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Interaction of cigarette smoke and house dust mite allergens on inflammatory mediator release from primary cultures of human bronchial epithelial cells

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Summary

Background Several studies have shown that exposure to cigarette smoke and/or house dust mite (HDM) can lead to increased airway inflammation in susceptible individuals. The underlying mechanisms, however, are not defined.

Objective To investigate the interaction between cigarette smoke and HDM allergen on mediator release from primary cultures of human bronchial epithelial cells.

Methods Confluent human bronchial epithelial cell cultures were exposed to cigarette smoke in the absence or presence of HDM allergen and investigated for the release of IL-8, IL-1β, and sICAM-1. Damage to the epithelial cells themselves was assessed by release of ^{51}Cr. On separate occasions, we investigated the effect of PTL11028, a highly potent and selective *Der p*1 inhibitor, on HDM allergen-induced release of IL-8, following activation of HDM allergen by incubation with cysteine. The effect of cigarette smoke exposure on the stability of these released mediators in prepared solutions in the absence/presence of reduced glutathione was also studied.

Results Both HDM allergens and short-term (20 min) cigarette smoke exposure led to a significantly increased release of IL-8, IL-1β and sICAM-1 from the epithelial cell cultures. Longer exposure (1–6 h) to cigarette smoke led to a dramatic decrease in the amount of these mediators detected in the culture medium. Whilst incubation of epithelial cultures with HDM allergen did not cause any significant change in the release of ^{51}Cr from pre-loaded cells, cigarette smoke on its own led to a marked, exposure and incubation-time dependent increase in the release of ^{51}Cr. Incubation with HDM allergen led to a significant, dose and time-dependent increase in the release of IL-8, which was further enhanced when the allergen extract was pre-activated with cysteine. This effect was completely abrogated by PTL11028, a novel *Der p*1 inhibitor. Prepared solutions of various concentrations of IL-8, IL-1β and sICAM-1 exposed to cigarette smoke demonstrated a dramatic exposure time-dependent decrease in the detectable amount of these mediators, an effect which was abrogated by GSH.

Conclusions HDM-induced airway inflammation may include *Der p*-mediated release of inflammatory mediators from epithelial cells. Additionally, short-term cigarette smoke exposure may induce airway inflammation by release of inflammatory mediators from these cells, an effect which may be potentiated by *Der p* allergens. Longer term cigarette smoke exposure may cause damage to epithelial cells and changes in the structure of inflammatory mediators.

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Introduction

Several studies have demonstrated that there is an association between exposure to cigarette smoke and airway inflammation. Many studies have demonstrated that children of smoking parents and in particular smoking mothers, have a higher frequency of respiratory illnesses [1–3]. Other investigators have shown a highly significant increase in wheezing, coughing and respiratory infections in children with smoking mothers and that this effect is directly proportional to the number of cigarettes smoked by the mother [4]. Cigarette smoking is also associated with increased airway responsiveness [5], exacerbation of asthma [6] and impaired pulmonary immune function.

Mechanistic studies have demonstrated that cigarette smoke condensate significantly increases rat lung epithelial permeability, associated with a significant recruitment of neutrophils into the air-spaces. Pulmonary epithelial permeability has also been shown to be increased in smokers when compared to non-smokers [7]. Other studies have demonstrated increases in interleukin (IL)-8 levels in bronchoalveolar lavage (BAL) fluid of smokers compared with that of non-smokers [8,9], and a significant correlation between IL-8 concentration and neutrophil count in bronchial samples of BAL fluid [9]. Moreover, Kuschner and colleagues [10] demonstrated that not only statistically greater concentrations of neutrophils, macrophages, IL-1β, IL-6, IL-8 and monocyte chemoattractant protein (MCP-1) in BAL were observed among smokers compared with non-smokers, but that cigarette smoking, categorized as 'pack daily' was predictive of BAL macrophages, neutrophils, IL-1β and IL-8.

Similarly, several studies have suggested that exposure to house dust mite (HDM) allergens may also lead to increased airway inflammation. Significant increases in IL-8 and eosinophil cationic protein (ECP) were observed in the nasal lavage fluid of HDM-allergic asthmatic children compared with both normals and allergic non-asthmatics. IL-8 was also found to be correlated significantly with ECP, indicating that IL-8 plays a role in the maintenance of airway inflammation [11].

Comoy and colleagues demonstrated that *Dermatophagoides pteronyssinus* 1 (*Der p*1), a major allergen of HDM, has the ability to modify the profile of an immune response by modulating the balance between the cytokines IL-4 and interferon-γ (IFN-γ). These authors also showed that inactivation of the protease activity of *Der p*1 reduced this ability, suggesting that this activity plays an important role in deviating the immune response [12]. *Der p* allergens have also been shown to induce a significantly increased expression of HLA-DR and enhanced release of IL-2, granulocyte-colony stimulating factor (GM-CSF), IL-1β, tumour necrosis factor-α (TNF-α) and IFN-γ from blood mononuclear cells of subjects sensitized to *Der p* [13].

In our previous study [14] we demonstrated that exposure of human bronchial epithelial cell (HBEC) cultures for 20 min to cigarette smoke (CS) did not significantly alter the permeability of these cultures, whilst exposure to cigarette smoke for longer periods of 1–6 h significantly increased the permeability of these cultures. Additionally, exposure to cigarette smoke led to significant epithelial cell damage, an effect which was augmented further when the cultures were incubated with *Der p*. We also showed that incubation of HBEC cultures with *Der p* led to a significant, time-dependent increase in the permeability of these cultures, and that these effects were enhanced significantly by exposure to cigarette smoke and abrogated by incubation with specific protease inhibitors and reduced glutathione (GSH) [14]. Also, the passage of *Der p* allergens across the epithelial layer progressively increased with time during incubation and this was augmented significantly by exposure to cigarette smoke [14].

Whilst *Der p*1 and *Der p*9 have been found to mediate the release of IL-8, IL-6 and GM-CSF from both HBEC cultures and bronchial epithelial cell lines [15], to date no data are available with regard to the effect of HDM allergens and cigarette smoke interaction on the release of inflammatory mediators from primary cultures of HBEC.

In this present study we have hypothesized that exposure to cigarette smoke may augment HDM allergen-induced airway inflammation by modulating increased release of pro-inflammatory mediators from the airway epithelial cells. To test this hypothesis, we have cultured HBEC as primary explant cultures and studied the effect of exposure to CS for 20 min, as well as 1, 3 and 6 h in the absence or presence of non-activated HDM allergens on the release of IL-8, IL-1β and sICAM-1 from these cells. Damage of HBEC following exposure to CS both in the absence or presence of HDM allergen was also assessed. We have also investigated the effect of PTL11028, a specific inhibitor of *Der p*1 on non-activated and activated HDM allergen-induced release of IL-8.

Materials and methods

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

Culture of HBEC

Bronchial tissue was obtained from 21 male and eight female patients, who underwent lobectomy for lung cancer at The London Chest Hospital, London, UK. All patients were smokers, not allergic to common allergens, and with a mean age of = 65.3 ± 3.1 years (range 60–78 years). Following resection, only tissue macroscopically free of tumour was processed further for culture.

Bronchial epithelial cells were cultured using the explant cell culture technique developed in our laboratory [16]. Briefly, the epithelium was dissected away from the underlying lamina propria and after further dissection into smaller sections of approximately 0.5 mm^3, the epithelium was washed three times with fresh sterile Medium 199. Single sections of the epithelium were explanted into 9 mm diameter Falcon® cell culture inserts (Becton Dickinson Ltd, Oxford, England, UK) and 100 μL aliquots of complete culture medium, comprising 2.5% fetal calf serum and a variety of growth factors, including human transferrin (2.5 μg/mL), epidermal growth factor (20 ng/mL), bovine pancreatic insulin (2.5 μg/mL), hydrocortisone (0.36 μg/mL), L-glutamine (0.02 mg/mL) and 1.5% (v/v) antibiotic and antimycotic solution composed of penicillin, streptomycin and amphotericin B, were added to each insert.

The inserts were placed in 24-well culture plates (Becton Dickinson Ltd, Oxford, England, UK) containing 400 μL of the complete culture medium in each well, and incubated at 37 °C in a humidified 5% CO_2 in air atmosphere. The medium in the insert and the well was replaced with culture medium containing 2.5% NUSERUM™ IV Culture Supplement (Universal Biologicals Ltd, London, England, UK) after 3 days, and then every 48 hours. The cultures were observed for epithelial cell outgrowth until the cells had grown to confluency. The explants were removed and the cultures incubated further until the area left barren by the removed explant was overgrown with epithelial cells.

The identity of the epithelial cells was confirmed in all cultures by light microscopy and in randomly selected cultures by electron microscopy and immunocytochemical staining for cytokeratin using monoclonal antibody preparation CAM 5.2 (Becton Ltd, Oxford, England, UK). Contaminating cell types were investigated by staining for T and B lymphocytes using monoclonal antibodies CD3 and CD37, respectively (Serotec Ltd, Oxford, England, UK) and fibroblasts, mast cells, neutrophils and macrophages using monoclonal antibodies 5B5, AA1, NP57 and Ber-MAC3, respectively (Dako Ltd, Bucks, England, UK).

Preparation of Der p solution

Aquagen® freeze-dried allergen extract (ALK, Horsholm, Denmark), which is known to contain several *Der p* subgroups and therefore maybe more relevant than a preparation containing a single allergen subgroup, was reconstituted in the specified diluent according to the manufacturer's instructions. This resulted in a stock solution at a concentration of 100 000 SQ-U/mL *Der p* (equivalent to 9000 ng/mL *Der p*), which was further diluted with Medium 199 containing serum free supplement (SF-1 medium) (Hyclone Europe Ltd, Northumberland, England, UK), to provide a working *Der p* solution at a concentration of 300 ng/mL. This concentration was chosen since it is approximately one-tenth the concentration deemed to pose a risk of sensitization *in vivo* [17]. For dose response studies solutions at concentrations of 1000 and 3000 ng/mL *Der p* were also prepared.

Preparation of PTL11028

The design and synthesis of PTL11028 (benzoyl-valanyl-alanyl-norleucyl-(2,6-bistrifluoromethyl)-benzyl-acyloxymethylketone), a novel highly potent and selective *Der p*1 protease inhibitor (Peptide Therapeutics Ltd, Cambridge, England, UK) is described elsewhere [18]. PTL11028 was diluted to 12.5 mM in DMSO, with further dilutions being prepared to a 250-μM stock in tissue culture media.

Exposure of HBEC to cigarette smoke

Exposure of HBEC to cigarette smoke was carried out in an exposure system which we have developed in our laboratory and described in detail elsewhere [14]. Briefly, a continuous, 850 mL/min flow of cigarette smoke was generated by burning commercially available cigarettes (a single brand with 12 mg tar, 1.1 mg nicotine) and leading the smoke stream into an airtight polycarbonate exposure chamber with a capacity of 5.5 L (Billups Rothenberg, Del Mar, CA, USA) containing confluent HBEC cultures. The continuous flow of cigarette smoke was achieved by the use of an Airchek 50 Sampling Pump (SKC Ltd, Dorset, England, UK) capable of generating 5–3000 mL/min flow. The pump was connected to the top of the exposure chamber and operated to create a negative pressure required to draw in the smoke from a lit cigarette connected to the bottom outlet of the exposure chamber. The incoming smoke was distributed evenly throughout the chamber by means of a small solar energy powered fan placed inside the chamber just below the HBEC cultures. The exposure chamber was

tilted gently, at intervals of 2.5 s, to an angle of 10° from the horizontal in each quarter of the horizontal plane on a Luckham 4RT rocking table (Luckham Ltd, Burgess Hill, England, UK), thereby providing adequate mixing of the culture medium with the cigarette smoke. To ensure that the temperature was kept stable at 37 °C during exposure, the entire system was placed in a 60-litre capacity acrylic SI.60 incubator (Stuart Scientific, Redhill, England, UK).

HBEC cultures were exposed to cigarette smoke for 20 min, 1 h, 3 h and 6 h, and appropriate controls were prepared by exposure to air for similar periods.

Effect of exposure to cigarette smoke in the absence or presence of Der p on IL-8, IL-1β and sICAM-1 release from HBEC cultures

Sets of eight separate confluent cultures, each from a different individual were washed and incubated overnight in SF-1 medium (Hyclone Europe Ltd, Northumberland, England, UK) and prior to further use, the cultures were gently washed three times with fresh medium. 500 μL aliquots of SF-1 medium were added into each insert and well, and the HBEC cultures were then exposed to cigarette smoke or air for 20 min, 1 h, 3 h and 6 h, either in the absence or presence of 300 ng/mL *Der p*, as described above. Immediately after exposure the medium was collected from each insert well, and 100 μL medium was removed from each insert. 500 μL of fresh medium was added into each well, and the cultures were incubated further at 37 °C in a 5% CO_2 in air atmosphere for up to 24 h, during which time this cycle of collecting and adding fresh medium into the well was repeated at 1, 3, 6 and 24 h after exposure. The medium samples collected were centrifuged for 10 min at 500 g**,** stored at − 70 °C, until analysis for IL-8, IL-1ß and sICAM-1 using commercially available ELISA kits (R & D Systems, Abingdon, England, UK). The insert membrane, containing the attached epithelial cells, was removed and stored by freezing at − 70 °C in 250 μL 1 M NaOH, until analysis for total cellular protein, by the method of Lowry and colleagues [19] and all results were expressed as pg IL-8 or IL-1ß/μg cellular protein, and ng sICAM-1/μg cellular protein.

Effect of PTL11028 on activated and non-activated Der p-induced release of IL-8 from HBEC cultures

To further explore the *Der p*-induced increase in IL-8 release from HBEC we sought to determine whether the *Der p*1 component had any significant contributory effect. This we examined in two ways, firstly by reducing the *Der p* extract with cysteine to ensure complete activation of the *Der p*1 component and secondly, by using PTL11028 to specifically inhibit the *Der p*1 component.

Therefore, sets of separate confluent HBEC cultures, each from a different individual, were incubated for 24 h in the absence or presence of either 300, 1000 or 3000 ng/mL *Der p* which had been pre-activated by incubation for 5 min with 1 mM cysteine, both in the absence and presence of saturating concentration of 1 μM PTL11028. All samples of culture media were evaluated for release of IL-8 and cellular protein, as described above.

Effect of cigarette smoke exposure on concentrations of IL-8, IL-1β and sICAM-1 in solution

Since our preliminary studies demonstrated that long-term exposure to cigarette smoke led to significantly decreased release of IL-8, IL-1β and sICAM-1 from HBEC cultures after 24 h of incubation, we hypothesized that this was a consequence of the degradative effects of cigarette smoke itself on these molecules, rather than the cytotoxic effects of cigarette smoke. To test this hypothesis, 500 μL of medium containing different concentrations of IL-8 (31.2–2000.0 pg/mL), IL-1β (3.9–250.0 pg/mL) and sICAM-1 (2.1–46.0 ng/mL) (R & D Systems, Abingdon, England, UK) were placed in 24-well culture plates (Becton Dickinson Ltd), and exposed to cigarette smoke for 20 min, 1 h, 3 h and 6 h, as described above. All plates were then incubated further for 24 h, and on termination the solutions were collected and stored at − 70 °C, until analysis for IL-8, IL-1ß and sICAM-1 using the same commercially available ELISA kits (R & D Systems). Appropriate controls were prepared by exposing the different mediator concentration solutions to air.

To determine whether the effect of cigarette smoke was due to its oxidizing characteristics, the above experiment was repeated with the test solutions containing 500 μM GSH, a concentration that was chosen to parallel concentrations of between 450 and 500 μM GSH found in the epithelial lining fluid *in vivo* [20].

Effect of exposure to CS on HBEC membrane damage

HBEC membrane damage was investigated by assessing the release of ^{51}Cr from cells radiolabelled with sodium chromate (Amersham International plc, Amersham, England, UK). Prior to exposure to CS, explants were removed from 2–3-week-old confluent HBEC cultures established on inserts and the cells incubated overnight in the presence of 1.0μCi[^{51}Cr] sodium chromate. Following this incubation the culture medium was decanted from both the inserts and the wells, and the cells were washed gently with Medium 199, to remove any radiolabel not incorporated into the cells. Fresh Medium 199 was added then into both the inserts and the insert wells, and the cultures were exposed to CS for 20 min in the absence or presence of

300 ng/mL *Der p* added into the inserts, as described above. At the end of exposure, all the medium in the wells and 50 μL aliquots of the medium in the inserts were removed. Fresh Medium 199 was added to the wells and the cultures were incubated further for up to 24 h at 37 °C in a 5% CO_2 in air atmosphere. During the incubation, the culture medium from the wells and inserts was collected at times 1, 3, 6 and 24 h, as described above.

After the final sampling of the medium from both the wells and the inserts at 24 h, 500 μL of 1 M NaOH was added into the inserts and the cell pellet lysed, prior to analysis for total radioactivity. 50 μL aliquots of all samples collected were mixed with 4.0 mL of Ready-Solv™ CP scintillation cocktail (Beckman-RIIC Ltd, High Wycombe, England, UK) and assessed for ^{51}Cr radioactivity, using a Beckman LS 6500 scintillation counter (Beckman-RIIC Ltd). All radioactivity released into the medium at any time point investigated was expressed as a percentage of total in the culture, according to the formula as shown below, and the results calculated as cumulative release of ^{51}Cr radioactivity at each time point.

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

Statistical analysis

Data were tested for normality using a normal probability plot and the Shapiro-Wilk test [21]. Since all data were found to be normally distributed, within group comparisons were made using two-way analysis of variance (ANOVA) and paired *t*-test, and between group comparisons were made using two sample *t*-tests.

Results

Culture and identification of HBEC

Microscopic examination of HBEC using Hoffman modulation contrast optics demonstrated large numbers of cells with polygonal morphology, typical of epithelial cells, growing out from the explants, which reached confluence after 3 weeks. Indirect immunoperoxidase staining with CAM 5.2, revealed that all the cells in the cultures stained for cytokeratin confirming the epithelial nature of the cells. Staining with specific monoclonal antibodies against contaminating cell types including endothelial cells, fibroblasts, muscle cells, macrophages, T and B lymphocytes, mast cells, eosinophils and neutrophils did not show any indication of these cells.

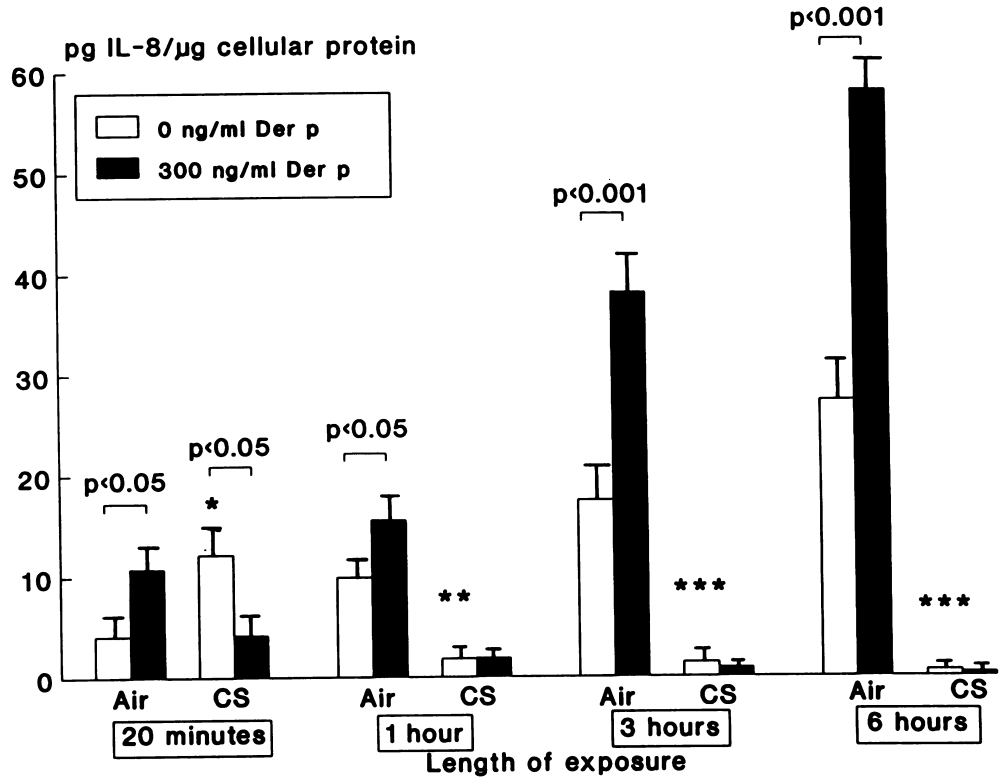


Fig. 1. Effect of exposure to cigarette smoke in the absence or presence of 300 ng/mL *Der p* on the release of IL-8 from HBEC cultures ($n = 8$ in each group; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. 0 ng/mL Derp + Air).

Although results were obtained at several time points during the 24 h incubation, only cumulative data after 24 h of incubation are shown; 1 μM PTL 11028 on its own had no effect on the release of any of the mediators investigated (data not shown).

Effect of exposure to cigarette smoke in the absence or presence of 300 ng/mL Der p on the release of IL-8 from HBEC cultures

Exposure of these cells for 20 min to cigarette smoke led to a significantly increased release of IL-8 compared to cultures exposed to air alone ($P < 0.05$).

Exposure to air in the presence of 300 ng/mL *Der p* for 20 min produced a significantly increased release of IL-8 compared to cultures exposed only to air ($P < 0.05$) (Fig. 1).

Cigarette smoke exposure for 20 min in the presence of *Der p* significantly decreased the release of IL-8 compared to both cultures exposed to 300 ng/mL *Der p* in the presence of air, and cultures exposed to cigarette smoke alone.

More prolonged cigarette smoke exposure for 1, 3 and 6 h, both in the absence or presence of *Der p*, led to a dramatic decrease in the amount of IL-8 detected in the culture medium. This was observed after each exposure time studied. In contrast, there was an incubation time-dependent increase in the release of IL-8 in the air exposed cultures which was further enhanced by incubation of the cultures with *Der p*.

Effect of exposure to cigarette smoke in the absence or presence of 300 ng/mL Der p on the release of IL-1β from HBEC cultures

Similar to our findings with IL-8 release, there was an incubation time-dependent increase in the release of IL-1β in the air exposed cultures which was further enhanced by incubation of the cultures with *Der p* (Fig. 2).

Contrary to our findings with IL-8, 1 h exposure to cigarette smoke also induced a significantly increased release of IL-1β when compared to cultures exposed to air ($P < 0.01$). However, the culture media of HBEC cultures exposed to cigarette smoke for 3 and 6 h, both in the absence or presence of *Der p*, contained significantly less detectable IL-1β than seen in the air exposed cultures, and the enhancing effect of *Der p* was not observed at exposure times of greater than 1 h.

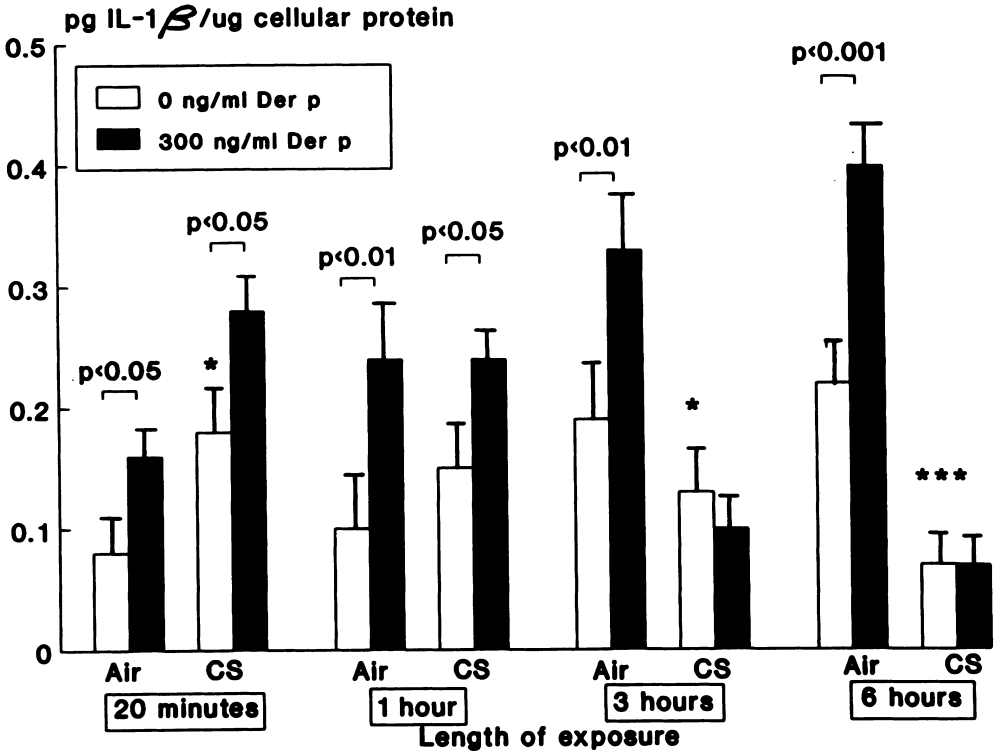


Fig. 2. Effect of exposure to cigarette smoke in the absence or presence of 300 ng/mL *Der p* on the release of IL-1β from HBEC cultures ($n = 8$ in each group; $^*P < 0.05$; $^{***}P < 0.001$ vs. 0 ng/mL Derp + Air).

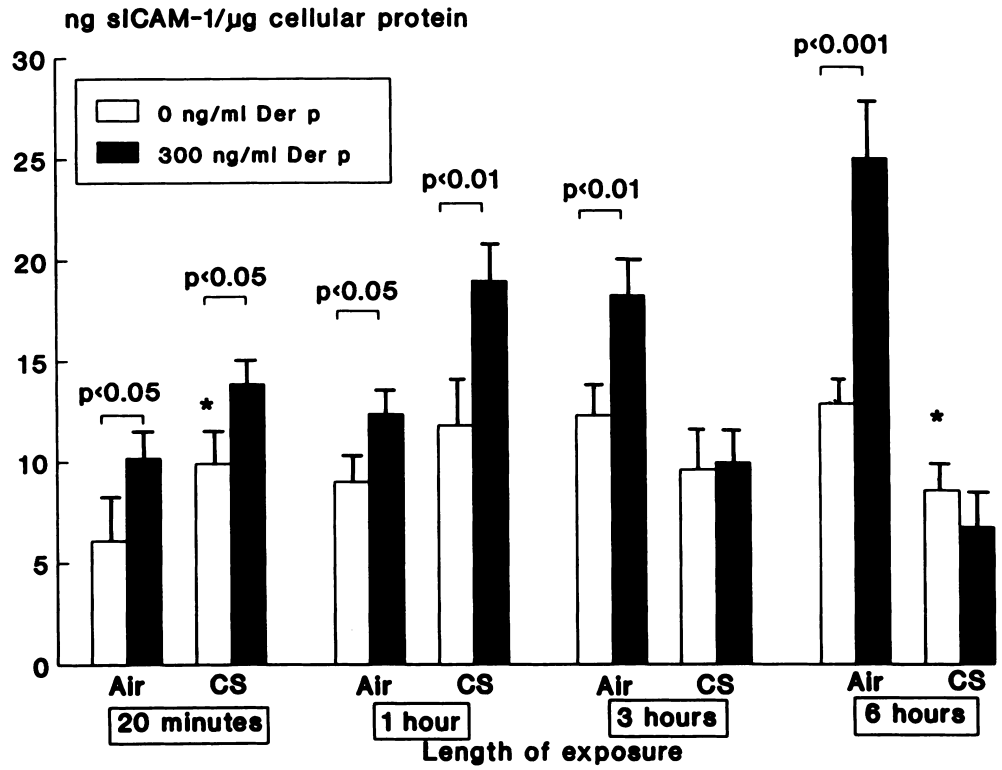


Fig. 3. Effect of exposure to cigarette smoke in the absence or presence of 300 ng/mL *Der p* on the release of sICAM-1 from HBEC cultures ($n = 8$ in each group; $^*P < 0.05$, vs. 0 ng/mL Derp + Air).

Effect of exposure to cigarette smoke in the absence or presence of 300 ng/mL Der p on the release of sICAM-1 from HBEC cultures

The pattern of sICAM-1 release in this experiment resembled that observed for IL-1β in that (a) the maximal release of this mediator occurred in air exposed cultures incubated in the presence of *Der p*, and (b) a significant decrease in the release of sICAM-1 occurred in cultures which were exposed for 3 or 6 h to cigarette smoke (Fig. 3).

Effect of PTL11028 on activated and non-activated Der p-induced increase in the release of IL-8 from HBEC cultures

Incubation with *Der p* significantly increased the release of IL-8 from HBEC cultures after 24 h, at all concentrations of 300–3000 ng/mL *Der p* investigated ($P < 0.05$), compared with release of IL-8 in the absence of *Der p* (Fig. 4). Incubation of HBEC cultures with *Der p* pre-activated with 1 mM cysteine further enhanced the release of IL-8, an effect which was significantly abrogated by incubation of *Der p* with the specific *Der p*1 inhibitor PTL 11028. Incubation of cultures with PTL11028 alone did not significantly influence the release of IL-8 from these cells.

Effect of cigarette smoke exposure on concentrations of IL-8, IL-1β and sICAM-1 in solution

Analysis of various concentrations of IL-8, IL-1β, and sICAM-1 solutions exposed for 1, 3, and 6 h to cigarette smoke have demonstrated that cigarette smoke exposure did cause a dramatic exposure time-dependent decrease in the detection of all the above mediators after 24 h of incubation (Figs 5,6,7). The magnitude of decrease in the amount of IL-8, IL-1β and sICAM-1 was not influenced by the starting concentrations of these mediators. The mean percentage changes for each mediator after exposure to cigarette smoke for 1, 3 and 6 h were: 28.8 ± 2.2%, 57.6 ± 2.5%, and 77.0 ± 3.3%, respectively, for IL-8 (Fig. 5); 66.8 ± 2.5%, 77.2 ± 1.6%, and 93.4 ± 1.6%, respectively, for IL-1β (Fig. 6); and 25.0 ± 1.4%, 37.6 ± 2.7%, and 57.2 ± 2.6%, respectively, for sICAM-1 (Fig. 7). Exposure of IL-8, IL-1β and sICAM-1 solutions in the presence of 500 μM GSH almost completely abrogated this effect of cigarette smoke. In contrast to the cigarette smoke-exposed mediator solutions, there was a

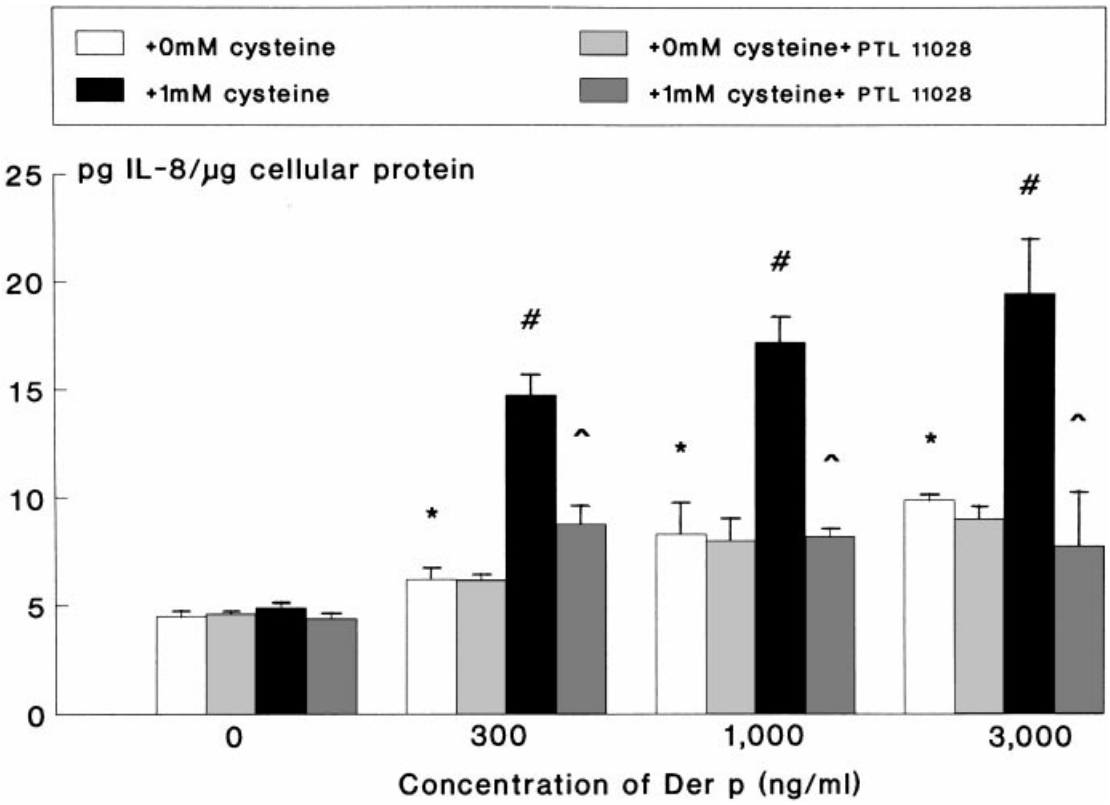


Fig. 4. Effect of PTL11028 on activated and non-activated *Der p*-induced increase in the release of IL-8 from HBEC cultures ($n = 9$ in each group; $*P < 0.05$ vs. 0 ng/mL *Der p*; $\#P < 0.001$ vs. 0 mM cysteine; $\wedge P < 0.01$ vs. 1 mM cysteine).

slight increase in the concentrations of the above mediators after exposure to air, due to the drying out effect of the air flow on the test solutions. However, when corrected for the evaporated fluid, there was no significant difference found between the starting and final concentrations of mediator.

Effect of exposure to CS in the absence or presence of 300 ng/mL Der p on HBEC membrane damage

Exposure of HBEC to CS for 20 min, 1 h, 3 h and 6 h in the absence of *Der p* demonstrated that this agent led to a significant, exposure and incubation time-dependent increase in the release of ^{51}Cr from these cells, when compared to cultures exposed to air (Fig. 8a).

In contrast, incubation of HBEC cultures in the presence of *Der p* did not significantly alter the release of ^{51}Cr, when compared to cultures exposed to air only (Fig. 8a-d).

Exposure of HBEC cultures to CS for 20 min in the presence of 300 ng/mL *Der p* did not significantly increase the release of ^{51}Cr at all time points studied when compared to cultures exposed to CS alone (Fig. 8a). Similarly, no such potentiating effect of *Der p* was observed in cultures exposed to CS for longer periods (Fig. 8 b, c, d).

Discussion

Our studies have demonstrated that incubation with *Der p* significantly increased the release of IL-8, IL-1β and sICAM-1 from HBEC cultures, and that incubation with pre-activated *Der p* further enhanced the release of IL-8, an effect which was blocked by pre-incubation with the *Der p*1 inhibitor PTL11028. Our studies also demonstrated that whilst short-term (20 min) exposure to cigarette smoke led to a significantly increased release of these mediators from the HBEC cultures, exposure for longer periods of 1–6 h led to a dramatic decrease in the amount of mediator detected in the culture medium. We have also demonstrated that whilst incubation of epithelial cultures with HDM allergen did not cause significant change in the release of ^{51}Cr from pre-loaded cells, CS on its own led to a marked increase in the release of ^{51}Cr. Collectively, these results suggest that whilst short-term exposure to cigarette smoke may augment HDM-induced mediator release from HBEC cultures, this is unlikely to occur when exposure to cigarette smoke takes place over longer periods.

Despite the lack of naturally occurring defences such as protective epithelial lining fluid and endogenous reducing agents which may limit the oxidant-induced cell damaging

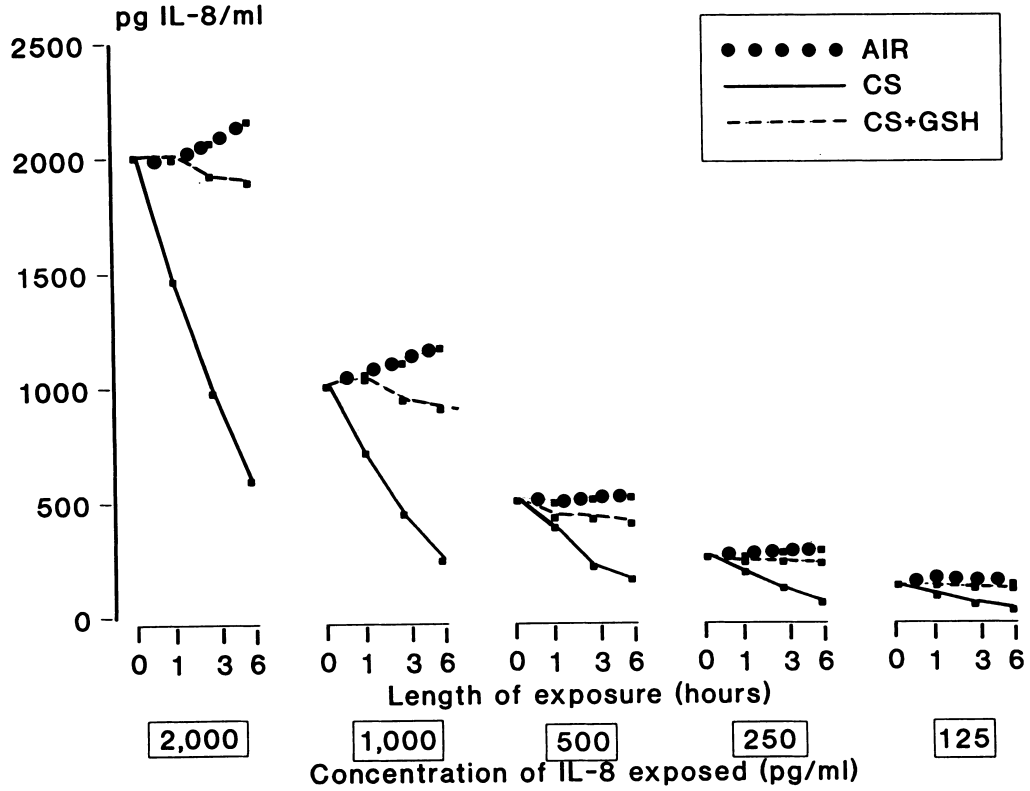


Fig. 5. Effect of cigarette smoke exposure on the stability of various concentrations of IL-8 ($n = 8$ for each concentration).

effect of cigarette smoke, primary cultures from human airway epithelial cells offer an *in vitro* model to study the effects of direct exposure to this agent and the mechanism/s underlying these effects in epithelial cells. The cell culture system can also be used to study the mechanisms by which allergens exhibiting proteolytic activity influence mediator release from epithelial cells. A well-balanced human epithelial lining fluid would be the optimal culture medium for use in such studies, but such standardized preparations are not available. A further limitation of this study is that epithelial cells were cultured from surgical tissue from lung cancer patients with a history of smoking; such cells might react differently to oxidant stress when compared to epithelial cells from healthy non-smokers [22].

Our observation of increased cytokine/mediator release from HBEC cultures following incubation with *Der p* are in agreement with the findings of King and colleagues [15]. These authors demonstrated that incubation with *Der p*1 and *Der p*9, produced release of GM-CSF, IL-6 and IL-8 from cultures of bronchial epithelial cells and BEAS-2B epithelial cell line, effects which could be inhibited by treatment of the cells with specific protease inhibitors. These authors also demonstrated greater release of IL-8 after treatment with *Der p*1 compared with *Der p*9, respectively, the HDM cysteine and serine proteolytic allergens. This suggested that cysteine protease activity was associated with IL-8 release, an interpretation supported by the observation that non-selective cysteine protease inhibitors such as E-64, blocked the *Der p*1-induced increase in the release of IL-8.

In our studies, activation of cysteine proteolytic activity with 1 mM cysteine led to an increased release of IL-8, whilst pre-incubation of *Der p* with the specific *Der p*1 inhibitor PTL11028 abrogated the *Der p*-induced release of IL-8. This effect of *Der p* on mediator release may be due to increased synthesis *de novo* since *Der p*1 and *Der p*9 have been found to increase the expression of IL-6 mRNA, an effect which was inhibited using appropriate protease inhibitors [15]. King and colleagues hypothesized that proteases interact with so-called 'proteinase activated receptors' to up-regulate a range of cellular functions including cytokine production and secretion, and cellular proliferation.

The allergen preparation used in our experiments contained a mixture of HDM allergen (*Der p*) subgroups (ALK, Horsholm, Denmark) containing at least three protease activities as we demonstrated previously [14]. The concentrations of *Der p* used in our experiments, 300–3000 ng/mL, are likely to be relevant to natural exposure since it has been demonstrated that specific bronchial

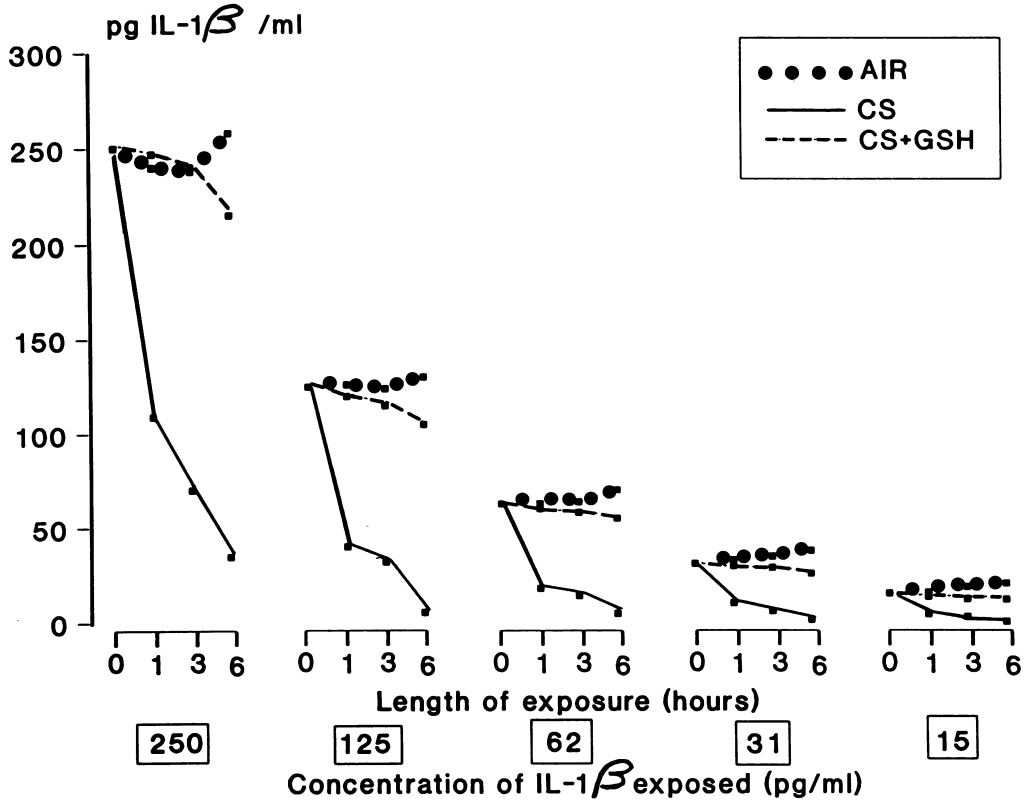


Fig. 6. Effect of cigarette smoke exposure on the stability of various concentrations of IL-1β ($n = 8$ for each concentration).

segmental challenge with 200 ng/mL *Der p* can induce local inflammatory responses [23], and mite faeces contain up to 10 mg *Der p*1/mL [24].

Our studies also demonstrated that whilst 20 min exposure to cigarette smoke led to a significantly increased release of IL-8, IL-1β and sICAM-1 from HBEC cultures, exposure for longer periods of 1–6 h led to a dramatic decrease in the amount of these mediators detected in the culture medium. Our observations of the effects of 20 min exposure to cigarette smoke have confirmed our previous preliminary findings [25], but the data obtained from studies of longer exposure periods were unexpected. We hypothesized that the observed decrease in the amounts of mediator with longer exposure to CS was in part the result of the oxidative effect of CS exposure on the structure of mediator molecules. We subsequently observed that solutions of IL-8, IL-1β and sICAM-1 exposed to CS demonstrated a dramatic exposure time-dependent decrease in the detectable amount of mediator, an effect which was abrogated by GSH. In cultures of HBEC, the magnitude of cigarette smoke-induced decrease in concentration of mediators in solution is likely to be modified by the release of glutathione from the cultures themselves [26].

Whilst it is plausible that the decrease in mediator concentration was due to the oxidizing properties of cigarette smoke, particularly since GSH reduced the effect so markedly, the precise underlying mechanism is undetermined. Cigarette smoke might alter the structure of cytokines, or alternatively modify the interaction of monoclonal antibodies with cytokines so reducing the amount detected. If the effect of cigarette smoke is on the structure of the mediators, then their function may be modified and future studies might usefully evaluate this. It is unlikely that there was significant adhesion of the investigated inflammatory mediators to the wall of culture plates or inserts as a result of prolonged exposure to cigarette smoke. Our previous experiments investigating exposure to cigarette smoke for up to 6 h [14] showed no adherence of proteins such as ^{14}C-BSA to the insert or well wall, whether the cultures were exposed to air or cigarette smoke.

Our data demonstrated that exposure to CS for between 20 min and 6 hours led to a marked increase in the release of ^{51}Cr, indicating epithelial cell membrane injury. In this study we have not assessed the effects of CS on apoptosis specifically. However, Vayssier and colleagues [27] have shown that exposure of human and rodent cell lines to CS for 4–16 h induced primarily cell necrosis, rather than apoptosis, as demonstrated by flow cytometry. These observations suggest that the primary effect of longer term CS exposure is cell damage, rather than apoptosis. This cell

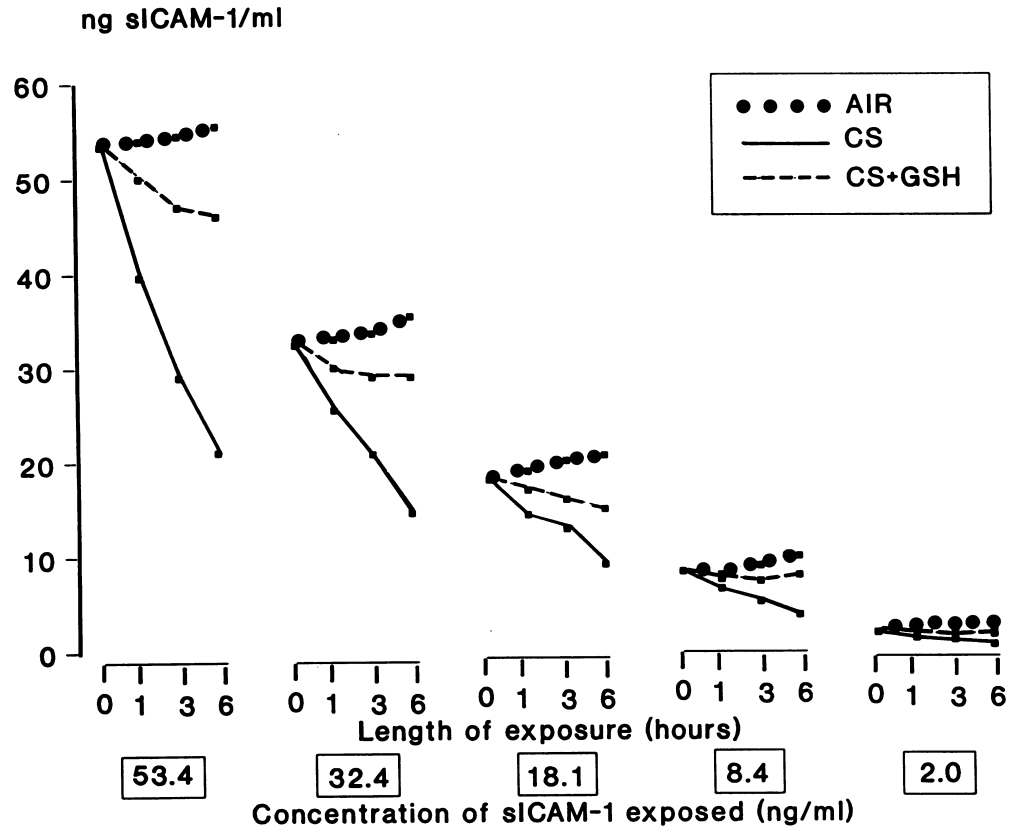


Fig. 7. Effect of cigarette smoke exposure on the stability of various concentrations of sICAM-1 ($n = 8$ for each concentration).

damage may in part be responsible for the decreased release of IL-8, IL-1β and sICAM-1. Collectively, these results suggest that short-term exposure to cigarette smoke may augment *Der p*-induced mediator release from HBEC. However, such augmentation is less likely to occur when exposure to cigarette smoke takes place over longer periods.

Others have reported similar findings. Witherden and colleagues investigated the effect of 1 and 5% cigarette smoke solution on the production of IL-8, macrophage inflammatory protein, monocyte chemoattractant protein and 'released on activation, normal T cell expressed and secreted' (RANTES) from primary cultures of human alveolar epithelial type II cells [28]. Cigarette smoke reduced levels of all these chemokines. In contrast, Mio and colleagues [9] using human bronchial epithelial cells obtained from brushings found that cigarette smoke extract induced a significant, concentration and time dependent increase in the release of IL-8. The different findings of Mio and colleagues' might be related in part to their use of a cell suspension whereas our study and that of Witherden *et al.* [28] employed confluent cultures of epithelial cells.

The results of our present study suggest that an important effect of cigarette smoke-exposure may be to cause release of ICAM-1 from the surface of the epithelial cells, since exposure caused an increased concentration of sICAM-1. Since the expression of adhesion molecules on airway epithelial cells facilitates their interaction with the underlying substratum and adjacent cells thereby forming functional permeability barriers, loss of surface ICAM-1 would render the epithelial barrier less tight [29] leading to increased permeability of the epithelial layer. This effect may be enhanced by *Der p* mediated cleavage of tight intercellular junctions [30]. In a more permeable epithelial layer with individual epithelial cells less tightly bound, the cells would have a larger surface interfacing with external stimuli such as other pollutants or allergens. As a result there might be a greater release of pro-inflammatory mediators. Indeed, this hypothesis is supported by our previous work, where we demonstrated that increased permeability was associated with increased release of IL-8, and IL-1β [22].

We conclude, that HDM-induced airway inflammation may include *Der p*-mediated release of inflammatory mediators from epithelial cells. Additionally, short-term cigarette smoke exposure may also induce release of inflammatory mediators from these cells. However, longer cigarette smoke exposure may be associated with a

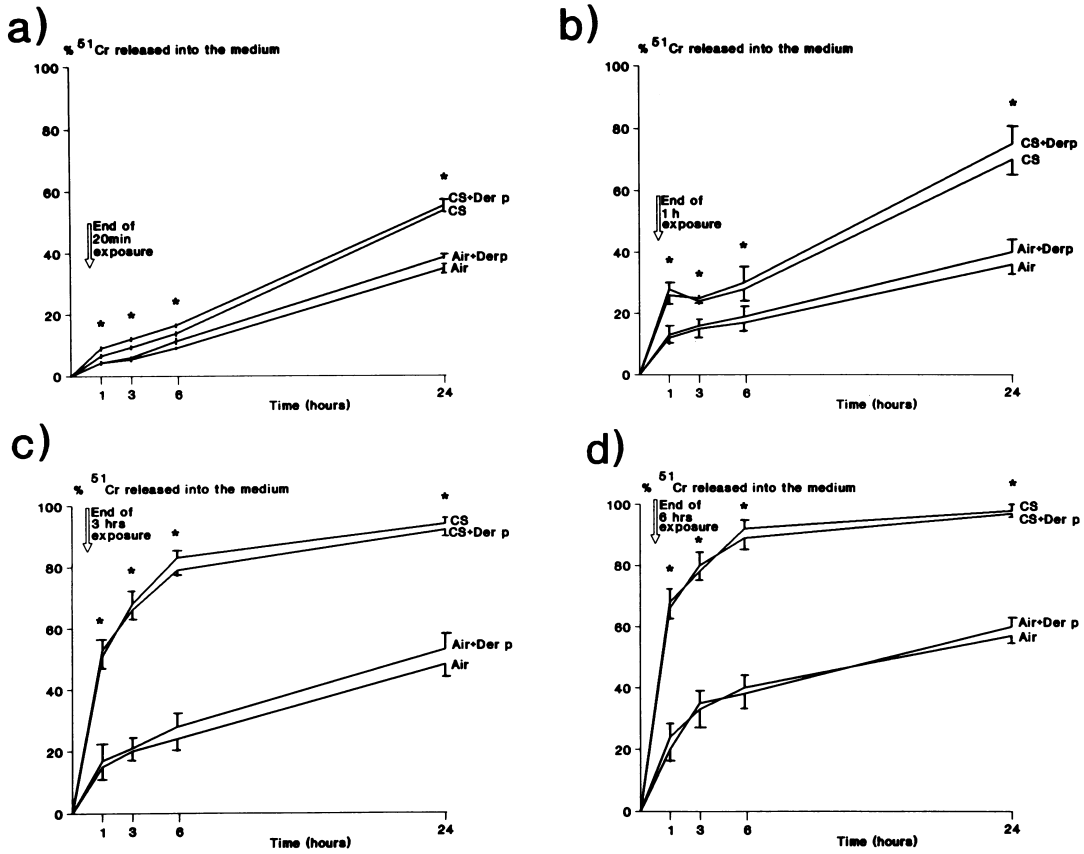


Fig. 8. Effect of (a) 20 min, (b) 1 h, (c) 3 h and (d) 6 h exposure to CS in the absence or presence of 300 ng/mL *Der p* on the release of ^{51}Cr from HBEC cultures over a period of 24 h (*$P < 0.001$ vs. air; $n = 7$ at each time point).

reduction in detectable concentrations of mediator and epithelial cell damage.

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