

ORIGINAL ARTICLE

Early timing of low-dose dexamethasone decreases inflammation in a murine model of eosinophilic airway disease

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Abstract

Conclusion. A very low dose of dexamethasone (DEX) was as equally as sufficient as a pharmacological dose to decrease eosinophil inflammation in airways and bone marrow. The timing of DEX treatment in relation to allergen challenge was strongly decisive for the outcome of the inflammatory response. **Objectives.** We aimed to study compartmental allergic airway inflammatory responses to classic pharmacological and also extremely low physiological DEX dosage, given at different time points close to allergen challenge in a murine model. **Materials and methods.** Ovalbumin-sensitized BALB/c-mice were exposed to intra-nasal ovalbumin. DEX was given i.p. as 1 µg/kg low-dose or 500 µg/kg pharmacological single-dose 2 h before, immediately before or 7 h after each of three challenges. Inflammatory cells were evaluated in bronchoalveolar lavage (BAL), lungs, nasal mucosa, and bone marrow. **Results.** Groups treated with low-dose DEX decreased eosinophilia in BAL to the same extent as the pharmacological dose, but only when administered before challenge. The most prominent decrease of eosinophils in BAL was seen in mice treated with the low dose 2 h before challenge. A similar response pattern as in BAL eosinophilia was detected in lung histopathology. DEX treatments had no obvious effects on inflammation in nasal mucosa.

Keywords: Airway, allergy, eosinophil, glucocorticoids, inflammation, low dose, ovalbumin

Introduction

Anti-inflammatory treatments with glucocorticoids (GCs) have been proven to be effective in animal models and in clinical studies of asthma/rhinitis [1]. Several murine studies have also been performed to investigate the role of dexamethasone (DEX) on airway allergy. Most of these use only one dose of GCs and treat at only one time point, primarily pretreatment in correlation to allergen provocation [2,3] and no rhinitis data are reported.

In the clinical setting, there has been a debate on the anti-inflammatory mechanisms of GCs doses given pretreatment or post-treatment in relation to allergen exposure or at exacerbations. Some studies have demonstrated that in patients with moderate asthma, the inflammation was adequately suppressed with a low dose of corticosteroid, and at exacerbations a temporary addition of inhaled ster-

oids was effective in controlling asthma [4,5]. Pipkorn et al. showed that both prophylactic and immediate pretreatment of allergic rhinitis with steroids in relation to allergen challenge gave a less pronounced allergic response in the clinical setting [6]. It is now considered optimal to start treatment early in each pollen season. However, the physiological background for this recommendation is not entirely corroborated by available experimental studies, as different activated eosinophil compartments, i.e. bronchoalveolar lavage (BAL), bone marrow, nose, and lung, will display an individual response pattern that will also react selectively at various stages of the disease [7]. As the time and dose response pattern is dependent on different cellular compartments displaying an individual sensitivity to GCs, it may be expected that further fine-tuning of steroid timing and dosage should have a clinical advantage.

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In atopic dermatitis, a hyporeactive hypothalamus-pituitary-adrenal (HPA) axis increases the susceptibility to chronic allergic inflammation [8]. This relatively blunted cortisol response indicates that even minor decreases in cortisol levels/reactivity may affect the propensity for allergic inflammation early in disease. A small exogenously distributed dose of GCs, lower than the corresponding systemic dose tried before in humans (2.5 mg prednisolone) or mice for allergic diseases, can thus be expected to have noticeable effects on both inflammatory parameters and, to a limited extent, on the HPA axis setting. In mice and rats, dosages of microgram quantities have been demonstrated to display peripheral physiological anti-inflammatory effects in other models of inflammation and also somewhat suppress the HPA axis, and are thus considered close to physiological release levels [9].

In this study, we aimed to perform a closer analysis of the timing dependency of the effects of microgram GC dosages in eosinophilic airway disease and compare them to a classical pharmacological, 500 times higher dose. The low dose was chosen to correspond to a physiological/low dose, close to a dose of endogenous production of corticosterone in mice. Specifically, the eosinophilia in selected compartments (BAL, bone marrow, and lungs), the morphological pathology in the lung and nose, and levels of leukotriene B₄ in BAL were analyzed.

Materials and methods

Animals

Male BALB/c mice were obtained from B&K Universal AB (Sollentuna, Sweden) and were 6–8 weeks old at the initiation of the sensitization protocol. They were allowed food and water ad libitum and kept in a room with a 12 h light/dark cycle. Twenty animals were allocated to each protocol group. The local committee on animal ethics approved all experiments (Dnr S79-00).

Sensitization

Mice were sensitized with 10 µg of OVA grade III (Sigma Chemical Co., St Louis, MO, USA) and 4 mg/ml Al(OH)₃ (Sigma) suspended in 0.2 ml PBS, given as an intraperitoneal (i.p.) injection in the morning on days 0, 7, and 14. The control group was sham-sensitized and received only 4 mg/ml Al(OH)₃ suspended in 0.2 ml PBS i.p.

Treatment groups

Three separate pharmacological intervention groups (2 h before, immediately before or 7 h after) of OVA-

sensitized and OVA-challenged mice were treated with an i.p. injection of water-soluble DEX 1 µg/kg (Dexamethasone-Water Soluble, Sigma) after each challenge. Another three groups were similarly treated with DEX 500 µg/kg (pharmacological dose) at the corresponding time points. Sham groups of OVA-sensitized and OVA-challenged mice were treated with PBS i.p. at each of the three corresponding time points (Figure 1).

Airway challenge

Antigen challenge procedures were performed in the morning on days 27, 28, and 29 in all groups by intranasal instillation; 100 µg OVA suspended in 50 µl PBS was administered into the nostrils using a micropipette under slight CO₂ anesthesia.

Tissue collection and preparation

The mice were sacrificed on day 30 (24 h after the last challenge) with a lethal injection of sodium pentobarbiturate (Apoteksbolaget, Umeå, Sweden) i.p.

Bronchoalveolar lavage

The trachea was exposed surgically and gently flushed with 400 µl PBS three times via a plastic cannula (outer diameter 1.2 mm) to collect bronchoalveolar cells. The BAL was centrifuged at 1200 rpm in an Eppendorf centrifuge (4515D) for 10 min. The supernatant was removed and stored at –70°C until leukotriene B₄ (LTB₄) analysis was performed. The cell pellet was resuspended in PBS and centrifuged in a cytopspin (Shandon Cytospin 2, Shandon Southern Products Ltd, UK) at 450 rpm for 5 min, air-dried, and stained with May-Grünwald Giemsa or Luxol fast blue (LFB). The slides for LFB staining were fixed with CAM (a combination citrate buffer (Sigma Diagnostics, St Louis, MO, USA), acetone (Histolab, Göteborg, Sweden), and methanol (Merck, Darmstadt, Germany)) for 5 min, and then rinsed in distilled water. Eosinophils were stained with Solvent Blue 38 (Sigma) in alcohol saturated with urea (Sigma) for 30 min. The staining shows eosinophils as bright blue-green. Counterstain with Mayer's hematoxylin was used.

A differential cell count was performed on the May-Grünwald Giemsa-stained slides and an eosinophil count on the LFB-stained slides, under light microscopy, counting 300 leukocytes in a blinded manner.

LTB₄ analysis

LTB₄ in BAL supernatant was measured using a commercially available EIA kit (Leukotriene B₄

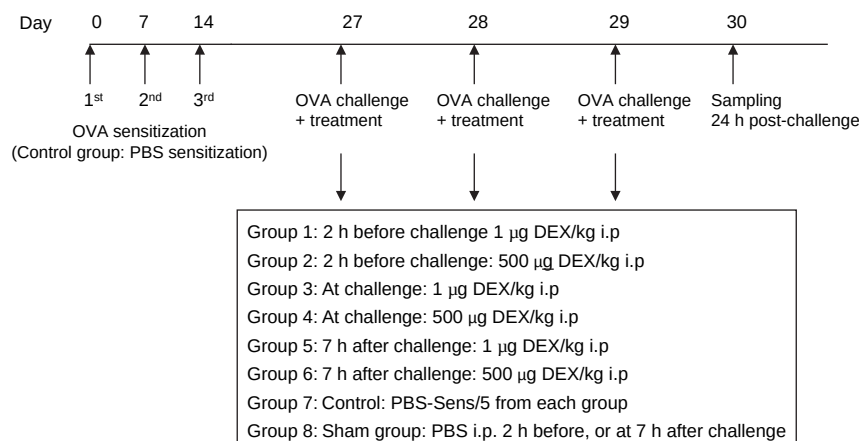


Figure 1. Illustration of OVA-sensitization, challenge, and dexamethasone (DEX) treatment protocol. All sampling was performed 24 h after the last challenge. Groups were treated with an low (1 µg/kg) or pharmacological (500 µg/kg) dose of DEX and in separate series, at 2 h before, immediately before or 7 h after each challenge.

enzyme immunoassay system, Biotrak, Amersham Pharmacia Biotech, Uppsala, Sweden). In brief, the BAL was incubated with rabbit anti-LTB₄ serum for 2 h in a microtiter plate precoated with donkey anti-rabbit IgG. Competing LTB₄ coupled to peroxidase was added to the plate and was incubated for 1 h, followed by thorough washing and substrate addition. At 30 min later the reaction was terminated with sulfuric acid and the optical density was determined at 450 nm. The lower limit of detection for LTB₄ was 6 pg/ml.

Lung histology

The lungs were removed surgically. One lobe was fixed in Stefaninis solution and the other lobe in 4% buffered formaldehyde. The formaldehyde-fixed lungs were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) and the slides were coded. Histological evaluation was performed in a blinded manner, and semi-quantitatively graded as: 0, no cell infiltration; 1+, few; 2+, moderate; or 3+, abundant.

The Stefaninis-fixed lungs were rinsed in Hanks's balanced salts solution after 24 h and snap-frozen in liquid nitrogen. They were then sectioned 10 µm thick and stained for eosinophilic peroxidase (EPO) [10]. The number of EPO-positive cells in the lungs were semi-quantitatively graded in a blinded manner as: 0, no EPO-positive cells; 1+, few; 2+, moderate; or 3+, abundant.

Nose complex

The entire nose complex was removed, trimmed, and fixed in 4% formaldehyde ($n=5$ from each group). The specimens were decalcified with 24% ethylenediaminetetraacetic acid (EDTA; Tritriplex

III, Merck) at 37°C for approximately 1 month. Following dehydration in ethanol and embedding in paraffin, the whole mounted complex was sectioned (5 µm) and stained with H&E and Alcian blue-periodic acid-Schiff for morphological studies or alkaline Congo red technique for eosinophil detection.

A histopathological examination of the nasal mucosa was performed in a blinded manner and a semi-quantified grading system was then employed regarding: (1) inflammatory cells, (2) ciliary loss, (3) epithelial desquamation, and (4) eosinophils. Each parameter was estimated separately over a three-graded scale as follows: 0, no increase; 1, mild increase; 2, moderate increase; and 3, pronounced increase. The scores for inflammatory cell influx, ciliary loss, and epithelial desquamation were added up, thus producing a maximum grading score of 9.

Bone marrow

The bone marrow (BM) cells were collected by carefully flushing 1 ml PBS via a needle (0.6 × 25 mm) from the end-opened right femur of each mouse. The cytocentrifugation and staining were performed in a similar way as for the BAL cells.

An eosinophil count was performed on the May-Grünwald Giemsa- and LFB-stained slides under light microscopy, counting 300 cells per staining in a blinded manner. Eosinophils were exclusively defined as cells containing eosinophilic granules.

Statistical analysis

The data were analyzed by two-way (group and time) repeated measurements analysis of variance (ANOVA). Individual groups were compared using Student's *t* test or Mann-Whitney U test (nasal

pathology). Correlation was made with Spearman's rank correlation test. A p value of <0.05 was considered statistically significant. Results were expressed as mean $\pm$ SEM. All statistical analysis was performed with Statistica 6.0 (StatSoft Inc, Tulsa, OK, USA).

Results

Bronchoalveolar lavage fluid

Virtually no eosinophils could be detected in the BAL fluid from the control animals ($0.5 \pm 0.3\%$), and OVA-sensitization (sham) increased the eosinophils significantly as compared with the control group ($p < 0.001$) (Figure 2). There were no differences in BAL fluid, lung histopathology, BM or nasal tissue in the different sham-treated groups, so they were pooled. Low-dose DEX treatments administered 2 h before or just before OVA challenge gave a significant decrease ($p < 0.05$) of BAL fluid eosinophilia compared with the sham-treated group. All treatments with the pharmacological dose of DEX gave a decreased eosinophilia as compared with sham-treated mice ($p < 0.05$) (Figure 2). Inhibition of eosinophils with $1 \mu\text{g/kg}$ DEX 2 h before challenge was 55% compared with sham-treated mice. The minimum inhibition with DEX treatment was 22% ($1 \mu\text{g/kg}$ DEX 7 h after challenge). When the $1 \mu\text{g/kg}$ DEX dose was given 2 h before challenge, the eosinophilia ($7.5 \pm 0.5\%$) was significantly lower than when the $500 \mu\text{g/kg}$ DEX dose was given ($10.2 \pm 0.7\%$, $p = 0.007$). The $1 \mu\text{g/kg}$ DEX

dose given 2 h before challenge led to a significant decrease in eosinophils compared with the same dose given at challenge or 7 h after ($p < 0.01$). The groups treated with $500 \mu\text{g/kg}$ DEX displayed the same effects on BAL eosinophils regardless of the timing of the treatment.

There was an increase in the proportion of lymphocytes in sham-treated OVA-sensitized as compared with PBS-sensitized controls, although it was not significant (sham $1.6 \pm 0.2\%$, control $1.1 \pm 0.2\%$, $p = 0.09$). No differences in the proportion of neutrophils or macrophages were observed between the groups

Leukotriene B_4 in bronchoalveolar lavage

There was a significant increase in LTB_4 levels in BAL in the sham-treated mice ($22.5 \pm 2.9 \text{ pg LTB}_4/\text{well}$) compared with the control group ($13.0 \pm 2.1 \text{ pg LTB}_4/\text{well}$, $p < 0.05$) (Figure 3). In the groups treated before or at challenge with GCs the levels were similar to those demonstrated in the control group, i.e. the treatment prevented the OVA-challenged induced LTB_4 mice. The decrease was significant compared with the sham-treated group ($p < 0.05$). The groups treated with DEX 7 h after challenge displayed a significantly high level of LTB_4 compared with all other groups, including the sham-treated group ($1 \mu\text{g/kg}$ DEX, $36.2 \pm 1.5 \text{ pg LTB}_4/\text{well}$; $500 \mu\text{g/kg}$ DEX, $27.8 \text{ pg LTB}_4/\text{well}$; $p < 0.05$ vs all other groups). No correlation was found between LTB_4 levels and the relative number of neutrophils

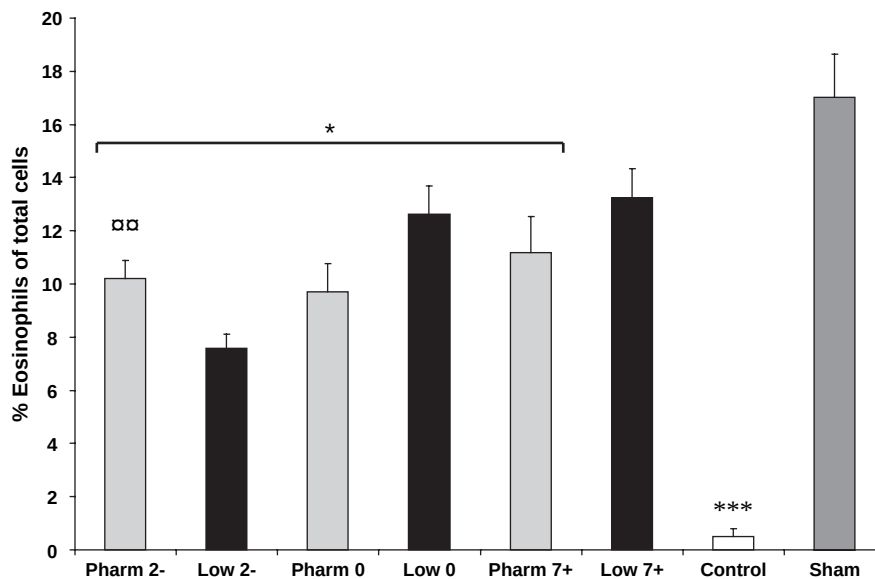


Figure 2. Eosinophil count (%) of total cells in bronchoalveolar lavage (BAL) in OVA-sensitized and challenged mice. Groups were treated with a low ($1 \mu\text{g/kg}$) or pharmacological (Pharm, $500 \mu\text{g/kg}$) dose of DEX and at three separate time points. Results are shown as mean $\pm$ SEM. * $p < 0.05$ *** $p < 0.001$ compared with the sham-treated group; aa $p < 0.01$ compared with the low-dose administered 2 h before challenge.

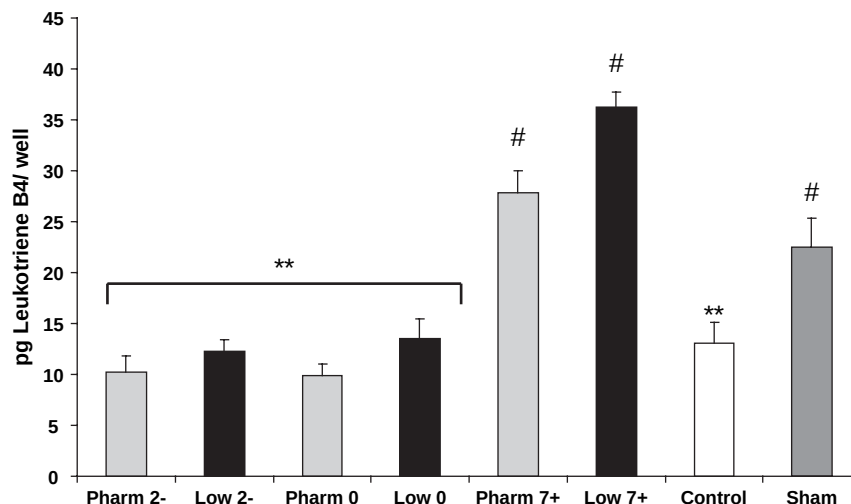


Figure 3. Leukotriene B₄ (LTB₄) levels in bronchoalveolar lavage (BAL). Groups were treated with a low (1 µg/kg) or pharmacological (Pharm, 500 µg/kg) dose of DEX and at three separate time points. Results are shown as mean ± SEM. ** $p < 0.01$ compared with the sham-treated group; # $p < 0.05$ compared with the control group.

or eosinophils for the various treatment groups in the BAL.

Lung tissue

In the lungs of OVA-sensitized and OVA-challenged mice, an increase of histopathology scores, mainly inflammatory cells in the peribronchial and perivascular areas, and also increased EPO-positive cells were seen as compared with the PBS-sensitized controls (Figure 4). The groups treated with 1 µg/kg DEX 2 h before or at challenge had a significant decrease ($p < 0.01$) in both EPO staining and histopathological scores compared with the sham group. When 1 µg DEX was administered 7 h after each challenge a significant decrease of EPO cells was seen as compared with sham-treated mice, but no significant effect on pathology scores was seen. The group treated with 500 µg DEX 2 h before challenge was significantly ($p = 0.001$) decreased in pathological scores compared with the sham-treated group, and the EPO staining was decreased but not significantly ($p = 0.06$). However, the 500 µg DEX groups displayed a significant ($p < 0.01$) decrease in both EPO staining and pathological findings when treated at challenge or 7 h after challenge. The EPO staining showed a weak but positive correlation ($r = 0.57$) with BAL eosinophils.

Nasal mucosa

The OVA-sensitized and OVA-challenged group displayed an increase ($p < 0.05$) of total inflammatory scores (inflammatory cells, ciliary loss, and epithelial deformation) (Figure 5) and eosinophilia when compared with the control group (results not

shown). As there were no differences between the pharmacological and low-dose treatments, they were pooled. The groups treated before challenge appeared to have decreased inflammatory scores compared with the sham group, although this was not significant (Figure 5). Treatment with DEX did not lead to any differences in eosinophils compared to the sham group (results not shown). Concerning the nasal mucosa, only minor and relative treatment effects were found, the general pattern of GC treatment on total inflammatory scores and eosinophilia being similar to that for the inflammatory scores in the lung.

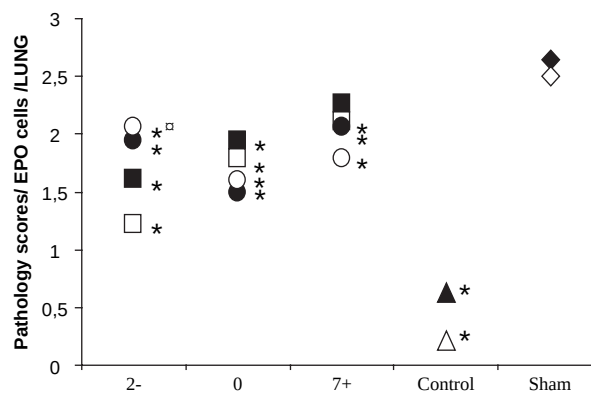


Figure 4. Semi-quantitative grading of lung histopathology scores and the number of eosinophil peroxidase (EPO)-positive cells in lung tissue. Groups were treated with a low (1 µg/kg) or pharmacological (Pharm, 500 µg/kg) dose of DEX and at three separate time points. Results are shown as mean ± SEM. Filled symbols (■▲●◆) represent the histopathological grading scores. Open symbols (□△○◇) represent the grading scores for EPO-positive cells. ■□, low-dose; ●○, pharmacological dose; ▲△, controls; ◆◇, sham-treated groups. * $p < 0.05$ compared with the sham-treated group; □△ $p < 0.05$ compared with the low dose administered 2 h before challenge.

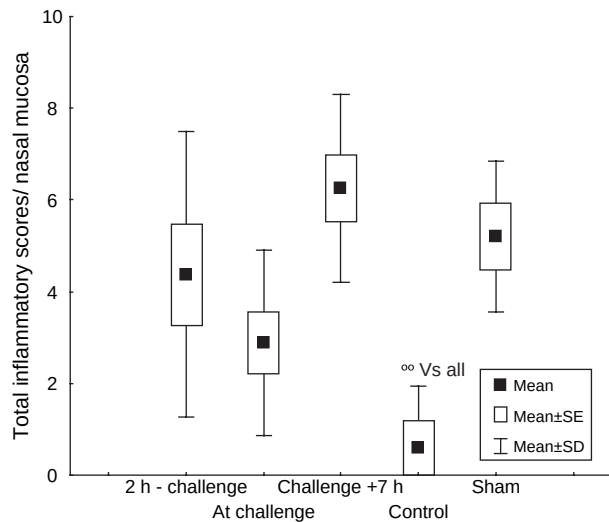


Figure 5. Total inflammatory scores (inflammatory cells, ciliary loss, epithelial desquamation) observed in nasal mucosa. Groups were treated with a low (1 µg/kg) or pharmacological (Pharm, 500 µg/kg) dose of DEX and at three separate time points. $oo p < 0.01$ compared with the control group.

Bone marrow

All OVA-sensitized groups had an increase in eosinophils compared with the control group ($p < 0.001$). DEX given at challenge or 7 h after (1 µg/kg or 500 µg/kg) decreased the BM eosinophilia significantly ($p < 0.001$) compared with both the sham-treated group and groups treated with DEX 2 h before challenge (Pharm 2: $14.3 \pm 0.9\%$, Phys 2: $13.2 \pm 0.7\%$, Pharm 0: $9.7 \pm 0.4\%$, Phys 0: $9.5 \pm 0.5\%$, Pharm 7+: 9.0 ± 0.8 , Phys 7+: $8.6 \pm 0.4\%$, control: $4.2 \pm 0.3\%$, sham: $14.3 \pm 0.7\%$). The maximum inhibition of eosinophils with DEX treatment reached 40% (1 µg/kg 7 h after challenge) compared with sham-treated mice. DEX given 2 h before allergen challenge showed no effect on bone marrow eosinophilia in mice compared with the sham-treated group. The group treated with 1 µg/kg DEX 2 h before challenge showed a more reduced number of eosinophils than the group treated with 500 µg/kg, but this difference was not significant.

Discussion

Dexamethasone pre- and co-treatment decreased the number of eosinophils in BAL from allergen-challenged mice. We were also able to observe a corresponding pattern in lung pathology and lung eosinophilia as demonstrated in BAL eosinophilia. The low dose had a better effect as compared with the pharmacological dose if given before the challenge and this was not seen when given after challenge. The effects seen with the pharmacological dose did not differ depending on when it was given.

This indicates that the timing effect is more important for eosinophilia when low-dose treatment is employed. When the pharmacological dose was used for pretreatment, the efficiency was not as prominent as when the low dose was used for pretreatment. One possible explanation for this may be that pre-challenge treatment with the pharmacological dose causes a negative feedback in the endogenous steroid production that probably occurs at challenge [11]. The low dose used is so close to the physiological level that it only increases the steroid levels and probably has a very limited HPA axis feedback effect. An alternative explanation could be an induction of a relative peripheral insensitivity to GCs in the initiated cell compartments pretreated with the pharmacological dose [11,12].

In the nasal mucosa, we detected a weak but significant increase in pathology scores and eosinophilia in the OVA-sensitized groups compared with the control group. We could not see any effect of the DEX treatment in any group. The pathology score pattern was similar to that for nasal eosinophilia. The nose is in the first line of defense and may not be as sensitive to allergens as the lungs [13], and in our model setting nasal inflammation might also be too weak to detect any differences between the allergen-sensitized groups.

It has been shown that even if most of the allergens are distributed in the nasal tissue the lungs develop a more prominent allergic reaction, and it has been indicated that the nose is the primary site for immune response and triggers the systemic response in the BM and then onward to the lungs [14]. In addition, the nasal mucosa of rodents, as compared with humans, has a relatively high normal inflammatory setting, and may thus be less sensitive to treatment with GCs [15].

We have previously shown that an inhibition of endogenous corticosteroids gives an increase in the eosinophilic response in the BM [16]. The eosinophilia in the BM could be more sensitive to variations of the endogenous glucocorticoids than DEX given exogenously. This may represent a hierarchy of effects by steroids in different compartments. The various cellular compartments respond individually to DEX, and this is probably a result of differences in sensitivity.

LTB₄ originates from multiple cell sources, mainly activated alveolar macrophages and neutrophils [17]. Post-challenge treatment had no effect on the LTB₄ levels, but all groups treated before or at challenge showed decreased levels compared with the sham-treated group. This lack of effect by late post-challenge treatment is probably the result of a relative lack of effect via DEX inhibition on late cellular activation, and further inhibition would then

be more dependent on the early timing of treatment inhibiting the cellular recruitment to the site of inflammation.

With the allergen challenge model, it has been shown that when using only one challenge a nasal inflammation is the main result. When using protocols with repeated challenges, a systemic response with inflammation in the BM and the lungs develops [18]. The repeated challenges in our model resulted in a systemic type of airway reaction, believed to be more similar to the human asthmatic reaction than the more acute inflammation after a single challenge [2]. Although complex kinetic responses take place after multiple challenges, this does not affect our observations of differential effects of DEX on cellular responses in different compartments in this model.

Interestingly, the early physiological low dose, not tested in allergy models previously, as compared with a 500 times higher dose that is the frequently used dose for systematic treatment in murine models of asthma, displayed an anti-eosinophilia effect that was most pronounced in the BAL and lungs. Several studies have shown that systemic and local DEX treatments prevent allergic eosinophilic reaction via partly different mechanisms concerning how allergen exposure can override the inhibitory effects of inhaled steroids [19,20]. The sensitivity to GCs in the tuning of allergic inflammation thus displays substantial differences in the local and systemic cellular compartments. This study suggests that in the human setting, systemic dosages below 1 mg of prednisolone may display substantial effects if the possibility to treat is given close to the point of exposure and challenge. The role of dosage and timing, both locally and systemically, will be even more important considering the differences in compartmental responses to steroids and how inflammatory cells respond to the interval of GC doses ranging from our low physiological dose to the higher doses needed when a disease is established. This study puts a focus on the necessity to provoke and develop different intervention strategies depending on disease-specific activation and pathology in the compartments involved, based on the GC sensitivity in specific cell populations during different stages of the allergic disease.

In conclusion, we have shown that DEX differentially affects eosinophilia in BAL, bone marrow, nasal mucosa, and lung pathology after an allergen challenge, depending on the dosage and timing of the treatment, where the responses in BAL, nasal tissue, and lungs appear to be dependent on both timing and dose and the BM more on the timing. The response pattern in different cellular inflammatory compartments is likely to involve the inhibition

of the release of eosinophils in peripheral storage pools, and probably also the differentiation, proliferation, and migration of eosinophils in the BM.

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