HBEC and 3) medium 199 containing HIE; and 4) medium 199 only. HEC were washed three times with medium 199, and incubated for a further 30 min at 37°C, in the presence of 0.5×10^6 neutrophils. The HEC were washed again as above, to remove the nonadherent neutrophils, and then incubated for 2 min at room temperature, in the presence of 0.5 ml tetramethylbenzidine (TMB) solution (2 mM TBM+0.1% (w/v) cetyltrimethylammonium bromide (CTAB) + 1 mM 3-amino-1,2,4 triazole, the selective eosinophil peroxidase inhibitor, in 0.1 M sodium acetate buffer, pH 4.2), as the substrate for neutrophil peroxidase. Hydrogen peroxide, 0.7 mM, was added to the HEC for 2 min, and the reaction was then stopped by the addition of 0.5 ml of 4 N acetic acid, containing 10 mM sodium azide. The optical density of the reaction colour developed was determined by measuring the absorbance at 620 nm. The number of neutrophils adhering to the HEC was calculated from a calibration curve prepared for the reaction colour developed from cell suspensions containing known numbers of neutrophils.

Statistical analysis

Individual data were analysed for skewness and expressed as mean \pm SEM for each treatment group. The significance of any differences in means of the different treatment groups, at each time-point, was compared by one way analysis of variance (ANOVA), followed by further analysis by Student's two sample t-test. Electrical resistance data were log-transformed and then subjected to similar statistical evaluation. All values of $p < 0.05$ were considered to be statistically significant.

Results

In accordance with our previous findings, these studies demonstrated that HBEC could be cultured to confluence on microporous membranes.

The Limulus amoebocyte lysate test demonstrated that the purified HIE was biologically active, as indicated by gelling of the lysate. Analysis of the total protein and nucleic acid contents of the endotoxin preparation revealed that these constituted 5.0 and 6.1% of the total preparation, respectively. Electrophoresis, followed by silver staining, further demonstrated that the HIE was composed of three constituents; a smaller component (No. 3) of molecular weight less than 26 kD, and two larger components (Nos 1 and 2) of molecular weight 58–84 kD (fig. 1). Comparison of this preparation with commercially available preparations demonstrated that the components of HIE had similar electrophoretic properties to those of *Escherichia coli* F583 (Rd[2] mutant) and *Klebsiella pneumoniae* lipopolysaccharides (fig. 1).

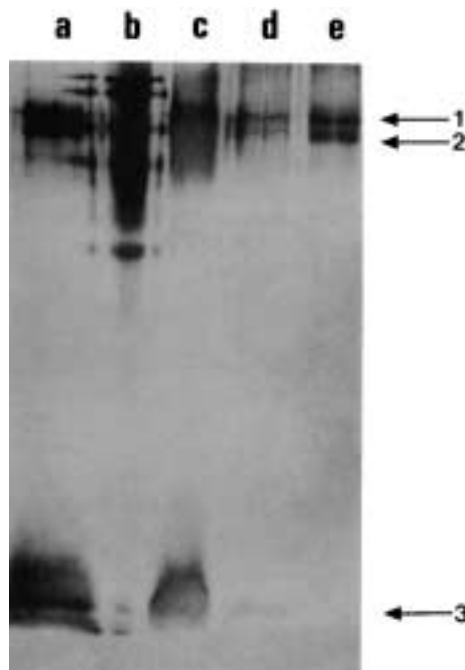


Fig. 1. – SDS-PAGE of *Haemophilus influenzae* endotoxin (HIE) preparation, followed by silver staining. Lane (a) 1,000 $\mu\text{g}\cdot\text{ml}^{-1}$ *Escherichia coli* F583 (Rd[2] mutant) lipopolysaccharide; lane (b) standard molecular weight (MW) markers (26–116 kDa); lane (c) 1,000 $\mu\text{g}\cdot\text{ml}^{-1}$ *Klebsiella pneumoniae* lipopolysaccharide; lane (d) 1,000 $\mu\text{g}\cdot\text{ml}^{-1}$ HIE; and lane (e) 100 $\mu\text{g}\cdot\text{ml}^{-1}$ HIE. Lane (d) demonstrates two larger components 1 and 2 (MW 58–84 kDa), and a smaller component 3 (MW <26 kDa). Components 1 and 2 are also seen in lane (e). These profiles are similar to those seen for *E. coli* and *K. pneumoniae* lipopolysaccharides. SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Movement of ^{14}C -BSA from the apical to the serosal aspects of HBEC incubated with 10 and 100 $\mu\text{g}\cdot\text{ml}^{-1}$ HIE for 24 h, respectively, was found to be $1.7 \pm 0.08\%$ and $1.8 \pm 0.09\%$ of the total ^{14}C -BSA added to the apical aspects of HBEC. This was not significantly different from the $2.0 \pm 0.6\%$ of total ^{14}C -BSA found in the insert wells of untreated HBEC, incubated simultaneously for the same period (fig. 2).

Incubation with 10 or 100 $\mu\text{g}\cdot\text{ml}^{-1}$ HIE, significantly increased the electrical resistance from 2.62 ± 0.08 and $2.6 \pm 0.15 \Omega \text{ cm}^2$, respectively, at beginning of incubation, to $2.99 \pm 0.1 \Omega \text{ cm}^2$ ($p < 0.05$) and $3.09 \pm 0.09 \Omega \text{ cm}^2$ ($p < 0.05$), respectively, after 24 h incubation. However, the electrical resistance was not altered significantly in the untreated HBEC at any time-point during incubation (fig. 3).

Analysis of IL-6 released by HBEC into the culture medium, demonstrated that this was significantly increased to $12.12 \pm 1.5 \text{ pg}\cdot\mu\text{g}^{-1}$ cellular protein ($p < 0.005$) by 100 $\mu\text{g}\cdot\text{ml}^{-1}$ HIE, compared to 0.09 ± 0.03 and $1.74 \pm 0.8 \text{ pg}\cdot\mu\text{g}^{-1}$ cellular protein released by untreated and 10 $\mu\text{g}\cdot\text{ml}^{-1}$ HIE-treated HBEC, respectively, after 24 h incubation (fig. 4). In contrast, analysis of IL-8 released from HBEC into the culture medium, demonstrated that incubation with 10 and 100 $\mu\text{g}\cdot\text{ml}^{-1}$ HIE, induced a significantly greater release of 10.2 ± 1.1 ($p < 0.05$) and $12.3 \pm 2.2 \text{ pg}\cdot\mu\text{g}^{-1}$ cellular protein ($p < 0.05$), respectively, compared to

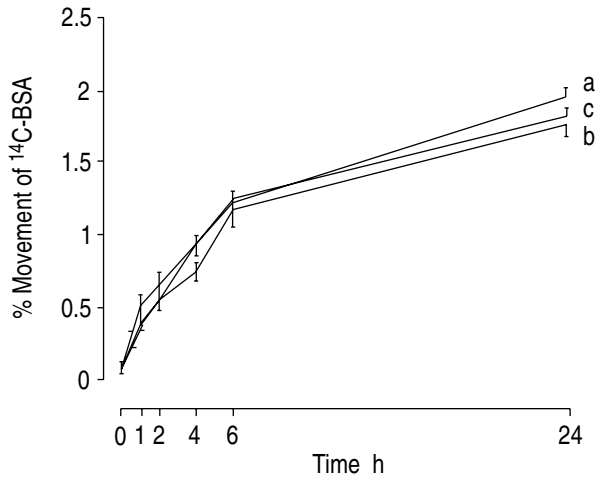


Fig. 2. – The effect of *Haemophilus influenzae* endotoxin (HIE) on the movement of ¹⁴C-bovine serum albumin (¹⁴C-BSA) across human bronchial epithelial cell cultures. a: control; b: 10 µg·ml⁻¹ HIE; c: 100 µg·ml⁻¹. Results are expressed as mean±SEM, (n=6 at each concentration).

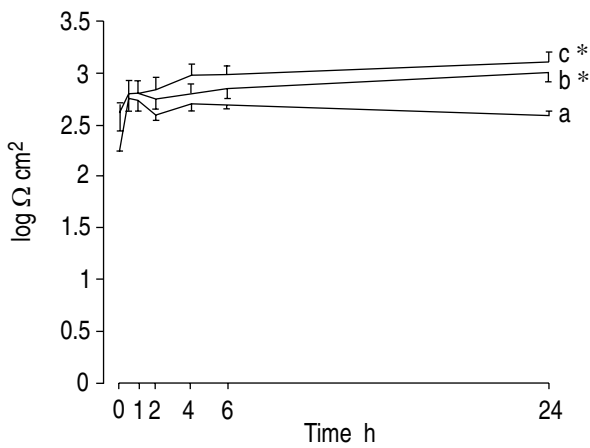


Fig. 3. – The effect of *Haemophilus influenzae* endotoxin (HIE) on the electrical resistance (log Ω) of human bronchial epithelial cell cultures. a: control; b: 10 µg·ml⁻¹ HIE; and c: 100 µg·ml⁻¹ HIE. Results are expressed as mean±SEM, (n=6 at each concentration). *: p<0.05 vs control.

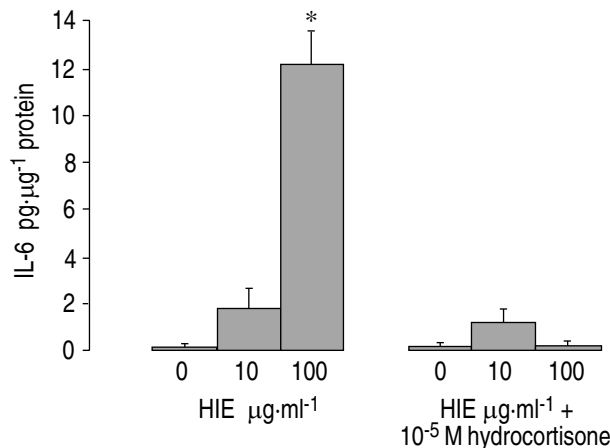


Fig. 4. – The effect of *Haemophilus influenzae* endotoxin (HIE) on the release of interleukin-6 (IL-6) by human bronchial epithelial cell cultures, and the influence of 10⁻⁵ M hydrocortisone. Results are expressed as mean±SEM, (n=6 at each concentration). *: p<0.005 vs untreated cells.

5.3±1.8 pg·µg⁻¹ cellular protein released from untreated HBEC after 24 h incubation (fig. 5). Similarly, analysis of TNF-α released into the culture medium demonstrated that this was significantly increased to 0.04±0.004 pg·µg⁻¹ cellular protein (p<0.001) on incubation of the HBEC with 100 µg·ml⁻¹ HIE, compared to 0.01±0.003 and 0.018±0.002 pg·µg⁻¹ cellular protein released from untreated and 10 µg·ml⁻¹ HIE-treated HBEC, respectively, (fig. 6). Incubation of HBEC in the presence of 10⁻⁵ M hydrocortisone, however, blocked HIE-induced release of IL-6, IL-8 and TNF-α.

The mean percentage of cells staining positively for ICAM-1, was significantly increased from 10.0±1.3%, in untreated HBEC, to 78.0±4.9% (p<0.001), in HBEC incubated with 100 µg·ml⁻¹ HIE (fig. 7). Although incubation with 10 µg·ml⁻¹ HIE resulted in a slightly increased number of 13.9±1.6% of cells staining positively for ICAM-1, this was not found to be significantly different from that in untreated HBEC. Incubation of HBEC with 10⁻⁵ M hydrocortisone significantly reduced

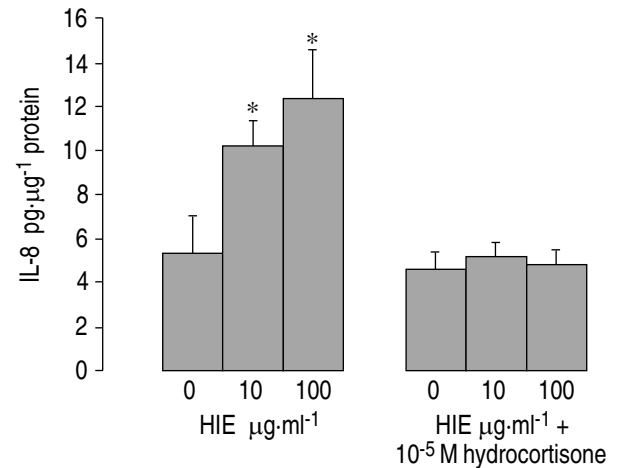


Fig. 5. – The effect of *Haemophilus influenzae* endotoxin (HIE) on the release of interleukin-8 (IL-8) by human bronchial epithelial cell cultures, and the influence of 10⁻⁵ M hydrocortisone. Results are expressed as mean±SEM, (n=6 at each concentration). *: p<0.05 vs untreated cells.

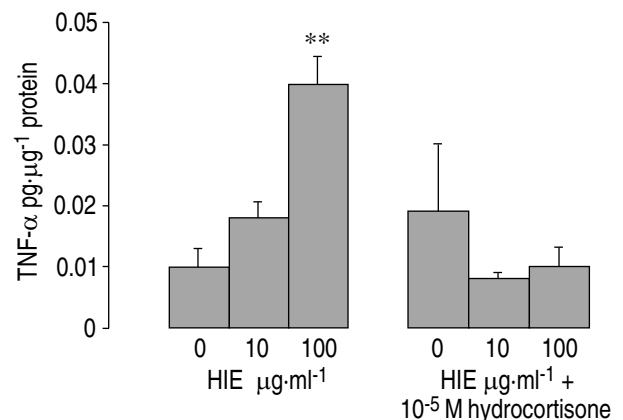


Fig. 6. – Effect of *Haemophilus influenzae* endotoxin (HIE) on the release of tumour necrosis factor-α (TNF-α) by human bronchial epithelial cell cultures, and the influence of 10⁻⁵ M hydrocortisone. Results are expressed as mean±SEM, (n=6 at each concentration). **: p<0.001 vs untreated cells.

the HIE-induced increase in the number of cells staining positively for ICAM-1 from 78.0 ± 4.9 to $43.5 \pm 4.2\%$ ($p < 0.001$), in cultures incubated with $100 \mu\text{g}\cdot\text{ml}^{-1}$ HIE (fig. 7). Similarly, 10^{-5} M hydrocortisone significantly reduced the basal expression of ICAM-1 from 10.0 ± 1.3 to $3.8 \pm 0.7\%$ ($p < 0.005$), in untreated HBEC.

To assess the biological relevance of HIE-induced release of inflammatory mediators, we investigated the effect of conditioned medium from cultures treated with $100 \mu\text{g}\cdot\text{ml}^{-1}$ HIE on neutrophil chemotaxis and adhesion to HEC, *in vitro*. Neutrophil chemotaxis was significantly increased from 28.3 ± 2.5 neutrophils $\cdot\text{hpf}^{-1}$, for conditioned medium from untreated HBEC, to 49.0 ± 8.0 neutrophils $\cdot\text{hpf}^{-1}$ ($p < 0.05$), for conditioned medium from HIE-treated cultures, and to 76.4 ± 10.5 for FMLP (fig. 8). Medium 199 \pm HIE, did not significantly alter the neutrophil chemotaxis. Similarly, conditioned medium

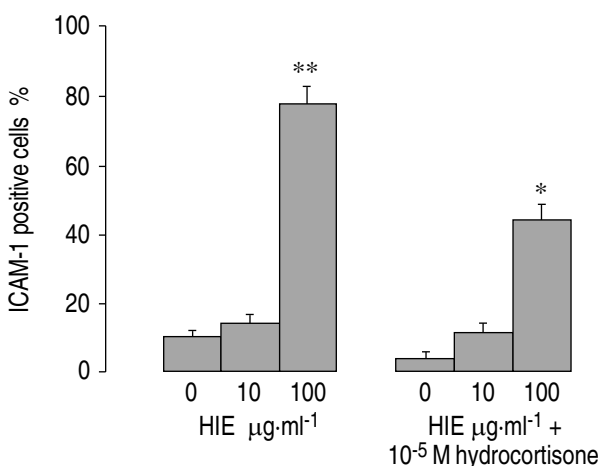


Fig. 7. – Effect of *Haemophilus influenzae* endotoxin (HIE) on the expression of surface intercellular adhesion molecule-1 (ICAM-1) by human bronchial epithelial cells (HBEC) cultures, and the influence of 10^{-5} M hydrocortisone. Results are expressed as mean \pm SEM, ($n=6$ at each concentration). **: $p < 0.001$ vs untreated cells; *: $p < 0.001$ vs HBEC treated with $100 \mu\text{g}\cdot\text{ml}^{-1}$ HIE.

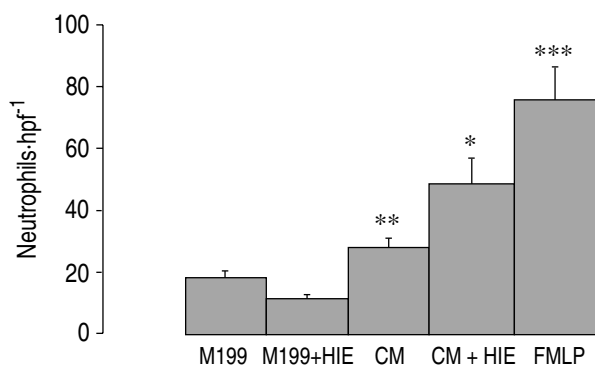


Fig. 8. – The effect of medium 199 (M199), medium 199 + *Haemophilus influenzae* endotoxin (HIE) (M199 + HIE), conditioned medium from human bronchial epithelial cells (HBEC) incubated for 24 h in the absence of HIE (CM), conditioned medium from HBEC incubated for 24 h in the presence of $100 \mu\text{g}\cdot\text{ml}^{-1}$ HIE (CM + HIE) and formyl-methionyl-leucyl-phenylalanine (FMLP) on neutrophil chemotaxis. Results are expressed as mean \pm SEM ($n=6$ at each concentration). *: $p < 0.05$ for CM vs CM + HIE; **: $p < 0.05$ CM vs M199/M199 + HIE; ***: $p < 0.01$ for CM vs FMLP. hpf: high power field.

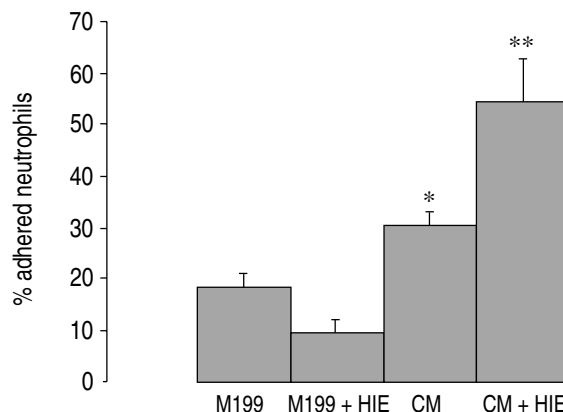


Fig. 9. – The effect of medium 199 (M199), medium 199 + *Haemophilus influenzae* endotoxin (HIE) (M199 + HIE), conditioned medium from human bronchial epithelial cells (HBEC) incubated for 24 h in the absence of HIE (CM) and conditioned medium from HBEC incubated for 24 h in the presence of $100 \mu\text{g}\cdot\text{ml}^{-1}$ HIE (CM + HIE), on neutrophil adhesion to cultured HEC. Results are expressed as mean \pm SEM ($n=6$ at each concentration). **: $p < 0.05$ for CM vs CM + HIE; *: $p < 0.05$ for CM vs M199/M199 + HIE.

from HIE-treated HBEC led to significantly increased adherence of $54.9 \pm 8.1\%$ ($p < 0.05$) of neutrophils to endothelial cells, compared with adherence of $30.8 \pm 2.3\%$ observed for conditioned medium from untreated cultures (fig. 9). Medium 199 \pm HIE, did not significantly increase neutrophil adherence.

Discussion

Although bacterial endotoxins have been shown to induce acute airway inflammation, leading to epithelial dysfunction and damage, the precise mechanisms underlying this inflammation are not clearly understood. 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 important colonizing strains isolated in chronic bronchitis and rarely the cause of lower respiratory tract infection in normal individuals.

To address these questions, we have prepared endotoxin from a nontypable strain of *H. influenzae* (strain NCTC 8143) and investigated the effect of this compound on epithelial permeability, release of proinflammatory cytokines and expression of ICAM-1, in HBEC *in vitro*.

Our studies have demonstrated that HIE does not increase the permeability of HBEC, as indicated by lack of differences in movement of ^{14}C -BSA across untreated or HIE-treated HBEC. Indeed, these studies have demonstrated that HIE significantly increases the electrical resistance of HBEC, and may actually lead to an initial decrease in epithelial permeability of HBEC *in vitro*. These findings are in accordance with the findings of WIENER-KORNISH *et al.* [21], who investigated the effect of *E. coli* endotoxin (ECE) on sheep lung epithelium *in vivo*. These authors demonstrated that although ECE produced a marked increase in interstitial pulmonary oedema and a tenfold increase in the number of leucocytes, particularly neutrophils, in the alveolar air spaces, it did

not affect the bidirectional movement of ^{125}I -labelled albumin across the alveolar epithelium, suggesting that alveolar epithelial permeability was not altered. Furthermore, these authors demonstrated that treatment with ECE did not lead to any detrimental morphological changes in the alveolar epithelium.

Our studies have demonstrated that HBEC have the ability to synthesize and release proinflammatory cytokines, including IL-6, IL-8 and TNF- α . This is in accordance with our previous findings, which demonstrated that HBEC express specific messenger ribonucleic acid (mRNA) for and immunoreactive interleukin-1 β (IL-1 β), IL-8, granulocyte macrophage colony-stimulating factor (GM-CSF), and TNF- α [6]. Our studies with HIE have demonstrated that this agent induces significant release of IL-6, IL-8 and TNF- α and increases the expression of ICAM-1 in HBEC. This may be of particular importance in bacterial-induced airway inflammation, since these mediators have been shown to exert a significant influence on neutrophil activity [7–9, 15]. Our studies of the effect of conditioned medium from HIE-treated HBEC have demonstrated that this significantly increases neutrophil activation *in vitro*, and suggests that bacterial endotoxin may indeed serve to enhance neutrophil infiltration of the airways. Animal studies have also suggested that exposure to endotoxin may lead to increased neutrophil activity [22], as a consequence of increased generation of leukotriene B₄ (LTB₄), 12-hydroxyeicosate-traenoic acids (12-HETEs) [23], and platelet-activating factor (PAF) [24], which may directly affect neutrophil activity.

Overall, our findings of HIE-induced release of cytokines is in accordance with the findings of others, who have demonstrated that several endotoxin-elicited effects may be mediated *via* generation of proinflammatory cytokines and adhesion molecules. Studies by HEREMANS *et al.* [25] have suggested that interleukin-6 (IL-6) may be involved in endotoxin-mediated reactions, since anti-IL-6 antibodies were found to protect mice against the generalized endotoxin-elicited Schwartzman reaction. Numerous activities have been ascribed to IL-6, including its ability to contribute to the stimulation of humoral and cellular defence mechanisms; to facilitate the terminal differentiation of B-cells into immunoglobulin-secreting cells; and to act as a helper factor in primary antigen-receptor-dependent T-cell activation and subsequent proliferation [26]. SMITH *et al.* [27] and DOHERTY *et al.* [28], have investigated the effects of ECE injected into rats and mice, respectively, and demonstrated that this results in a significant increase in serum TNF- α activity. The studies of DOHERTY *et al.* [28] further demonstrated that specific neutralizing antibodies both to TNF- α and interferon- γ (IFN- γ) protected the animals against lethality of the ECE, and suggested that TNF- α and IFN- γ may be acting synergistically to potentiate the toxicity of ECE. This finding of an endotoxin-mediated increase in TNF- α may be of particular relevance, since this cytokine has been shown to upregulate ICAM-1 [12], which is important in the recruitment and "trafficking" of inflammatory cells.

Whilst these studies have given an indication of the likely mechanisms underlying the neutrophilia observed in endotoxaemia, they have also suggested that steroids could have a role in the treatment of this condition, since hydrocortisone treatment was seen to block endotoxin-induced release of IL-6, IL-8 and TNF- α and attenuate the expression of ICAM-1 on HBEC. Indeed, these findings are in agreement with those of others, who have also demonstrated that steroids, at concentrations of 10^{-7} – 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* [29, 30].

In summary, our studies have demonstrated that human bronchial epithelial cells are capable of expressing and releasing potent proinflammatory cytokines and adhesion molecules, and that HIE significantly increases their expression. Although the specific mechanism(s) of *H. influenzae* induced damage to the airways are not fully understood, the finding that HIE can release cytokines from human airway epithelial cells and that this can be blocked by steroids is of particular significance, since it suggests that an interaction between bacterial products and airway epithelial cells may be an important stage in the development of bacterial-induced respiratory disease.

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