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# Effects of dexamethasone on airway hyper-responsiveness to the adenosine $A_1$ receptor agonist cyclo-pentyl adenosine in an allergic rabbit model

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**1** New Zealand White (NZW) rabbits were immunized within 24 h of birth with *Alternaria tenuis* in aluminium hydroxide (Al $(OH)_3$) (i.p.) or sham immunized (saline plus Al $(OH)_3$ i.p.) and subsequently injected with the allergen (i.p.) or sham-immunized for the next 3 months. At 3 months of age, baseline airway responsiveness was assessed using cyclo-pentyl adenosine (CPA). Bronchoalveolar lavage (BAL) was performed in all animals and samples of peripheral blood were collected from some animals for estimation of dexamethasone levels. In some animals, blood was collected at the end of the experiment and cellular function was assessed by measurement of *ex vivo* proliferation of mononuclear cells in response to phytohaemagglutinin (PHA).

**2** Allergen immunization significantly increased baseline airway responsiveness to inhaled CPA ($P<0.05$) in comparison with sham-immunized animals, at 3 months after immunization. Dexamethasone (0.5 mg $kg^{-1}$ $day^{-1}$) treatment for 1 month did not modify this established airway hyper-responsiveness to CPA. Dexamethasone treatment did not affect either total or differential cell numbers in BAL fluid during the 4 week period, although significant plasma levels of dexamethasone were achieved in dexamethasone treated animals.

**3** Treatment of rabbits with dexamethasone (0.1 mg $kg^{-1}$ i.p.), 6 h prior to each allergen injection from the neonatal stage, significantly reduced baseline airway hyper-responsiveness to CPA measured at 3 months ($P<0.05$). There was no significant difference in either total or differential cell numbers in BAL fluid, or any difference in mitogen-induced proliferation of mononuclear cells between dexamethasone and vehicle treated rabbits.

**4** These results suggest that introduction of glucocorticosteroids in early life can prevent baseline airway hyper-responsiveness to inhaled CPA in allergic rabbits. However, once established, such underlying airway hyper-responsiveness is difficult to resolve, even with prolonged treatment with glucocorticosteroids.

**Keywords:** Baseline airway hyper-responsiveness; glucocorticosteroid; allergic inflammation; adenosine $A_1$ receptor

**Abbreviations:** AHR, airway hyper-responsiveness; BAL, bronchoalveolar lavage; CPA, cyclo pentyl adenosine; GCS, glucocorticosteroid; PHA, phytohaemagglutinin; TPP, trans-pulmonary pressure

## Introduction

Airway hyper-responsiveness (AHR) is a characteristic feature of asthma and is defined as an increased sensitivity and reactivity of the airways to numerous chemical and physical stimuli (Sterk & Bel, 1989), although the mechanisms underlying AHR are far from resolved. Nonetheless, changes in airway wall morphology (Cho *et al*., 1996), smooth muscle contractility (Schellenberg & Foster, 1984; De Jongste *et al*., 1987) and airway neural control (reviewed by Barnes *et al*., 1991) have all been suggested to contribute to this phenomenon.

Some investigators have reported a striking 10–100 fold difference in baseline airway responsiveness to histamine and methacholine between normal and asthmatic individuals (Cockcroft *et al*., 1977; Woolcock *et al*., 1984). Other investigators have reported that inhalation of adenosine 5′-monophosphate by normal subjects has no effect on airway function, whilst having a profound effect in subjects with asthma (Mann & Holgate, 1985) and such observations have led to the suggestion that the AHR characterizing asthma is heterogeneous (Crowther *et al*., 1997). Baseline airway responsiveness appears not to be normalized following chronic treatment with glucocorticosteroids (Dutoit *et al*., 1987; Kerrebijn *et al*., 1987), even when administered for up to 10 years (Lundgren *et al*., 1988). These clinical results suggest that inflammation is unlikely to contribute to the maintenance of baseline AHR, and other studies have found no evidence for a correlation between indices of inflammatory cell activation and baseline AHR (Djukanovic *et al*., 1990; Lozewicz *et al*., 1988; Ollerenshaw & Woolcock, 1992).

We, and others, have shown previously that, following immunization from birth, adult allergic rabbits can have an increase (1.8–3.0 fold) in baseline airway responsiveness to inhaled histamine or methacholine, which has been shown to persist for up to 12 months of age, a time when there was no overt airways inflammation present (Minshall *et al*., 1993). We, and others, have also shown recently that normal rabbits do not exhibit significant airway responses to inhaled adenosine or its analogue, CPA, whereas adult allergic rabbits immunized from birth exhibit an increased airway responsiveness to inhaled CPA *via* an $A_1$ mediated mechanism (Ali *et al*., 1994;

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El-Hashim *et al*., 1996), suggesting an up-regulation of $A_1$ receptors in allergic conditions and that CPA may, therefore, be a better marker of baseline AHR.

Given the similarities between this allergic rabbit model and the responses to inhaled substances seen in human asthma (Herd & Page, 1996), we have utilized the model to investigate, firstly, whether GCS can reverse baseline AHR, once it has been established, and, secondly, whether treatment with GCS from birth can prevent the development of baseline AHR.

## Methods

### *Animals*

Male and female New Zealand White (NZW) rabbits (Froxfield Farms, Petersfield, Hampshire) were used throughout the study.

### *Immunization protocol*

The immunization of neonatal NZW rabbits was performed using a modification (Coyle *et al*., 1989) of a previously described method (Shampain *et al*., 1982). Allergen-immunized rabbits were injected on the day of birth with 0.5 ml (i.p.) Of *Alternaria tenuis* extract at 40,000 protein nitrogen units (PNU) $ml^{-1}$ in aluminium hydroxide (Al $(OH)_3$) moist gel adjuvant and 0.9% sterile saline in the ratio of 2 : 1 : 1 v $v^{-1}$ $v^{-1}$. Littermate controls were injected with the adjuvant and saline (sham-immunized) in the ratio of 1 : 3. The i.p. administration of allergen and/or adjuvant was repeated weekly for the first month and then biweekly for the remaining 2 months. The methods described in this study were subject to Home Office approval and performed under the Animal (Scientific Procedures) Act, 1986.

### *Pulmonary function measurement*

Rabbits were prepared for measurement of pulmonary function at 3 months of age, 4–7 days after their last intraperitoneal injection. All rabbits were sedated with diazepam (2.5 mg $kg^{-1}$, i.p.) and then anaesthetized with Hypnorm (0.4 ml $kg^{-1}$, i.m.; a mixture of fentanyl citrate 0.315 mg $ml^{-1}$ and fluanisone 10 mg $ml^{-1}$). This regime produces a neuroleptanalgesia and is recommended for recovery procedures in laboratory rabbits (Flecknall, 1987). Animals were placed in a supine position and intubated with a 3.00 mm endotracheal tube (Mallinckrodt Laboratories, Athlone, Ireland). The cuff was inflated and the tube attached to a heated Fleisch pneumotachograph. Flow was measured with a Validyne differential transducer (Validyne Engineering Corp., Northridge, CA, U.S.A.) and inspiration was taken, by convention, to register positive flow. The flow was continuously integrated to give tidal volume. Pleural pressure was estimated by placing an oesophageal balloon, connected to a Validyne pressure transducer, in the lower third of the oesophagus. The difference between atmospheric and pleural pressure was taken as a measure of transpulmonary pressure (TPP). The position of the balloon, after adjustment to obtain the maximum index of TPP, was maintained throughout the experiment. Measurements of total lung resistance ($R_L$) and dynamic compliance ($C_{dyn}$) were calculated by a computer assisted on-line respiratory analyser (PMS version 7.1, Mumed Ltd., London, U.K.) according to the method of Von Neergaard & Wirtz (1927). Resistance is defined as the difference in flow values divided by the difference in TPP measured at half the tidal volume. $C_{dyn}$ is defined as the change in lung volume per unit change in pressure. $C_{dyn}$ measurements were determined every breath, by dividing the change in tidal volume by the change in transpulmonary pressure between the points of zero flow. The contribution made to total lung resistance, $R_L$, by the endotracheal tube was negligible at flow rates between 0 and 60 ml $min^{-1}$ and was therefore not taken into consideration.

### *Airway responsiveness to cyclopentyl adenosine (CPA)*

Airway responsiveness to CPA was determined by performing a cumulative dose-response curve to inhaled CPA (0.078–10 mg $ml^{-1}$) after establishing baseline responses to the vehicle (50% ethanol). Aerosols were generated with a turbo turret jet nebulizer generating particle sizes 0.3–3 $\mu$m. Following each 2 min aerosol of CPA, 10 breaths were recorded and the mean values of $R_L$ and $C_{dyn}$ were calculated.

### *Bronchoalveolar lavage (BAL)*

BAL was performed following the administration of the final concentration of CPA. A polythene catheter was inserted *via* the endotracheal tube into the lungs, until resistance was met. Five ml of sterile saline were injected into the lungs and then almost immediately aspirated by gentle suction under vacuum and collected on ice. Total cell counts were determined under a light microscope using an improved Neubauer haemocytometer. For differential cell counts, cytospin preparations were made. Seventy-five $\mu$l aliquots of BAL fluid were centrifuged onto microscope slides at 1200 r.p.m. for 1 min using a Shandon Cytospin (Shandon Southern Instruments, Sewickley, PA, U.S.A.) at room temperature. Cells were then stained with a combination of haematoxylin and chromotrope 2R to allow differentiation between leukocytes.

### *Measurement of dexamethasone levels*

Blood was collected from a marginal ear vein of each rabbit in dosing regimen 1 (see below) into 5 ml heparinized vials. The blood was spun at 1200 r.p.m. for 5 min and the plasma was removed and stored at $-20^{\circ}C$ for subsequent measurements of dexamethasone levels. Dexamethasone levels in plasma were measured using an extraction radioimmunoassay. Aliquots (300 $\mu$l) of individual plasma samples were extracted with diethylether, the organic phase was evaporated to dryness under air and the extracts were reconstituted in 300 $\mu$l 0.05 M phosphate buffered saline containing 0.1% (w $v^{-1}$) bovine serum albumin. Normal rabbit plasma samples, to which known amounts of dexamethasone had been added (standards), were treated in identical fashion. One hundred $\mu$l sheep polyclonal anti-dexamethasone serum and 100 $\mu$l tritiated ($^3H$) dexamethasone were added to 100 $\mu$l of each extract in polystyrene tubes. The tubes were incubated at 4°C for 1 h after which 500 $\mu$l cold, continuously mixed charcoal suspension (0.5% (w $v^{-1}$) in buffer) was added and mixed to separate free and bound hormone. Following incubation at 4°C for 15 min, the tubes were centrifuged at 1500 × *g* for 30 min at 4°C. The supernatants were decanted into scintillation vials, scintillant was added and the amount of $^3H$ in each vial was determined by liquid scintillation counting. A calibration curve was constructed from the counts obtained with the standard samples and the dexamethasone levels in the test plasma samples were interpolated from this curve. The

imprecision of the assay was less than 10% over the working range of the assay.

### *Measurement of proliferation of mononuclear cells* ex vivo

*Preparation of peripheral blood mononuclear cells* Peripheral venous blood (approximately 10 ml) was drawn from each rabbit in dosing regimen 2 (see below) and collected in tubes coated with the anti-coagulant ethylenediamine tetraacetate (EDTA). Mononuclear cells were then isolated by Ficoll-Paque gradient centrifugation as described previously (Banner *et al*., 1995) prior to being suspended in Spinner modified minimal essential medium (SMEM) containing L-glutamine (2 mM), non-essential amino acids (1% v $v^{-1}$), sodium pyruvate (1 mM), penicillin (100 u $ml^{-1}$), streptomycin (100 $\mu$g $ml^{-1}$) and HEPES (20 mM). Total cell counts were performed using an improved Neubauer haemocytometer and cell viability was assessed by trypan blue exclusion. The volume of cells was then adjusted to $10^6$ cells $ml^{-1}$ in culture medium containing 10% Ultroser (serum substitute). Approximately $1-1.5\times10^6$ cells per ml of blood were routinely obtained and cell viability was always $>95\%$. Platelet contamination was less than one platelet per nucleated cell.

*Preparation of plates* Mononuclear cells from either vehicle or dexamethasone treated rabbits ($10^5$ 100 $\mu l^{-1}$ per well) were seeded in 96 well plates. Ten min later, phytohaemagglutinin (PHA) (0–20 $\mu$g $ml^{-1}$) in a volume of 50 $\mu$l was added to each well. The cells were then incubated at 37°C in a 95% air, 5% $CO_2$ atmosphere for 24 h.

*Assessment of cell proliferation* The cells were pulsed with [$^3$H]-thymidine (0.1 $\mu$Ci per well) for a further 24 h period. Cells were then harvested onto glass fibre filters using a cell harvester (ICN Flow, Buckinghamshire, U.K.) and the incorporated radioactivity counted in a $\beta$ scintillation counter to quantify cell proliferation.

## *Materials*

All reagents were of analytical grade. Drugs and chemicals used were Dexamethasone 21-phosphate (Sigma Chemical Company, Poole, Dorset, U.K.); CPA (Semat Technical (U.K.) Ltd, St Albans, Hertfordshire, U.K.); Dexamethasone pellets (RBI, Sarasota, Florida, U.S.A.). *Alternaria tenuis* was obtained from Greer Laboratories Inc. Lenoir, N.C., U.S.A. (40,000 PNU $ml^{-1}$) and aluminium hydroxide (Al $(OH)_3$) moist gel from FSA laboratory supplies, (Loughborough, U.K.); Hypnorm (Janssen Pharmaceutical Ltd., High Wycombe, U.K.). SMEM, L-glutamine, non-essential amino acids (10X), sodium pyruvate, penicillin, streptomycin (Gibco, Paisley, U.K.), HEPES, Histopaque, trypan blue, chromotrope 2R, Erhlichs Haematoxylin, (Sigma diagnostics, Dorset, U.K.), Ultroser (Jones Chromatography, Mid Glamorgan, U.K.), PHA (Biochrom, Berlin, Germany), [6-$^3$H]-thymidine, [$^3$H]-$PGE_2$ (Amersham International, Buckinghamshire, U.K.).

## *Experimental protocols*

*Effect of dexamethasone treatment on established baseline airway hyper-responsiveness (dosing regimen 1)* From each litter, *Alternaria tenuis* immunized and sham-immunized rabbits were prepared for use in this part of the study exactly as described under *Immunization protocol* above.

At the end of the 3 months of immunization (week 0), baseline airway responsiveness of both *Alternaria tenuis* immunized and sham-immunized rabbits to CPA was assessed. Five ml of blood was collected from the marginal ear vein after completion of the dose response curve to CPA. BAL procedure was also performed after completion of the dose response curve. Ten min later, rabbits were implanted with pellets containing either dexamethasone (45 mg, releasing 0.5 mg $kg^{-1}day^{-1}$) or vehicle (biodegradable material). Implantation was performed with a trochar in the scruff of the neck.

In weeks 1, 2, 3 and 4, CPA dose response curves and BAL were performed as for week 0 and blood was again withdrawn in week 1.

*Effect of dexamethasone treatment from birth on the development of baseline airway hyper-responsiveness to CPA (dosing regimen 2)* Rabbits on this dosing regime were treated with dexamethasone, 0.1 mg $kg^{-1}$ (i.p.), or vehicle, 6 h prior to the first (neonatal) injection of *Alternaria tenuis* or sham. Dexamethasone or vehicle treatment was then repeated weekly for the first month and biweekly for the next 2 months 6 h prior to each immunization procedure. A similar dosing regime was used for sham-immunized rabbits.

At the end of the 3 months of immunization and dexamethasone (or vehicle) treatment, baseline airway hyperresponsiveness to CPA was assessed as described earlier. BAL was performed and 10 ml of blood (used to assess cell proliferation) was withdrawn after completion of the CPA dose response curve.

## *Expression and analysis of results*

Statistical comparisons were considered significant if $P<0.05$. All statistical analysis was performed on GraphPad Instat™ (GraphPad Software, California, U.S.A.) using either one way or two way ANOVA, as appropriate, followed by Tukey's test.

*Airway responsiveness* Airway responsiveness to inhaled CPA has been expressed as the percentage change in $R_L$ and $C_{dyn}$ from baseline values in response to increasing doses of the inhaled CPA. The maximum percentage increase in $R_L$ ($R_{L\,max}$) and decrease in $C_{dyn}$ ($C_{dyn\,max}$) within the studied dose range were recorded and used as indices of airway responsiveness.

*Dosing regime 1* A two way ANOVA was used to compare differences in responsiveness between different groups at week 0 and the effect of drug or vehicle over the 4 week period.

*Dosing regime 2* A one way ANOVA was used to assess differences in both $R_{L\,max}$ and $C_{dyn\,max}$ between the different treatment groups.

*Bronchoalveolar lavage* A total of 200 cells were counted and classified as mononuclear cells, neutrophils or eosinophils according to standard morphological criteria. Total and differential cell counts are expressed as the number of cells per millilitre of fluid recovered. A one way ANOVA test was used for comparing differences in total and differential cell numbers for both dosing regimes as described above.

*Proliferation* Cell proliferation data was measured as d.p.m. $^3$H-thymidine incorporation in response to phytohaemagglutinin (PHA) and mean ± s.e.mean values were calculated.

Differences between PHA concentration response curves obtained with cells from control and dexamethasone treated animals were analysed using ANCOVA (Kenakin, 1987).

## Results

*Effect of dexamethasone (0.5 mg kg$^{-1}$ day$^{-1}$) on established AHR in 3 month old rabbits*

*Airway responsiveness to CPA* There was a significant increase in airway responsiveness to inhaled CPA, in terms of both $R_{L\,max}$ and $C_{dyn\,max}$, in allergen immunized, in comparison to sham immunized, vehicle treated rabbits at 3–4 months following immunization (Figure 1; $P<0.05$), full DRC for $R_L$ and $C_{dyn}$ are show for weeks 0 and 4; Figure 2). Dexamethasone treatment of allergen immunized rabbits failed to significantly alter the airway responsiveness to inhaled CPA over the 4 week period in comparison to allergen immunized rabbits treated with vehicle (Figures 1 and 2; $P>0.05$). Allergen immunized rabbits treated with dexamethasone were also more responsive to inhaled CPA at 3–4 months in comparison to sham immunized rabbits treated with either vehicle or dexamethasone (Figures 1 and 2; $P<0.05$).

*Bronchoalveolar lavage* There were no significant differences in either total or differential cell numbers between vehicle treated allergen and sham-immunized rabbits at week 0 or any of the other weeks (Table 1; $P>0.05$). Dexamethasone treatment did not significantly affect either total or differential cell numbers over the 4 weeks in comparison to vehicle treatment in either allergen or sham immunized rabbits (Table 1; $P>0.05$).

*Measurement of dexamethasone levels* There were significant differences in plasma dexamethasone levels prior to treatment (week 0) and after dexamethasone treatment in week 1 (Figure 3; $P<0.05$ paired $t$-test). There was also a significant difference in dexamethasone levels between dexamethasone and vehicle treated rabbits in week 1 (Figure 3; $P<0.05$ unpaired $t$-test).

*Effect of dexamethasone (0.1 mg kg$^{-1}$ i.p.) on development of baseline airways responsiveness to CPA*

*Airway responsiveness to CPA* There was a significant increase in airway responsiveness to inhaled CPA in allergen-immunized, compared with sham immunized, rabbits treated with vehicle (Figures 4 and 5; $P<0.05$). Dexamethasone treatment significantly inhibited the increase in airway responsiveness to inhaled CPA in allergen immunized rabbits in terms of both $R_{L\,max}$ and $C_{dyn\,max}$ (Figures 4 and 5; $P<0.05$).

*Bronchoalveolar lavage* There was no significant difference in either total or differential cell numbers obtained from allergen or sham immunized rabbits (Table 2; $P>0.05$) treated with vehicle. Dexamethasone treatment of allergen immunized rabbits did not significantly affect either total or differential cell numbers in comparison with either vehicle treated allergen or sham immunized rabbits (Table 2; $P>0.05$).

*Proliferation of mononuclear cells to PHA* There was no significant difference in PHA induced proliferation of mononuclear cells obtained from either dexamethasone or vehicle treated rabbits ($P>0.05$; results not shown).

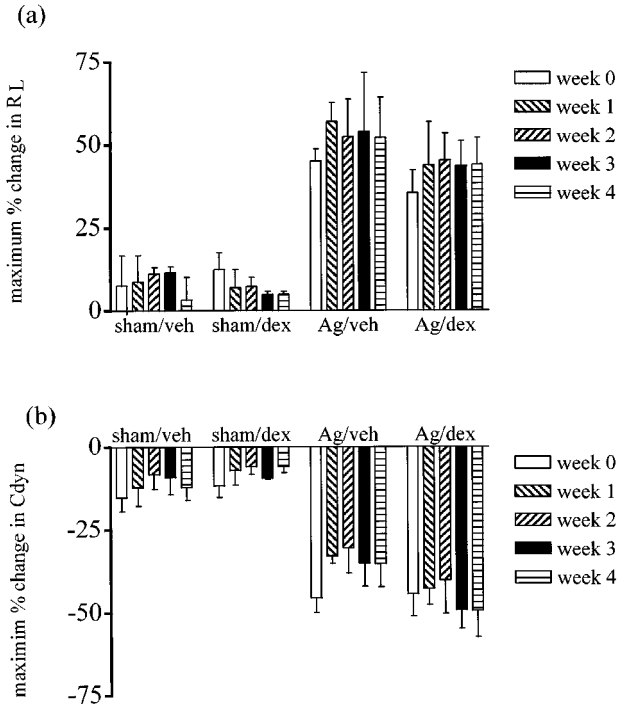


**Figure 1** Maximum percentage change in (a) $R_L$ and (b) $C_{dyn}$ to inhaled CPA in sham and allergen (Ag)-immunized rabbits before treatment (week 0) and during the 4 weeks treatment with vehicle (veh) or dexamethasone (dex). There was a significant difference in both $R_{L\,max}$ and $C_{dyn\,max}$ between sham and allergen-immunized rabbits at week 0 and during all the other weeks following treatment with the vehicle ($P<0.05$). Dexamethasone treatment of allergen-immunized rabbits did not significantly change either the $R_{L\,max}$ or $C_{dyn\,max}$ ($P>0.05$). Values are means±s.e.mean, $n=5-11$.

## Discussion

Our data shows that immunization of rabbits from birth with *Alternaria tenuis* results in adult rabbits with baseline AHR to inhaled CPA which is evident for at least 4 weeks. This confirms our previous finding that a qualitative difference in airway responsiveness between adult allergen and sham-immunized rabbits is seen with inhaled CPA as a marker (El-Hashim *et al*., 1996), suggesting that this substance may be a useful molecule for the assessment of baseline airway responsiveness. Our work further supports the suggestions that the $A_1$ receptor is upregulated in allergic conditions (Ali *et al*., 1994) and that these $A_1$ receptors may play an important role in the airways of allergic animals (Nyce & Metzger, 1997). Long-term treatment of adult rabbits with dexamethasone did not modify this baseline AHR to CPA, once it had been established, whereas treatment of allergic rabbits with dexamethasone from birth significantly attenuated the AHR to inhaled CPA expected to occur in the adult population, at a dose which did not affect the mitogen-induced proliferation of mononuclear cells in peripheral blood. In addition, neither dosing regime had any effect on either total or differential cell numbers, as assessed by BAL in the adult rabbits. These findings provide further evidence for AHR to inhaled CPA in this animal model, support clinical studies which report a lack of effect of long-term treatment with GCS on chronic AHR and, furthermore, show that very early intervention with GCS can prevent the induction of baseline AHR.

The absence of evidence for an increase in either total or differential cell numbers in allergen, compared to sham, immunized vehicle treated rabbits at 3–4 months in the

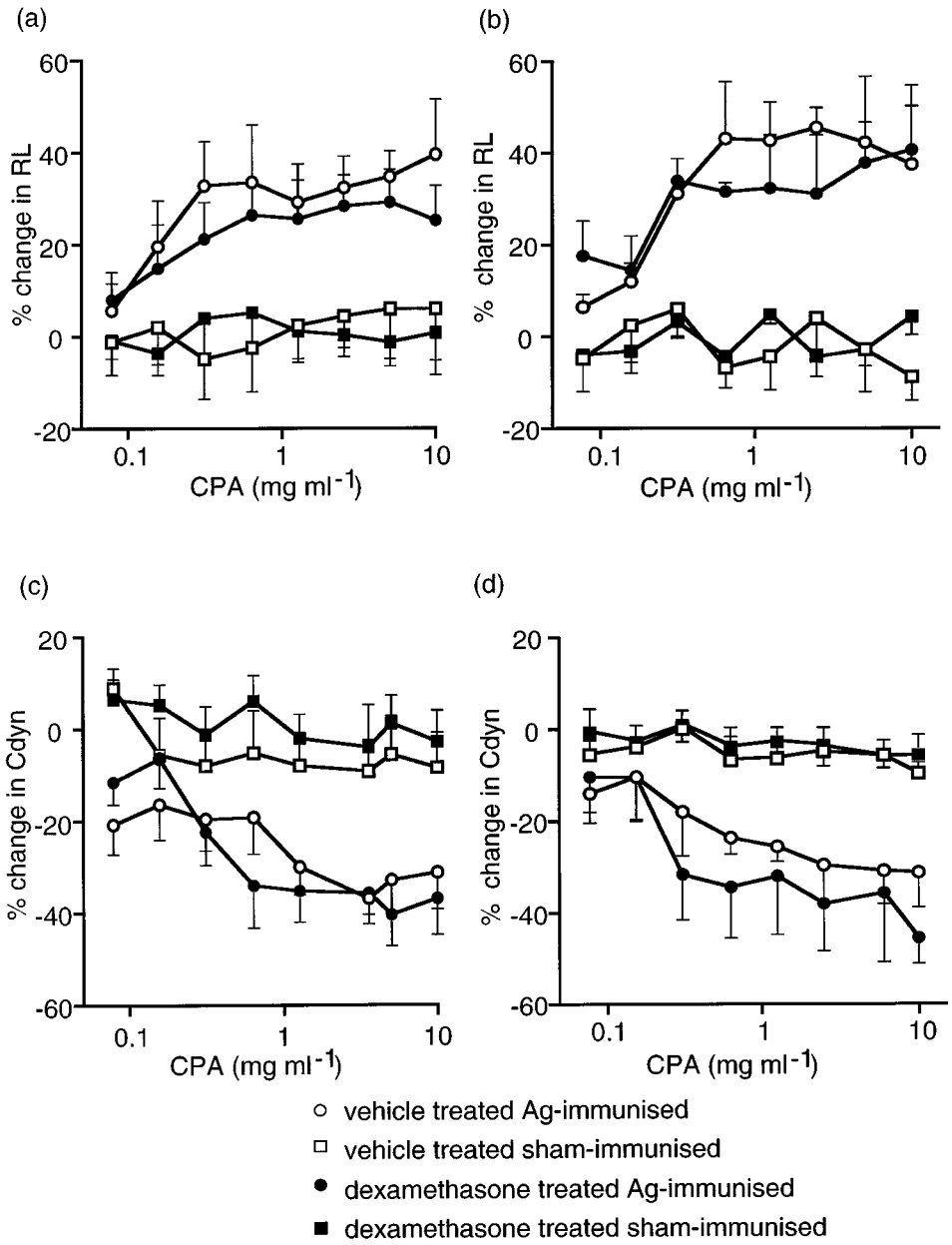


**Figure 2** Percentage change in $R_L$ (a and b) and $C_{dyn}$ (c and d) in response to inhaled CPA before treatment (a and c; week 0) in allergen (Ag)-immunized and sham-immunized rabbits and after treatment (b and d; week 4) in vehicle treated allergen-immunized, dexamethasone treated allergen-immunized, vehicle treated sham-immunized and dexamethasone treated sham-immunized animals. There was a significant difference in responsiveness between allergen and sham immunized rabbits at week 0 ($P<0.05$; ANOVA). Dexamethasone treatment did not affect the difference in airway responsiveness between the groups ($P>0.05$; ANOVA). Values are means ± s.e.mean, $n=5-11$.

present study would not support a role for airway inflammation as a major mechanism underlying baseline AHR to inhaled CPA, at least in this model, supporting our previous studies (Minshall *et al*., 1993). Nonetheless, it can be argued that the BAL procedure may not be sensitive enough to detect subtle inflammatory reactions within the airway tissue of allergen-immunized rabbits which could contribute to this baseline AHR. However, treatment for 1 month with dexamethasone did not reduce this baseline AHR to CPA, an observation which is unlikely to be due to the insensitivity of the rabbit to GCS, as we have shown previously that Budesonide is a very potent drug at inhibiting allergen induced inflammatory cell influx and acute exacerbations of airway responses in this model (Gozzard *et al*., 1996). In addition, this lack of effect of dexamethasone cannot be attributed to either the dose or the duration of treatment, as this dose was shown previously to exert potent anti-inflammatory effects (Elwood *et al*., 1992) and the duration is long enough for GCS to exert their effects (Cockcroft & Murdock, 1987; reviewed by Lipworth, 1993). Moreover, the plasma levels of dexamethasone achieved would suggest that the systemic bioavailability of dexamethasone was high enough to maintain potent anti-inflammatory effects. The fact that our findings demonstrate that, once-established, baseline AHR is resistant to potent anti-inflammatory therapy would question the central role of overt inflammatory changes in the airways as a major underlying cause for baseline AHR. However, it remains plausible that the airways were inflammed before the cell

**Table 1** Effect of dexamethasone treatment (regimen 1) on total and differential cell numbers recovered from BAL fluid over the 4 week treatment period

| | *Sham/veh* | *Sham/dex* | *Ag/veh* | *Ag/dex* |
|---|---|---|---|---|
| | | $\times 10^4$ *cells* $ml^{-1}$ *BAL* | | |
| *Week 0* | | | | |
| Total cells | 24.3±0.9 | 26.4±3.3 | 22.8±4.3 | 23.5±3.2 |
| mononuclear | 24.1±0.9 | 26.2±3.4 | 22.5±4.2 | 23.2±3.1 |
| neutrophils | 0.2±0.06 | 0.2±0.12 | 0.27±0.09 | 0.2±0.1 |
| eosinophils | 0 | 0 | 0 | 0 |
| *Week 1* | | | | |
| Total cells | 22.7±0.9 | 23.8±5.6 | 21.0±6.7 | 26.9±2.7 |
| mononuclear | 22.6±0.8 | 23.5±5.5 | 20.6±6.9 | 26.4±2.5 |
| neutrophils | 0.2±0.05 | 0.3±0.07 | 0.3±0.1 | 0.4±0.3 |
| eosinophils | 0 | 0 | 0.03±0.02 | 0.02±0.02 |
| *Week 2* | | | | |
| Total cells | 20.9±4.2 | 22.4±5.0 | 20.7±3.2 | 23.8±4.8 |
| mononuclear | 20.4±4.4 | 22.3±5.0 | 20.4±3.3 | 23.6±4.7 |
| neutrophils | 0.5±0.4 | 0.50±0.06 | 0.29±0.1 | 0.16±0.06 |
| eosinophils | 0 | 0 | 0.075±0.06 | 0 |
| *Week 3* | | | | |
| Total cells | 22.2±3.8 | 28.0±6.9 | 23.9±2.8 | 25.7±6.3 |
| mononuclear | 21.6±3.9 | 27.8±6.8 | 23.8±2.7 | 25.5±6.2 |
| neutrophils | 0.62±0.36 | 0.20±0.07 | 0.1±0.07 | 0.17±0.05 |
| eosinophils | 0 | 0 | 0 | 0.008±0.008 |
| *Week 4* | | | | |
| Total cells | 21.2±0.5 | 21.25±1.6 | 21.50±2.5 | 28.0±5.5 |
| mononuclear | 21.10±0.6 | 21.2±1.6 | 21.35±2.5 | 27.8±9.4 |
| neutrolphils | 0.15±0.03 | 0.05±0.05 | 0.15±0.02 | 0.20±0.06 |
| eosinophils | 0 | 0 | 0 | 0 |

There was no significant difference in either total or differential cell numbers between the different groups at week 0. Dexamethasone (dex) treatment of either allergen (Ag) or sham-immunized rabbits did not significantly alter either total or differential cell numbers in comparison to either vehicle (veh) treated allergen or sham-immunized rabbits at any time point ($P<0.05$; one way ANOVA). Values are means±s.e.mean for $n=5-11$ observations.

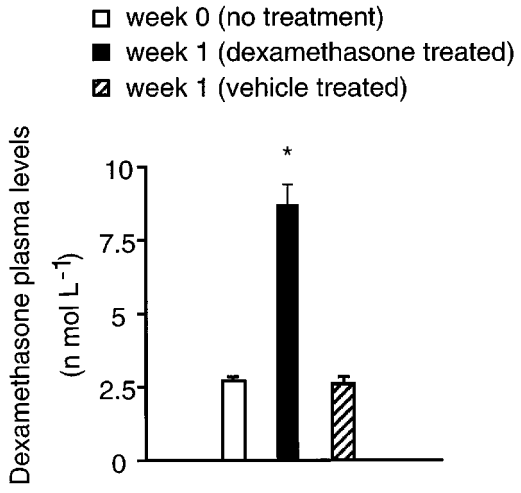


**Figure 3** Plasma levels of dexamethasone prior to dexamethasone treatment (week 0, no treatment), and 1 week after dexamethasone or vehicle treatment. There were significantly greater dexamethasone levels following dexamethasone treatment (week 1) than prior to (week 0) treatment ($P<0.05$, un-paired *t*-test). There were also significantly greater dexamethasone levels at week 1 in dexamethasone treated animals compared with vehicle treated animals ($P<0.05$, un-paired *t*-test). $n=5-11$.

measurements were made, and that this inflammation led to other changes in the airways, such as remodelling, which are not readily reversed by GCS.

Our data are in keeping with clinical findings. Easton (1981) reported no change in the methacholine responsiveness, after 4 months of treatment with 400 $\mu$g of beclomethasone diphosphate (BDP), in patients with moderately severe asthma. Also, Svendsen *et al.* (1987) have found no significant change in airway responsiveness to histamine after 8 weeks of treatment with 400 $\mu$g of BDP per day. However, although several other studies (Dutoit *et al.*, 1987; Kerrebijn *et al.*, 1987) have reported statistically significant improvement in AHR to histamine or methacholine, the mean levels of change in airway responsiveness in these studies were small and remained outside the range obtained in normal subjects. Moreover, the improvement in AHR did not seem to be a result of improvement in lung function as no correlation between baseline $FEV_1$ and $logPD_{20}$ has been noted (Kerrebijn *et al.*, 1987; Juniper *et al.*, 1990). Indeed, in a longitudinal study where biopsies of bronchial mucosa from asthmatic patients were assessed before and after 10 years' treatment with inhaled steroids, it has been reported that, in contrast to the considerable inflammation prior to treatment, the mucosa after treatment was not significantly different from that of normal controls in numbers of inflammatory cells, thickness of epithelium or thickness of basement membrane (Lundgren *et al.*, 1988). Despite this the geometric mean of the airway responsiveness was 0.24 mg $ml^{-1}$, suggesting that the individuals were still considerably hyper-responsive and, thus, that a part of the chronic airway responsiveness was resistant to steroid therapy. The persistence of AHR in the absence of overt inflammation in our rabbits and in asthmatics suggests that there must be factors other than inflammation contributing to chronic AHR.

In contrast to the lack of effect of GCS on established baseline AHR, our results show that treatment of rabbits during the sensitization phase in early life significantly

attenuates baseline AHR to inhaled CPA at 3 months. This suggests that early treatment with GCS interferes with the development of the mechanisms that lead to baseline AHR and lends support to clinical observations that early intervention with GCS in asthmatics has a significant impact on the subsequent outcome of the disease (Haatela *et al*., 1994). Furthermore, from our work, this effect of GCS seems to be achieved at a dose that does not affect mononuclear cell proliferation *ex vivo*, although we cannot rule out an inhibitory effect on cytokine release from inflammatory cells, and it remains possible that dexamethasone could affect the process of allergen presentation, as dexamethasone treatment has been shown to favour $Th_1$, and not $Th_2$, cytokine release (Krouwels *et al*., 1996).

We have reported previously that treatment of allergic rabbits with capsaicin also significantly inhibits the development of baseline AHR in this rabbit model (Riccio *et al*., 1993), suggesting the possible involvement of neural mechanisms in this phenomenon. It is of considerable interest, therefore, that $A_1$ receptor agonists have recently been observed to activate peripheral sensory nerves (Dowd *et al*., 1998; Hong *et al*., 1998), a process hypothesized to have many similarities with the AHR characterizing asthma (Adcock & Garland, 1993).

Some studies have suggested that adenosine may be producing its effect through activation of mast cells (Church & Holgate, 1993). However, our data would question the role

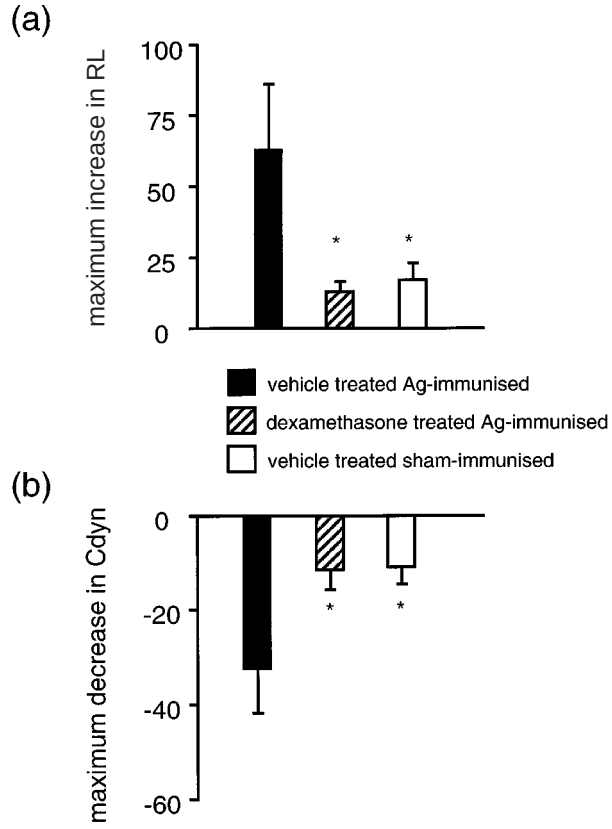


**Figure 4** Maximum percentage increase in (a) $R_L$ and decrease in (b) $C_{dyn}$ induced by CPA in vehicle treated/allergen (Ag)-immunized ($n=8$), dexamethasone treated/allergen-immunized ($n=8$) and vehicle treated/sham-immunized rabbits ($n=8$). *$P<0.05$ compared with allergen-immunized/vehicle treated rabbits (one way ANOVA). Values are means±s.e.mean.

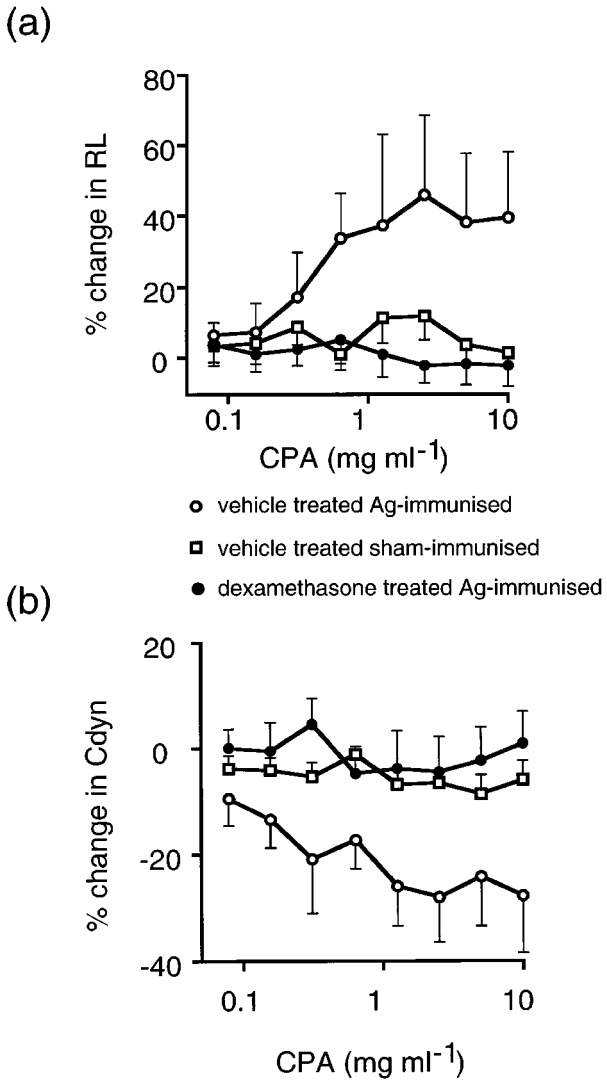


**Figure 5** Percentage change in (a) $R_L$ and (b) $C_{dyn}$ in response to inhaled CPA in vehicle treated/allergen (Ag)-immunized ($n=8$), dexamethasone treated/allergen-immunized ($n=8$) and vehicle treated/sham-immunized ($n=8$) rabbits. Values are expressed as means ±s.e.mean.

**Table 2** Effect of dexamethasone treatment (regimen 2) on total and differential cell numbers recovered from BAL fluid

| | $\times 10^4$ $ml^{-1}$ BAL | | | |
|---|---|---|---|---|
| | *Total* | *Mononuclear* | *Neutrophils* | *Eosinophils* |
| Sham/veh ($n=8$) | 24.8±2.9 | 23.9±2.7 | 0.8±0.65 | 0 |
| Ag/veh ($n=7$) | 19.1±2.7 | 18.5±2.75 | 0.6±0.53 | 0 |
| Ag/dex ($n=8$) | 24.8±0.75 | 24.4±0.67 | 0.4±0.35 | 0 |

There was no significant difference in either total or differential cell numbers between the treatment groups ($P<0.05$; one way ANOVA). Values are means±s.e.mean for $n$ observations given in parentheses.

of mast cells in the AHR to inhaled CPA as chronic dosing of adult rabbits failed to modify AHR to CPA, whereas it has been reported previously that chronic treatment with GCS reduced mast cell mediated responses (Barnes, 1990). Furthermore, recent evidence has shown that the adenosine $A_1$ receptor elicits bronchospasm in the absence of mast-cell activation (Meade *et al*., 1996) and purified bronchial airway smooth muscle (ASM) membranes were shown to have $A_1$ receptors which would suggest that adenosine has the potential to directly activate ASM (Ali *et al*., 1994; Nyce & Metzger, 1997) as well as airway nerves (Hong *et al*., 1998). Furthermore, in support of a role for $A_1$ receptors in neurally mediated mechanisms, there is evidence to suggest that adenosine, and adenosine analogues, can modulate airway neural transmission. *In vitro* studies of guinea-pig airway tissue have shown that adenosine significantly enhanced the airway narrowing induced by capsaicin in both normal and vagotomized animals (Nieri *et al*., 1996), thus suggesting adenosine works through enhancement of peptidergic nerves. Furthermore, in rabbit bronchial smooth muscle, Gustafsson *et al*. (1986) have demonstrated an enhancement of the constrictor response to transmural nerve stimulation. However, in another study chronic treatment with capsaicin did not result in any significant protection against adenosine induced enhancement of the bronchocontractile effect of histamine (Breschi *et al*., 1994). Nonetheless, Polosa *et al*. (1991) have suggested that adenosine 5′-monophosphate induces bronchoconstriction in man through release of neuropeptides.

The ineffectiveness of chronic dexamethasone treatment in resolving established AHR to CPA would suggest that, whatever the mechanisms underlying this AHR, once established they are not readily reversed with potent GCS therapy. Adenosine $A_1$ receptor expression has been shown to be increased with dexamethasone treatment (Gerwins & Fredholm, 1991), a finding which may, at least partly, explain the lack of effect of GCS on AHR to CPA.

In conclusion, our findings show that baseline AHR to inhaled CPA is chronic, is not associated with overt airway inflammation and, once established, is not readily reversed with chronic GCS therapy. However, early intervention with GCS therapy prevents baseline AHR through mechanisms that remain to be elucidated.

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