Br. J. Pharmacol. (1993), **109**, 553–561

A comparison of the effects of polyarginine and stimulated eosinophils on the responsiveness of the bovine isovolumic bronchial segment preparation

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1 The bovine isovolumic bronchial segment preparation has been used to study the sensitivity and responsiveness of bronchial smooth muscle after various manipulations.

2 Addition of acetylcholine (ACh) to the lumen of the segments elicited an increase in intraluminal pressure as a result of contraction of the airway smooth muscle. However, the increases in intraluminal pressure were greater when the ACh was added to the adventitial surface of the preparation.

3 Addition of polyarginine to the bronchial lumen for 60–120 min resulted in an increased magnitude of response and greater than 100 fold increase in sensitivity to ACh administered into the lumen. Depolarizations induced by KCl were similarly enhanced when the solution was added into the lumen. In contrast, the sensitivity and responsiveness to ACh or K^+-induced depolarization administered adventitially was unchanged.

4 The mechanical disruption of the epithelium produced a 32 fold increase in sensitivity to ACh introduced via the lumen, whereas the sensitivity to ACh added adventitially remained unaltered.

5 Addition of polyarginine to the adventitial bathing medium resulted in no change in the responsiveness or sensitivity to ACh, irrespective of whether the ACh was given intraluminally or adventitially.

6 Histological examination revealed that polyarginine caused extensive disordering of the normal architecture of the bronchial epithelium. Taken together with the unaltered responsiveness to adventitial ACh (i.e. lack of change in intrinsic muscle sensitivity) these observations suggest that the effect of polyarginine was most likely due to disruption of a diffusion barrier.

7 In contrast to the effects of polyarginine, the only effect of stimulated eosinophils was to produce a small diminution in the responsiveness to ACh that had been added adventitially.

Keywords: Polyarginine; bronchial responsiveness; eosinophil; bronchial segment preparation

Introduction

In diseases such as bronchial asthma or following certain viral infections, the consequences of the mucosal immune response result in the acquisition of enhanced bronchial responsiveness (Empey *et al.*,1977; Jennings *et al.*, 1987; Cockroft, 1989). The mechanistic relationships between the functional manifestation of bronchial hyperresponsiveness and histological changes occurring in the airway mucosa are currently unknown. However, the apparent association between the presence of activated inflammatory cells, injury of the airway epithelium and the appearance of bronchial hyperresponsiveness have fostered the belief that airway inflammation is of primary and causal significance in its pathophysiological development. Superficially, this viewpoint is attractive because inflammatory cells certainly do have the potential to liberate chemical mediators, such as free radicals, cytotoxic proteins and proteolytic enzymes that are known to elicit cell injury (Gleich *et al.*, 1988; Gleich, 1990). This site-directed injury might result in the exposure of intraepithelial nerve endings, cause a reduction in the production or release of epithelium-derived inhibitory factors and facilitate the destruction of a cellular diffusion barrier; events which together might account for augmented bronchial responsiveness.

In studies of patients with mild asthma Beasley and colleagues (1989) reported an inverse correlation between the number of exfoliated epithelial cells in bronchoalveolar lavage fluid and the provocation concentration to methacholine. Other workers have reported the existence of an inverse correlation between the proportion of eosinophils present in bronchoalveolar lavage fluid (Lam *et al.*, 1987; Wardlaw *et al.*, 1988) or the airway mucosa (Ohashi *et al.*, 1992) and the degree of bronchial responsiveness to inhaled methacholine. Likewise, in asthmatic patients treated with a topically active corticosteroid, data have been presented which apparently link a reduction in airway eosinophil numbers of a diminution (but not ablation) of bronchial hyperresponsiveness (Djukanović *et al.*, 1992). Similar evidence associating inflammatory cell infiltration with heightened bronchial responsiveness exists in experimental animals (Marsh *et al.*, 1985; Murphy *et al.*, 1986). On the basis of these studies it has been argued that there is likely to be a causal link between eosinophils, epithelial injury and the acquisition of heightened bronchial responsiveness.

However, several investigations in man and experimental animal models have provided contrary evidence which dissociates the presence of increased numbers of eosinophils in the airway from the development of bronchial hyperresponsiveness, or at least casts doubt on the obligatory and dominant role of the eosinophil in this process (Sanjar *et al.*, 1990; Chapman *et al.*, 1991; Djukanović *et al.*, 1990; 1992; Elwood *et al.*, 1992). The implication of these experiments is that airway inflammation is not causally related to bronchial hyperresponsiveness, or that the link between them is more complex than originally believed. Thus, the relationships

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between eosinophils, epithelial damage and smooth muscle responsiveness require further appraisal.

In the present paper we report the results of experiments in which we have continued our mechanistic investigations into mucosal injury and its relationship to functional changes in the airway. Specifically we have compared the effects on smooth muscle responsiveness of treatment with the poly-basic polymer polyarginine (a surrogate for the arginine-rich eosinophil granule proteins), treatment with eosinophils or mechanical denudation of the epithelium. A preliminary account of this work was presented to the British Pharmacological Society (Omari *et al.*, 1991a).

Methods

Isovolumic bronchial segment preparation

Fresh bovine lungs were obtained from a local abattoir and used not more than 1 h *post mortem*. On arrival at the laboratory, bronchial segments, approximately 2 cm in length and with a distal internal diameter of 0.3 cm, were dissected from the upper lobes and cannulated at both ends. The segments were placed in a modified organ bath (Omari & Sparrow, 1992) and bathed at 37°C in Krebs solution (composition, mM: NaCl 121, KCl 5.4, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.5 and $CaCl_2$ 2.5) and gassed with 5% CO_2 in O_2. Krebs solution was also perfused through the lumen of the segment from an external reservoir. The Krebs solution bathing both the luminal and adventitial surfaces was exchanged for fresh solution at regular intervals throughout the experiment. Changes in muscle tone were recorded by closing off proximal and distal taps leaving the segment in series with a differential pressure transducer (type MPX10DP, Motorola Semiconductors, Phoenix, USA). Responses were recorded on a Rikadenki R-50 chart recorder.

Agonists were introduced either on to the adventitial surface of the segments or into the lumen, by use of a syringe and 3-way tap situated at the distal end of the preparation. The segments were also prepared to permit electrical field stimulation (EFS) with platinum ring electrodes and a Grass S44 stimulator. The tissues were maintained at a resting intraluminal pressure of 6 cmH_2O, this being determined as optimal by studying the relationship between EFS stimulation and response at different resting pressures (Figure 1). Cumulative responses were recorded to acetylcholine (ACh) added to the adventitial surface, while responses to ACh introduced into the lumen were recorded non-cumulatively. In some cases the tissues were also challenged with a KCl depolarizing solution that contained 121 mM KCl in place of NaCl.

In all experiments control responses to agonists and EFS were first determined before undertaking a variety of manipulations when the tissues had resumed their resting tone and showed consistent responses to EFS.

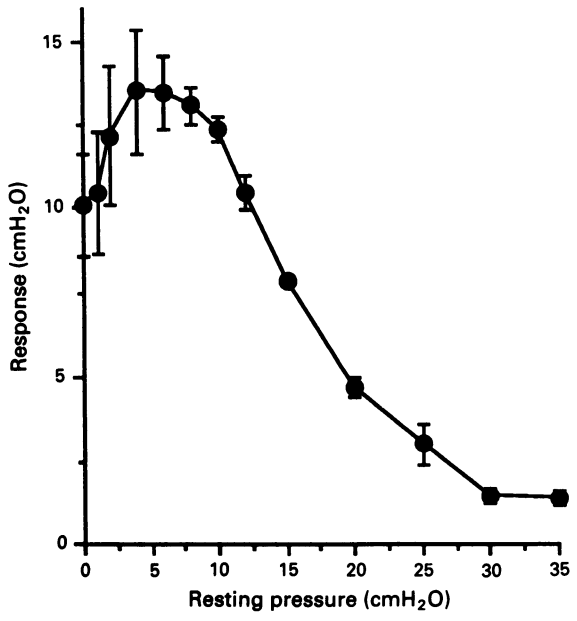

Figure 1 Responses of the bovine isovolumic bronchial segment preparation to electrical field stimulation (60 V, 0.5 ms, 20 Hz) via platinum ring electrodes after being maintained at a range of resting intraluminal pressures. Data are mean ± s.e.mean of 4 separate experiments.

Mechanical injury of bronchial epithelium

As a control for other manipulations, we examined the responsiveness of bronchial segments that had been subjected to mechanical injury of their epithelium. The epithelium was damaged by inserting into the lumen a cotton applicator tip soaked in Krebs solution and rubbing it along the length of the segment.

Treatment with polyarginine

Polyarginine was dissolved in Krebs solution each day and diluted to appropriate concentrations for use. To determine the effect of polyarginine when added to the lumen, exposures of 1 mg ml^{-1} for 1 h as well as 10 and 100 $\mu g\ ml^{-1}$ for 2 h were tested. In experiments comparing the adventitial and luminal administration of polyarginine, an exposure of 100 $\mu g\ ml^{-1}$ was used. In this set of experiments, after obtaining control responses to EFS and ACh, the segments were exposed to polyarginine via the adventitial surface and the effects on the responses of the tissue observed. After complete recovery, the lumen was then exposed to polyarginine and the responses to ACh studied once again. Thus, in contrast to tissues in which the epithelium had been injured mechanically, these studies incorporated within segment rather than between segment control responses.

Experiments with inflammatory cells

For each experiment, paired segments from the same lung were used. After control responses had been obtained, each segment was exposed to either stimulated or unstimulated cells that had been obtained as described below.

Stimulation of eosinophils and neutrophils was achieved with 5 μM ionophore A23187, a concentration that we have previously shown to activate these cell types (Herbert *et al.*, 1991). The ionophore (or its dimethylsulphoxide (DMSO) vehicle at a final concentration of 1.6%) was added to the cells 1 min before they were introduced into the lumen of the segments. After an exposure period of 1 h the segments were then flushed with fresh Krebs solution and the perfusate collected for the measurement of leukotriene B_4 by radioimmunoassay.

Preparation of inflammatory cells

A peritoneal eosinophilia was established in guinea-pigs by injecting male Dunkin-Hartley strain animals i.p. with 1 mg ml^{-1} polymyxin B in saline twice weekly for 4 weeks. At 24 h after the last injection, the animals were anaesthetized with metofane and a 16G cannula (Critikon Ltd, Ascot, Berks.) inserted into the peritoneal cavity. The cavity was lavaged with 50 ml sterile saline and the lavage return collected into a polypropylene tube. The lavage fluid was centrifuged at 150 *g* for 10 min at room temperature and resuspended in 2 ml of Dulbecco's phosphate buffered saline (DPBS, Ca^{2+}- and Mg^{2+}-free). Erythrocytes were lysed by use of hypotonic saline and an initial differential cell count then performed by

use of eosin/pontamine sky blue. In some experiments the cells were resuspended in an appropriate volume of Krebs solution and used without further purification. In those experiments which used purified eosinophils, further enrichment was obtained by resuspending the cells in Tyrode solution containing gelatin and deoxyribonuclease (DNase) and subjecting the cells to density centrifugation on Metrizamide gradients.

The washed lavage cells were overlaid onto density gradients which consisted of a cushion of 2 ml Maxidens upon which a discontinuous gradient of 16, 18, 20, 22, 24, 26 and 30% Metrizamide in Tyrode solution (supplemented with gelatin and DNase) had been prepared. Each step in the gradient had a volume of 2 ml and a maximum of 10^8 cells was loaded onto each gradient. Centrifugation was performed at 1200 *g* for 45 min at room temperature. The eosinophil-rich band at the 24–26% Metrizamide interface was carefully aspirated., washed in DPBS and the number and purity of cells determined by use of eosin/pontamine sky blue. Viability was determined by exclusion of trypan blue and was generally >90%.

Human neutrophils were obtained from the blood of volunteers who had taken no medication within at least one week prior to donation. Eight volumes of citrate-anticoagulated blood were treated with 1 volume of 6% dextran (M_r 500,000) and 1 volume of normal saline. Erythrocytes were sedimented at 1 *g* for 30 min, after which the plasma layer was carefully aspirated, mixed with an equal volume of phosphate-buffered saline and layered as 5 ml aliquots onto 5 ml of Mono-Poly resolving medium. After centrifugation at 400 *g* for 30 min the polymorphonuclear cell layer was aspirated, the cells washed in Krebs solution and an aliquot taken for a differential cell count by use of Wright's stain and an assessment of cell viability with trypan blue.

Histology

At the end of the experiment segments were fixed for 48 h in 4% formaldehyde solution. Small pieces were then dissected and embedded in Cryo-M-Bed prior to the preparation of 10 μm cryostat sections. Sections were stained with haematoxylin and eosin and the appearance of the airway mucosa assessed by light microscopy (Zeiss Axiovert 10 or Leitz Laborlux 12).

Data presentation

Data are shown as either the increase in intraluminal pressure above the resting value of 6 cmH_2O or as a percentage of the maximal response to 10 mM ACh. The sensitivities of the segments to adventitially added ACh were compared by determining the EC_{50} values. The responses to intraluminal ACh rarely exceeded 30% of maximum and consequently the sensitivities were compared by calculating concentrations corresponding to EC_{20} of the maximum adventitial response. Results are expressed as the mean ± s.e.mean of *n* preparations. The significance of any difference between means was evaluated with Student's *t* test, with $P \leqslant 0.05$ being considered statistically significant. Tests for paired samples were used to compare data obtained within individual segments (i.e. control versus subsequent manipulation), whereas unpaired tests were used to evaluate data between experiments.

Materials

The following were purchased from Sigma (Poole, Dorset): acetylcholine, ionophore A23187, polyarginine (M_r 11,600), polymyxin B sulphate, dimethysulphoxide, gelatin (from bovine skin) and DNase. DPBS and Mono-Poly resolving medium were obtained from Flow Laboratories (Rickmansworth, Herts). Metrizamide and Maxidens were purchased from Nycomed (Birmingham). Metofane (methoxyfluorane) was obtained from C-Vet Ltd (Bury St Edmunds, Suffolk). Reagents for the radioimmunoassay of leukotriene B_4 were purchased from NEN-Du Pont Research Products (Stevenage, Herts). Stains for microscopy and all other laboratory reagents were purchased from BDH (Poole, Dorset).

Results

Responses of the preparation

The responses of a bovine bronchial segment maintained at a resting transmural pressure of 6 cmH_2O are shown in Figure 2. Electrical field stimulation (EFS) at 60 V with a duration of 0.5 ms and frequency of 20 Hz produced an increase in intraluminal pressure which relaxed readily upon cessation of the stimulus (Figure 2a). Also illustrated in Figure 2a is a typical cumulative response to ACh that had been applied to the adventitial surface, whereas Figure 2b displays the significantly smaller and non-cumulative dose-response characteristics for ACh injected intraluminally. For example, 10 mM ACh applied adventitially elicited a contraction that produced a mean increase in pressure of 33.0 ± 1.8 cmH_2O ($n = 45$), whereas introduction of ACh into the lumen produced a pressure change of only 12.1 ± 1.2 cmH_2O ($n = 45$, $P \leqslant 0.01$).

Effect of polyarginine

In these experiments, luminal or adventitial surfaces of bovine bronchial segments were exposed to several concentrations of polyarginine. By itself, polyarginine altered neither the resting tone of the bronchi, nor the responses to EFS. However, the responses of bronchial segments to ACh added intraluminally were significantly enhanced by prior exposure of the lumen to 100 μg ml^{-1} polyarginine for 2 h (Figure 3a). For example, the response to 10 mM ACh added into the lumen was increased from 7.3 ± 1.6 to 28.9 ± 3.9 cmH_2O ($n = 4$, $P \leqslant 0.01$). This effect was not seen if the polyarginine was added to the adventitial bathing medium (Figure 3a). In contrast, the responses to ACh added adventitially were unchanged by exposure of either the luminal or adventitial surfaces to polyarginine when using the same treatment regime (Figure 3b, $n = 4$ for each type of exposure). In the case of EFS, the responses of bronchial segments were unchanged by polyarginine, even after 1 h exposure to 1 mg ml^{-1} (21.7 ± 6.2 versus 22.2 ± 7.4 cmH_2O in control and treated tissues respectively, $n = 4$). However, it was of interest that the responses to depolarization by KCl

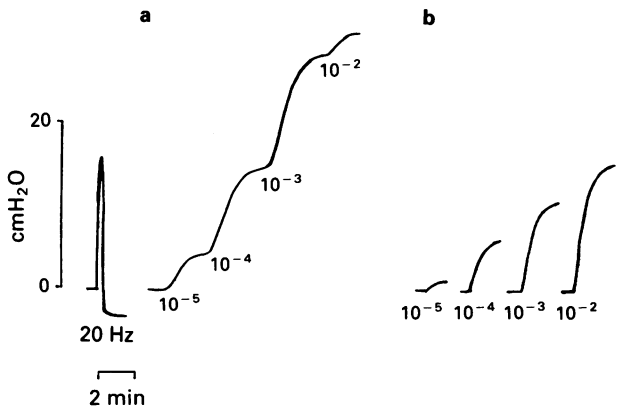

Figure 2 Increases in the intraluminal pressure of a bronchial segment contracting isovolumically to electrical field stimulation (EFS) and acetylcholine (ACh) from a resting pressure of 6 cmH_2O: (a) illustrates the response to EFS and cumulative responses to ACh added to the adventitial surface; (b) illustrates single responses to ACh administered intraluminally.

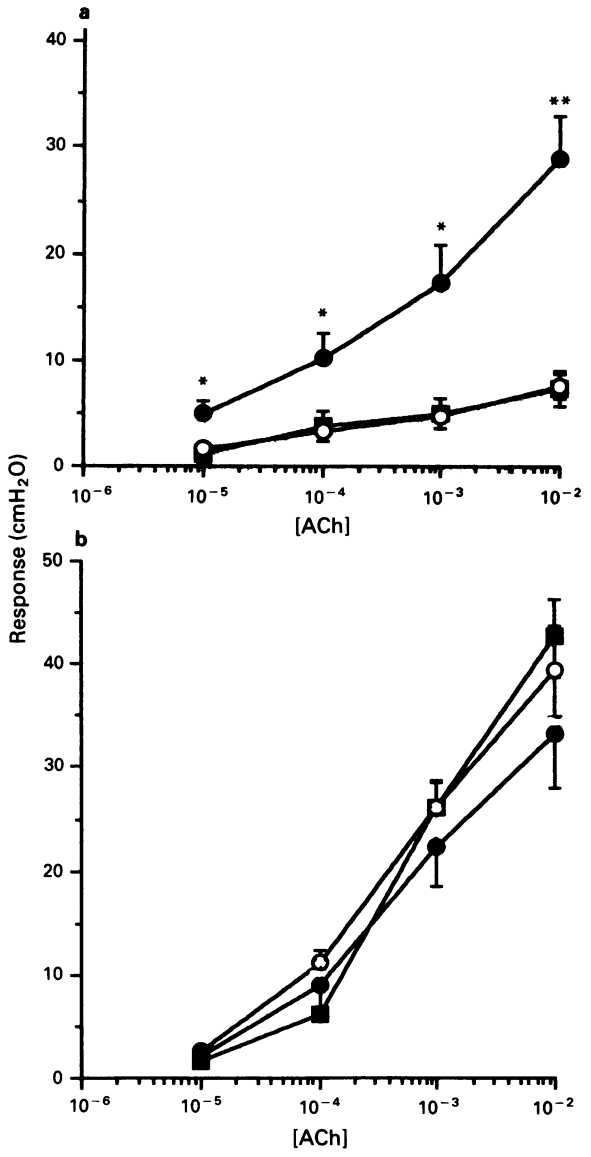

Figure 3 Effect of polyarginine on the responsiveness to acetylcholine (ACh). In (a), ACh was added into the lumen. In (b), ACh was added to the adventitial surface. Each tissue served as its own control (■) before exposure of the lumen (●) or the adventitial surface (○) to 100 μg ml^{-1} polyarginine for 2 h. Data are mean ± s.e.mean of 4 separate experiments. *$P \leqslant 0.05$; **$P \leqslant 0.01$ with respect to appropriate control.

were enhanced in a similar way to those of ACh (Figure 4).

Table 1 summarizes the effect of different treatments with polyarginine, as well as mechanical injury, on the EC_{20} and EC_{50} values which indicate the sensitivity to luminally and adventitially applied ACh respectively. Exposure to 10 μg ml^{-1} polyarginine for 120 min did not alter the sensitivity to ACh at all, whereas higher concentrations in the lumen (100 μg ml^{-1} and 1 mg ml^{-1}) enhanced the sensitivity to luminally-applied ACh by 110 and 135 fold respectively. The EC_{50} values for ACh applied to the outside of the tissue were unchanged by these treatments. Similarly, tissues that had been subjected to mechanical trauma of the epithelium were on average 32 fold more sensitive to ACh added into the lumen when compared with epithelium-intact segments for the same lung. However, there was no change in sensitivity to ACh added to the adventitial side of mechanically injured preparations (Table 1). Overall, there was no significant difference in the changes in tissue sensitivity evoked by polyarginine or mechanical trauma. That mechanical injury appeared to be less effective than polyarginine treatment is probably a reflection of the limitations of the different experimental protocols used and the way in which control re-

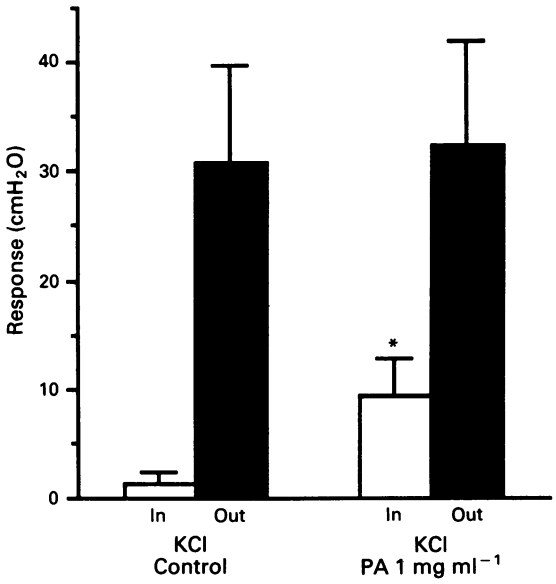

Figure 4 Effect of polyarginine (PA, 1 mg ml^{-1}, 1 h in the lumen) on the response to electrical field stimulation (EFS) and depolarization evoked by K^+. Data are mean ± s.e.mean from 4 separate experiments. *$P \leqslant 0.01$ with respect to appropriate control.

Table 1 Calculated EC_{20} and EC_{50} values for acetylcholine (ACh) added to either the luminal or adventitial sides of bovine bronchial segments before and after exposure to polyarginine: also included are values obtained from tissues where the epithelium had been injured mechanically and their corresponding epithelium-intact controls from the same lungs

Treatment	*log_{10} EC_{20} ACh* (luminal)	*log_{10} EC_{50} ACh* (adventitial)
Polyarginine (10 μg ml^{-1}, 2 h)		
pre-exposure	− 2.66 ± 0.17	− 3.55 ± 0.11
post-exposure	− 2.93 ± 0.32	− 3.30 ± 0.22
Polyarginine (100 μg ml^{-1}, 2 h)		
pre-exposure	− 2.12 ± 0.11	− 3.22 ± 0.12
post-exposure	− 4.18 ± 0.26**	− 3.29 ± 0.08
Polyarginine (1 mg ml^{-1}, 1 h)		
pre-exposure	− 2.05 ± 0.36	− 3.55 ± 0.12
post-exposure	− 4.18 ± 0.33*	− 3.48 ± 0.08
Epithelium-intact segments	− 2.48 ± 0.26	− 3.36 ± 0.09
Mechanically injured segments	− 3.99 ± 0.12†	− 3.34 ± 0.20

Asterisks indicate significant differences between pre- and post-exposure values, *$P \leqslant 0.05$; **$P \leqslant 0.01$. †Significantly different from intact segments, $P \leqslant 0.01$. All data are mean ± s.e.mean values from four experiments in each case.

sponses were determined (see Methods). It is also possible that mechanical injury failed to remove significant amounts of the epithelium.

Effect of inflammatory cells

Bronchial segments were exposed to ionophore A23187 (5 μM, intraluminally) to establish whether A23187 could be used as a means of activating inflammatory cells without interfering with the responsiveness of bronchial smooth muscle. The ionophore produced a slow increase in bronchial tone that reached 50% of the maximal response after approximately 3 h (Figure 5). Despite the change in tone, the smooth muscle responsiveness to ACh applied either intraluminally or adventitially was unaltered by a 60 min exposure to 5 μM A23187 in the lumen (Figure 6).

When bronchial segments were exposed to either stimulated or unstimulated guinea-pig peritoneal eosinophils ($15.9 \pm 0.1 \times 10^6$ ml^{-1} for 60 min) there were no significant effects on the responsiveness to ACh administered intraluminally when this was compared with the corresponding control responses made prior to the addition of cells (Figure 7). This was true irrespective of whether the eosinophils were used as unfractionated peritoneal lavage cells or whether they had been purified by density gradient centrifugation. However, when ACh was applied adventitially after exposure to eosinophils it was noted that there was a small reduction in the magnitude of response compared with the pre-eosinophil control values. In segments exposed to stimulated eosinophils this tendency was statistically significant at all but the highest dose of ACh (Figure 7). Pre- and post-exposure EC_{20} and EC_{50} values derived from these dose-response curves are illustrated in Table 2. In one series of studies we incubated bronchial segments with eosinophil populations at 91.6×10^6 ml^{-1} and purity of 92.6% (with and without ionophore stimulation) and even under these conditions there was no increase in muscle responsiveness (data not shown). As judged by the release of immunoreactive leukotriene B_4, the eosinophils were activated for mediator release by A23187, with a net release of 1.39 ± 0.29 ng LTB_4 per 10^6 eosinophils being detected in the fluid returned from the bronchial lumen.

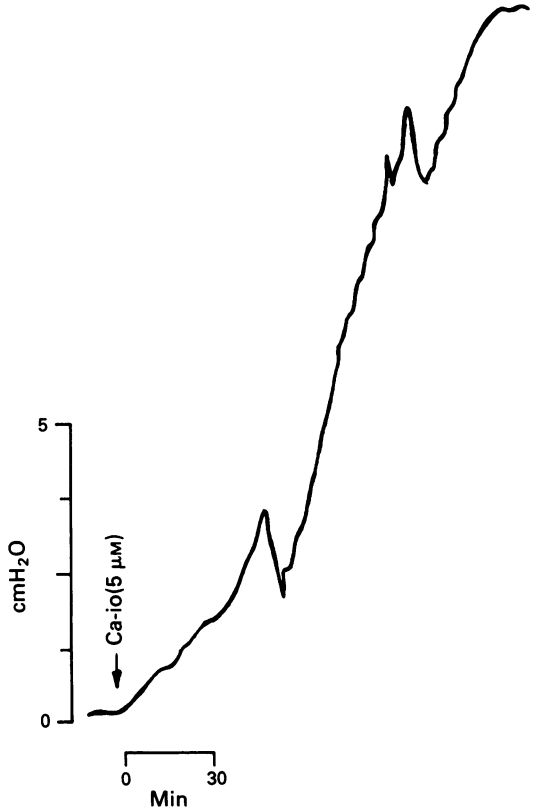

Figure 5 A tracing of changes in intraluminal pressure in a bovine bronchial segment that had been exposed to 5 μM A23187 (Ca-io) on the luminal side.

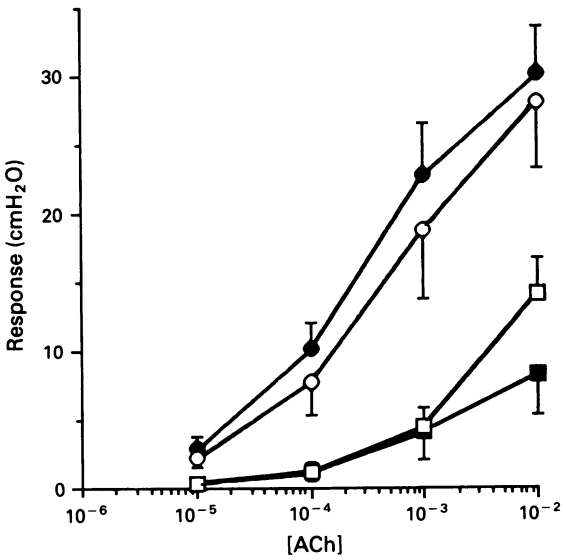

Figure 6 Concentration-response curve to acetylcholine (ACh) added either luminally (●) or adventitially (○) under control conditions. Tissues were then exposed to 5 μM A23187 added into the lumen (□) or into the adventitial bathing medium (■) for 60 min before retesting tissue responsiveness. Data are mean ± s.e.mean of six experiments.

Similar experiments were performed with human neutrophils with 60 min exposures of bronchial segments to cell numbers ranging from $6-330 \times 10^6$ neutrophils ml^{-1} at an average purity of 88.6 ± 4.2%. In no case was the responsiveness of the tissues materially altered by these treatments (data not shown). These cells exhibited a net release of leukotriene B_4, (LTB_4), confirming their stimulation by A23187, with a net 2.81 ± 2.27 ng LTB_4 per 10^6 neutrophils being detected in the fluid recovered from the bronchial lumen.

Histological effects

Cryostat sections prepared from control bronchial segments or tissues that had been exposed to 10 μg ml^{-1} polyarginine for 60 min showed preservation of a relatively intact, healthy epithelium. At high magnification, abundant cilia were evident on cells, and focal intercellular contact was a notable feature between suprabasal epithelial cells. In contrast, those segments exposed to higher concentrations of polyarginine exhibited cytoplasmic shrinkage, loss of interepithelial adhesion and evidence of exfoliation (Figure 8). Interestingly, the areas where exfoliation was noted showed retention of basal cells and cellular debris on the matrix substratum.

Bronchial segments that had been exposed to either unstimulated or ionophore-stimulated eosinophils and neutrophils were also examined. In contrast to the effects of polyarginine, the exposure to inflammatory cells was characterized by little effect on the gross morphology of the airway mucosa.

Discussion

In this series of experiments we have demonstrated that exposure of the bronchial lumen to the basic amino acid

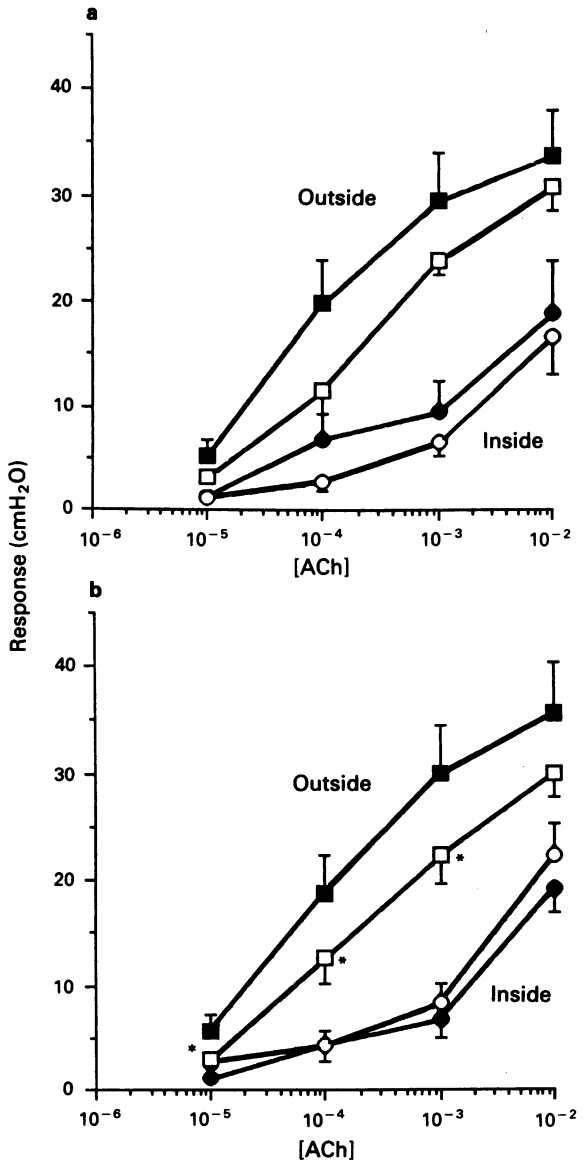

Figure 7 The effect of exposure of bronchial segments to either unstimulated (a) or ionophore stimulated (b) eosinophils. Control concentration-response curves to acetylcholine (ACh) (solid symbols) were initially undertaken before addition of the unfractionated peritoneal eosinophils to the bronchial lumen ($15.9 \pm 0.1 \times 10^6$ ml at an average purity of $68.6 \pm 0.1\%$, $n = 5$). Responses after exposure to eosinophils are denoted by open symbols. In each experiment one tube of each pair dissected from the lung received unstimulated eosinophils, the other received cells which had been activated by the ionophore. Data are mean ± s.e.mean of 5 experiments and asterisks denote significant differences from the appropriate control values.

Table 2 Calculated EC_{20} and EC_{50} values for acetylcholine (ACh) added luminally or adventitially before and after exposure of bronchial segments to guinea-pig peritoneal eosinophils (15.9×10^6 ml^{-1})

Treatment	*log_{10} EC_{20} ACh* (luminal)	*log_{10} EC_{50} ACh* (adventitial)
Unstimulated eosinophils		
pre-exposure	-3.38 ± 0.47	-4.06 ± 0.22
post-exposure	-3.00 ± 0.16	-3.72 ± 0.12
Stimulated eosinophils		
pre-exposure	-3.21 ± 0.41	-4.01 ± 0.23
post-exposure	-3.52 ± 0.38	-3.52 ± 0.27

Data are from five experiments in each case.

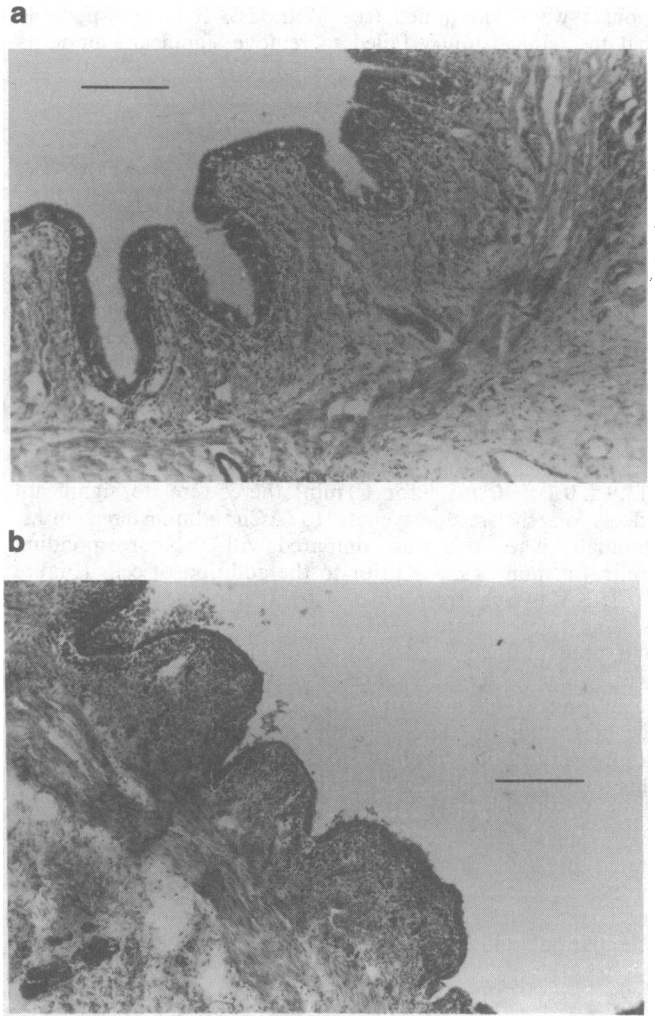

Figure 8 Sections of bronchial segments following (a) control treatment, or (b) exposure for 2 h to 100 μg ml^{-1} polyarginine. The scale bars represent 200 μm in each case.

polymer, polyarginine, results in a relatively rapid augmentation of the responsiveness of the underlying smooth muscle to ACh and KCl-induced depolarizations, but not to EFS. This enhancement was not observed if the adventitial surface of the preparation was similarly treated. A further feature of the 'sidedness' of this phenomenon was that only the responses to agonists introduced into the bronchial lumen were enhanced. Responses to agonists applied adventitially were unchanged, even in those bronchial segments which had been treated with polyarginine in the bronchial lumen.

This pattern of alteration in the responsiveness of the bronchial smooth muscle was similar to that seen in segments in which the bronchial mucosa had been injured mechanically (this study; Munakata *et al.*, 1989; Sparrow & Mitchell, 1991; Omari & Sparrow, 1991) and is consistent with the effects seen after exposure of the mucosa to proteinases (Omari *et al.*, 1991b; Omari & Sparrow, 1992). Collectively, these similarities suggest that polyarginine may be causing disruption of the airway muscosa, a view that was verified experimentally by undertaking a histological evaluation before and after treatment with various concentrations of polyarginine. Tissues that had not been exposed to polyarginine, or those which had experienced only low concentrations, exhibited a normal appearance with an essentially intact epithelium. In contrast, tissues incubated with concentrations of polyarginine that significantly augmented the smooth muscle responsiveness were characterized by evidence of cytolytic damage, cytoplasmic shrinkage and frank exfoliation. It was

of interest that areas of mucosa that had been denuded of epithelial cells in this way still retained a significant number of basal cells. This pattern of tissue injury is thus in many ways similar to that produced by stimulated eosinophils in an *in vitro* model of mucosal injury (Herbert *et al.*, 1991) which has been suggested to represent a convenient model of the mucosal lesion which characterizes lung diseases such as bronchial asthma (Herbert *et al.*, 1991; Montefort *et al.*, 1992). The retention of basal cells in injury of the airway mucosa may arise because the desmosomal attachment of columnar epithelial cells to basal cells is more susceptible to cleavage than is the adhesion of basal cells to the biomatrix (Montefort *et al.*, 1992).

As the airway epithelium functions, in part, as a protective barrier it seems plausible that one effect of polyarginine is to render the mucosa more permeable to solutes as a consequence of the cellular injury and loss of interepithelial tight junctions. In support of this view Herbert *et al.* (1991) demonstrated that polyarginine ($1\ mg\ ml^{-1}$) significantly increased the permeability of the bovine bronchial mucosa to serum albumin. The failure of a lower concentration of polyarginine to produce a significant effect on the net unidirectional flux of albumin, compared to the present results in smooth muscle, probably relates to the fact that Herbert and colleagues focused their attention on macromolecular solute permeability rather than highly diffusible low molecular mass drugs.

The airway epithelium has also been suggested as a source of poorly-characterized factors which inhibit the responsiveness of the underlying smooth muscle (Barnes *et al.*, 1985; Vanhoutte, 1988; Spina *et al.*, 1992). It is therefore possible that removal of such inhibitory factors may contribute to the heightened responsiveness seen in the present experiments. Whilst the bovine bronchial epithelium does release substances, such as prostaglandin E_2 (PGE_2), which have relaxant effects on airway smooth muscle (Herbert & Robinson, unpublished), we discount this as an explanation for our observations in this study. Moreover, we also argue against the possibility that polyarginine enhances responsiveness by releasing bronchoconstrictor agents (e.g. histamine from mast cells). The principal evidence against these hypotheses is that addition of polyarginine to the bronchial lumen does not change the responses of the tissue to agonists added adventitially, even though the responses to intraluminal ACh are enhanced. Furthermore, in a study of mast cell heterogeneity in bovine lung Hunt *et al.* (1991) demonstrated the refractoriness of bovine mast cells to polybasic secretagogues. Thus, we favour the view that the principal effect of polyarginine is to disrupt the epithelial diffusion barrier. The behaviour of the airway epithelium as a diffusion barrier capable of modulating the actions of pharmacological agonists has been suggested on the basis of other studies with bronchial segments (Small *et al.*, 1990; Sparrow & Mitchell, 1991; Omari & Sparrow, 1992; Omari *et al.*, 1993).

One of our reasons for studying polyarginine in the present experiments is that it may be a useful model of the arginine-rich cytotoxic proteins of the eosinophil (Butterworth *et al.*, 1979; Uchida *et al.*, 1990). Attention has been focussed on the role of eosinophil-derived proteins, in particular the major basic protein (MBP), in the pathogenesis of mucosal injury (Gleich *et al.*, 1988). Deposits of MBP have been found adjacent to areas of epithelial exfoliation in the asthmatic lung (Filley *et al.*, 1982) and at relatively high concentrations *in vitro* it is known to cause disruption of nasal and bronchial epithelium (Frigas *et al.*, 1980; Hastie *et al.*, 1987; Ayars *et al.*, 1989; Motojima *et al.*, 1989) and a modest increase in the responsiveness of airway smooth muscle (Flavahan *et al.*, 1988). Other studies have demonstrated that MBP causes an epithelium-dependent increase in bronchial tone in the guinea-pig and monkey (White *et al.*, 1990; Gundel *et al.*, 1990) and an augmentation of responses to agonists administered intravenously (Brofman *et al.*, 1989; White *et al.*, 1990). However, the mechanism(s) of these changes are not understood. In our studies polyarginine had no effect on the resting tone of the bronchial segments, nor did we observe any change in the response of the tissue to ACh added adventitially. These data suggest that there may be important differences between the action of MBP and basic amino acid polymers, implying that the interaction between MBP and target cells is more subtle than that defined by cationic nature alone. Clearly, further work is necessary to establish exactly how polyarginine, MBP and other eosinophil proteins cause such an apparently targeted injury of epithelial cells.

In view of the established toxicity of eosinophil granule proteins and other eosinophil-derived mediators towards airway epithelium it was surprising to find that guinea-pig peritoneal eosinophils that had been stimulated by A23187 produced only a small decrease in the responsiveness to ACh added to the adventitial surface of the preparation. The basis of this effect remains unknown. Relatively small numbers of eosinophils were used in the present studies and it is conceivable that the quantities of eosinophil-derived mediators released were inadequate to affect airway function over the time course of the experiment. Interestingly, Jongejan (1991) did not observe any change in the sensitivity of human bronchial rings to methacholine after exposure to granulocytes ($1-5 \times 10^6$ of which $>90\%$ were neutrophils) that had been activated by zymosan. However, bronchial rings incubated with larger numbers of granulocytes ($10-20 \times 10^6$) exhibited a decreased responsiveness. Similarly, McAlpine and colleagues (1988) found that zymosan-activated neutrophils did not affect the responsiveness of rabbit bronchi. The stimulation of the eosinophils and neutrophils was confirmed in our own experiments by demonstrating a net release of leukotriene B_4 in the fluid recovered from the segments after incubation with the cells. The most likely sources of this leukotriene are the eosinophils and neutrophils because, in contrast to the leukocytes bovine bronchial mucosa is only a poor source of this eicosanoid (Sun *et al.*, 1989; Herbert, 1992).

In contrast to the unexpected effect on bronchial responsiveness in this study, relatively small numbers of guinea-pig eosinophils and human neutrophils ($3-10 \times 10^6\ ml^{-1}$) have been shown to increase the net unidirectional flux of albumin across the bovine bronchial mucosa by causing the exfoliation of epithelial cells (Herbert *et al.*, 1991; Herbert, 1992). In the present work, histological examination of the bronchial segments that had been exposed to stimulated leukocytes showed considerably less disruption than those exposed to polyarginine. These findings are in marked contrast to the tissue morphology reported previously after exposure to eosinophils obtained and stimulated by methods identical to those used in the present study (Herbert *et al.*, 1991). However, there are several important differences which may account for the absence of eosinophil-induced tissue injury. Firstly, in the current study the eosinophils were added to the bronchial lumen and not the adventitial side of the isolated epithelium as reported by Herbert *et al.* (1991). Secondly, no special measures were taken to promote eosinophil adhesion to the target tissue within the bronchial lumen. This is in contrast to the experiments performed by Herbert *et al.* (1991) in which eosinophils were incubated in a small volume under conditions which promoted settling onto a limited and defined area of bronchial mucosa. Moreover, these authors presented specific evidence which indicated that the eosinophil-dependent injury to the mucosa was a contact-dependent phenomenon (Herbert *et al.*, 1991).

In summary, these studies have shown that polyarginine is able to augment the responsiveness of bronchial smooth muscle to ACh in an effect which is dependent on the removal of the diffusion barrier presented by the airway epithelium. Eosinophils stimulated by the ionophore A23187 were unable to reproduce this phenomenon, suggesting that if eosinophils are responsible for the bronchial hyperreactivity seen in asthma then their ability to injure the airway mucosa

is a more chronic process than that produced in this *in vitro* system. Alternatively, certain aspects of the interaction between eosinophils and the airway epithelium may be different in certain types of clinical asthma. In view of the findings reported by Herbert *et al.* (1991) one such difference may involve the nature of the physical contact between eosinophils and the mucosal microenvironment.

We thank the British Council, The Medical Research Council and The British Lung Foundation for financial support. Thanks are also due to Dr Carolyn Herbert for helpful discussion.

References

AYARS, G.H., ALTMAN, L.C., MCMANUS, M.M., AGOSTI, J.M., BAKER, C., LUCHTEL, D.L., LOEGERING, D.A. & GLEICH, G.J. (1989). Injurious effect of the eosinophil peroxide-hydrogen peroxide-halide system and major basic protein on human nasal epithelium *in vitro*. *Am. Rev. Respir. Dis.*, **140**, 125–131.

BARNES, P.J., CUSS, F.M. & PALMER, J.B. (1985). The effect of airway epithelium on smooth muscle contractility in bovine trachea. *Br. J. Pharmacol.*, **86**, 685–691.

BEASLEY, R., ROCHE, W.R., ROBERTS, A.J. & HOLGATE, S.T. (1989). Cellular events in the bronchi in mild asthma and after bronchial provocation. *Am. Rev. Respir. Dis.*, **139**, 806–817.

BROFMAN, J.D., WHITE, S.R., BLAKE, J.S., MUNOZ, N.M., GLEICH, C.J. & LEFF, A.R. (1989). Epithelial augmentation of trachealis contraction caused by major basic protein of eosinophils. *J. Appl. Physiol.*, **66**, 1867–1873.

BUTTERWORTH, A.E., WASSOM, D.L., GLEICH, G.J., LOEGERING, D.A. & DAVID, J.R. (1979). Damage to shistosomula of schistosoma mansoni induced directly by eosinophil major basic protein. *J. Immunol.*, **122**, 221–229.

CHAPMAN, I.D., BOUBEKEUR, K. & MORLEY, J. (1991). Airway eosinophilia and airway hyperresponsiveness parallel other sequential events in the guinea pig. In *New Drugs For Asthma*. ed. Morley, J. & Anderson, G. Basle: Birkhauser-Verlag.

COCKROFT, D.W. (1989). Airway hyperresponsiveness: therapeutic implications. *Ann. Allergy*, **59**, 405–414.

DJUKANOVIĆ, R., WILSON, J.W., BRITTEN, K.M., WILSON, S.J., WALLS, A.F., ROCHE, W.R., HOWARTH, P.H. & HOLGATE, S.T. (1990). Quantitation of mast cells and eosinophils in the bronchial mucosa of symptomatic atopic asthmatics and healthy control subjects using immunohistochemistry. *Am. Rev. Respir. Dis.*, **142**, 863–871.

DJUKANOVIĆ, R., WILSON, J.W., BRITTEN, K.M., WILSON, S.J., WALLS, A.F., ROCHE, W.R., HOWARTH, P.H. & HOLGATE, S.T. (1992). Effect of an inhaled corticosteroid on airway inflammation symptoms in asthma. *Am. Rev. Respir. Dis.*, **145**, 669–674.

ELWOOD, W., LÖTVALL, J.O., BARNES, P.J. & CHUNG, K.F. (1992). Effect of dexamethasone and cyclosporin A on allergen-induced airway hyperresponsiveness and inflammatory cell responses in sensitized brown norway rats. *Am. Rev. Respir. Dis.*, **145**, 1289–1294.

EMPEY, D.W., LAITINEN, L.A., JACOBS, L., GOLD, W.M. & NADEL, J.A. (1977). Mechanisms of bronchial hyperreactivity in normal subjects after upper respiratory tract infections. *Am. Rev. Respir. Dis.*, **113**, 131–139.

FILLEY, W.V., HOLLEY, K.E., KEPHART, G.M. & GLEICH, G.J. (1982). Identification by immunofluorescence of eosinophil granule major basic protein in lung tissue of patients with bronchial asthma. *Lancet*, **ii**, 11–16.

FLAVAHAN, N.A., SLIFMAN, N.R., GLEICH, G.J. & VANHOUTTE, P.M. (1988). Human eosinophil major basic protein causes hyperreactivity of respiratory smooth muscle. Role of the epithelium. *Am. Rev. Respir. Dis.*, **138**, 685–688.

FRIGAS, E., LOEGERING, D.A. & GLEICH, G.J. (1980). Cytotoxic effects of the guinea pig eosinophil major basic protein on tracheal epithelium. *Lab Invest.*, **42**, 35–42.

GLEICH, G.J. (1990). The eosinophil and bronchial asthma: current understanding. *J. Allergy Clin. Immunol.*, **85**, 422–436.

GLEICH, G.J., FLAVAHAN, N.A., FUJISAWA, T. & VANHOUTTE, P.M. (1988). The eosinophil as a mediator of damage to respiratory epithelium: a model for bronchial hyperreactivity. *J. Allergy Clin. Immunol.*, **81**, 776–781.

GUNDEL, R.H., LETTS, L.G. & GLEICH, G.J. (1991). Human eosinophil major basic protein induces airway constriction and airway hyperresponsiveness in primates. *J. Clin. Invest.*, **87**, 1470–1473.

HASTIE, A.T., LOEGERING, D.A., GLEICH, G.J. & KUEPPERS, G. (1987). The effect of purified eosinophilic major basic protein on mammalian ciliary activity. *Am. Rev. Respir. Dis.*, **135**, 848–853.

HERBERT, C.A. (1992). The effects of leukocytes, local hormones and selected peptides on the permeability of the bronchial mucosa to albumin *in vitro*. *Ph.D. Thesis. University of Southampton.*

HERBERT, C.A., EDWARDS, D., BOOT, J.R. & ROBINSON, C. (1991). *In vitro* modulation of the eosinophil-dependent enhancement of the permeability of the bronchial mucosa. *Br. J. Pharmacol.*, **104**, 391–398.

JENNINGS, L.C., BARNS, G. & DAWSON, K.P. (1987). The association of viruses with acute asthma. *N.Z. Med. J.*, **100**, 488–490.

JONGEJAN, R.C. (1991). Responsiveness of isolated human airways. Modulation by inflammatory cells, mediators and physical stimuli. *Ph.D. Thesis. Erasmus University, Rotterdam, The Netherlands.*

LAM, S., LERICHE, J., PHILLIPS, D. & CHAN-YEUNG, M. (1987). Cellular and protein changes in bronchial lavage fluid after late asthmatic reaction in patients with red cedar asthma. *J. Allergy Clin. Immunol.*, **80**, 44–50.

MARSH, W.R., IRVIN, C.G., MURPHY, K.R., BEHRENS, B.L. & LARSEN, G.L. (1985). Increases in airway reactivity to histamine and inflammatory cells in bronchoalveolar lavage after the late asthmatic response in an animal model. *Am. Rev. Respir. Dis.*, **131**, 875–879.

MCALPINE, L.G., MCGRATH, J.C., THOMSON, N.C. & WILSON, E.P. (1988). Effect of activated human neutrophils on histamine response of rabbit secondary bronchi smooth muscle *in vitro*. *Br. J. Pharmacol.*, **95**, 526P.

MONTEFORT, S., HERBERT, C.A., ROBINSON, C. & HOLGATE, S.T. (1992). The bronchial epithelium as a target for inflammatory attack in asthma. *Clin. Exp. Allergy*, **22**, 511–520.

MOTOJIMA, S., FRIGAS, E., LOEGERING, D.A. & GLEICH, G.J. (1989). Toxicity of eosinophil cationic proteins for guinea pig tracheal epithelium *in vitro*. *Am. Rev. Respir. Dis.*, **139**, 801–805.

MUNAKATA, M., HUANG, I., MITZNER, W. & MENKES, H. (1989). The protective role of the epithelium in the guinea pig. *J. Appl. Physiol.*, **66**, 1547–1552.

MURPHY, K.R., WILSON, M.C., IRVIN, C.G., GLEZEN, G.S., MARSH, W.R., HASLETT, C., HENSON, P.M. & LARSEN, G.L. (1986). The requirement for polymorphonuclear leukocytes in the late asthmatic response and heightened airways reactivity in an animal model. *Am. Rev. Respir. Dis.*, **134**, 62–68.

OHASHI, Y., MOTOJIMA, S., FUKUDA, T. & MAKINO, S. (1992). Airway hyperresponsiveness, increased intracellular spaces of bronchial epithelium, and increased infiltration of eosinophils and lymphocytes in bronchial mucosa in asthma. *Am. Rev. Respir. Dis.*, **145**, 1469–1476.

OMARI, T.I., HERBERT, C.A., SPARROW, M.P., CHURCH, M.K., HOLGATE, S.T. & ROBINSON, C. (1991a). Effects of polyarginine on bronchial mucosal permeability and its relationship to smooth muscle contraction in isovolumic bronchial segments. *Br. J. Pharmacol.*, **102**, 179P.

OMARI, T.I., MITCHELL, H.W. & SPARROW, M.P. (1991b). Epithelial damage and responsiveness of bronchial segments. *Clin. Exp. Pharmacol. Physiol.*, **18** (Suppl.) 47.

OMARI, T.I. & SPARROW, M.P. (1992). Epithelial disruption by proteases augments the responsiveness of porcine bronchial segments. *Clin. Exp. Pharmacol. Physiol.*, **19**, 785–794.

OMARI, T.I., SPARROW, M.P. & MITCHELL, H.M. (1993). Responsiveness of human isolated bronchial segments and its relationship to epithelial loss. *Br. J. Clin. Pharmacol.*, **35**, (in press).

SANJAR, S., AOKI, S., KRISTERSSON, A., SMITH, D. & MORLEY, J. (1990). Antigen challenge induces pulmonary airway eosinophil accumulation and airway hyperreactivity in sensitized guinea pigs: the effect of antiasthma drugs. *Br. J. Pharmacol.*, **99**, 679–686.

SMALL, R.C., GOOD, D.M., DIXON, J.S. & KENNEDY, I. (1990). The effects of epithelium removal on the actions of cholinomimetic drugs in opened segments and perfused tubular preparations of guinea pig trachea. *Br. J. Pharmacol.*, **100**, 516–522.

SPARROW, M.P. & MITCHELL, H.W. (1991). Modulation by the epithelium of the extent of bronchial narrowing produced by substances perfused through the lumen. *Br. J. Pharmacol.*, **103,** 1160–1164.

SPINA, D., FERNANDES, L.B., PREUSS, J.M.H., HAY, D.W.P., MUCCITELLI, R.M., PAGE, C.P. & GOLDIE, R.G. (1992). Evidence that epithelium-dependent relaxation of vascular smooth muscle detected by co-axial bioassays is not attributable to hypoxia. *Br. J. Pharmacol.*, **105,** 799–804.

SUN, F.F., CZUK, C.I. & TAYLOR, B.M. (1989). Arachidonic acid metabolism in guinea pig eosinophils: synthesis of thromboxane B_2 and leukotriene B_4 in response to soluble or particulate activation. *J. Leukocyt. Biol.*, **46,** 152–160.

UCHIDA, D.A., LARSEN, G.L. & IRVIN, C.G. (1990). Cationic charge may be responsible for the increase in airways responsiveness associated with major basic protein. *Am. Rev. Respir. Dis.*, **141,** A174.

VANHOUTTE, P.M. (1988). Epithelium-derived relaxing factor: myth or reality? *Thorax*, **43,** 665–668.

WARDLAW, A.J., DUNETTE, S., GLEICH, G.J., COLLINS, J.V. & KAY, A.B. (1988). Eosinophils and mast cells in bronchoalveolar lavage in subjects with mild asthma. *Am. Rev. Respir. Dis.*, **137,** 62–69.

WHITE, S.R., OHNO, S., MUNOZ, N.M., GLEICH, G.J., ABRAHAMS, C., SOLWAY, J. & LEFF, A.R. (1990). Epithelium-dependent contraction of airway smooth muscle caused by eosinophil MBP. *Am. J. Physiol.*, **259,** L294–L303.

(Received November 18, 1992
Revised January 28, 1993
Accepted February 18, 1993)