

Original article

Down-regulated IL-5 receptor expression on peripheral blood eosinophils from budesonide-treated children with asthma

Background: The expression and function of cytokine receptors on peripheral blood eosinophils (PBE) from healthy and asthmatic children are poorly characterized.

Methods: The PBE count and expression of IL-5 receptor (R) and GM-CSFR positive PBE was analyzed in nonsteroid-treated asthmatic children ($n=13$), budesonide-treated asthmatic children ($n=24$) and healthy children ($n=16$) by flow cytometry. Alterations in intracellular EG2-epitope expression were used to measure the *in vitro* responsiveness of PBE to recombinant IL-5 and GM-CSF.

Results: The PBE count was increased ($P<0.05$) in both asthmatic groups, independent of treatment, as compared to healthy children. The IL-5R expression on PBE, as well as the *in vitro* responsiveness of PBE to recombinant IL-5, was reduced ($P<0.05$), in budesonide-treated asthmatic children compared to nonsteroid-treated asthmatic children and healthy children. The proportion of GM-CSFR positive PBE and *in vitro* responsiveness of PBE to recombinant GM-CSF were not different between the groups. *In vitro* treatment with budesonide did not down-regulate the proportion of IL-5R positive PBE.

Conclusions: Budesonide-treatment of asthmatic children induces a selectively reduced IL-5R expression on PBE, concomitant with a reduced *in vitro* responsiveness of PBE to IL-5. We suggest that this budesonide-related down-regulation of the IL-5R might be a mechanism by which steroid treatment inhibits the action of IL-5 on eosinophil accumulation and activation *in vivo*.

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Asthma is characterized by episodic airway obstruction and increased bronchial responsiveness to nonspecific irritants (1). The eosinophils have been shown to be important effector cells during asthma and allergic inflammation (2) and an increased number of activated eosinophils in both peripheral blood (3–6) and airways (2, 7–10) have been noted in the allergic inflammatory response. Eosinophils contribute to the pathophysiology of asthma through the release of cytotoxic basic granule proteins like eosinophil cationic protein (ECP) (7, 11). The magnitude and nature of the activation state can be judged by different activity markers. For example, the EG2-epitope on intracellular ECP is expressed in all eosinophils and is augmented upon activation, both *in vivo* (12, 13) and *in vitro* (14).

The cytokines interleukin (IL)-5 and granulocyte macrophage-colony stimulating factor (GM-CSF) are hematopoietic growth factors with profound effects on eosinophil differentiation, activation and survival. IL-5 acts specifically on eosinophils, while GM-CSF acts on many different cell types (15). Both cytokines have been detected at the protein level in sera and bronchoalveolar lavage (BAL) fluid from asthmatic individuals (16, 17),

and as mRNA expression in the airways of asthmatics (18, 19). The biological signal from IL-5 and GM-CSF is mediated through receptors consisting of a specific ligand-binding α -chain, and a signalling common β -chain. When the β -chain associates with any of the specific IL-5 or GM-CSF receptor α -chains, a high affinity complex is formed (20, 21).

The immunopathology of atopic asthma has been proposed to be very similar, if not identical, in childhood and adult asthma, but the exact underlying cellular mechanisms of these disease entities are not clearly characterized (1, 8). However, treatment strategies are similar and inhaled corticosteroids constitutes a first line therapy in both asthmatic children and adults (8, 22). It has previously been shown that budesonide treatment suppress eosinophilic airway inflammation (23–25), and decreases both the number and the state of activation of peripheral blood eosinophils (PBE) in adults as well as in children (6, 22, 24). In line with these data is also the observation that withdrawal of inhaled steroids in adults results in an augmented PBE count (26).

The aim was to compare PBE counts and expression

of IL-5 and GM-CSF receptor positive eosinophils, between nonsteroid-treated asthmatic children, budesonide-treated asthmatic children and healthy children. We also investigated whether the *in vitro* responsiveness of PBE to recombinant human IL-5 and GM-CSF differed between these three groups.

Material and methods

Subject characterization and study design

This study was performed off-pollen season from October to the beginning of April in two consecutive years. Children (age 7–16) with a positive history of mild-to-moderate IgE-mediated asthma (27), and allergy to animal dander participated in the study (Table 1). All children had a forced expiratory volume in one second (FEV₁) over 80% of calculated normal value combined with positive skin prick test (SPT) (Soluprick 10.000 SQ) (ALK, Hørsholm, Denmark) for cat and/or dog. The budesonide-treated asthmatic children ($n=24$) had been treated with inhaled steroids (budesonide 200 or 400 µg/day) for more than 3 months. The nonsteroid-treated asthmatic children ($n=13$) had been off budesonide for more than 3 months. Age-matched healthy children with negative SPT for cat, dog, horse, house-dust mite, moulds, birch, grass and mugwort served as controls (Table 1). Blood samples were collected from patients and controls. This study was approved by the local ethics committee. All children and parents gave their informed consent.

Preparation of peripheral blood leukocytes

Peripheral blood from asthmatic children and healthy children was collected in tubes containing EDTA (Vacutainer, 5 ml, with 50 µl of 21% EDTA; Terumo, Leuven, Belgium). Peripheral blood (150 µl) was haemolyzed in 3 ml + 4°C isotonic lysing solution (154 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.2). The suspensions were incubated for 5 min at + 15°C and then centrifuged at 300 g for 6 min at + 4°C. The leukocytes were washed with 2 ml + 4°C PBS-EDTA at 300 g for 5 min and kept on ice.

In vitro stimulation of leukocytes with recombinant IL-5 or GM-CSF

Mixed leukocytes from 150 µl haemolyzed blood were resuspended in 100 µl of 100 ng/ml recombinant human IL-5 (Immunokontakt, Frankfurt, Germany), or 200 ng/ml recombinant GM-CSF (Immunokontakt) diluted in HEPES (10 mM)-buffered RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% heat-inactivated calf serum (RPMI). As a control, leukocytes were incubated in 100 µl RPMI only. The suspensions were incubated for 60 min at + 37°C and washed twice in PBS-EDTA.

In vitro incubation of peripheral blood with budesonide

Whole blood samples from three untreated adult atopic patients were incubated in 24-well plates (800 µl blood/well) with or without budesonide (10^{-6} M and 10^{-9} M) (AstraZeneca R & D, Lund, Sweden), for 2 h and 20 h at + 37°C in 5% CO₂. The suspensions were washed in PBS-EDTA, haemolyzed in lysing solution (see above), followed by one additional wash.

Immunofluorescence staining of leukocytes

Surface antigens were immunostained prior to FOG treatment (see below), by adding the respective monoclonal antibodies (mAb) to isolated leukocytes from 150 µl blood. Leukocytes were treated by the FOG-method before staining in order to analyze intracellular ECP. Antibodies used were: nonconjugated mAb against interleukin-5 receptor α (IL-5R) (10 µg/ml) (Clone: A14), GM-CSFR (20 µg/ml)

Table 1. Characteristics of healthy and asthmatic children

	Healthy children	Nonsteroid-treated asthmatic children	Budesonide-treated asthmatic children
<i>n</i>	16	13	24
Mean age (years)	13.3	11.7	11.3
Sex (males/females)	6/10	5/8	13/11
Budesonide (400/200 µg/day)	0	0	19/5
Positive SPT for cat	0	13	24
Positive SPT for dog	0	11	22
PBE count/µl (medians)	247	597*	528*

* $P < 0.05$, as compared to healthy children.

(Clone: SCO6) (Beckman Coulter Inc., Fullerton, CA) and fluorescein isothiocyanate (FITC)-conjugated mAb EG2 (40 µg/ml) (Clone: 593) (Pharmacia & Upjohn, Uppsala, Sweden). Cells were incubated with antibodies for 30 min at + 4°C, and then washed in PBS-EDTA. Leukocytes incubated with nonconjugated mAb were secondarily immunostained with FITC-conjugated rabbit anti-mouse immunoglobulin, F(ab')₂ (50 µg/ml) (Code: F313) (DAKO A/S), for 30 min at + 4°C and washed with PBS-EDTA. To determine the nonspecific binding, isotype-matched control antibodies were used in corresponding concentrations. Cells were finally diluted in 0.5 ml PBS-EDTA and analyzed by flow cytometry.

Cell membrane fixation and permeabilization (the FOG method)

Leukocytes were fixed and permeabilized according to the FOG method, as previously described (5, 28, 29), to enable detection and analysis of eosinophils in unseparated blood by flow cytometry and to measure surface and intracellular antigens.

Analysis by flow cytometry

The final preparations of leukocytes were analyzed in an EPICS XL-MCL (Beckman Coulter) flow cytometer. Leukocyte subpopulations were detected as well separated populations based on their different light-scattering properties, as previously described (5). Eosinophil identification was based on their high scatter signals and the expression of CD9 and EG2-epitope on intracellular ECP (12, 29). Discrimination gates were set around the eosinophil cell population and a minimum of 1000 cells was analyzed. Isotype-matched control antibodies were used to define the cut-off for positively labeled cells. The quantitative level of IL-5R and EG2 was expressed as mean fluorescence intensity (MFI) ratio and MFI, respectively. MFI ratio was calculated by dividing MFI for the specific antibody binding with MFI for the nonspecific antibody binding. The flow cytometer was calibrated daily with Flow Check and Flow Set (Beckman Coulter).

Statistics

Nonparametric methods were used; data were first analyzed by one-way ANOVA, followed by Mann-Whitney *U*-test between groups and Wilcoxon paired within groups. $P < 0.05$ were considered significant. Results are presented as medians (interquartile ranges), mean $\pm$ SD and MFI.

Results

Eosinophils in peripheral blood

The absolute number of PBE was significantly higher in nonsteroid-treated asthmatic children, 597 PBE/µl (212–684) ($P < 0.01$) and in budesonide-treated asth-

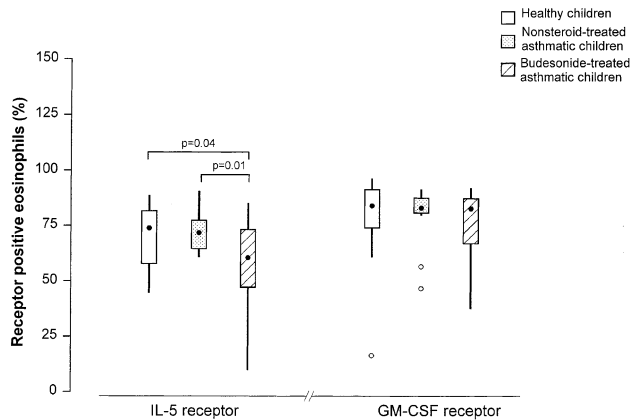


Figure 1. Proportion of IL-5R and GM-CSFR positive eosinophils in peripheral blood from healthy children ($n=16$), nonsteroid-treated asthmatic children ($n=13$) and budesonide-treated asthmatic children ($n=24$). Results are presented as median, interquartile range, min/max and outlier (open circle).

matic children, 528 PBE/ μ l (330–716) ($P<0.001$) as compared to healthy children, 247 PBE/ μ l (139–293) (Table 1).

Surface expression of IL-5R and GM-CSFR on PBE

In budesonide-treated asthmatic children, the proportion of IL-5R positive PBE was significantly decreased as compared to nonsteroid-treated asthmatic children ($P=0.01$) and healthy children ($P=0.04$) (Fig. 1). In addition, the MFI ratio for the IL-5R expression on PBE was significantly decreased in budesonide-treated asthmatic children, 1.6 MFI (1.5–1.7) when compared to nonsteroid-treated asthmatic children, 1.7 MFI (1.6–1.8) ($P=0.04$) and healthy children, 1.8 MFI (1.6–1.9) ($P=0.02$). No differences were found between the groups when the proportion of GM-CSFR positive PBE was analyzed (Fig. 1).

Budesonide (10^{-6} M) did not down-regulate the proportion of IL-5R positive PBE when peripheral blood cells were incubated for 2 h ($64\% \pm 19$) and for 20 h ($52\% \pm 11$), as compared to the control ($61\% \pm 16$, $51\% \pm 17$, respectively). No down-regulation was observed at a concentration of 10^{-9} M, when cells were incubated for 20 h ($74\% \pm 6$), as compared to the control ($51\% \pm 17$).

No correlations were found between the proportion of IL-5R and GM-CSFR positive PBE and asthma severity, which was defined as budesonide dosage and FEV₁ predicted (data not shown). These data should be interpreted carefully due to the low number of subjects studied.

PBE response to recombinant IL-5 and GM-CSF *in vitro*

The EG2-epitope on intracellular ECP was analyzed before and after *in vitro* stimulation with recombinant human IL-5 and GM-CSF. No significant differences

were found between nonsteroid-treated asthmatic children, budesonide-treated asthmatic children and healthy controls after incubation with buffer (RPMI) alone (Fig. 2). Recombinant IL-5 and GM-CSF induced a significantly increased EG2-epitope expression in PBE from nonsteroid-treated asthmatic children ($P=0.01$, $P=0.001$) and healthy controls ($P=0.003$, $P=0.001$), as compared to RPMI alone (Fig. 2). In contrast, in budesonide-treated asthmatic children, GM-CSF, but not IL-5, induced significantly ($P<0.001$) increased EG2-binding. A weak correlation ($r=0.45$, $P=0.03$) was found in budesonide-treated asthmatic children between the change in EG2 expression by recombinant IL-5 and the proportion IL-5R positive PBE.

Discussion

The PBE count was significantly higher in asthmatic children, independent of treatment, as compared to healthy controls. The IL-5R expression on PBE was significantly lower in budesonide-treated asthmatic children, when compared to nonsteroid-treated asthmatic children and healthy children. In addition, only in budesonide-treated asthmatic children, the *in vitro* responsiveness of PBE to recombinant IL-5, judged as EG2-epitope expression, was reduced. No differences in the proportion of GM-CSFR positive PBE and PBE responsiveness to recombinant GM-CSF were observed between the different groups.

In this study, we found an increased number of PBE in both nonsteroid-treated asthmatic children and budesonide-treated asthmatic children when compared to healthy children. This is in line with previous studies showing that asthmatics have increased numbers of PBE (3–6). However, the PBE count was not down-regulated in budesonide-treated asthmatic children as compared to nonsteroid-treated children, which is in contrast to previous studies showing that budesonide treatment decreases the number of PBE (6, 22, 24). We suggest that the differences between the studies might be explained by patient selection and individual dose-responses to budesonide.

Important factors that influence the outcome of eosinophil activation and survival are the cytokines IL-5 and GM-CSF (15). Since these cytokines exert their action by binding to the IL-5R and GM-CSFR, studies of the expression of these receptors on PBE from asthmatic children, and the impact of budesonide-treatment, are of significant importance. We found that the IL-5R expression was decreased on PBE from budesonide-treated asthmatic children, as compared to nonsteroid-treated asthmatic children and healthy children. However, no differences were found in the proportion of GM-CSFR positive PBE between these three groups. These results suggest that budesonide-

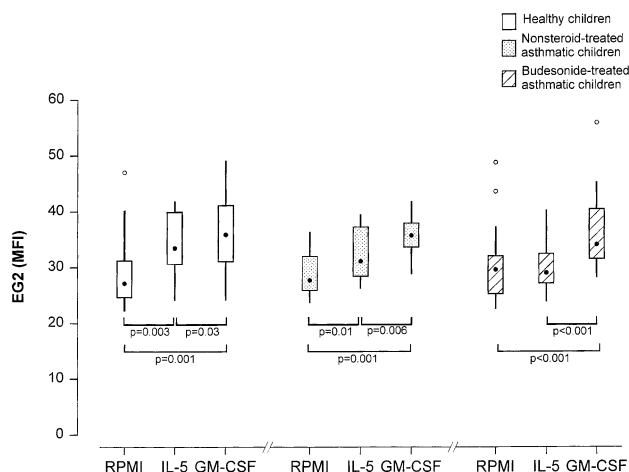


Figure 2. Expression of intracellular EG2-epitope on ECP (EG2) in peripheral blood eosinophils from healthy children ($n=16$), nonsteroid-treated asthmatic children ($n=13$) and budesonide-treated asthmatic children ($n=24$) after stimulation with RPMI, recombinant IL-5 (100 ng/ml) or GM-CSF (200 ng/ml) at 37°C for 60 min. Results are presented as median, interquartile range, min/max and outliers (open circles).

treatment down-regulates the IL-5R on PBE from asthmatic children. These data are in line with other studies in adults showing that there are no differences in IL-5 and GM-CSF receptor expression on PBE between atopic subjects and healthy subjects (30).

To further examine our finding we incubated whole blood from untreated atopic patients with budesonide *in vitro*. In this experiment budesonide did not down-regulate the proportion of IL-5R positive PBE, suggesting that the down-regulated IL-5R is not a direct effect of budesonide, but mediated through indirect mechanisms acting *in vivo*. Indeed, other studies have demonstrated that steroid treatment inhibits the increased IL-5 mRNA production observed in asthmatics (31, 32), and this might contribute to a down-regulated IL-5R. Interestingly, it has previously been shown that the IL-5 receptor can be dynamically regulated. For example, IL-5 has been shown to up-regulate the IL-5R on cord blood cells (33), and *in vitro* activation of eosinophils with recombinant GM-CSF has been shown to down-regulate the IL-5R (34). Moreover, migration of eosinophils from peripheral blood to the peritoneal cavity down-regulates the proportion of IL-5R positive eosinophils (35). A decreased expression of the IL-5R on PBE could be the result of a switch from the membrane-bound form of the receptor to the soluble form, which neutralizes the effects of soluble IL-5 (36, 37). We suggest that the budesonide-related down-regulation of IL-5R observed

in our study might be a mechanism by which steroid treatment inhibits the action of IL-5 on eosinophil accumulation and activation *in vivo*.

Eosinophil responsiveness to stimuli is poorly characterized in asthmatics and little is known about the effects of corticosteroid-treatment. Since the IL-5R was down-regulated in budesonide-treated asthmatic patients we hypothesized that the PBE from these patients had an altered responsiveness towards selected stimuli. Indeed, an important finding in our study was that recombinant IL-5 did not induce an up-regulation of the EG2-epitope in PBE from budesonide-treated asthmatics, whereas recombinant GM-CSF did. In contrast, in PBE from nonsteroid-treated asthmatic children and healthy controls both IL-5 and GM-CSF induced a significant up-regulation of the EG2-epitope expression. These results suggest that budesonide treatment induces a reduced PBE responsiveness towards IL-5. The biological consequence of having circulating eosinophils with a low responsiveness to IL-5 can only be speculated on, but one might hypothesize that it could result in decreased PBE activation and transmigration to inflammatory foci, since IL-5 has been shown to be important for these actions in eosinophils (15). This hypothesis is in line with other studies showing that budesonide decreases the activation and infiltration of eosinophils into the airways of asthmatics (23–25). In addition, since IL-5 is important for eosinophil survival (38) the existence of IL-5 low-responding eosinophils may result in decreased survival. Indeed, budesonide and other glucocorticoids have been shown to induce apoptosis in eosinophils (38, 39).

In conclusion, our data suggest that budesonide-treatment induces down-regulation of the IL-5R on PBE concomitant with decreased *in vitro* responsiveness of PBE to IL-5. However, budesonide treatment did not effect the proportion of GM-CSFR positive PBE or the responsiveness of PBE to GM-CSF. We suggest that our results describe a potential mechanism by which budesonide could act *in vivo*, and this mode of action might explain the inhibition of eosinophil activation and decreased eosinophil survival, observed in budesonide-treated asthmatics.

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