Dissociation by steroids of eosinophilic inflammation from airway hyperresponsiveness in murine airways

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Abstract

Background: The link between eosinophils and the development of airway hyperresponsiveness (AHR) in asthma is still controversial. This question was assessed in a murine model of asthma in which we performed a dose ranging study to establish whether the dose of steroid needed to inhibit the eosinophil infiltration correlated with that needed to block AHR.

Methods: The sensitised BALB/c mice were dosed with vehicle or dexamethasone (0.01–3 mg/kg) 2 hours before and 6 hours after each challenge (once daily for 6 days) and 2 hours before AHR determination by whole-body plethysmography. At 30 minutes after the AHR to aerosolised methacholine the mice were lavaged and differential white cell counts were determined. Challenging with antigen caused a significant increase in eosinophils in the bronchoalveolar lavage (BAL) fluid and lung tissue, and increased AHR.

Results: Dexamethasone reduced BAL and lung tissue eosinophilia (ED₅₀ values of 0.06 and 0.08 mg/kg, respectively), whereas a higher dose was needed to block AHR (ED₅₀ of 0.32 mg/kg at 3 mg/ml methacholine. Dissociation was observed between the dose of steroid needed to affect AHR in comparison with eosinophilia and suggests that AHR is not a direct consequence of eosinophilia.

Conclusion: This novel pharmacological approach has revealed a clear dissociation between eosinophilia and AHR by using steroids that are the mainstay of asthma therapy. These data suggest that eosinophilia is not associated with AHR and questions the rationale that many pharmaceutical companies are adopting in developing low-molecular-mass compounds that target eosinophil activation/recruitment for the treatment of asthma.

Introduction

Airway inflammation and hyperresponsiveness (AHR) are recognised as major characteristics of bronchial asthma; however, their relationship is still poorly understood. Exposure to allergen causes an increase in airway responsiveness that is associated with an influx of inflammatory cells, particularly eosinophils, into the airways in allergic humans [1] and sensitised mice [2], which suggests a causal relationship between airway inflammation and AHR [3,4]. However, there is also much published literature...
suggesting that there is no relationship between allergic airway inflammation and AHR.

In this study we wished to determine whether there was a dissociation between the effective dose of a steroid, dexamethasone, needed to affect antigen-induced AHR in comparison with that needed to affect airway inflammation in the mouse. We have previously described a murine model of asthma that includes non-specific AHR and associated eosinophilia in the airways [5]. In the present study we followed the same sensitising and challenging protocol but decided to determine AHR in conscious, spontaneously breathing, unrestrained mice by whole-body plethysmography [6–9]. Airway responsiveness was expressed as enhanced pause ($P_{\text{enh}}$), a calculated value, which is an indirect measurement that is correlated with measurement of airway resistance, impedance and intrapleural pressure in the same animal [6]. This method was chosen instead of our previously used invasive method because it might offer several potential advantages: it is technically less demanding, it allows repeated measurements over a long period and it avoids the use of anaesthetic and mechanical ventilation. However, one possible disadvantage is that one cannot rule out a contribution by the nose and upper respiratory tract to the parameters measured. This method of antigen-induced airway inflammation and AHR is very similar to that of Dohi et al. [9] in which they report a strong correlation between $P_{\text{enh}}$ and eosinophil number in bronchoalveolar lavage (BAL) fluid.

**Materials and methods**

**Animals**

Male Balb/C mice (14–16 g, 5 weeks old), were obtained from Harlan (Bicester, Oxon., UK), and housed for 1 week before experiments were initiated. Food and water were supplied ad libitum. Experiments were performed in accordance with the UK Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) Act 1986.

**Study design**

The aim of this study was to determine whether there was a dissociation between the effective dose of a steroid needed to affect antigen-induced airway inflammation and AHR.

**Sensitisation and antigen challenge protocol**

Mice were immunised on days 0 and 14 by intraperitoneal (i.p.) injection of 10 µg of ovalbumin (Grade V; Sigma-Aldrich, Poole, Dorset, UK), in 0.2 ml of saline (Fresenius Kabi, Warrington, Cheshire, UK) with 20 mg of aluminium hydroxide (Merck, Lutterworth, Leics., UK) in distilled water) or dexamethasone (Sigma-Aldrich) was administered twice daily by the oral route in a dose volume of 10 ml/kg (0.01–3 mg/kg), the day before the first ovalbumin challenge, 2 hours before and 6 hours after subsequent challenges and on the morning of the AHR determination.

**Administration of dexamethasone**

Vehicle (1% carboxymethylcellulose [Merck, Lutterworth, Leics., UK] in distilled water) or dexamethasone (Sigma-Aldrich) was administered twice daily by the oral route in a dose volume of 10 ml/kg (0.01–3 mg/kg), the day before the first ovalbumin challenge, 2 hours before and 6 hours after subsequent challenges and on the morning of the AHR determination.

**Airways mechanics measurements in nonrestrained, conscious mice**

Twenty-four hours after the last ovalbumin challenge, mice were placed in a whole-body plethysmograph to facilitate the measurement of lung function as described by Tsuyuki et al. [7]. Bronchoconstriction to aerosolised methacholine (MCh) (3 or 10 µg/ml for 60 seconds with 5 minute intervals) (Sigma-Aldrich) was determined.

**Inflammatory cells in the lung**

One hour after the last MCh challenge the mice were killed by anaesthetic overdose (pentobarbitone sodium, 200 mg/kg; Rhone Merieux, Harlow, Essex, UK). BAL was performed with 3 and 0.3 ml aliquots of Roswell Park Memorial Institute medium (RPMI 1640; Life Technologies, Paisley, Renfrewshire, UK). The lungs were removed, and were then cleaned and finely chopped after blood had been perfused out. The chopped tissue was then digested enzymatically to obtain inflammatory cells, as described by Underwood et al. [10]. Total counts of cells recovered in the BAL fluid and tissue digest were made with an automated cell counter (Sysmex F-820; Sysmex UK, Linford Wood, Bucks., UK). Differential counts of cells (eosinophils, neutrophils, macrophages, monocytes and lymphocytes) recovered in the samples were made by light microscopy, of cytocentrifuge preparations (100 µl aliquots spun at 700 rpm for 5 minutes at low acceleration) (Cytospin; Shandon Scientific, Runcorn, Cheshire, UK), which had been stained with Wright-Giemsas stain (Sigma-Aldrich), with a Hematek 2000 (Ames Co., Elkhart, Indiana, USA).

**Statistical analysis**

All values are presented as means ± SEM per group with $n$ = 10. ED$_{50}$ values stated are defined as the amount of drug required to elicit 50% of the maximum inhibition. Statistical analysis was made by analysis of variance with a correction for multiple comparisons. $P < 0.05$ was considered to be statistically significant.
Results

**Inflammatory cells in the lung**

Antigen challenge caused a significant increase in eosinophils recovered in the BAL fluid and lung tissue. Dexamethasone evoked a significant dose-related inhibition of antigen-induced eosinophilia in the BAL fluid and lung tissue, with ED$_{50}$ values of 0.08 and 0.06 mg/kg, respectively (Fig. 1 and Table 1). The higher doses of dexamethasone almost completely abolished BAL eosinophilia but inhibited tissue eosinophilia only by about 50%.

Antigen challenge also significantly increased neutrophil, monocyte and lymphocyte numbers in BAL fluids, and neutrophil, macrophage, monocyte and lymphocyte numbers in lung tissue (Table 2). This increase in numbers of inflammatory cells was significantly inhibited by dexamethasone treatment, although the effect on tissue neutrophilia did not reach statistical significance (Tables 1 and 2).

**Airway responsiveness**

There was no change in basal $P_{\text{enh}}$ after multiple antigen challenge when compared with saline controls and there was no effect of dexamethasone treatment on basal $P_{\text{enh}}$ at the doses tested. Antigen challenge significantly increased airway responsiveness to inhaled MCh compared with saline controls. Dexamethasone treatment significantly inhibited AHR (Fig. 2A depicts peak changes after 3 mg/ml MCh). Figure 2B represents an effective dose of dexamethasone (1 mg/kg) on all of the concentrations of MCh including positive and negative controls. A higher dose of dexamethasone was needed to block AHR than eosinophilia when ED$_{50}$ values are compared (Table 1).

**Discussion**

In this study we have shown for the first time that there is dissociation between the dose of steroid needed to affect antigen-induced BAL and lung tissue eosinophilia and that needed to affect AHR. The ED$_{50}$ dose of dexamethasone required to inhibit AHR is higher than that needed to inhibit eosinophilia. It is possible that eosinophilia has to be completely inhibited to have an effect on AHR; indeed, at 1 mg/kg dexamethasone, eosinophil infiltration into the BAL fluid following challenge is almost completely blocked and at the same dose AHR is also completely reversed. Lung tissue eosinophilia, however, is only inhibited by about 50% at 1 mg/kg dexamethasone, which further indicates the dissociation between eosinophilia and AHR. De Bie et al. [11] showed that dexamethasone (0.5 mg/kg) inhibited both antigen-induced AHR and airway eosinophilia in the mouse; however, using similar doses we found only an effect on eosinophilia. In the study by De Bie et al. [11] they administered the steroid intraperitoneally and employed a different way of measuring AHR, which might account for the difference.

Throughout the literature there are reports of various interventions that affect both allergic AHR and eosinophilia. Antibodies against interleukin-5 (IL-5) have been shown to inhibit both AHR and eosinophilia in the mouse [12–14]. Both allergic AHR and eosinophilia have been shown to be reduced in the following cases: in mice deficient in ICAM-1 (intercellular cell-adhesion molecule-1) [15] by treatment with an anti-B7-2 (CD86) monoclonal antibody [7,16] and with an anti-CTLA4-IgG [17]; in Vβ$^+$-deficient mice and BALB/c mice treated with antibodies against Vβ8 [18]; in mice lacking a functioning 5-lipoxygenase enzyme [19]; in interferon-β-treated mice [20]; in IL-12 treated mice [21,22]; and in mice treated with an immunosuppressive agent, FK-506 [8].
There are reports of interventions inhibiting allergic eosinophilia but not AHR: in humans, an IL-5-blocking monoclonal antibody [23]; in mice, antibodies against IL-5 [24–26] and IL-5 knockout animals [27]. Other interventions have been shown to have the reverse effect, inhibiting allergic AHR without affecting eosinophilia: antibodies against interferon-γ in mice [26], antibodies against IL-16 in mice [28], IL-10-deficient mice [29] and mast-cell-deficient mice [24,25]; Tournoy et al. [30] showed that by lowering the allergic challenge eosinophilia was lost but AHR remained.

Treatment with dexamethasone inhibited other leukocytes measured in the lung with ED50 values comparable to those determined for eosinophilia (Table 1). This would suggest that these inflammatory cells are also not associated with AHR; indeed, neutrophil numbers in the BAL fluid and tissue were not reduced to unchallenged levels by any dose of steroid used here (Table 2), whereas AHR was completely reversed. The involvement in AHR of other leukocytes measured here cannot be completely ruled out because it might be necessary to completely inhibit their infiltration into the lung before any impact on AHR is observed. Increased levels of macrophages, monocytes and lymphocytes in the lung were only completely inhibited at 1 mg/kg of dexamethasone, which is the corresponding dose needed to block AHR.

There is therefore a wealth of literature on the association between allergic eosinophilia and AHR that is sometimes

### Table 1: Effect of dexamethasone treatment on inflammatory cell numbers in bronchoalveolar lavage (BAL) fluid and lung tissue after the last antigen challenge in sensitised mice

| Parameter | Eosinophils | Neutrophils | Macrophages | Monocytes | Lymphocytes | MCh challenge (3 mg/ml) |
|-----------|-------------|-------------|-------------|-----------|-------------|------------------------|
| BAL ED50  | 0.08        | 0.14        | -           | 0.09      | 0.10        | -                      |
| Tissue ED50 | 0.06       | -           | 0.13        | 0.13      | 0.08        | -                      |
| AHR peak changes, ED50 | -           | -           | -           | -         | 0.35        | -                      |
| AHR AUC changes, ED50 | -           | -           | -           | -         | -           | 0.32                   |

Results are expressed as ED50 values, in mg/kg of dexamethasone. AHR, airway hyperresponsiveness; AUC, area under the curve; MCh, methacholine.

### Table 2: Effect of dexamethasone treatment on inflammatory cell numbers in bronchoalveolar lavage (BAL) fluid and lung tissue after the last antigen challenge in sensitised mice

| Cell type       | Vehicle saline | Vehicle OA | Dex 0.01 OA | Dex 0.03 OA | Dex 0.1 OA | Dex 0.3 OA | Dex 1 OA | Dex 3 OA |
|-----------------|---------------|------------|-------------|-------------|------------|------------|----------|----------|
| BAL eosinophils | 0.6 ± 0.2     | 180.3 ± 34.6a | 239.8 ± 76.5 | 129.9 ± 45.3 | 66.9 ± 20.6 | 43.6 ± 11.5a | 4.6 ± 1.7a | 3.6 ± 2.1a |
| BAL neutrophils | 0.7 ± 0.3     | 449.4 ± 76.7a | 617.9 ± 196.7 | 335.2 ± 93.9 | 218.8 ± 59.5 | 173.6 ± 46.8 | 38.5 ± 11.2a | 29.8 ± 10.2a |
| BAL macrophages | 104.4 ± 16.5  | 68.6 ± 12.7 | 79.9 ± 14.2 | 91.5 ± 14.2 | 57.0 ± 11.3 | 65.5 ± 5.5 | 71.7 ± 10.5 | 70.9 ± 4.7 |
| BAL monocytes   | 10.2 ± 2.0    | 62.4 ± 8.5a | 68.6 ± 19.5 | 52.2 ± 14.0 | 37.7 ± 9.2 | 25.8 ± 6.5 | 6.1 ± 1.0a | 6.6 ± 1.6a |
| BAL lymphocytes | 6.2 ± 1.8     | 80.4 ± 12.9a | 111.5 ± 39.1 | 62.3 ± 18.0 | 37.4 ± 13.0 | 28.2 ± 6.7 | 5.6 ± 1.2a | 6.9 ± 1.7a |
| Tissue eosinophils | 823 ± 108   | 4944 ± 715a | 5480 ± 1323 | 4033 ± 734 | 3479 ± 713 | 2703 ± 328a | 2557 ± 519a | 2739 ± 476a |
| Tissue neutrophils | 4948 ± 622  | 21869 ± 2756a | 22884 ± 4686 | 22261 ± 5450 | 16166 ± 2473 | 14520 ± 1767 | 15188 ± 1750 | 18619 ± 1805 |
| Tissue macrophages | 571 ± 95    | 4721 ± 1277a | 4590 ± 1266 | 3602 ± 754 | 2887 ± 841 | 2267 ± 714 | 689 ± 239a | 311 ± 125a |
| Tissue monocytes | 367 ± 78     | 6889 ± 1316a | 7946 ± 2196 | 5524 ± 1524 | 3923 ± 993 | 2737 ± 1168 | 304 ± 69a | 196 ± 75a |
| Tissue lymphocytes | 1069 ± 98   | 8277 ± 1327a | 8292 ± 2638 | 5912 ± 1696 | 3990 ± 959 | 3440 ± 959 | 707 ± 156a | 691 ± 116.5a |

The concentration of cells in BAL fluid was 10³/ml (the volume of BAL recovered in the lavage in this experiment was 0.6 ml from each animal) and that of tissue cells was 10³/mg of tissue. Results are means ± SEM (n = 10). Asterisks indicate a significant difference (P < 0.05) from the relevant vehicle-dosed control group. OA, Ovalbumin.
confusing and contradictory. This is the first study that has addressed this question with a range of doses of corticosteroid, compounds known to block AHR and eosinophilia in all animal models of asthma and to affect inflammation and AHR in asthmatics in a clinical setting. We feel that this novel pharmacological approach has revealed a clear dissociation between eosinophilia and AHR in the same animal and this concurs with a study in humans showing no correlation between AHR and the number of inflammatory cells in sputum or bronchoalveolar lavage [31]. These data question the rationale that many pharmaceutical and biotechnology companies have adopted in embarking on drug discovery programmes that target the eosinophil activation/infiltration signalling pathways (e.g. IL-5, VLA-4 and CCR-3).

These data suggest that other factors, such as airway wall remodelling, activation state of the eosinophils, T-cell activation or autonomic dysfunction, might be more important in the development of AHR.

**Conclusion**

Dissociation was observed between the dose of steroid needed to affect AHR compared with that required to affect inflammation, suggesting that AHR is not a direct consequence of eosinophilia. This novel pharmacological approach has revealed a clear dissociation between eosinophilia and AHR by using steroids that are the mainstay of asthma therapy. If the eosinophil is not associated with AHR, as this result suggests, the information described here is vitaly important given that many pharmaceutical companies are engaged in developing low-molecular-mass compounds that target eosinophil activation/recruitment for the treatment of asthma.

**Abbreviations**

AHR = airway hyperresponsiveness; BAL = bronchoalveolar lavage; IL = interleukin; i.p. = intraperitoneal; MCh = methacholine; $P_{enh}$ = enhanced pause.

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