Active sensitization of guinea-pig airways in vivo enhances in vivo and in vitro responsiveness


ABSTRACT: A plethysmographic method was employed to assess the airway resistance of conscious, free-breathing guinea-pigs. Using this method animals sensitized by inhalation of ovalbumin and appropriate controls were assessed for their responsiveness to histamine and methacholine in vivo. The cough frequency on exposure to citric acid mist in the two groups was also assessed. Tracheal spirals from these animals were subsequently tested for their responsiveness to histamine, methacholine and prostaglandin D₂. Sensitization increased responsiveness to histamine, methacholine and citric acid in vivo but only histamine responses were affected in vitro. These changes were accompanied by a significant eosinophilia in the airways as assessed by bronchoalveolar lavage. We conclude that sensitization of the airways to ovalbumin results in responsiveness changes in bronchial smooth muscle accompanied by signs of airway inflammation. Eur Respir J., 1988, 1, 839-845.

Increased responsiveness of the airways to a wide variety of stimuli is a feature of asthma that underlies much of its symptomatology [1]. Since asthma is commonly found in association with enhanced IgE reactions to common environmental allergens [2], it is likely that sensitization of the airways with allergen-specific IgE plays a crucial role in the pathogenesis of hyperresponsiveness and accompanying inflammatory events. From studies in animals it is clear that sensitization of the airways followed by antigen challenge can induce bronchial hyperresponsiveness to inhaled histamine [3, 4]. Similar observations have been made in man from studies of naturally occurring asthma and asthma secondary to occupational exposure to small molecular weight chemicals [5]. Indeed, following allergen challenge of sensitized subjects, the airways become hyperresponsive to histamine, methacholine, cold air and exercise, a state which may last for many weeks after a single allergen exposure [4]. The mechanisms involved in the disordered airway physiology associated with hyperresponsiveness are diverse and involve the participation of inflammatory leucocytes with release of potent pro-inflammatory chemical mediators [1, 5-7]. In particular, eosinophilia is a marked feature of the human airways response to antigen challenge [8, 9].

In studies of the cellular and molecular mechanisms underlying bronchial hyperresponsiveness, one finding that has been difficult to explain is the failure to reproduce in vitro airways hyperresponsiveness to an agonist in tissues isolated from lungs of animals or human subjects in whom hyperresponsiveness to the same agonist had been shown in vivo [10-13]. Recent work by Soubra and co-workers [14-17] has shown that sensitization of guinea-pigs in vivo with antigen can lead to alterations in the electrophysiology of isolated tracheal smooth muscle in vitro and these alterations are associated with increased responsiveness to histamine in vitro.

In the present study we have extended this observation by studying the effects of local sensitization of the airways of guinea-pigs in vivo on airway responsiveness of free-breathing non-anaesthetized animals to inhaled histamine, methacholine and citric acid in vivo and to histamine, methacholine and prostaglandin D₂ in vitro. We have also examined the cellular content of bronchoalveolar lavage (BAL) fluid during the process of active sensitization in order to test the hypothesis that airway inflammation is associated with the development of hyperresponsiveness.

Methods

Airways responsiveness in vivo

Pathogen-free male Dunkin Hartley guinea-pigs, weighing 450–550 g, were actively sensitized by exposing them for 3 min to aerosolized ovalbumin (10 mg·ml⁻¹ in 0.9% sterile sodium chloride). The aerosol rate of 0.7 ml·min⁻¹ and mass median particle diameter of 4.0 μm was generated by a Wright’s nebulizer driven by compressed air at 8 l·min⁻¹ [18]. Seven days after the first ovalbumin exposure, the pulmonary sensitization procedure was repeated. A separate group of control animals was subjected to similar inhalations of sterile saline. One week after the second exposure animals were tested for...
their bronchial response to inhaled methacholine, histamine or citric acid. Earlier experiments [18] have shown that animals sensitized as described above and subsequently challenged with ovalbumin respond with such a vigorous bronchoconstriction that antihistamine administration is required (mepyramine, 10 mg·ml⁻¹) to prevent fatality. Such early reactions are followed by late reactions in more than 90% of animals. No early or late responses are observed in unsensitized animals or sensitized animals challenged with aerosolized saline [18].

The airways responses to methacholine and histamine were determined in conscious guinea-pigs by measuring airway function by whole body plethysmography [16]. Briefly, animals were placed in the plethysmograph and allowed a 10 min equilibration period. Four baseline measurements of thoracic gas volume and airways resistance were taken at one minute intervals and computed to give specific airways conductance (sGaw). Animals were then provoked by inhalation of methacholine (0.5 mg·ml⁻¹) or histamine (0.1 mg·ml⁻¹) freshly prepared in sterile 0.9% sodium chloride, aerosolized and delivered to the animals as described above. Following provocation, pulmonary function measurements were repeated at one minute intervals for 4 min. The procedure was then repeated with increasing concentrations of agonists up to 1.0 mg·ml⁻¹ and 2.5 mg·ml⁻¹ for histamine and methacholine, respectively. Airway calibre was allowed to return to within 5% of baseline before a further concentration of agonist was given.

The cough response to citric acid was determined by placing each animal in a perspex chamber (3.9 l) into which a citric acid mist (75 mg·ml⁻¹) was generated by a Wright’s nebulizer as described above. The number of times the animal coughed during a 3 min exposure to citric acid was recorded [19].

**Tracheal responsiveness in vitro**

Responsiveness of isolated tracheal preparations was studied using tissue from animals actively sensitized to ovalbumin or appropriate saline-exposed controls as described above. Experiments were performed on animals that had either undergone histamine provocation testing in vivo 24 h previously, or had received no additional treatment other than the two exposures to ovalbumin to induce sensitization or saline as control.

After allergen or saline exposure the animals were sacrificed by stunning and exsanguination. The trachea was then removed, cut circularly and suspended in a tissue bath containing Kreb’s solution (composition mM, KCl 4.69, KH₂PO₄ 1.18, MgSO₄ 1.03, NaCl 118.1, NaHCO₃ 25.0, glucose 11.1, CaCl₂ 2.5) maintained at 37°C and gassed with 95% O₂:5% CO₂. Tracheal preparations were placed under an initial isometric tension of 2 g and allowed to equilibrate at 37°C for 1 h. Tension developed by the tissues was measured isometrically using a Lectromed Multi­trace 6-channel recorder. Each preparation was initially tested for its response to a supramaximal (100 μM) concentration of methacholine. If consistent responses were obtained to this concentration of methacholine on two or three occasions, cumulative concentration-response curves were then constructed for histamine (0.1–320 μM), methacholine (0.1–320 μM) and prostaglandin D₂ (0.1–32 μM), a 7 min contact time being allowed for each contraction to develop before addition of the next concentration.

**Cytology**

Cellular infiltration of the airways associated with sensitization was assessed by light microscopical examination of the cells recovered by BAL [18]. For BAL, guinea-pigs were anaesthetized with pentobarbitone (50 mg·kg⁻¹ i.p.), a tracheostomy performed and the trachea cannulated. Animals were then exsanguinated by severing the carotid arteries. Towards the end of exsanguination, BAL of both lungs was performed by introducing 5 ml of sterile saline at 37°C through the tracheal cannula and recovering the lavage fluid by gentle aspiration. A second lavage was performed with a further 5 ml of saline and the two fluid collections combined and filtered through surgical gauze. The combined cell suspension was immediately centrifuged at 200 g for 10 min at 4°C and the cell pellet resuspended in 1 ml Eagle’s Minimum Essential Medium (MEM). Total leucocyte counts were made on an aliquot of cell suspension stained with May-Grünwald-Giemsa and counted in a Neubauer counting chamber. Differential leucocyte counts were performed on cytospin (Shandon model 2 cytocentrifuge) preparations stained with May-Grünwald-Giemsa. A minimum of 300 cells were counted using standard morphological criteria to classify them into macrophages, lymphocytes, eosinophils and neutrophils.

**Analysis of results**

Changes in airway calibre in vivo following inhalation of histamine or methacholine were expressed as a percentage change from the mean control baseline sGaw value. Non-cumulative concentration-response curves were constructed by least squares linear regression analysis and the potencies of agonists expressed as the EC₅₀ value (the concentration calculated to cause a 50% fall in sGaw). Constrictions of tracheal smooth muscle in response to each of the three agonists were expressed as grams tension developed. Cumulative concentration-response curves were constructed by least squares linear regression analysis and the potencies of agonists expressed as the EC₅₀ value (the concentration calculated to produce 50% of the maximum contraction observed with that agonist). The possibility of statistically significant differences between concentration-response curves obtained in different groups of animals was assessed by analysis of covariance and testing for coincidence of regression lines by Student’s t-test. Cough frequency with citric acid and absolute cell numbers in BAL fluid in sensitized and non-sensitized animals were compared by a two-tailed Student’s t-test for unpaired
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data. A probability of \( p < 0.05 \) was considered to be statistically significant.

Materials

Histamine, methacholine and ovalbumin were obtained from Sigma (Poole, Dorset, UK), citric acid from BDH Chemicals Ltd (Poole, Dorset, UK), Evans blue dye, 5% aqueous solution, from William Warner & Co. (Eastleigh, Hampshire, UK), sodium pentobarbital from May and Baker Ltd (Dagenham, Essex, UK) and Eagle’s Minimum Essential Medium from Gibco Europe Ltd (Paisley, Scotland, UK). In vitro solutions were made up freshly in Kreb’s buffer except for prostaglandin \( \text{D}_2 \), which was diluted from a \( 5 \) mg·ml\(^{-1} \) stock solution in methanol stored at -20°C.

Results

Airway responsiveness in vivo

The mean basal sGaw values (±SEM) for groups of ten sham-sensitized and ten ovalbumin-sensitized animals were 0.059±0.002 and 0.060±0.002 cmH\(_2\)O·s\(^{-1} \), respectively. These values were not significantly different. However, compared to sham-sensitized controls, ovalbumin-sensitized animals showed an increased bronchial responsiveness to histamine and methacholine and an increased cough response to citric acid. Sensitization produced parallel shifts to the left in the concentration-response curves for both histamine and methacholine (fig. 1). The geometric mean PC\(_{20}\) for histamine fell 4.1-fold from 0.77 (0.37-1.60) mg·ml\(^{-1} \) to 0.19 (0.13-0.27) mg·ml\(^{-1} \) (95% confidence limits in parentheses). This reduction in PC\(_{20}\) was statistically significant \( (p<0.001) \).
Similarly, sensitization produced a 1.6-fold reduction in the geometric mean PC$_{20}$ for methacholine from 1.36 (0.94–1.97) mg·ml$^{-1}$ to 0.83 (0.57–1.20) mg·ml$^{-1}$. This difference was again highly significant (p<0.001).

Sensitization also significantly increased (p<0.05) the cough frequency in response to inhalation of 75 mg·ml$^{-1}$ citric acid. The number of coughs per 3 min (mean±SEM) in groups of eight animals was 8.6±0.9 in sham-sensitized and 13.0±1.0 in ovalbumin-sensitized animals.

**Table 1.** – Effect of histamine exposure in vivo on the subsequent in vitro sensitivity of tracheal strips to histamine, methacholine and prostaglandin D$_2$

<table>
<thead>
<tr>
<th>Agonist</th>
<th>EC$_{50}$ control</th>
<th>EC$_{50}$ histamine pretreatment</th>
<th>Change in sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-sensitized animals</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histamine</td>
<td>14.4 (7.7–27.0)</td>
<td>50.0 (28.8–86.7)</td>
<td>–3.5 (p&lt;0.05)</td>
</tr>
<tr>
<td>Methacholine</td>
<td>9.4 (3.5–24.9)</td>
<td>3.2 (1.9–5.4)</td>
<td>+2.9 (NS)</td>
</tr>
<tr>
<td>Prostaglandin D$_2$</td>
<td>3.6 (0.8–16.2)</td>
<td>1.4 (0.3–6.2)</td>
<td>+2.6 (p&lt;0.05)</td>
</tr>
<tr>
<td><strong>Sensitized animals</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histamine</td>
<td>7.6 (4.0–14.7)</td>
<td>32.1 (9.0–115.0)</td>
<td>–4.2 (p&lt;0.05)</td>
</tr>
<tr>
<td>Methacholine</td>
<td>5.3 (2.4–11.7)</td>
<td>4.8 (1.7–13.3)</td>
<td>+1.1 (NS)</td>
</tr>
<tr>
<td>Prostaglandin D$_2$</td>
<td>1.9 (0.9–4.2)</td>
<td>0.5 (0.1–2.8)</td>
<td>+3.8 (NS)</td>
</tr>
</tbody>
</table>

The table shows the EC$_{50}$ values for histamine, methacholine and prostaglandin D$_2$ in tracheal strips from control animals and animals receiving histamine provocation 24 h earlier. EC$_{50}$ concentration giving 50% maximum contraction; NS: not statistically significant. Each response is the mean of values obtained in 7–10 animals; ±: with 95% confidence limits.

**Tracheal responsiveness in vitro**

The effect of in vivo sensitization of guinea-pigs on the responsiveness of airways smooth muscle in vitro was examined using isolated tracheal preparations from groups of eight to ten animals. Ovalbumin sensitization increased the responsiveness to histamine in vitro by 1.9-fold (fig. 2), the geometric mean EC$_{50}$ values (with 95% confidence limits) being 7.6 (4.0–14.7) µM and 14.4 (7.7–27.0) µM for ovalbumin- and sham-sensitized animals, respectively. This change was statistically significant (p<0.01). Sensitization also caused an apparent increase in the sensitivity of tracheal strips to methacholine by 1.7-fold and prostaglandin D$_2$ by 1.8-fold, the EC$_{50}$ values falling to 5.3 (2.4–11.7) µM from 9.4 (3.5–25.9) µM and to 1.9 (0.9–4.2) µM from 3.6 (0.8–16.2) µM, respectively. These changes failed, however, to reach statistical significance.

Administration of aerosolized histamine to groups of seven to ten animals in vivo 24 h before sacrifice and removal of tissues for in vitro study also significantly influenced the responsiveness of the tracheal strips to histamine and prostaglandin D$_2$, but not methacholine, in vitro (table 1). Histamine treatment in vivo significantly decreased (p<0.05) the contractile responsiveness of the tracheal preparations to histamine from both sham- and ovalbumin-sensitized animals, the EC$_{50}$ values being increased 3.5-fold (p<0.05) in sham-sensitized and 4.2-fold (p<0.05) in actively-sensitized animals, respectively (table 1). In contrast, the tracheal responsiveness to prostaglandin D$_2$ was increased in vitro in tissues from animals exposed to histamine in vivo, the EC$_{50}$ values falling 2.6-fold (p<0.05) and 3.8-fold (NS) in sham- and ovalbumin-sensitized guinea-pigs, respectively.

**Changes in the cellular composition of BAL fluid**

The effect of sensitization on cellular infiltration into the airway lumen, as assessed by BAL in groups of six guinea-pigs, revealed a significant eosinophilia.

**Table 2.** – The effect of sensitization on cells recovered by bronchoalveolar lavage (BAL)

<table>
<thead>
<tr>
<th>Cell type</th>
<th>No. of cells recovered (x10$^6$)</th>
<th>Change from control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>controls</td>
<td>sensitized animals</td>
</tr>
<tr>
<td>Macrophages</td>
<td>1.53±0.27</td>
<td>2.46±0.40</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.15±0.05</td>
<td>0.49±0.09</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0.03±0.01</td>
<td>0.05±0.01</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0.07±0.03</td>
<td>0.11±0.03</td>
</tr>
</tbody>
</table>

Number of cells recovered by BAL of sensitized and sham-sensitized guinea-pigs, figures are mean of six experiments ±SEM.
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eosinophil numbers being increased 3.3-fold in the sensitized animals (p<0.05) (table 2). Recovery of lavage fluid was consistently between 70 and 80% of the 10 ml instilled and macrophages were the dominant cell type, constituting between 64.3 and 92.5% of total cells recovered. No significant differences were observed between the two groups with respect to macrophage, lymphocyte or neutrophil numbers.

Discussion

Sensitization by inhalation of ovalbumin increased responsiveness of the airways of conscious fre­
breathing guinea-pigs to methacholine, histamine and citric acid in vivo. In vitro, the responsiveness of tracheal spirals to histamine was increased, although to a lesser degree than observed in vivo, whilst the EC50 values of methacholine and prostaglandin D2 were not significantly altered. The changes in airway responsiveness were accompanied by a 3.3-fold increase in the number of eosinophils recovered from the lungs of sensitized animals. Exposure of animals to histamine in vivo reduced the in vitro potency of histamine but increased that of prostaglandin D2.

Airway inflammation may increase bronchial responsiveness, either by interference with the integrity of the bronchial epithelium, allowing greater penetration of mediators to the muscle [20], or by inflammatory mediators themselves altering smooth muscle responsiveness [6, 7, 21, 22]. In this model, the airways of sensitized animals showed a significant eosinophilia as assessed by BAL; this is analogous to the situation in human asthmatics where BAL reveals eosinophilia following antigen challenge [8, 9]. Activated eosinophils can produce an array of cytotoxic principles, such as eosinophil cationic protein [23] and major basic protein [24], which damage airway epithelial cells. Furthermore, eosinophils have been shown to produce both leukotriene C4 [25] and PAF-acether [26], two lipid mediators that have been shown to increase airways responsiveness to histamine. The increase in airway responsiveness induced by sulphidopeptide leukotrienes in guinea-pigs is, however, dependent upon the route of administration of the agonist in vivo and occurs only at unphysiologically low calcium concentrations in vitro [6, 21]. PAF-acether has also been shown to increase smooth muscle responsiveness in vitro and in vivo [22], the latter event being accompanied by an eosinophil infiltration into the bronchial lumen [27]. The failure to observe hyperresponsiveness in vitro could therefore be explained if the continuous presence of a mediator is required to maintain increased responsiveness and this mediator is lost when the trachea is removed from the animal and washed in the tissue bath.

One possible explanation for the differences between in vivo and in vitro responsiveness is an effect on nervous modulation of airway constriction [28], which would not be apparent in vitro. Certainly the increased cough frequency in sensitized animals on citric acid exposure in vivo may be unrelated to smooth muscle hyperresponsiveness of the airways, as the cough reflex is triggered by irritant-sensitive receptors in the airways [29].

It has been reported [14-17] that both passive and active sensitization increase the smooth muscle cell resting membrane potential of guinea-pig airways smooth muscle cells, thus causing an increased responsiveness of preparations to histamine. It is claimed that this change results from a direct effect of antibodies on the smooth muscle, since it is unaffected by inhibitors of inflammation, including diphenhydramine, methysergide, indomethacin and FPL 55712 [17]. It has been suggested that IgE, which may bind to a variety of cell types [30], binds to smooth muscle [14, 15] where it increases Na+/K+ ATPase activity in a manner similar to that seen following its binding to macrophages [31]. Although we have produced non-specific hyperresponsiveness in vivo by sensitization alone, we find the picture less clear in vitro. The responsiveness of tracheal spirals to histamine was significantly increased by sensitization, whilst responsiveness to methacholine and prostaglandin D2, although slightly increased, was not significantly changed from the control. It seems likely, therefore, that there are factors affecting the in vivo response of the airways to agonists which are in addition to any direct effect of sensitization on smooth muscle. Loss of mediators and neuronal influences have been discussed. Additionally the sensitization procedure employed here compared to the longer, more chronic sensitization employed by McCay and Souhrada [32] may account for the small effects on isolated muscle. Furthermore, it is possible that the maximal effects of airway hyperresponsiveness are not evident in the trachea but only in smaller peripheral airways [33].

The apparent tachyphylaxis of isolated tracheal preparations to histamine in vitro, following histamine treatment in vivo, may be significant in the light of reported difficulties in correlating in vivo and in vitro reactivities [11, 12, 23] and a longer interlude between in vivo and in vitro testing may be needed in these situations. The action of histamine exposure in vivo on the response to prostaglandin D3 in vitro is less easy to explain. Actively-sensitized and sham-sensitized animals treated with histamine in vivo showed an increased responsiveness to prostaglandin D3 although in the actively-sensitized animals this change did not attain statistical significance due to the large variability in in vitro responses to prostaglandin D3 in this group. Furthermore, although sensitization alone did not alter the sensitivity of tracheal spirals to prostaglandin D3, the combined effects of histamine exposure in vivo and sensitization did produce a significant increase in responsiveness. This would suggest an additive effect of histamine and sensitization on responsiveness to prostaglandin D3. It has previously been reported that prostaglandin D3 enhances airway responsiveness to histamine in asthmatic subjects in vivo [34, 35] although other workers have not found this to be the case [36].

In conclusion, it would seem that sensitization of the airways by two exposures to ovalbumin aerosol produces an increased responsiveness to agonists in vivo, which is accompanied by signs of airway inflammation. These changes in responsiveness are not seen to the same degree
in isolated tracheal strips. This difference may be the result of the loss of factors modulating airway calibre in vivo such as autonomic nervous tone or inflammatory mediators. The finding that histamine exposure in vivo decreases histamine responsiveness in vivo whilst responsiveness to prostaglandin D$_2$ is increased indicates how exposure to mediators may alter the responsiveness of the airways in an unpredictable fashion. It seems likely that in vivo bronchial hyperresponsiveness is a consequence of a complex interplay of a number of factors including direct effects of sensitization on the muscle, effects of inflammatory mediators and alterations in neuronal control of the airway.

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References


RÉSUMÉ: Une méthode pléthysmographique a été utilisée pour apprécier la résistance des voies aériennes de cobayes conscients, respirant librement. Avec cette méthode, on a apprécié, pour leur réactivité à l’histamine et à la méthacholine in vivo, les animaux sensibilisés par inhalation d’ovalbumine et les contrôle appropriés. L’on a également estimé la fréquence de la toux lors d’une exposition à un aérosol d’acide citrique dans les deux groupes. Les spirales trachéales provenant des mêmes animaux ont été ultérieurement testées pour leur réactivité à l’histamine, la méthacholine et la prostaglandine D, in vitre. La sensibilisation a augmenté la réactivité à l’histamine, à la méthacholine et à l’acide citrique in vitre, mais in vitre seules les réponses à l’histamine ont été influencées; ces modifications ont été accompagnées d’une eosinophilie significative, décelée par lavage broncho-alvéolaire au niveau des voies aériennes. Nous concluons que la sensibilisation des voies aériennes à l’albumine entraîne des modifications de la réactivité du muscle lisse bronchique, accompagnées de signes d’inflammation des voies aériennes.