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ORIGINAL ARTICLE

## The Anti-inflammatory Effects of Inhaled Corticosteroids versus Anti-leukotrienes on the Lymphocyte P-Glycoprotein (PGP) Expression in Asthmatic Children

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**Background.** Inhaled corticosteroids (ICS) are used in asthma therapy for their anti-inflammatory effects. P-glycoprotein (PGP) is a transmembrane efflux pump for many drugs, including corticosteroids. Expression of PGP is associated with therapy resistant disease. **Objective.** The purpose of this study was to compare expression levels of PGP in blood lymphocytes of pediatric asthma patients either on ICS or on the leukotriene inhibitor montelukast medication. **Patients and Methods.** The evaluation of lymphocyte PGP expression was performed on a sample of 99 children (66 boys and 33 girls) aged 2–18 (median 12) years with intermittent or persisting mild asthma, (as defined by GINY 2002). The asthmatic children were divided into 3 groups: (1) treated by ICS budesonide 200–400  $\mu\text{g}$  per day ( $n = 27$ ) more than 1 year; (2) treated by oral montelukast 4 or 5 mg at an age-based dose ( $n = 16$ ); and (3) treated by inhaled corticosteroids and montelukast ( $n = 45$ ). Reference PGP values were obtained from a group of 64 healthy children (23 boys and 29 girls) aged 2–15 years (median, 10 years). The expression of lymphocyte PGP was determined on fixed and permeabilized blood mononuclear cells using indirect immunofluorescence staining technique by flow cytometry modified according to Boer et al., 1997. **Results.** Based on the weighted medians for PGP expression in peripheral blood lymphocytes, we found a significant difference ( $p < 10^{-3}$ ) between the group of asthma patients ( $n = 99$ ), ( $619 \pm 5.3$ ) and healthy controls ( $n = 64$ ) ( $446.9 \pm 4.4$ ). Second, there was a lower level of PGP expression in the group treated by ICS ( $548.6 \pm 9$ ) than the group treated by montelukast and montelukast with budesonide ( $643.2 \pm 6.3$ ) ( $p < 10^{-6}$ ). **Conclusions.** The anti-inflammatory activity of ICS is more effective in decreasing the production of pro-inflammatory mediators and results in reduced multidrug resistance (MDR-1) gene activity and expression of lymphocyte PGP in asthmatic children.

**Keywords** bronchial asthma, children, P-glycoprotein, inhaled corticosteroids, montelukast

### INTRODUCTION

The main pathogenic mechanism of bronchial asthma (AB) is a special form of chronic eosinophil-based inflammation of the bronchial mucosa. The inflammatory process involves increased expression of a wide variety of pro-inflammatory chemokines, cytokines, growth factors, lipid mediators, adhesion molecules, enzymes and increased resident and invading inflammatory cells. This milieu leads to persistent or recurrent symptoms that require daily anti-inflammatory treatment to control the symptoms and life quality. Inhaled corticosteroids (ICSs) and systemic leukotriene receptor antagonists are widely used for this purpose. Although current anti-asthma therapy is highly effective, individual patients respond differently to identical drugs and there is still a significant proportion of children with unstable and/or therapy resistant disease (1).

However, the successful pharmacotherapy of various diseases including cancer and inflammatory diseases is often limited by the phenomenon of multidrug resistance which is particularly important in “barrier tissues” like the lungs, trachea, stem and some immune cells that have been shown to have high transcriptional activity for a variety of

ATP-binding-cassette (ABC) transporters, for instance P-glycoprotein (PGP)(2). PGP is a glycoprotein consisting of approximately 1280 amino acids coded by the multidrug-resistant gene ABCB1 (MDR1;7q21.12)(3). In the lungs, PGP is expressed on the apical side of ciliated epithelial cells or ciliated collecting ducts, and on apical and lateral surfaces of serous cells of the bronchial glands but not in mucus-secreting goblet cells. PGP is a transmembrane efflux pump for different endogenous substrates, exotoxins, lipophilic drugs, corticosteroids including the budesonide used in our study (4). Thus, upregulation of PGP may provide another explanation for reduced glucocorticosteroid response complementary to more frequently described factors such as the functionality of glucocorticoid receptors, impaired pathways and tissue glucocorticosteroid accessibility (5). In addition to PGP, another ABC transporter, the multidrug resistant protein 1 (MRP1), is involved in transportation of key AB inflammatory mediators, leukotrienes (6). It is also known that expression of PGP and MRP1 is regulated by numerous stimuli, such as heat shock and irradiation which both induce cellular stress, cytokine release and/or inflammation (7, 8).

Another important AB therapeutic approach is based on cell response modulation to leukotrienes. Leukotrienes are inflammatory lipid mediators, generated by lipoxygenation of membrane-bound arachidonic acid, which act via specific membrane receptors expressed on target cells.

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One of the leukotriene receptors, cysteinyl leukotriene receptor 1 (CysLTR1), occurs on the surface of mast cells, macrophages, smooth muscle cells, monocytes, B-lymphocytes, as well as eosinophils (9), where it activates the cells upon exposure to leukotrienes. In order to prevent proinflammatory activities of LTs mediated via CysLTR1, its competitive agonist called montelukast (MK571) was developed and it is clinically successful (10, 11).

To better understand the role of MDR transporters in AB, we studied the effects of the inhaled corticosteroid budesonide and the CysLTR1 antagonist montelukast on expression of PGP, in peripheral blood lymphocytes of asthmatic versus age matched healthy children. Both drugs are known to decrease pro-inflammatory cytokines and reduce the inflammatory response.

#### PATIENTS

Our study included a group of 99 children (66 boys and 33 girls) aged 2–18 (median 12) years with intermittent or persisting mild asthma, (as defined by GINA 2002)(12). A detailed analysis of their medical history was taken along with skin prick testing and eosinophil blood counts (Eo). Serum levels of IgE (S-IgE), specific IgE (spec.S-IgE), serum eosinophil cationic protein (S-ECP) were also determined (12). All patients had a positive skin test (wheal  $\geq 3$  mm) to at least one of the most common local aeroallergens (house-dust-mites, grass pollen, birch pollen, cat and dog dander and/or mould mixture) and they were referred to our department between 2003 and 2005. Concomitant drugs e.g., antibiotics – rifampicin, that are known to induce PGP expression, vitamins and dietary supplements were withheld for at least 1 month prior to blood collections. Since the budesonide, is a dual substrate for P-glycoprotein and cytochrome P450 3A (CYP3A), the children were also not permitted to ingest fresh grapefruit or grapefruit juice one month before inclusion in the study, as they are known to affect cytochrome P450 3A activity (13).

The asthmatic children were divided into three groups: (i) treated by inhaled budesonide 200–400  $\mu\text{g}$  per day ( $n = 27$ )  $\geq 1$  year; ii) treated by montelukast 4 or 5 mg at an age-based dose ( $n = 16$ ) for  $\geq 1$  year; iii) treated with ICSs and montelukast ( $n = 45$ )  $\geq 1$  year. The control expression of lymphocyte PGP was also determined in 64 age matched healthy children (29 boys and 35 girls) aged 2–15 years (median 10), who had no history of allergy or medication use. None of the healthy children had been previously treated with ICSs and/or montelukast. The same examinations were conducted in healthy controls as in asthmatic patients.

#### METHODS

##### *Flow Cytometry Analysis of PGP Expression*

Briefly, PBL were separated by density centrifugation on Ficoll Hypaque (density 1.077 g/ml; Pharmacia Sweden) and washed twice in  $1 \times$  phosphate-buffered solution (PBS). After the second wash, the cell pellet was fixed in ice cold methanol and stored in the freezer at  $-20^\circ\text{C}$ . Expression of PGP on fixed and permeabilized cells was analyzed by flow cytometry indirect immunofluorescence staining technique modified according to Den Boer et al. (14). Fixed cells were washed with PBS, permeabilized with permeabilizing

solution (FACS Permeabilizing solution 2, Becton Dickinson), washed in PBS with 0.5% BSA + 0.1% NP-40 and blocked in solution containing FC receptor saturation reagent (Immunotech).

Another washing step was followed by incubation with specific mouse monoclonal antibodies against PGP (clone F4, Sigma) and isotypic control of mouse IgG1 (Sigma). Antibodies were used in a concentration of  $0.5 \mu\text{g}/10^6$  cells/100  $\mu\text{l}$  of staining solution. The secondary anti-mouse fluorescein labelled F(ab')<sub>2</sub> antibody fragment (Sigma) was then applied. Cells were analyzed by FACS Calibur flow cytometry and Cell Quest software, where percentage and median of PGP positive cells were included in the analysis. All statistical methods were based on weighted moments, i.e., median of PGP-expression weighted by percentage of positive cells.

Blood eosinophils were examined using an automatic GenS<sup>TM</sup> System 2 device by Coulter, USA and Sysmex<sup>TM</sup> device by Sysmex Corporation, USA.

Nephelometry was used for total S-IgE determination. S-IgE values  $> 150$  IU/ml were defined as significant for allergy.

Serum ECP was determined using a fluorescent enzyme immunoassay (FEIA) CAP System (Pharmacia, Sweden). Blood for ECP was collected into Vacutainer test tubes with activator (Beckton-Dickinson, England). Samples were incubated for 60–120 minutes at room temperature, subsequently centrifuged and the aspirated serum was frozen prior to complete processing. S-ECP  $< 15 \mu\text{g/l}$  was defined as physiological. Allergen-specific serum IgE concentration was determined a fluorescent enzyme immunoassay (FEIA) CAP System (Pharmacia, Sweden). Positive concentration for specific IgE was 0, .35 IU/ml.

##### *Statistical Analysis*

STATISTICA 7, StatSoft CR was used for statistical analysis. Comparisons between groups were made using unpaired Student's *t*-tests and Pearson correlation coefficients were calculated. A  $p < 0.05$  was considered statistically significant.

#### RESULTS

The study groups included 99 asthmatic children with intermittent or persisting mild bronchial asthma and 52 healthy age matched controls. Quantification of PGP expression on human lymphocytes via weighted fluorescence intensity means (intensity fluorescence weighted by percentage of positive cells) was highly reproducible with minimum variation across individual patients in the same group, since the standard error was below 10% of the mean in most groups.

The PGP expression in peripheral blood lymphocytes was substantially increased ( $p < 10^{-3}$ ) in bronchial asthma patients ( $n = 99$ ,  $619 \pm 5.3$ ) compared to healthy controls ( $n = 64$ ,  $446.9 \pm 4.4$ ). Interestingly we found a highly significant difference in the lymphocyte PGP expression in children treated with the inhaled corticosteroid budesonide ( $n = 27$ ,  $548.6 \pm 9$ ) and the PGP expression in the group treated with montelukast and montelukast with budesonide ( $n = 72$ ,  $643.2 \pm 6.3$ ,  $p < 10^{-6}$ ) (Figure 1).

We found an association between age and PGP expression in peripheral blood lymphocytes in the group of asthmatic

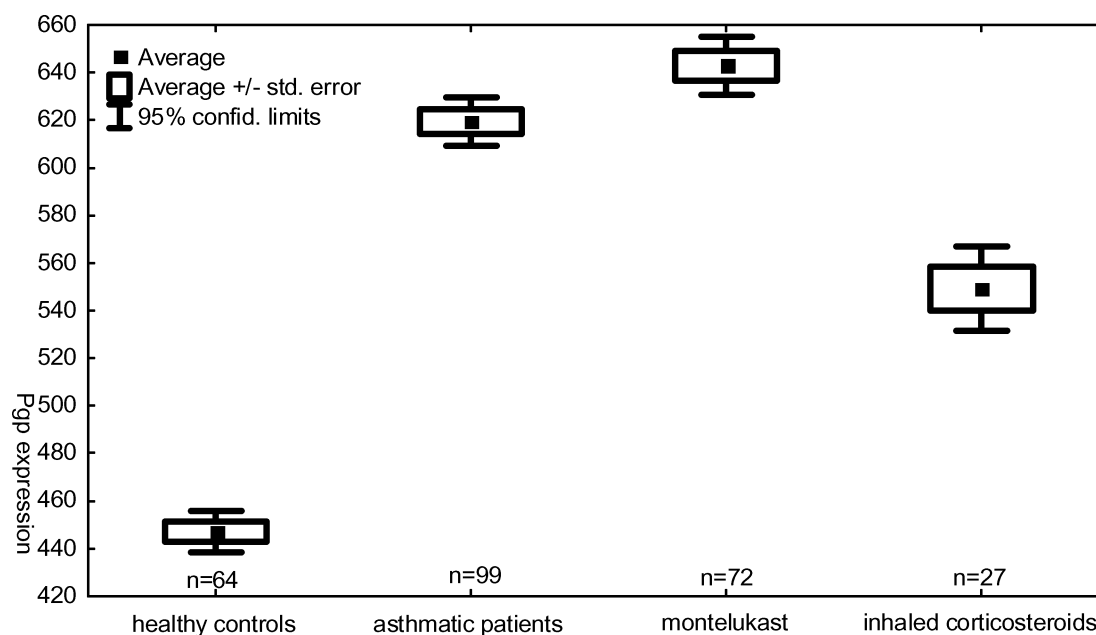


FIGURE 1.—Based on the weighted of PGP expression on peripheral blood lymphocytes we proved significant difference ( $p < 10^{-3}$ ) for group of asthma patients ( $n = 99$ ), ( $619 \pm 5.3$ ) and healthy controls ( $n = 64$ ) ( $446.9 \pm 4.4$ ) and lower level of PGP-median in group treated by inhaled budesonide ( $548.6 \pm 9$ ) compared to the group treated by montelukast and montelukast with budesonide weighted median of PGP expression ( $643.2 \pm 6.3$ ) ( $p < 10^{-6}$ ).

patients ( $r = 0.09$ ,  $p < 10^{-4}$ ) and controls ( $r = 0.18$ ,  $p < 10^{-4}$ ) (Figure 2).

#### DISCUSSION

ICSs effectively and reproducibly suppress the inflammatory processes in the airways of most asthmatics. The versatile anti-inflammatory activity of ICSs down-regulates most pathways involved in the synthesis of cytokines and other inflammatory mediators, with the exception of leukotrienes. Their clinical benefits include decreased asthma symptoms, fewer exacerbations, fewer hospitalizations, decreased airway hyperresponsiveness, improved pulmonary function, and decreased exhaled nitric oxide (15).

ATP-binding cassette (ABC) transporters are a family of transmembrane proteins that can transport a wide variety of substrates across biological membranes in an energy-dependent manner. Overexpression of ABC transporters such as PGP and the multidrug resistance-associated protein 1 (MRP1) were initially detected in tumour cell lines. Currently we know that ABC transporters are virtually ubiquitous and play central roles in physiology. The prominent expression of PGP and MRP1 in the human lung suggests that these transporters may be pivotal in the protection against endogenous or exogenous toxic compounds entering the lung. The delivery of pulmonary drugs to reach the site of action may also depend on the presence and activity of ABC transporters.

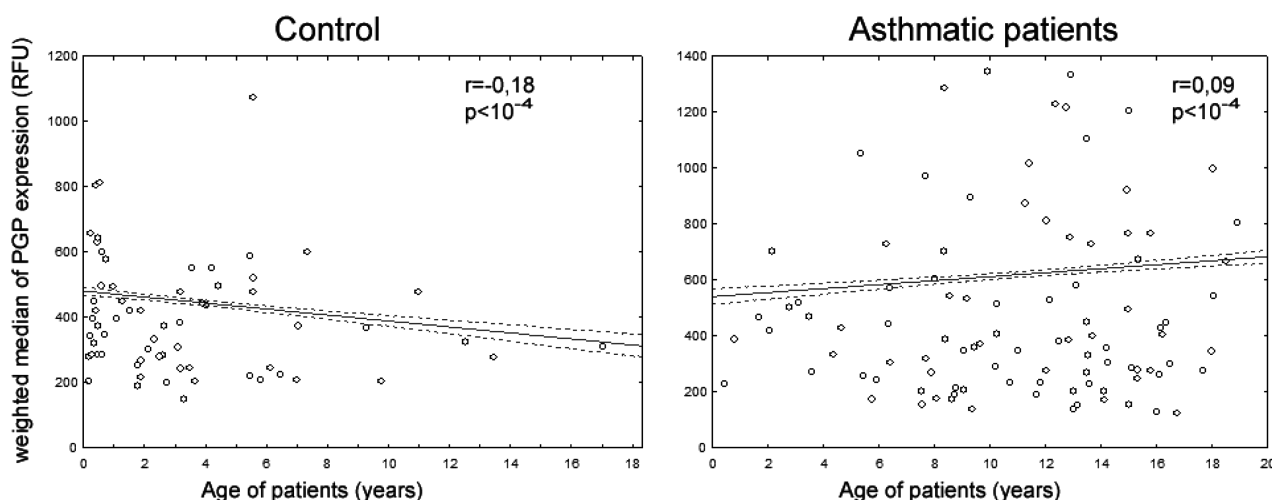


FIGURE 2.—The association between PGP expression in peripheral blood lymphocytes and age of asthmatic patients/controls.

Over-expression of PGP results in reduced intracellular concentrations of xenobiotics, drugs and poisons, such as Ritonavir and glucocorticoids. Thus, PGP appears to be a double-edged sword, involved both in protecting cells from these drugs and in the development of resistance to them (16).

Glucocorticoid insensitivity presents a profound problem in asthma patients with multidrug resistance to other therapies as well. Glucocorticoids, acting through the glucocorticoid receptor (GR), are able to selectively repress inflammatory gene expression by using several distinct mechanisms targeting nuclear factor- $\kappa$ B, activator protein-1 activation complexes and by effects on mitogen-activated protein kinases. Thus, the abnormal activation of many signaling pathways may affect corticosteroid responsiveness in patients with corticosteroid-resistant asthma. Moreover, it is possible that the stimulation of metabolism and excretion of inhaled drugs by bronchial tissues leads to reduced drug efficacy (17).

Regulation of PGP can occur at many levels including transcriptional, mRNA, protein and post-translational. The transcription of ABCB1 gene is directly regulated by the human Y box-binding protein-1, an ABCB1 transcription factor (18, 19). Alterations in the expression and activity of the PGP are seen in a large number of tissues during the inflammatory response. As the ABCB1 gene has no glucocorticoid-reactive elements in the regulatory region, mediators other than GRE may play a role.

An experimental model of acute inflammation induced by endotoxins has been shown to induce a down-regulation of the hepatic expression and activity of PGP in rats and mice. It has been hypothesized that the pro-inflammatory cytokines (IL-1 $\beta$ , IL-6 and TNF  $\alpha$ ) are involved in PGP regulation. Kuzuya et al. investigated transporter stimulation (PGP, MRP1) in bronchial cells by beclomethasone dipropionate (BPD) an inhaled steroid (20). Their study demonstrated that the BPD induced the expression of uridine 5'-diphosphate glucuronosyl transferase and transporters in bronchial epithelial cells. The molecular mechanisms of corticosteroid metabolism are in fact complicated. Transcriptional regulation, "transactivation" of gene transcription, epigenetic factors and the activity of the chronic bronchial wall eosinophilic inflammation might be implicated in the individual responses to steroid treatment (21, 22).

Based on the weighted median (median of fluorescence intensity weighted by percentage of positive cells) of PGP expression on peripheral blood lymphocytes, we demonstrated significantly higher PGP expression in asthmatic patients than healthy controls. We hypothesize that inflammatory cytokines of eosinophilic inflammation influence the activity of transcription factors to increase expression of PGP.

In our study we showed lower expression of PGP in patients treated by ICSs. We speculate that the anti-inflammatory activity of corticosteroids may have decreased the production of mediators of inflammation and decreased MDR-1 gene activity and expression of PGP.

LTs are derived from the metabolic breakdown of arachidonic acid and consist of cysteinyl leukotrienes (CysLTs) and B4 leukotrienes (LTB4). Biosynthesis of LTs requires cellular activation by which membrane phospholipids are converted into arachidonic acid by the action of a family of enzymes

termed phospholipase A2. Following activation, the enzyme 5-lipoxygenase (5-LO) translocates from the cytosol to the inner nuclear membrane where, in association with 5-lipoxygenase-activating protein (FLAP), arachidonic acid is oxygenated to form 5-hydroperoxy-eicosatetraenoic acid (5-HPETE) and then dehydrated to generate leukotriene A4 (LTA4). LTA4 is unstable, and it is converted to leukotriene B4 (LTB4) by LTA4 hydrolase; alternatively, LTA4 can be metabolized into the cysteinyl leukotriene LTC4 by the enzyme LTC4 synthase (LTC4S) or by the related enzyme microsomal glutathione transferase II. LTC4 is exported from the cell via the multi-drug transporter MRP1 where hydrolysis and the removal of the amino acid glutamate occur by the action of serum  $\gamma$ -glutamyl transferase. This gives rise to leukotriene D4 (LTD4). Collectively, LTC4, D4, and E4 constitute the CysLTs (23).

A number of studies have revealed positive actions of the cysteinyl receptor antagonist, montelukast, in children with asthma. Montelukast provides clinical benefit to patients with chronic asthma by decreasing bronchial hyper-responsiveness. Montelukast causes a statistically significant decrease in serum concentrations of cytokine, intercellular adhesion molecule (ICAM-1), eosinophil cationic protein (ECP) and peripheral blood counts (24). Recent research on the mechanism of action of CysLT1 receptor antagonists suggests they might indirectly lower leukotriene levels by decreasing leukotriene-producing inflammatory cells (25).

We were unable to determine any significant regulatory effect of montelukast on PGP expression. At this level of knowledge it is hard to say whether the observed higher level of PGP expression is due to the inflammatory process per se or treatment. We can only speculate that montelukast stimulates PGP pump expression. Further studies with patients from the start of the pharmacotherapy with follow up measurements of PGP expression are needed.

The lower lymphocyte PGP expression in the patient group treated by ICSs confirm their anti-inflammatory activity, suppression of pro-inflammatory cytokine production and mediators. Reduced expression of PGP can indirectly increase the efficacy of ICSs.

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#### DECLARATION OF INTEREST

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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