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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 (ED_{50} values of 0.06 and 0.08 mg/kg, respectively), whereas a higher dose was needed to block AHR (ED_{50} 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 responsive-

ness 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_{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_{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 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, Leicester, UK). From day 21 the animals were challenged with aerosolised ovalbumin (5% in saline) or vehicle (saline) for 20 minutes per day on six consecutive days. Aerosol generation was

achieved by use of an air-driven nebuliser (System 22; Medic-aid, Pagham, West Sussex, UK).

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

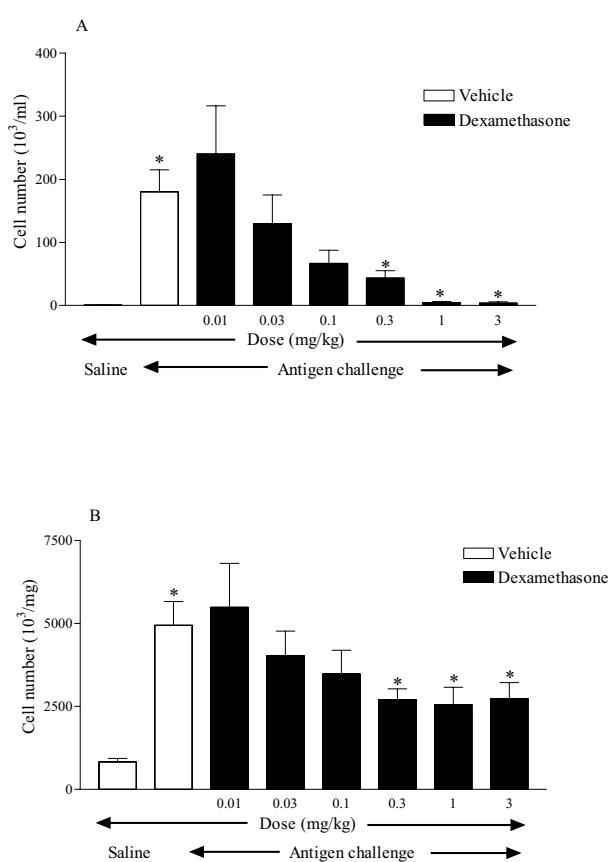
Twenty-four hours after the last ovalbumin challenge, mice were placed in a whole-body plethysmograph to facilitate the measurement of lung function as described by Tsuyuki *et al.* [7]. Bronchoconstriction to aerosolised methacholine (MCh) (3 or 10 mg/ml for 60 seconds with 5 minute intervals) (Sigma-Aldrich) 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 three 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 cyt centrifuge 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-Giemsa stain (Sigma-Aldrich), with a Hematek 2000 (Ames Co., Elkhart, Indiana, USA).

Statistical analysis

All values are presented as means ± SEM per group with $n = 10$. ED₅₀ 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.

**Figure 1**

Effect of dexamethasone treatment on BAL (A) and lung tissue (B) eosinophil number 24 hours after the last antigen challenge in sensitised mice. Results represent mean \pm s.e.m. ($n = 10$). * $P < 0.05$ compared with relevant vehicle dosed control group.

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_{enh} after multiple antigen challenge when compared with saline controls and there was no effect of dexamethasone treatment on basal P_{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 V β 8 $^{+}$ -deficient mice and BALB/c mice treated with antibodies against V β 8 [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].

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 ED ₅₀	0.08	0.14	-	0.09	0.10	-
Tissue ED ₅₀	0.06	-	0.13	0.13	0.08	-
AHR peak changes, ED ₅₀	-	-	-	-	-	0.35
AHR AUC changes, ED ₅₀	-	-	-	-	-	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 ± 34.6*	239.8 ± 76.5	129.9 ± 45.3	66.9 ± 20.6	43.6 ± 11.5*	4.6 ± 1.7*	3.6 ± 2.1*
BAL neutrophils	0.7 ± 0.3	449.4 ± 76.7*	617.9 ± 196.7	335.2 ± 93.9	218.8 ± 59.5	173.6 ± 46.8	38.5 ± 11.2*	29.8 ± 10.2*
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 ± 8.5*	68.6 ± 19.5	52.2 ± 14.0	37.7 ± 9.2	25.8 ± 6.5	6.1 ± 1.0*	6.6 ± 1.6*
BAL Lymphocytes	6.2 ± 1.8	80.4 ± 12.9*	111.5 ± 39.1	62.3 ± 18.0	37.4 ± 13.0	28.2 ± 6.7	5.6 ± 1.2*	6.9 ± 1.7*
Tissue eosinophils	823 ± 108	4944 ± 715*	5480 ± 1323	4033 ± 734	3479 ± 713	2703 ± 328*	2557 ± 519*	2739 ± 476*
Tissue neutrophils	4948 ± 622	21869 ± 2756*	22884 ± 4686	22261 ± 5450	16166 ± 2473	14520 ± 1767	15188 ± 1750	18619 ± 1805
Tissue macrophages	571 ± 95	4721 ± 1277*	4590 ± 1266	3602 ± 754	2887 ± 841	2267 ± 714	689 ± 239*	311 ± 125*
Tissue monocytes	367 ± 78	6889 ± 1316*	7946 ± 2196	5524 ± 1524	3923 ± 993	2737 ± 1168	304 ± 69*	196 ± 75*
Tissue lymphocytes	1069 ± 98	8277 ± 1327*	8292 ± 2638	5912 ± 1696	3990 ± 959	3440 ± 959	707 ± 156*	691 ± 116.5*

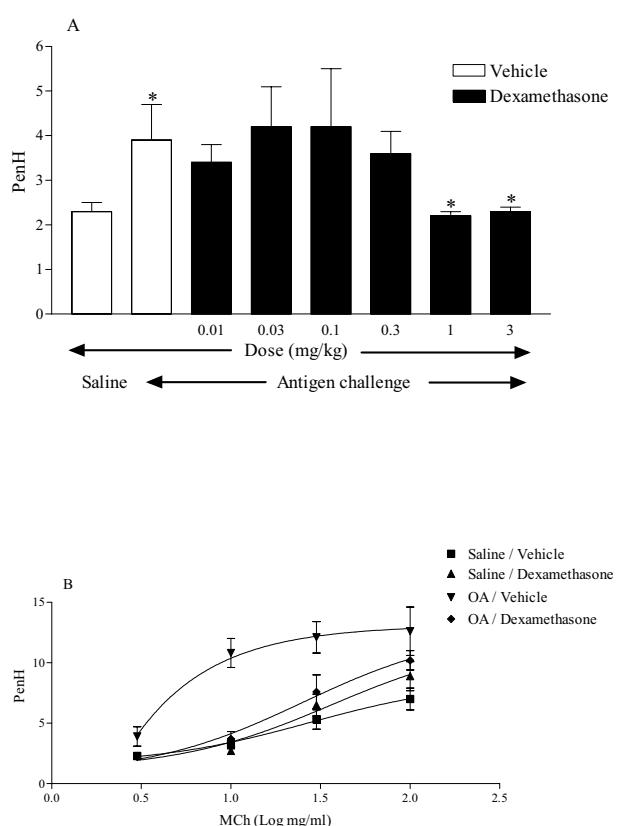
The concentration of cells in BAL fluid was 10³/ml (the volume of BAL recovered in the lavage in this experiment was 0.6 ml from each animal) and that of tissue cells was 10³/mg of tissue. Results are means ± SEM (n = 10). Asterisks indicate a significant difference (P < 0.05) from the relevant vehicle-dosed control group. OA, Ovalbumin.

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

**Figure 2**

Effect of dexamethasone (0.01 - 3 mg/kg) on peak changes in PenH measured after aerosolised methacholine (3 mg/ml for 60sec) 24 hours after the last antigen challenge in sensitised mice (Figure 2A). Effect of dexamethasone (1 mg/kg) on peak changes in PenH measured after aerosolised methacholine (3 - 100 mg/ml for 60sec) 24 hours after the last antigen challenge in sensitised mice (Figure 2B). Results represent mean \pm s.e.m. ($n = 10$). * $P < 0.05$ compared with relevant vehicle dosed control group.

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 vitally 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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