

Eosinophil and mast cell parameters in children with stable moderate asthma

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Mast cells and eosinophils are important cells that contribute to the process of inflammation in asthma either by activating other cells or by secreting products which are potentially toxic to the respiratory epithelium. The influx of these cells in the airways and the secretion of toxic products by these cells is abrogated by inhaled corticosteroids.

Methods – In a double blind randomised, placebo controlled, study in children with stable moderate asthma (N = 34, 15 children received fluticasone propionate (FP), an inhaled corticosteroid, and 19 children used a placebo), we investigated the influence of treatment with FP 100 µg bd on various parameters of inflammation: number of eosinophils, secretory products of eosinophils i.e. ECP and EDN (in serum and urine) and a secretory product of mast cells, histamine, which is determined as the compound to which histamine is converted and excreted by the human body: N^T-methyl-histamine.

Results – Previously we reported that lung function increased and bronchial hyperresponsiveness decreased in the 30 children that completed the study during treatment with FP. In these children we found that none of the laboratory parameters of inflammation changed significantly during treatment with either FP or placebo. However, the decrease in urinary EDN almost reached significance (P = 0.07).

Conclusions – Our results indicate that the number of eosinophils, serum ECP and EDN and urinary EDN as well as urinary N^T-methyl-histamine do not reflect asthma disease activity in children with stable moderate asthma. Our data on urinary EDN warrant further study of the use of this parameter to monitor asthma in children.

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Inhaled corticosteroids are increasingly used as maintenance treatment in childhood asthma. Several studies have shown that inhaled corticosteroids play an important role in the control of asthma (1). These drugs are effective in reducing the process of inflammation, which is reflected in a decrease in bronchial hyperresponsiveness (2). Eosinophils (3) and mast cells (4) are involved in the process of inflammation. Eosinophils degranulate and secrete proteins that can damage respiratory epithelium. Examples of eosinophil derived proteins are Eosinophil Cationic Protein (ECP) and Eosinophil Derived Neurotoxin (EDN). (5) Mast cells contribute to the process of inflammation by secreting several mediators including histamine, which causes vasodilatation and is largely metabolised to N^T-methyl-histamine which is secreted in the urine (4). Recently, we reported the clinical results of treatment with the inhaled corticosteroid, fluticasone propionate (FP) and we observed that bronchial hyperresponsiveness was reduced during treatment with FP, which suggests that FP reduces airway

inflammation (6). Recently, we also observed that certain parameters of inflammation (the number of eosinophils, serum ECP and EDN and urinary EDN) were increased in asthmatic children compared with healthy controls (7). We hypothesized that treatment with FP, which dampens airway inflammation, would lead to a decrease in parameters of inflammation. Therefore, the effect of treatment with FP on the number of eosinophils, on serum ECP, EDN, and on urinary EDN N^T-methyl-histamine was assessed in the current study.

Methods

Study design

The study was performed in a double blind randomised, placebo controlled, parallel manner. The study duration was 4.5 months and consisted of a run-in period (2 weeks), a treatment period (3 months) and a follow-up period (1 month). All con-

current medication was stopped 4 weeks prior to the start of the run-in period, with the exception of β_2 -mimetics. β_2 -mimetics other than salbutamol were stopped at the start of the run-in period. After the run-in period of 2 weeks, the patients who were eligible to enter the study, were randomly assigned to one of the two intervention groups, using study medication (twice daily either dry powder FP 100 μ g or placebo, to be used with a Diskhaler®) for a period of 3 months. The study medication was then stopped and the patients finished the study with a follow-up period of 4 weeks. During the study the patients attended the clinic on 6 occasions. Salbutamol (200 μ g per Diskhaler®) was used twice daily throughout the whole study (run-in period, treatment period (Salbutamol was used preceding the inhalation of FP or placebo) and follow-up period) and could be used a maximum of 6 times daily (equals 1200 μ g) for symptom relief.

Subjects

A group of 34 children aged 8 to 14 years with the diagnosis allergic asthma (according to the criteria of the American Thoracic Society (8)) with perennial symptoms requiring maintenance treatment participated in the study. They were recruited from the out-patient department of the pediatric university hospital and from general practitioners' practices in Groningen. Children who used inhaled corticosteroids 4 weeks prior to the study or systemic corticosteroids on more than 3 occasions in the 6 months prior to the start of the study were excluded. Allergy was defined as the presence of an increased total IgE (related to age) and at least one increased specific IgE against a major allergen. Lung function at the onset of the study of all participants had to show mild bronchus obstruction (defined as a value of $FEV_1\%pred > 75\%$ or $FEV_1\%VC > 65\%$), bronchial hyperresponsiveness (defined as a PC_{20} -histamine < 8 mg/ml) and reversibility of airway obstruction on salbutamol of at least 10%.

Symptom score

In a Daily Record Card respiratory symptoms as wheezing, cough, sputum production, dyspnea and exercise induced asthma (during physical activities) were recorded on a 0 to 3 point scale. Asthma attacks, supplementary use of rescue salbutamol and absence from school were noted.

Peak Flow Recording (PEFR)

PEFR was measured at home each day in the morning and in the evening using a mini Wright peak flow meter during the whole study period before

taking the medication. On two consecutive days in the week before the clinic visit PEFR was measured at 04.00 a.m. PEFR was performed in triplicate and the best values were selected for calculation. The $\Delta PEFR_{em}$ (the difference between the evening and the morning PEFR) was calculated by subtracting the morning from the evening PEFR.

Lung function

Patients were asked to refrain from taking study medication and salbutamol 12 hours prior to the clinic visit which took place between 8 and 10 am. After measuring FEV_1 and VC (on a water-sealed spirometer by Lode, The Netherlands) bronchial hyperresponsiveness was determined by histamine provocation test, as previously described (9). Briefly, PC_{20} -histamine was measured by inhalation of histamine-diphosphate in doubling dosages according to a standardised protocol. Each concentration of histamine was nebulized with a separate deVilbiss 646 nebulizer attached to a Rosenthal-French dosimeter. Inhaled concentrations of histamine were doubled from 0.25 up to 16 mg/ml maximum. The effect of each dose was assessed 3 minutes after administration. The PC_{20} -histamine was defined as the concentration of histamine causing a fall of 20% in FEV_1 . The exact PC_{20} -histamine was assessed by linear interpolation of the last two points of the log concentration response curve. Reversibility testing was performed when at least 95% of the pre-test FEV_1 value was reached in order to avoid being biased by the histamine provocation test. Reversibility of airway obstruction was determined after administration of 800 μ g of salbutamol. Twenty minutes after inhalation, FEV_1 was measured and reversibility was calculated as was previously described: $FEV_1 \text{ post-inhalation} - FEV_1 \text{ pre-inhalation} / FEV_1 \text{ pred}$ (10).

Laboratory parameters of inflammation

A blood specimen was drawn and morning urine was collected four times during the study: at the beginning of the treatment period, one month after the start of the treatment period, at the end of the treatment period (three months after the onset of the study) and one month after cessation of the study medication.

Number of eosinophils

EDTA blood was diluted 10 times and stained with an eosin-formalin solution, consisting of 2.5 ml formalin and 25 ml eosin 1% per 250 ml aqua dest. Dilution was performed with a Finnpiptette Diluter (Labsystems). Erythrocytes were thus lysed and eosinophils were stained. Eosinophils were counted with an improved Neubauer counting chamber.

IgE concentration.

Total IgE antibody concentration and specific IgE antibody concentrations (for house dust mite, grass pollen, tree pollen, dog dander and cat dander) were determined on visits two, three and five with Immuno CAP Technique (Pharmacia, Uppsala, Sweden). The range of IgE with this method was 2 to 2000 U/ml. Values above 2000 U/ml were further quantified by dilution.

Determination of Eosinophilic Cationic Protein (ECP) and Eosinophil Derived Neurotoxin (EDN) (EDN is also referred to as Eosinophil Protein X (EPX))

The blood specimen was directly transferred to the laboratory, where blood clotting was allowed for 1 hour at room temperature. Serum was subsequently collected by centrifugation, twice at $1450 \times g$ for 10 minutes. Serum was stored and frozen at -20°C . Urine specimen were taken in the morning at home after awakening, transferred to the hospital at room temperature in a plastic reservoir which was supplied at the previous visit. Directly after arrival in the hospital, the urine specimen was frozen at -20°C . The urine specimen of all the participants were defrosted only prior to determination of EDN.

Serum ECP, serum EDN and urinary EDN

Serum ECP, serum EDN and urinary EDN concentrations were determined with a radioimmunoassay (Pharmacia, Uppsala, Sweden) as described (11). With this method, the range of ECP was 0–200 $\mu\text{g/l}$ and the range of EDN was 0–400 $\mu\text{g/l}$. Values above the upper limits were further quantified by dilution.

Determination of N^T -methyl-histamine in urine

N^T -methyl-histamine in the urine was determined by gas chromatography and mass spectrometry, according to a previously described method (12).

Data analyses

Before the analyses were done, all variables with a non-Gaussian distribution (blood eosinophils, the percentage of hypodense eosinophils, serum EDN, ECP and IgE, urinary EDN, EDN per mmol creatinine, N^T -methyl-histamine) were log-transformed in order to approximate a Gaussian distribution. All variables were analysed with repeated measurements analysis of variance, using PROC MIXED of the SAS package (13). After testing for interaction between time and treatment, the treatment effect was estimated as the average difference between FP and the placebo group during the treatment period, corrected for the baseline measurement recorded at the randomisation visit (i.e. without interaction between visit and treatment, with baseline measure-

ment as covariate). The possible relation between a laboratory parameter and a lung function parameter was investigated by adding the laboratory parameter as a covariate to a repeated measurements analysis of variance model with the lung function parameter as dependent variable and visit and intervention group as factors. The significance level was set at $P = 0.05$. All variables are given as means $\pm$ the standard error of the mean (SEM).

Informed consent

The study was approved by the Medical Ethics Committee of the University Hospital of Groningen and by the Central Committee of the general practitioners of Northern Netherlands. The patients and their parents gave informed consent.

Results

Thirty of the 34 children completed the study. Four children allocated to the placebo group were withdrawn due to an exacerbation of their asthma symptoms, which was treated with a short course of oral steroids (2 children 4 weeks and 2 children 8 weeks after randomisation). The available data of these 4 children were used in the analyses. No relevant differences in baseline characteristics, regarding laboratory parameters, were observed between the two intervention groups (Table 1).

Symptom score, Peakflow, Lung function and bronchial hyperresponsiveness

These parameters were previously described and are summarized in Table 2 (6).

Symptom score

During the treatment period symptom scores were significantly decreased. The change in morning and evening wheezing was significantly different in the FP group compared with the placebo group. No difference in cough, sputum production and dyspnea were found between the two intervention groups. When the scores of wheeze, cough, sputum production and dyspnoea for morning and evening were added, this cumulative symptom score was significantly different between the two intervention groups ($P = 0.05$). After adjustment for the run-in period score, the P-value became 0.08. During the treatment period, the total symptom score decreased significantly in both intervention groups. During the treatment period supplementary salbutamol was used less often in the FP group than in the placebo group ($P < 0.05$). After cessation of FP, morning and evening wheezing increased significantly in the FP

Table 1. Baseline characteristics of children by intervention group (mean values $\pm$ SD).

	Fluticasone propionate (n=15)	Placebo (n=19)
Gender (m:f)	11/4	15/4
Age (yrs)	10.6 (1.8)	11.0 (1.8)
Cumulative symptom score (0–32)	3.2 (2.40)	3.2 (2.40)
VC (ml)	2.671 (.6373)	2.497 (.6301)
FEV ₁ pred (%)	92 (12.4)	88 (11.7)
FEV ₁ /VC (%)	81 (7.4)	80 (9.4)
² logPC ₂₀ (mg/ml)	1.17 (1.085)	0.89 (1.615)
Reversibility (%)	14.5 (6.7)	14.7 (8.25)
Serum glucose (mmol/l)	3.9 (.46)	3.8 (.46)
Serum cortisol (nmol/l)	383 (168.3)	398 (152.3)
Urinary cortisol (nmol/l)	102.5 (93.75)	85.3 (56.23)

[] = units, () = Standard Deviation

group and a significant rise in the total symptom score was only observed in the FP group. The number of school absence days was small in both groups and not significantly different.

Peakflow

During the treatment period morning and evening PEFR increased significantly in the FP group compared with the placebo group. The nocturnal PEFR increased in the FP group. The Δ PEFR_{em} decreased in the FP group by 8.6 l/min (95% CI: 1.0–16.2 l/min, $P < 0.05$). Between the two intervention groups, no differences in the nocturnal PEFR and the diurnal PEFR were observed during the treat-

ment period. After cessation of FP all PEFR values returned to pre-treatment values.

Lung function and bronchial hyperresponsiveness

During the treatment period FEV₁ and FEV₁%VC increased significantly in the FP group compared with the placebo group. PC₂₀ increased in both intervention groups, however only the increase in the FP group (1.52 doubling dose (DD)) was significant (95% CI: 1.01–2.03 DD, $P < 0.00001$). The PC₂₀-histamine was on average 0.93 DD higher (95% CI: 0.19 to 1.70 DD, $P = 0.015$) in the FP group compared to the placebo group. Reversibility decreased significantly in the FP group. After cessation of FP all parameters of lung function returned to pre-treatment values.

Laboratory parameters of inflammation

Baseline values (visit 2) of the different laboratory parameters, values after the third month of treatment (visit 5) and after the follow-up period (visit 6) in both intervention groups are shown in Table 3. During the treatment period and after cessation of the study medication, no significant change was observed in either intervention group in number of eosinophils, serum concentration of ECP and EDN and urinary concentration of NT-methyl-histamine. The only laboratory parameter that changed during treatment with FP was urinary EDN, which decreased during the treatment period in the FP group, whereas it remained constant in the placebo group. However, the difference in urinary EDN between

Table 2. Symptom scores, PEFR- and lung function-values in FP group (FP) and placebo group (P). (mean values $\pm$ SD)

Visit number	2	5	6	2	5	6
Group	FP	FP	FP	P	P	P
Parameter (SD)						
Symptom score:	0.22	0.07	0.33	0.29	0.21	0.23
morning wheezing	(0.281)	(0.257)	(0.610)	(0.297)	(0.289)	(0.299)
(0–4)						
Cumulative	3.23	1.92	3.22	3.16	1.84	1.97
symptom score	(2.42)	(2.45)	(4.00)	(2.36)	(1.86)	(1.79)
(0–34)						
Morning PEFR	322	365	347	310	328	332
(l/min)	(54.7)	(47.2)	(53.5)	(91.0)	(80.0)	(88.4)
Nocturnal PEFR	317	352	326	294	330	302
	(65.2)	(67.8)	(65.9)	(107.8)	(90.4)	(133.4)
FEV ₁ %pred	92	101	89	88	90	86
(%)	(12.4)	(13.3)	(10.6)	(9.4)	(10.2)	(10.6)
FEV ₁ /VC	81	86	79	80	82	80
(%)	(7.4)	(9.4)	(9.1)	(9.4)	(7.5)	(6.5)
² logPC ₂₀	1.17	3.15	1.44	0.89	1.92	1.26
(mg/ml)	(1.085)	(1.242)	(1.084)	(1.615)	(1.517)	(1.793)

Visit 2 = baseline; visit 5 = end treatment; visit 6 = end follow-up.

Number of participants: visit 2–15 FP and 19 placebo; visit 5 and 6: 15 FP and 15 placebo.

Table 3. Laboratory parameters in FP group (FP) and placebo group (P). (mean values $\pm$ SEM)

Visit number	2	5	6	2	5	6
Group	FP	FP	FP	P	P	P
Parameter (SEM)						
Eosinophils (/ μ l)	638 (89.8)	689 (107.9)	761 (105.6)	721 (88.6)	726 (101.4)	732 (100.7)
ECP (μ g/l)	11.5 (.93)	10.4 (1.34)	12.1 (2.65)	17.7 (4.19)	13 (1.93)	13.1 (1.98)
EDN _s (μ g/l)	36.4 (3.05)	32.2 (2.62)	39.2 (4.93)	45.5 (8.1)	40 (4.78)	40.3 (4.06)
EDN _u (μ g/l)	2324 (282.2)	1568 (251.0)	2534 (446.9)	1523 (282.8)	1735 (330.0)	2141 (347.3)

Visit 2 = baseline; visit 5 = end treatment; visit 6 = end follow-up.

Number of participants: visit 2–15 FP and 19 placebo; visit 5 and 6–15 FP and 15 placebo.

the two intervention groups was not significant ($P = 0.10$). When urinary EDN was expressed per mmol (urine) creatinine, the difference between the FP group and the placebo group was not significant ($P = 0.07$) (Fig. 1). After cessation of FP urinary EDN increased in both intervention groups, especially in the FP group where the increase was significant ($P < 0.05$).

Correlation laboratory parameters and lung function parameters

No relationship was found between number of eosinophils, IgE, the concentration of N^T-methyl-histamine, serum ECP and EDN and urinary EDN and lung function (i.e. VC, FEV₁, PC₂₀-histamine and reversibility of FEV₁).

Discussion

In this placebo controlled study in children with moderate stable asthma we did not observe a significant change in the parameters of inflammation after three months treatment with FP 100 μ g twice daily.

Results on the course of inflammation parameters during corticosteroid treatment are conflicting. In agreement with the present study, Zimmerman reported increased baseline values for the number of eosinophils, serum ECP and EDN in asthmatic children compared to healthy controls (14, 15). However, slightly higher baseline values for serum ECP and EDN compared to the present study were reported in asthmatic children (14, 16). The baseline values of urinary N^T-methyl-histamine reported by others were comparable to our results (17). In contrast with our observations, a decrease in eosinophils was shown in adults with stable asthma, treated with inhaled budesonide 800 μ g daily for 4 weeks, and in children with stable mild asthma, treated with inhaled budesonide 1600 μ g daily for 2

weeks (18, 19). A decrease in ECP and EDN during treatment with inhaled or oral corticosteroid was shown in asthmatic children who were either symptomatic or treated with inhaled budesonide 800 μ g daily (14, 15). The discordance between these studies and the present study may be caused either by a difference in the severity of asthma or by a difference in the dosage, the route of administration or the type of corticosteroid used. Furthermore, with respect to ECP it is possible that differences in results may be attributable to a difference in the handling of blood specimens. It has been suggested that the concentration of ECP released during the clotting procedure is temperature dependent, but the various reports on this issue are not conclusive (20, 21). Since blood specimens are not always kept at similar laboratory temperatures, the data on ECP of different

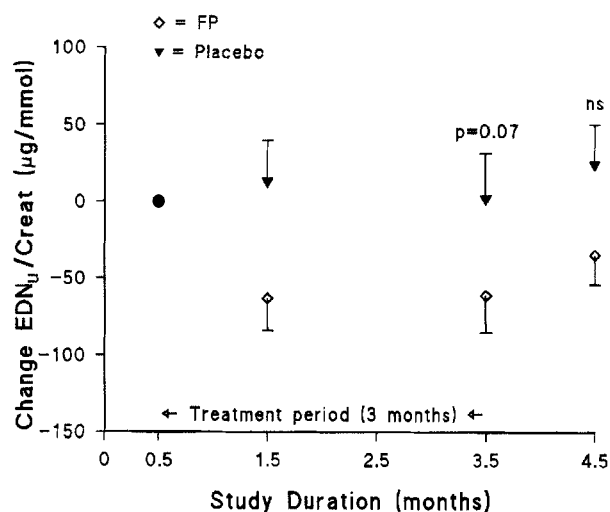


Fig. 1. The change in fasting urinary concentration of EDN ($\pm$ SEM) in the FP group (open symbols) and in the placebo group (closed symbols).

laboratories cannot be easily compared.

The major question resulting from this study is how to interpret the absence of a change in laboratory parameters during the use of FP, while symptom scores decreased, lung function increased and bronchial hyperresponsiveness increased. Although there is ongoing discussion about the precise meaning of bronchial hyperresponsiveness (22), it is generally assumed that a decrease in bronchial hyperresponsiveness reflects a decrease in inflammation (23). Therefore, the decrease in bronchial hyperresponsiveness during treatment with FP may indicate a decrease in airway inflammation. Several explanations can be given for the absence of a change in blood inflammation parameters during this study. Firstly, this study may indicate that treatment with FP 100 µg bd has a local rather than a systemic effect on the inflammatory process. Comparable to our findings, Levy et al. found that parameters of cellular immunity in children with asthma did not change during prolonged daily use of 600 µg inhaled beclomethasone dipropionate (24). Secondly, it is possible that changes in the inflammatory process are not well reflected in peripheral blood. Induced sputum and bronchoalveolar lavage may be more promising alternatives, because they may better reflect the target organ, although these techniques cannot easily be applied in children for ethical reasons. Lastly, the number of children may have been too small to find a change in the inflammatory parameters that were studied. A power calculation could not be made due to the absence of normal values of ECP and EDN in children at the time the study was initiated. Therefore, insufficient power cannot entirely be excluded.

This study suggests there is a discrepancy between the course of serum and urinary concentration of EDN during the treatment period. While no change in EDN_s was seen, results on EDN_u, after correction for creatinine, showed a decrease during treatment with FP, although statistical significance was not reached ($P = 0.07$). It is interesting to hypothesize why a change in EDN_u would be observed, while EDN_s remained constant during treatment. The first source of serum EDN may be the content of the eosinophils which is released in the serum during the process of clotting. Secondly, the physiologic process of eosinophil turnover in the body contributes to the level of EDN_s. And lastly, leakage from the target organs, after eosinophil degranulation takes place, can be a source of EDN_s. The former source seems to be the major contributor, since plasma levels of ECP and EDN are very low. (Data not shown in this study) Thus, EDN_s seems to be merely the result of an *in vitro* effect, reflecting eosinophil priming. On the other hand EDN_u is not related to the clotting process. It can be

speculated that EDN_u is derived from eosinophil turnover, plus leakage from the target organs. It can thus be hypothesized that EDN_u is a more accurate reflection of the inflammatory process in the lung compared with EDN_s. The significant rise of EDN_u after cessation of FP can be regarded as supportive evidence for the use of EDN_u as a parameter of inflammation. Based on the present results this conclusion is preliminary and further studies on this issue are needed.

In conclusion, we found that in children with moderate asthma, blood inflammatory parameters did not change during treatment with fluticasone propionate 100 µg bd, while bronchial hyperresponsiveness decreased. This may indicate either that FP has a local rather than a systemic effect or that eosinophil and mast cell parameters in peripheral blood do not reflect asthma disease activity in children with stable moderate asthma. The results of the present study warrant further evaluation of the possible use of urinary EDN to monitor asthma.

Abbreviations and definitions

Eosinophils	Number of eosinophils in peripheral blood per µl
FP	Fluticasone propionate
VC	Vital capacity in liters
FEV ₁	Forced expiratory volume in 1 second in liters
IgE	Concentration of IgE antibody in U/l
ECP	Eosinophilic Cationic Protein in µg/l
EDN/EPX	Eosinophil Derived Neurotoxin/Eosinophil Protein X in µg/l

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