

The influence of intravenous hydrocortisone on cytokine levels in children with asthma

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Nocturnal airway obstruction occurs frequently in childhood asthma and results from increased airway inflammation. Lymphocytes are believed to be key effector cells of airway wall inflammation, releasing pro-inflammatory mediators and cytokines. A previous study showed that hydrocortisone infusion, an effective anti-inflammatory treatment, improves nocturnal and daytime FEV₁ values. This study in 16 children with moderate asthma was designed to assess whether there exists day and night differences in IL-4, IL-5, IL-8, and IFN- γ production of concanavaline A stimulated peripheral blood mononuclear cells. Furthermore, we investigated whether substitution of low serum cortisol levels with intravenous hydrocortisone would affect those parameters. Saline (as a placebo) or hydrocortisone (30 μ g/m² body surface area/24 h) was intravenously administered in a randomized, double blind, cross-over design. Measurements under saline or hydrocortisone infusions were separated by 1 wk. At 04:00 and 16:00 hours 10 ml blood was taken for determination of peripheral blood mononuclear cell isolation and stimulation, and an eosinophil count. Hydrocortisone infusion significantly reduced the nocturnal fall in FEV₁. Median values of IFN γ , IL-4, IL-5, and IL-8 produced by peripheral blood mononuclear cells did not significantly differ at 04:00 and 16:00 hours, both with saline and hydrocortisone infusion. Our results suggest that FEV₁ improvement is not due to suppression of circulating peripheral blood mononuclear cell activation. We hypothesize that it is rather due to its effect on local lung tissue epithelial and/or fibroblasts thereby reducing airway inflammation and vascular leakage.

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Nocturnal airway obstruction occurs frequently in childhood asthma. Its pathogenesis is still not fully elucidated, yet most studies envisage that increased airway inflammation is one of the contributing factors (1–4). Eosinophils and lymphocytes are believed to be key effector cells of airway wall inflammation in asthma, releasing pro-inflammatory mediators and cytokines. The cytokines produced by T-helper lymphocytes (Th-cells) can differentiate into subtypes, e.g., Th₁- and Th₂-cells. The Th₁-cells in particular secrete IFN- γ that may down-regulate IL-4 expression of the Th₂-cells and promotes IgG production by B-cells. The Th₂-cells, in contrast, mainly produce IL-4, IL-5, and IL-13 promoters

of the eosinophilic inflammatory response. The IL-4 stimulates B cells for isotype switching to produce IgE and may inhibit Th₁-cell activity. The major functions of IL-5 are differentiation, maturation, and activation of eosinophils, while IL-13 acts on the expression of adhesion molecules on tissue cells. Several studies have shown IL-4 and IL-5 to be increased, and IFN- γ decreased in patients with allergic asthma (5–8).

Next to eosinophils and lymphocytes, neutrophils may play a role, especially during exacerbations of asthma and viral respiratory infections (9–11). The IL-8 may contribute to the clinical severity of asthma since it is a well-known chemoattractant for neutrophils, and primed

eosinophils and is increased during asthma exacerbations (12, 13). Activated monocytes and/or epithelial cells may serve as a source of the chemokine IL-8.

Furthermore, priming and/or activating substances from the lung compartment (cytokines and/or chemokines) are released in peripheral blood during an exacerbation of asthma, thereby changing the activation state of circulating blood lymphocytes. This type of changes has been shown after allergen induced asthmatic responses (14). We hypothesize that in patients with nocturnal asthma activating chemokines and/or cytokines may be released that subsequently activate circulating lymphocytes in a similar manner.

Corticosteroids are known to suppress virtually every step of the immunological response to allergens. Endogenous cortisol, a glucocorticoid produced by the adrenal gland, shows a circadian rhythm with its peak around 08:00 hours and its nadir around midnight. Decrease of serum cortisol levels at night may be of great importance in the loss of control on the inflammatory response. We have previously shown that cortisol levels are significantly lower in children with nocturnal asthma than in healthy children and particularly so at night (15). Cortisol substitution by low dose intravenous hydrocortisone, meant to abolish the nocturnal fall in endogenous cortisol, increased FEV₁ values both during day- and night-time, and significantly so at 08.00 hours (16). We therefore hypothesized that intravenous hydrocortisone might not only prevent the nocturnal fall in FEV₁, but also the corresponding activation of cytokines, as well as reduce the number of eosinophils. The present results contribute to a part of the previous double blind study with low dose, intravenous hydrocortisone infusion over 24 h. In this study we have investigated the release of IL-4, IL-5, IFN- γ , and IL-8 in concanavaline-A stimulated peripheral blood mononuclear cell (PBMC) cultures at 04:00 and 16:00 hours and assessed the effect of hydrocortisone infusion on release of these cytokines.

Methods

Subjects with asthma

We studied 28 children with stable asthma, aged 7–16 yr, with a history of recurrent wheezing, allergy for at least one aero-allergen (determined by a positive specific immunoglobulin E (IgE) for house dust mite, cat, dog, grasses, or trees) and increased airway responsiveness to histamine (PC₂₀ < 8 mg/ml histamine) (15). Sixteen

out of the 28 patients with a complete data set of stimulated PBMC cultures entered this study after they had stopped their inhaled corticosteroids for 2 wk, and long-acting β_2 agonists for 5 days before the onset of the study. Short-acting β_2 agonists were withheld 8 h before the study. No oral corticosteroids were used during at least 2 month before the study. Informed consent was obtained from all children and their parents. The study is approved by the Medical Ethical Committee of the University Hospital of Groningen.

Study design

Saline (as a placebo) or hydrocortisone (30 $\mu\text{g}/\text{m}^2$ body surface area/24 h) was intravenously administered in a randomized, double blind, cross-over design. The intervention dose of hydrocortisone of 30 mg/m² body surface, when converted to oral prednisone approximates a total dose of 7.5 mg/24 h. We aimed to administer a dose that was sufficiently high to abolish the physiologic nocturnal decrease in endogenous cortisone. Measurements under saline or hydrocortisone infusions were separated by 1 wk. The pharmacist who prepared the different solutions also performed the randomization procedure.

Children arrived at the hospital at 19:30 hours and remained until 17:00 hours the next day. An intravenous line was placed for taking blood samples at arrival. The intravenous line was kept open with continuous infusion of 0.9% saline or the hydrocortisone solution, administered with a portable CADD + pump (Continuous Ambulant Drug Delivery; Sinus Graseby Medical BV, The Netherlands). At 04:00 and 16:00 hours 10 ml blood was taken for determination of PBMC isolation and stimulation, and an eosinophil count.

Measurements

Serum cortisol was measured by a chemiluminescence immunoassay method (University Hospital Groningen, The Netherlands) every 4 h over 24 h starting at 20:00 hours. At 04:00 and 16:00 hours blood eosinophil numbers were counted in a coulter counter. The FEV₁ values were obtained every 4 h over 24 h by a pneumotachograph, Masterscreen IOS (Jaeger, Wurzburg, Germany). The FEV₁ was measured until three reproducible recordings were obtained, the best of three being used for analysis. Reference values for the FEV₁ are those of Zapletal et al. (17).

PBMCs were isolated using density gradient centrifugation as described before (5). In short, cells were resuspended in enriched RPMI and the number of cells were counted (RPMI 1640 medium with L-glutamine and with 25 mmol HEPES (M-[2-hydroxy-ethyl] piperazine-*N'*-[2-ethanesulfonic acid], Life Technologies BV, Breda, The Netherlands). Cytospins were prepared and differential cell counts of the cytopins were determined after May Giemsa Grünwald staining. Viability was assessed by cellular exclusion of trypan blue. Subsequently, the cells were cultured in quadruplo in a 24 wells plate containing 1 million cells per well in the presence of a stimulus: 10 μ g concanavaline A (ConA), obtained from Sigma Chemie, Bornum, Belgium. The cells stimulated with ConA were incubated for 24 h at 37°C with 5% CO₂. After incubation the suspension was centrifugated at 590 rcf at room temperature, the supernatant collected and stored at -80°C.

ELISA for IL-4, IL-5, IL-8, and IFN- γ

Cell supernatants were determined for IL-4, IL-8, and IFN- γ by an ELISA test (Sanquin blood supply foundation, division CLB, Amsterdam, The Netherlands). The detection limit for IL-4 was 0.2 pg/ml, for IFN- γ 1 pg/ml and for IL-8 1 pg/ml. The IL-5 were determined by a home-made sandwich ELISA as described before (5), its detection limit being 6 pg/ml.

Statistical analysis

Data were analyzed using Statistical Package for Social Science (SPSS; Chicago, USA) Windows Version 10.0. Normal distribution was checked visually by probability plots of the residuals and tested formally by the Kolmogorov-Smirnov test. Differences between groups were tested

using parametric or non-parametric tests as appropriate. The effect of hydrocortisone substitution during 24 h on IL-4, IL-5, IL-8, and IFN- γ at 04:00 and 16:00 hours were tested with Wilcoxon Signed Ranks tests. All tests were performed two-sided. A $p < 0.05$ was considered significant.

Results

Subjects

Sixteen children (six boys), mean age 12.9 ± 1.7 yr, with moderate asthma participated in the study and received intravenous hydrocortisone.

Serum cortisol levels

Intravenous hydrocortisone infusion resulted in significantly higher serum cortisol levels ($p < 0.05$) compared to the saline day at all time points apart from the 16:00 hours (mean $\pm$ s.d.) value (saline vs. hydrocortisone 175 ± 65 vs. 340 ± 130 nmol/l, $p > 0.05$).

Lung function and eosinophils

During saline infusion the mean daytime (16:00 hours) FEV₁% predicted value was significantly higher than at 04:00 hours (Table 1 and Fig. 1). This significant difference disappeared during hydrocortisone infusion ($93.3 \pm 14.1\%$ predicted and $91.8 \pm 13.7\%$ predicted, respectively) (Table 1). The number of eosinophils showed the same pattern. During saline infusion we observed significantly higher eosinophil numbers at 04:00 hours than at 16:00 hours (median [range] $0.62 [0.05-1.42] \times 10^9/l$ and $0.46 [0.01-1.56] \times 10^9/l$, respectively) (Fig. 2). The significant difference disappeared under hydrocortisone infusion (Table 1).

Table 1. Differences between night (04:00 hours) and day (16:00 hours) with saline or hydrocortisone infusion

	Saline		Hydrocortisone	
	16:00 hours	04:00 hours	16:00 hours	04:00 hours
FEV ₁ % predicted†	$92.6 \pm 11.9^*$	$88.1 \pm 14.5^*$	93.3 ± 14.1	91.8 ± 13.7
Eos $\times 10^9/l$ ‡	$0.46 [0.01-1.56]^*$	$0.62 [0.05-1.42]^{**}$	$0.31 [0.07-0.85]$	$0.36 [0.05-0.83]^{**}$
IFN- γ (pg/ml)‡	$267.6 [60.5-1309.0]$	$323.8 [69.1-806.3]$	$221.5 [29.6-1261.0]$	$274.2 [28.5-771.5]$
IL-4 (pg/ml)‡	$60.5 [27.6-321.6]$	$68.8 [12.5-291.9]$	$74.4 [22.1-411.8]$	$77.1 [21.8-247.5]$
IL-5 (pg/ml)‡	$162.7 [64.8-431.9]$	$242.6 [67.5-416.0]$	$199.5 [87.4-627.8]$	$237.7 [113.6-448.0]$
IL-8 (pg/ml)‡	$144.5 [45.2-236.0]$	$102.0 [51.0-349.0]$	$141.5 [70.1-298.0]$	$133.0 [31.7-323.0]$

†Values expressed as mean $\pm$ s.d.

‡Values expressed as median [range].

Eos, eosinophils.

* $p < 0.05$; 04:00 vs. 16:00 hours.

** $p < 0.05$; saline vs. hydrocortisone.

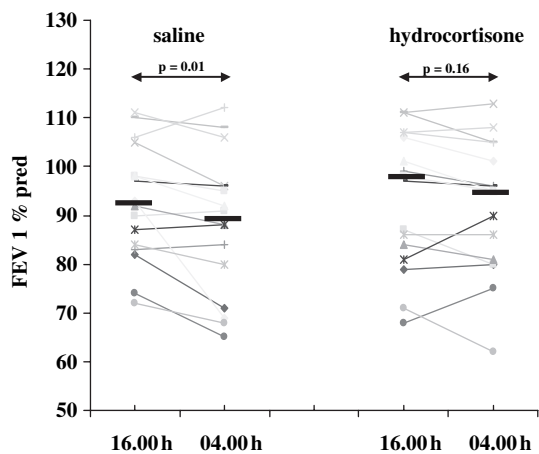


Fig. 1. Individual FEV₁% predicted values at 16:00 and 04:00 hours with saline and hydrocortisone infusion. Median values are indicated with horizontal lines.

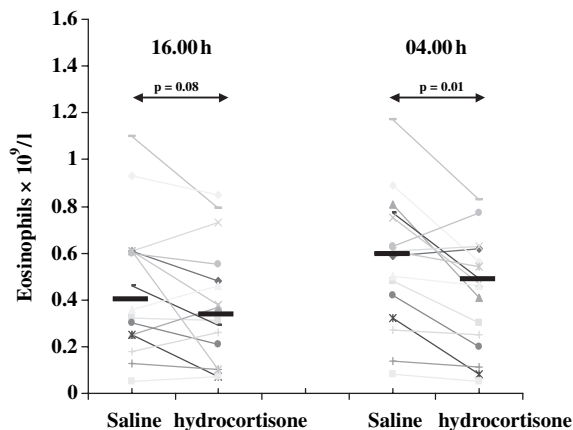


Fig. 2. Individual eosinophil values with saline and hydrocortisone infusion at 16:00 and 04:00 hours. Median values are indicated with horizontal lines.

Production of IFN γ , IL-4, IL-5, and IL-8 by PBMCs

Values of IFN γ , IL-4, IL-5, and IL-8 produced by PBMCs, using ConA as a stimulus, did not significantly differ between 04:00 and 16:00 hours, neither with saline, nor with hydrocortisone (Table 1).

Median (range) values of IFN γ , IL-4, IL-5, and IL-8 produced by ConA stimulated PBMCs did not significantly differ between the measurements with hydrocortisone and with saline at both time points. All values are summarized in Table 1.

Discussion

The suppression of inflammatory responses by inhaled or systemic corticosteroids is well known

and world wide used in clinical practice for the treatment of allergic asthma (18). Already in 1971 Booij-Noord et al. (19) described that steroids primarily affect the allergic late-phase reaction. We have previously shown that cortisol levels are significantly lower in children with asthma than in control subjects and particularly so at night. We, therefore, hypothesized lower cortisol values to be linked with a lack of suppression of airway inflammation (15). In this study we substituted low endogenous serum cortisol levels with intravenous hydrocortisone to enhance the anti-inflammatory activities of endogenous glucocorticosteroids. We found that FEV₁ values improved overnight in association with a reduction in the number of circulating eosinophils. In contrast to these improvements in lung function and numbers of eosinophils, no differences were found in production of IL-4, IL-5, IL-8, and IFN γ by stimulated cultures of PBMCs neither at 04:00 hours at the nadir of cortisol, nor at 16:00 hours upon hydrocortisone infusion.

Our observations are apparently in contrast to some *in vitro* studies regarding effects of steroids on PBMCs. Results of Krouwels et al. in adults with asthma (20) showed that addition of physiological concentrations of dexamethasone *in vitro* to T-cell clones, derived from broncho-alveolar lavage and blood, inhibited the production of IL-4 and IL-5 more strongly than IFN γ . These authors suggested that the *in vitro* effects of glucocorticosteroids could be explained by induction of a shift of the cytokine production profile towards the Th₁ type lymphocyte response. In another *in vitro* study (21) dexamethasone also suppressed IL-5 production of PBMCs *in vitro* in a dose dependent manner. This appears not to be the case *in vivo* in PBMCs obtained from peripheral blood in asthmatic children who received low dose intravenous hydrocortisone for a limited period with subsequent significant increase in serum cortisol at 4:00 hours. A more direct contact and/or a higher degree of up-regulation and binding may explain this difference to glucocorticosteroid receptors in the *in vitro* studies. Moreover, a difference in dosage and effectiveness of the different glucocorticosteroids that were used in the different studies may be another important explanatory factor.

A network of interacting cytokines regulates airway inflammation. In this network, IL-4 and IL-5 play a prominent role in allergic inflammation (22). We expected that the reduction in cytokine production by PBMCs upon hydrocortisone infusion would be associated with the

improvement in FEV₁, based on previous studies in the literature (23–25). Borger et al. (23) showed a significant inverse correlation between IL-5 mRNA expression by PBMCs and FEV₁ in adults with moderate to severe asthma. The latter study therefore suggested a direct relationship between airway obstruction and inflammation. Bentley et al. (25) found a beneficial effect of oral systemic steroids on FEV₁ levels in allergic adult asthmatic patients. Moreover, they measured a reduction of the number of cells expressing mRNA for IL-4 and IL-5 and an increase in mRNA for IFN γ , and concluded that corticosteroid treatment may act by modulation of cytokine expression with consequent inhibition of the local bronchial inflammatory infiltrate and tissue eosinophilia. Similar findings were observed in children with asthma (26). Another study investigating asthmatic patients, who were operated because of lung cancer, showed that mRNA for IL-5 in lung tissue appeared to be completely suppressed by a high dose of methylprednisolone (20 mg 2 day and 80 mg at the day of surgery) (27). All above studies are difficult to compare with our investigation given the fact that cytokines were either measured as lymphocyte mRNA expression, production by PBMCs or serum levels. Furthermore, the children in our study received a low dosage of intravenous hydrocortisone, only in order to abolish their nocturnal fall in endogenous cortisol levels. The differences in dosages, site of deposition and type of glucocorticosteroids used could explain the discrepancies in results between our study and the other *in vivo* studies.

Airway inflammation in asthma is characterized not only by infiltration and activation of eosinophils, but also by neutrophils. Therefore we also measured the levels of IL-8 of stimulated PBMCs. Norzila et al. (9) measured levels of IL-8 in induced sputum during an acute exacerbation of asthma and 2 wk later after treatment with oral prednisolone. They found high levels of IL-8 during the exacerbation, but a poor response to corticosteroid therapy. This is in agreement with our observation, despite the differences in steroid dose and study design.

An explanation of the divergent effect of infused hydrocortisone on lung function and cytokine production may well be the state of disease in these asthmatic children. Assuming that peripheral PBMCs in stable asthmatic patients are in a resting state, PBMC activity may not be influenced by low concentrations of corticosteroids. Positive *in vivo* and *in vitro* findings in the literature with respect to cyto-

kine suppression have been reported during exacerbations, after allergen challenge (28) and in a severe state of asthma when systemic steroids were being used (27). The PBMCs are in these situations in a so-called 'primed' state as supported by the observation that peripheral blood neutrophils and/or eosinophils have increased expression of cell surface markers after allergen challenge (29–31). Our data may suggest that inflammatory cells that are present in the airway wall and lung tissue may, in contrast to circulating cells, be in a 'primed' state. They are thus influenced by locally applied corticosteroids. Our data are also in line with the observations of Heijink et al. in adults (14). They suggest that lymphocytes are not activated during phases of nocturnal asthma in contrast to observations after allergen challenges. This may suggest that during nocturnal airway obstruction no chemokines are released, or the reverse may be postulated, that chemokine release is specific for an allergen induced asthmatic response, possible due to the allergen epithelial activation and corresponding release of chemokines such as TARC and RANTES. Finally, it is important to mention that glucocorticosteroids also have an inhibitory effect on tissue cells such as epithelial cells and fibroblasts. Previous studies have shown that low concentrations of corticosteroids more strongly suppress the expression of adhesion molecules (ICAM and VCAM) on fibroblasts (32–33). This, as well as the associated reduction in airway wall edema, may explain the improvement seen in lung function values after hydrocortisone.

In conclusion, this study in children with moderate asthma does not show significant differences between median values of IFN γ , IL-4, IL-5, and IL-8 produced by stimulated PBMCs at 04:00 and 16:00 hours. This was the case both during *in vivo* treatment with hydrocortisone and with saline. Our previous findings of improvement in nocturnal and morning FEV₁ values after substitution of low endogenous cortisol levels with a low dose intravenous hydrocortisone cannot be explained by suppression of cytokine release of peripheral blood PBMCs by hydrocortisone. Based on the literature we hypothesize that the improved FEV₁ may be due to effects on local tissue epithelial cells and fibroblasts, thereby suppressing airway inflammation and vascular leakage.

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