

Original article

Treatment of asthmatic patients with a cysteinyl leukotriene receptor-1 antagonist montelukast (Singulair), decreases the eosinophil survival-enhancing activity produced by peripheral blood mononuclear leukocytes *in vitro*

Background: Montelukast (Singulair, MSD) has been shown to have a beneficial effect on the clinical symptoms of asthma. We aimed to investigate the effect of montelukast treatment on the production of eosinophil survival-enhancing cytokines by peripheral blood mononuclear cells (PBMNC).

Methods: PBMNC obtained from 15 grass-allergic patients (7 treated with montelukast and 8 with a placebo) were cultured for 72 h. Eosinophils from allergic patients were cultured with MNC supernatants alone or with addition of neutralizing antibodies, and the proportion of living cells was assessed by flow cytometry. In another experiment PBMNC from 6 allergic patients were cultured *in vitro* in the presence of montelukast or vehicle. Following stimulation the production of GM-CSF in monocytes was assessed.

Results: Eosinophil survival in the MNC supernatants from the placebo-treated patients was significantly ($P < 0.05$) higher than in supernatants from montelukast-treated patients. GM-CSF was the predominant cytokine responsible for the eosinophil survival-enhancing activity (ESEA). *In vitro* production of GM-CSF by allergen-stimulated monocytes was significantly suppressed by addition of montelukast.

Conclusion: Treatment of patients with montelukast decreased the production of MNC-derived cytokines, particularly GM-CSF. We suggest that cysteinyl leukotriene receptor-1 (CysLT-R₁) antagonists may act, at least partially, by diminishing the production of GM-CSF from PBMNCs.

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Key words: cysteinyl leukotriene receptor-1 antagonist; eosinophil; GM-CSF; IL-5; montelukast; survival.

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Accepted for publication 17 June 2002

A common characteristic of allergic disease is the increased presence of eosinophils in the affected tissues and frequently a peripheral blood eosinophilia. Eosinophils produce and release cytotoxic proteins, oxygen metabolites and lipid mediators such as cysteinyl leukotrienes (CysLTs), which are considered to cause prominent tissue damage and increased bronchial hyperresponsiveness (BHR) in asthmatic patients (1). Indeed,

in vivo and *in vitro* evidence supports the role of CysLTs as important mediators of asthma; they are able to contract bronchial smooth muscle, enhance mucus secretion, increase microvascular permeability, and stimulate proliferation of fibroblasts, and smooth muscle cells (2). Montelukast is a new type of antiasthmatic drug aimed at inhibiting the effects of leukotrienes (3). This specific antagonist of cysteinyl leukotriene receptors acts by blocking CysLT receptor-1 (CysLT-R₁). In clinical studies, montelukast has been shown to attenuate both the early and late asthmatic responses (4), prevent exercise-induced bronchoconstriction (5), reduce the number of asthmatic exacerbations (6), and permit dose reduction of inhaled steroid (7). Moreover, in some studies montelukast treatment decreased the number of eosinophils in peripheral blood and sputum of asthmatic patients suggesting an antiinflammatory effect (8, 9). Only one *in vitro* study has been performed that showed an inhibitory effect of the CysLT-R antagonist pranlukast

Abbreviations: BHR: bronchial hyperresponsiveness; CysLTs: cysteinyl leukotrienes; CysLTR₁: cysteinyl leukotriene receptor-1; ELISA: enzyme-linked immunoabsorbent assay; ESEA: eosinophil survival-enhancing activity; FDA: fluorescein-diacetate; FEV₁: forced expiratory volume in 1 sec; GM-CSF: granulocyte/macrophage-colony-stimulating factor; IFN: interferon; LTC₄: leukotriene C₄; MNC: mononuclear cell; PBMNC: peripheral blood mononuclear cell; PHA-L: phytohemagglutinin (leucoagglutinin); PC₂₀: concentration of methacholine that causes a 20% fall in FEV₁; RAST: radioallergosorbent test; TNF: tumor necrosis factor.

on the production of cytokines IL-4, IL-5, and GM-CSF by MNC (10).

The amount of eosinophils circulating in the blood and accumulated in the tissue is regulated at different levels and depends on several factors including production from bone marrow (11), life span in the blood, selective recruitment from the circulation (12), and survival in the tissues (13). T-cell-, monocyte- and macrophage-derived cytokines such as IL-3, IL-5 or GM-CSF are not only greatly responsible for eosinophil accumulation and activation (14), but are also able to modulate their viability by interfering with programmed cell death (15, 16).

The aim of this study was to evaluate the production of eosinophil survival-enhancing activity (ESEA) by peripheral blood mononuclear cells (MNC) from allergic patients treated with montelukast or placebo. Further, selected cytokines with suggested ESEA in cell-culture supernatants were analyzed and the *in vitro* effect of montelukast on intracellular GM-CSF production in monocytes was determined.

Material and methods

Patients

Fifteen patients, nine men and six women, of mean age 32 (range 25–51 years) with a history of seasonal rhinoconjunctivitis and asthma (17) during last two season participated in the study. They all had a positive skin prick test, positive radioallergosorbent assay (RAST; class 2 or 3) to grass pollen and no relevant perennial allergy. The symptoms from the lower airways included wheeze, dyspnea, and exercise-induced symptoms. Seven of 15 patients received Singulair (sodium montelukast) (Merck Sharp & Dome AB, Sollentuna, Sweden), 10 mg daily for 6 weeks and the other eight patients received placebo. The only rescue medication for upper-airway symptoms were antihistamines as needed (Semprex tablets; acrivastine) and β_2 -agonists for lower airways. The groups were matched with regard to age, sex, and severity of allergic disease (size of skin prick test and *in vitro* specific-IgE levels). Blood samples (80 ml) were collected during the grass pollen season. Peripheral blood samples (30 ml) from six additional grass-allergic patients who were not part of a placebo controlled study, used for stimulation of MNC *in vitro* were also collected during grass pollen season.

This double-blind, placebo-controlled study was assessed and accepted by the Ethics Committee of Göteborg University.

MNC isolation and culture

Heparinized blood diluted 1 : 1 with phosphate buffered saline (PBS) was applied to isotonic Percoll (density 1.084 g/ml) (Amersham Pharmacia Biotech AB, Uppsala, Sweden). After centrifugation for 30 min at $1000 \times g$ the mononuclear leukocytes were carefully removed from the interface, washed in PBS and resuspended at the density of 2×10^6 cells/ml in RPMI–Hepes 20 mM medium, supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine, 100 μ g/ml gentamycin (Gibco BRL Life Technologies Ltd, Paisley, UK) and 50 μ M 2-mercaptoethanol (complete medium). The cells were cultured for 72 h at 37°C in a humidified atmosphere with 5% CO₂ in a total volume of 1 ml.

Cell suspensions were then collected, centrifuged and the supernatants were frozen at –70°C for later use in the eosinophil survival assay.

Eosinophil isolation

Eosinophils were purified from the peripheral blood of allergic patients by negative immunoselection using the MACS system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) (18,19). Briefly, heparinized blood diluted 1 : 1 with PBS was applied to isotonic Percoll (density 1.084 g/ml) (Amersham Pharmacia). After centrifugation for 30 min at $1000 \times g$ the mononuclear leukocytes and the Percoll were discarded. The erythrocytes were lysed by hypotonic shock performed using ice-cold water. Anti-CD16 MicroBeads (Miltenyi Biotec) were added to the cell pellet containing neutrophils and eosinophils and after incubation for 1 h at 4°C the cell suspension was applied to a depletion column in a magnetic field. The CD16-negative eosinophils were eluted from the column, collected by centrifugation and resuspended in complete RPMI medium (without 2-mercaptoethanol). The purity of the eosinophils was 98–99%, as assessed by May–Grunewald/Giemsa staining. The viability of the cells was 98–99%, as assessed by trypan blue exclusion.

Eosinophil survival assay

Freshly isolated eosinophils were seeded in 96-well plates and the diluted MNC culture supernatants were added to wells to a final concentration of 5% (v/v). Cells were cultured in a total volume of 100 μ l at a density of 1×10^6 cells/ml. As a control, eosinophils were cultured in complete RPMI medium alone, without addition of MNC supernatants. The eosinophils were incubated for 48 and 72 h at 37°C in a humidified atmosphere with 5% CO₂.

Cell viability was assessed by the use of fluorescein-diacetate (FDA) and propidium iodide staining. The proportion (%) of living (FDA positive) and dead (propidium-iodide positive) cells was determined using a flow cytometer (FACS Calibur, Becton Dickinson, San Jose, CA, USA). In each analysis 10 000 cells were counted. Duplicate samples were run in parallel and the results are expressed as a mean of two experiments.

Inhibition assay

In separate experiments, neutralizing antibodies against IL-5 (0.5 μ g/ml), GM-CSF (0.5 μ g/ml) (PharMingen Int., San Diego, CA, USA), IL-3 (0.25 μ g/ml), IFN- γ (0.25 μ g/ml) and TNF- α (0.25 μ g/ml) (R & D Systems Europe Ltd, Abingdon, UK) were added alone or in combination to the diluted (10%, v/v) MNC supernatants and the mixtures were then incubated for 1 h at 37°C. In parallel, supernatants were preincubated with the relevant control antibodies; specific rat IgG_{2a} antibody (PharMingen Int.) for anti-IL-5 and GM-CSF, and goat IgG antibody (R & D Systems Europe Ltd) for anti-IL-3, IFN- γ and TNF- α . The pretreated supernatants were added to freshly isolated eosinophils at a final concentration of 5% (v/v) and the cells were cultured at 37°C in a humidified atmosphere with 5% CO₂. Control eosinophils were cultured in complete RPMI medium alone, without addition of MNC supernatants. After 72 h the eosinophil viability was assessed as described above and the relative number of viable eosinophils (eosinophil survival) was calculated as the proportion (%) of FDA-positive cells.

The eosinophil survival values were obtained by subtracting the survival of control eosinophils (with medium alone) from the sur-

vival of eosinophils in the presence of supernatants. The results presented are the mean values from two experiments.

GM-CSF and IL-5 determination

The concentration of GM-CSF and IL-5 in the MNC supernatants was determined by the use of a sandwich enzyme-linked immunosorbent assay (ELISA). The commercial ELISA kits (R & D Systems Europe Ltd) were used according to the manufacturer's instructions. The sensitivity of the assays was A2.8 pg/ml and A3.0 pg/ml, respectively.

Stimulation of MNC and intracellular staining for GM-CSF

Mononuclear cells separated from peripheral blood of six grass-pollen allergic patients were resuspended in RPMI medium (supplemented with 5% FCS, glutamine and gentamicin) at the concentration of 2×10^6 cells/ml. Cells were preincubated with montelukast sodium (0.25 µg/ml, 0.5 µg/ml and 1 µg/ml) (a gift from Dr A. E. Naidoff from Merck & Co., Inc; Rahway, NJ, USA) or vehicle for 1 h at 37°C. Subsequently, grass extract 30 µg/ml (PHI p, ALK Abello, Horsholm, Denmark) was added to part of the cultures. After four hours of incubation, PHA-L (10 µg/ml) (Sigma-Aldrich, St Louis, USA) and brefeldin A (Golgi Stop, PharMingen Int.) were added to all cultures and cells were further incubated for 14 h at 37°C in a humidified atmosphere with 5% CO₂. Following culture cells were collected and washed with PBS-FCS 1% at 4°C. All further procedures were carried on ice. Fc-receptors were blocked with 1% of mouse and rat serum (Dako A/S, Glostrup, Denmark) for 30 min at 4°C and cells were preceded for surface staining with anti-CD14 antibodies. Subsequently cells were fixed with phosphate-buffered paraformaldehyde (PFA) 2% for 20 min at 4°C and permeabilized with saponin (Perm-Wash solution, PharMingen Int.). Saponin solution was also used for incubation with monoclonal PE-conjugated anti-GM-CSF antibodies (PharMingen Int.) (0.12 µg/10⁶ cells). After 30 min cells were washed, resuspended in PBS-PFA 1% and kept at 4°C until analysis on flow cytometer (FACS Calibur, Becton Dickinson).

The percentage of GM-CSF positive monocytes after allergen stimulation was calculated by subtracting the number of GM-CSF positive cells following PHA stimulation (basic level) from the numbers of monocytes producing GM-CSF cells after stimulation with allergen and PHA.

Statistical analysis

Comparison of results from the two groups of patients was performed using the Mann-Whitney *U*-test. The Wilcoxon matched-pairs test was used to evaluate the results of the inhibition assay. Correlations between different parameters were evaluated by the Spearman rank order correlation test. A *P* value of < 0.05 was considered to be statistically significant. All statistical tests were performed on a personal computer by the use of the STATISTICA software package (StatSoft Inc. Tulsa, OK, USA).

Results

Eosinophil survival assay

Culture of eosinophils with mononuclear cell supernatants significantly ($P < 0.05$) increased their survival compared with culture in medium alone. Eosinophil

survival in MNC supernatants from the placebo-treated patients was significantly enhanced both after 48 and 72 h of culture compared with survival in MNC supernatants from the montelukast-treated patients (Fig. 1).

Inhibition assay

To test the involvement of different cytokines in the increased eosinophil survival in the presence of MNC supernatants, specific neutralizing antibodies against GM-CSF, IL-5, IL-3, IFN-γ, and TNF-α were used. Preincubation with the antibody against GM-CSF (Fig. 2A) significantly and markedly decreased the ESEA of the supernatants from all patients, both placebo and montelukast-treated. The antibody against IL-5 markedly decreased the ESEA in one of the eight supernatants in the placebo group, while a minor—but significant—effect was observed in case of the other supernatants (Fig. 2B). Antibody against IFN-γ induced a marked decrease of the ESEA of one supernatant from another patient in the placebo group, and a minor but significant inhibition of the ESEA of the other supernatants from the placebo group (Fig. 2C). The addition of antibody against anti-IL-3 (Fig. 2D) as well as TNF-α (results not shown) induced a minimal decrease of the ESEA, which was significant in case of supernatants from the placebo group.

Antibody against GM-CSF induced a significantly ($P < 0.05$ – $P < 0.01$) greater decrease in the ESEA than anti-IL-3, anti-IL-5, anti-IFN-γ and anti-TNF-α, respec-

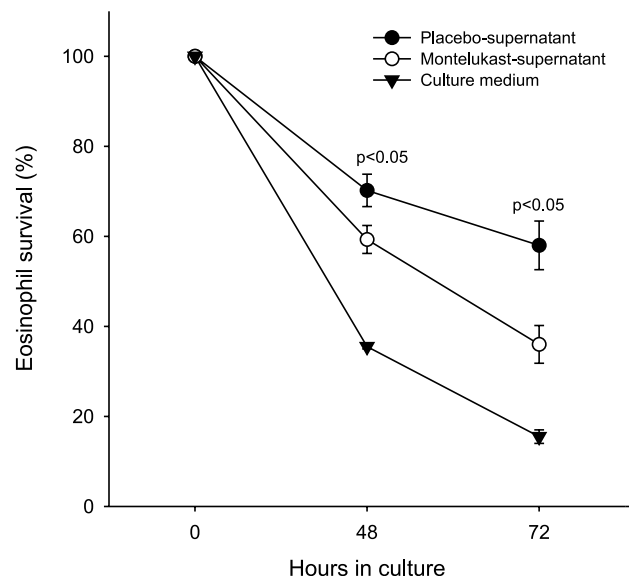


Figure 1. Eosinophil survival in (MNC) supernatants and culture medium. Significant differences between eosinophil survival-enhancing activity (ESEA) in MNC supernatants from placebo- and montelukast-treated patients are indicated. The results shown are medians $\pm$ SEM.

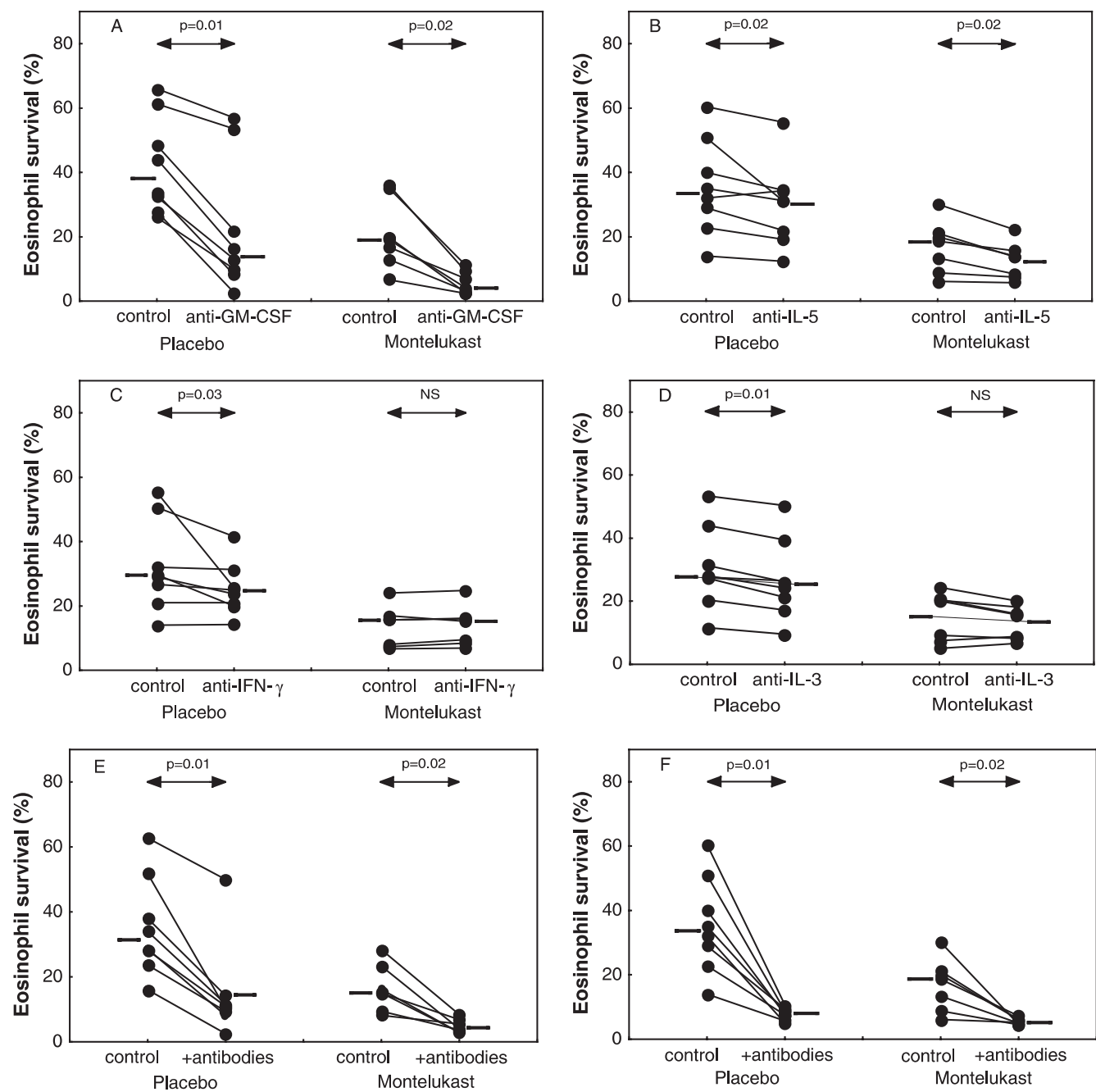


Figure 2. Eosinophil survival-enhancing activity (ESEA) in mononuclear cell (MNC) supernatants treated with control or neutralizing antibodies against (A) granulocyte/macrophage colony-stimulating factor (GM-CSF); (B) interleukin (IL)-5; (C) interferon (IFN)- γ ; (D) IL-3; (E) GM-CSF and IL-5; (F) GM-CSF, IL-5, and IFN- γ . Statistical comparisons between the effect of control antibody and the respective neutralizing antibody are indicated. Horizontal bars indicate median values. The zero level of eosinophil survival denotes the survival in culture medium only.

tively, in supernatants from all patients (Table 1). Anti-GM-CSF combined with anti-IL-5 (Fig. 2E), and the combination of anti-GM-CSF, anti-IL-5 and anti-IFN- γ (Fig. 2F) significantly and markedly decreased the ESEA of the supernatants from all patients, both placebo-

treated and montelukast-treated. The level of ESEA after treatment with the combination of anti-GM-CSF, anti-IL-5 and anti-IFN- γ was significantly ($P < 0.05$) lower than that after treatment with anti-GM-CSF alone, in supernatants from the placebo group.

Table 1. Inhibitory effect of neutralizing antibodies on the eosinophil survival-enhancing activity (ESEA) of mononuclear cell (MNC) supernatants. Δ : difference in ESEA in the presence of the control antibody compared with the respective neutralizing antibody

Antibody	Δ ESEA	
	Placebo (n = 8)	Montelukast (n = 7)
Anti-GM-CSF	22.3 (7.7–27.7)	15.1 (4.3–26.9)
Anti-IL-3	3.5* (1.1–6.1)	2.1** (– 1.6–4.6)
Anti-IL-5	4.5** (– 2.3–19.7)	5.0** (0.4–7.9)
Anti-IFN- γ	3.5* (– 0.2–29.6)	– 0.6** (– 1.5–1.8)
Anti-TNF- α	3.1** (– 0.2–9.4)	2.5** (– 1.4–4.4)
Anti-GM-CSF + anti-IL-5	18.9 (12.8–41.6)	12.1 (2.7–20)
Anti-GM-CSF + anti-IL-5 + anti-IFN- γ	26.8 (8.3–49.9)	11.4 (0.9–25.5)

The results are shown as medians and ranges. Significant differences between the inhibitory effect of anti-GM-CSF and the other antibodies as calculated by Mann–Whitney *U*-test are indicated by *P* values.

* *P* < 0.05.

** *P* < 0.01.

GM-CSF and IL-5 levels in MNC supernatants

The mean GM-CSF level in the MNC supernatants from patients receiving placebo was significantly higher than that in supernatants from montelukast-treated patients (Fig. 3). A considerable amount of IL-5 was detected in the MNC supernatant from one patient in the placebo group. The same MNC supernatant showed the highest inhibition of ESEA upon the addition of anti-IL-5 antibody. In the other MNC supernatants, the amount of IL-5 was low—in many cases below the detection level (results not shown).

Correlation between GM-CSF level and eosinophil survival

The concentration of GM-CSF in the supernatants shown a significant correlation to the ESEA ($r = 0.804$, $P < 0.001$), when the results from all patients were evaluated together. When evaluated separately for each group of patients the correlation for the placebo group was $r = 0.762$, $P < 0.05$, and for the montelukast group $r = 0.720$, $P = 0.06$.

Intracellular staining for GM-CSF

The production of GM-CSF by monocytes in response to stimulation of allergen *in vitro* was decreased by the montelukast at all investigated concentrations, reaching significance ($P = 0.03$) at the highest concentration used (1 $\mu\text{g/ml}$) (Fig. 4).

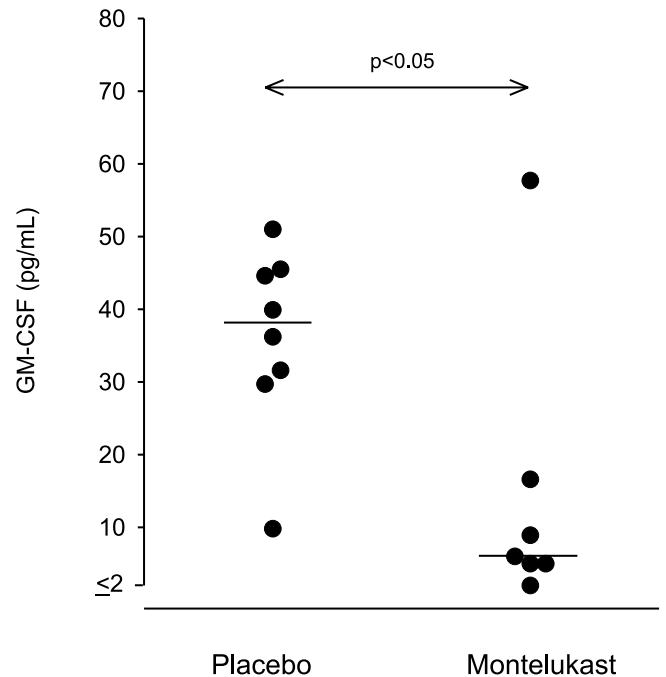


Figure 3. Levels of granulocyte/macrophage colony-stimulating factor (GM-CSF) in mononuclear cell (MNC) supernatants. The results of the statistical comparison between MNC supernatants from placebo- and montelukast-treated patients are indicated. Horizontal bars indicate median values.

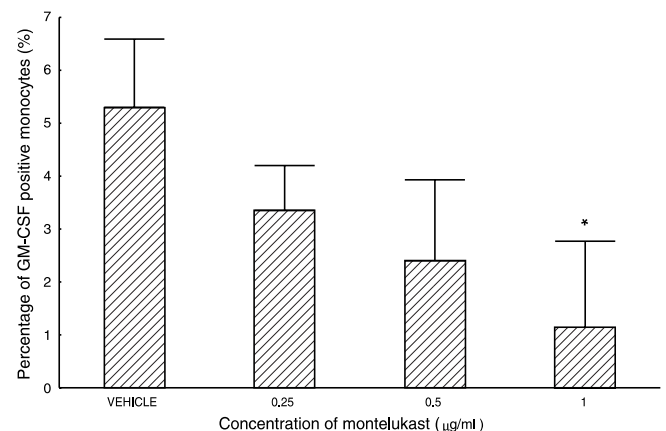


Figure 4. The percentage of granulocyte/macrophage colony-stimulating factor (GM-CSF) positive monocytes after culture *in vitro* with vehicle or montelukast. Results are shown as median and SEM. Significant differences ($P < 0.05$) between vehicle and montelukast concentrations are indicated.

Discussion

Prolonged survival could be one of the major mechanisms responsible for the raised number and possibly the increased activity of eosinophil granulocytes in asthma. Therefore treatments that interfere with the production of eosinophil survival-enhancing activities by MNCs are

of special interest. Our study shown that the production of eosinophil survival-enhancing activity (ESEA) by peripheral blood MNCs of asthmatic patients is decreased after treatment *in vivo* with a leukotriene receptor-1 antagonist (montelukast). The dominant ESEA in the MNC supernatants, both from the placebo- and the montelukast-treated group, was identified as GM-CSF. In addition, IL-5 and IFN- γ contributed, to a lesser extent, to the ESEA in the MNC supernatants.

GM-CSF and IL-5 are both well known to stimulate different aspects of eosinophil function *in vitro* (20, 21). In the present study GM-CSF, and not IL-5, was found to be the predominant cytokine in MNC supernatants responsible for prolonged eosinophil survival, in agreement with previous investigations (22, 23). The total ESEA correlated with the concentration of GM-CSF in the supernatants and both the ESEA and GM-CSF concentration were significantly higher in the placebo group compared with montelukast-treated group. The concentration of IL-5 in the MNC supernatants was similar in both patient groups. However, this result is probably influenced by the sensitivity of the assay, as concentration of IL-5 was below the detection limit in most supernatants.

IL-3 and IFN- γ have previously been reported to prolong eosinophil survival *in vitro* (24, 25). In agreement with other studies (26), we also found that IL-3 contributed only to a minor part of the ESEA in MNC supernatants, and its effect was only detectable in the placebo group. The inhibition of ESEA in placebo group supernatants by antibody against IFN- γ indicated that this cytokine also contributes to the ESEA, although to a lesser extent than GM-CSF. TNF- α can prolong eosinophil survival either directly by delivering antiapoptotic signals (27), or indirectly by stimulating eosinophils to produce and release GM-CSF, thus increasing their viability (28). In this study, the inhibitory effect of anti-TNF- α antibody was minimal compared with that of anti-GM-CSF and was seen only in supernatants from the placebo group. Furthermore, considerable amounts of GM-CSF were present in the supernatants before culture with eosinophils, and the levels of GM-CSF correlated to the ESEA. Accordingly, we suggest that the GM-CSF that constituted the major part of ESEA in the MNC supernatants was produced by the MNC and not induced by TNF- α stimulation of eosinophils.

Administration of CysLT-R antagonists results in improvement of pulmonary function and better control of asthmatic symptoms but exact mechanisms underlying these effects are not fully understood (29). Addition of CysLT-R antagonist pranlukast *in vitro* to antigen stimulated PBMNCs obtained from asthmatics significantly decreased the amount of Th2-type cytokines (IL-4, IL-5, IL-3 and GM-CSF) (10) compared with medium alone. Another study indicated that montelukast diminishes the levels of IL-5 mRNA and the secretion of

CysLTs from MNC (30). The exact cellular source of the examined cytokines has not been established. The recent characterization of the human CysLT-R₁ identified peripheral blood cells that could possibly respond to CysLT signaling, such as eosinophils, basophils, B lymphocytes, and monocytes (macrophages) (31). Although the mRNA expression of CysLT-R₁ has been confirmed in T lymphocytes (32), the protein was detected only in a small percentage of T cells (4%) (31).

We examined *in vitro* the effect of montelukast on GM-CSF production by monocytes, as this population of cells is clearly a potent producer of GM-CSF. In addition, a recent report shows that monocytes constitutively express low levels of CysLTR₁ mRNA and this expression is markedly increased after stimulation with Th2 type cytokines IL-4 and IL-13, at both mRNA and protein levels (33). Indeed, using intracellular staining we showed that montelukast suppressed the allergen-induced production of GM-CSF by monocytes *in vitro*. The observed decrease of GM-CSF production was specific and not from lower viability of mononuclear cells, as the latter was unaffected by the incubation with varying concentrations of montelukast.

We have not observed complete inhibition of GM-CSF production. GM-CSF remained an eosinophil survival factor also after montelukast treatment. We suggest that montelukast may act by decreasing GM-CSF production from monocytes. Obviously monocytes are not the only source of GM-CSF in peripheral blood, however the production of GM-CSF by other cell populations has not been examined in this study. In an *in vitro* study, by comparison, addition of corticosteroids decreased GM-CSF production from monocytes down to 16% of control levels, but complete inhibition could still not be observed (34). Similarly, although treatment with intravenous prednisolone reduced production of GM-CSF from LPS-stimulated PBMNC, the levels still remained detectable (35).

Previous *in vitro* studies suggested that anti-inflammatory effect of CysLTR₁ antagonists could be mediated through reduced cytokine production by MNC (29,30). Accordingly, we have shown a decrease in the ESEA produced by PBMNC of patients treated with montelukast. We suggest that the observed decrease is due to a general reduction in production of selected cytokines. One possible mechanism behind this effect could be binding of the drug to the CysLT-R₁ on PBMNC, possibly on monocytes, thus preventing their activation by CysLTs and subsequent production of cytokines. The primary clinical effect of CysLT-R₁ antagonists is considered to be located to the lung. Results of this study point to the possibility that montelukast affects the ongoing inflammatory process by inhibiting the cytokine production by cells in lung tissue.

Another possible mechanism could be that CysLT-R₁ antagonists inhibit the direct effect of CysLTs on eosinophils. LTC₄ and LTD₄ and not only prolong eosinophil

life span, but also mediate GM-CSF-induced survival signals (36). However, this effect was not investigated in our study.

The degree of clinical effect of antiasthma drugs varies between asthmatic patients. CysLTs are potent mediators contributing to many features of asthma. But given the multifactorial character of the disease, a larger number of patients or a more dynamic disease model is required to show distinct clinical benefits of a relatively selective agent (5–7). The model of asthma is of major importance. For example it is easier to show a clinical effect in patients with moderate-to-severe disease (9), or in the experimental setting of allergen challenge (8), compared with mild seasonal asthma. Despite lack of clinical effect in montelukast-treated patients compared to placebo in our small, unpublished seasonal study ($n = 35$), a significant increase in eosinophil number was observed in the placebo but not treated group. The number of patients and degree of allergen exposure was not sufficient to show a clinical difference between the montelukast- and placebo-treated groups. Interindividual variations in the effect of the CysLT- R_1 antagonist were clearly shown when the inhibitory effect of another receptor antagonist, zafirlukast, on early and late allergic reaction (37), and the effect of montelukast on adenosine monophosphate bronchial challenge were studied (38). Nevertheless decrease in eosinophil number in peripheral blood and sputum is a consistent

finding in both smaller and larger studies (9, 39). Cellular changes seem a more sensitive marker of an effect than perception of the symptomatic changes by the patients.

The ability to process large numbers of samples is another limiting factor in mechanistic studies.

The pattern of cytokines produced by MNCs of asthmatic patients also seems to be individual. The variation in patterns of cytokine release, as regulatory factors directing effector cell functions, could at least partly explain individual responses to a particular drug.

In summary, this investigation has shown that treatment of patients with montelukast decreases the production of MNC-derived cytokines, such as GM-CSF, IL-5, and INF- γ , where GM-CSF was the predominant cytokine responsible for the ESEA. Moreover, the *in-vitro* results suggest that CysLT- R_1 antagonists may act by suppressing GM-CSF production from monocytes in the circulation.

Acknowledgments

We thank Yvonne Bäckberg and Yvonne Andersson for their skilful assistance in the clinical part of the study. This work was supported by the Swedish Foundation for Health Care and Allergy Research, the Medical Faculty of the University of Göteborg, and the Medical Faculty of the University of Uppsala.

References

1. WALSH GM. Advances in the immunobiology of eosinophils and their role in disease. *Crit Rev Clin Laboratory Sci* 1999;**35**:453–496.
2. HENDERSON WR Jr. The role of leukotrienes in inflammation. *Ann Intern Med* 1994;**121**:684–697.
3. CLAESSON HE, DAHLÉN SE. Asthma and leukotrienes: antileukotrienes as novel anti-asthmatic drugs. *J Intern Med* 1999;**245**:205–227.
4. ALTMAN LC, MUNK Z, SELTZER J, et al. MONTELUKAST ASTHMA STUDY GROUP. A placebo-controlled, dose-ranging study of montelukast, a cysteinyl leukotriene-receptor antagonist. *J Allergy Clin Immunol* 1998;**102**:50–56.
5. LEFF JA, BUSSE WW, PEARLMAN D, et al. Montelukast, a leukotriene-receptor antagonist, for the treatment of mild asthma and exercise-induced bronchoconstriction. *N Engl J Med* 1998;**339**:147–152.
6. MALMSTRÖM K, RODRIGUEZ-GOMEZ G, GUERRA J, et al. MONTELUKAST/BECLOMETHASONE STUDY GROUP. Oral montelukast, inhaled beclomethasone, and placebo for chronic asthma. A randomized, controlled trial. *Ann Intern Med* 1999;**130**:487–495.
7. LÖFDAHL CG, REISS TF, LEFF JA, et al. Randomised, placebo controlled trial of effect of a leukotriene receptor antagonist, montelukast, on tapering inhaled corticosteroids in asthmatic patients. *Br Med J* 1999;**319**:87–90.
8. DIAMANT Z, GROOTENDORST DC, VESELIC-CHARVAT M, et al. The effect of montelukast (MK-0476), a cysteinyl leukotriene receptor antagonist, on allergen-induced airway responses and sputum cell counts in asthma. *Clin Exp Allergy* 1999;**29**:42–45.
9. PIZZICHINI E, LEFF JA, REISS TF, et al. Montelukast reduces airway eosinophilic inflammation in asthma: a randomized, controlled trial. *Eur Respir J* 1999;**14**:12–18.
10. TOHDA Y, NAKAHARA H, KUBO H, HARAGUCHI R, FUKUOKA M, NAKAJIMA S. Effects of ONO-1078 (pranlukast) on cytokine production in peripheral blood mononuclear cells of patients with bronchial asthma. *Clin Exp Allergy* 1999;**29**:1532–1536.
11. DENBURG JA, WOOL L, GAUVREAU G, SEHMI R, INMAN MD, O'BYRNE PM. Bone marrow contribution to eosinophilic inflammation. *Mem Inst Oswaldo Cruz* 1997;**92**:33–35.
12. WARDLAW AJ, WALSH GM, SYMON FA. Adhesion interactions involved in eosinophil migration through vascular endothelium. *Ann NY Acad Sci* 1996;**796**:124–137.
13. SIMON HU, YOUSEFI S, SCHRANZ C, SCHAPOWAL A, BACHERT C, BLASER K. Direct demonstration of delayed eosinophil apoptosis as a mechanism causing tissue eosinophilia. *J Immunol* 1997;**158**:3902–3908.
14. KON OM, KAY AB. T cells and chronic asthma. *Int Arch Allergy Immunol* 1999;**118**:1333–1335.

15. TAI PC, SUN L, SPRY CJ. Effects of IL-5, granulocyte/macrophage colony stimulating factor (GM-CSF) and IL-3 on the survival of human blood eosinophils in vitro. *Clin Exp Immunol* 1991;**85**:312–316.
16. SIMON HU, BLASER K. Inhibition of programmed cell death: a key pathogenic event for eosinophilia? *Immunol Today* 1995;**16**:53–55.
17. JOHANSSON SG, HOURIHANE JO, BOUSQUET J, et al. A revised nomenclature for allergy. An EAACI position statement from the EAACI nomenclature task force. *Allergy* 2001;**56**:813–824.
18. HANSEL TT, DE VRIES IJ, IFF T, et al. An improved immunomagnetic procedure for the isolation of highly purified human blood eosinophils. *J Immunol Meth* 1991;**145**:105–110.
19. IDE M, WEILER D, KITA H, GLEICH GJ. Ammonium chloride exposure inhibits cytokine-mediated eosinophil survival. *J Immunol Meth* 1994;**168**:187–196.
20. LOPEZ AF, WILLIAMSON DJ, GAMBLE JR, et al. Recombinant human granulocyte-macrophage colony-stimulating factor stimulates in vitro mature human neutrophil and eosinophil function, surface receptor expression and survival. *J Clin Invest* 1986;**78**:1220–1228.
21. LOPEZ AF, SANDERSON CJ, GAMBLE JR, CAMPBELL HD, YOUNG IG, VADAS MA. Recombinant human interleukin-5 is a selective activator of human eosinophil function. *J Exp Med* 1988;**167**:219–224.
22. WALKER C, VIRCHOW JC Jr, BRUIJNZEEL PL, BLASER K. T-cell subsets and their soluble products regulate eosinophilia in allergic and nonallergic asthma. *J Immunol* 1991;**146**:1829–1835.
23. BURKE LA, HALLSWORTH MP, LITCHFIELD TM, DAVIDSON R, LEE TH. Identification of the major activity derived from cultured human peripheral blood mononuclear cells, which enhances eosinophil viability, as granulocyte macrophage colony-stimulating factor (GM-CSF). *J Allergy Clin Immunol* 1991;**88**:226–235.
24. ROTHENBERG ME, OWEN WF JR, SILBERSTEIN DS, et al. Human eosinophils have prolonged survival, enhanced functional properties, and become hypodense when exposed to human interleukin-3. *J Clin Invest* 1988;**81**:1986–1992.
25. OCHIAI K, TANABE E, ISHIHARA C, et al. Role of JAK2 signal transductional pathway in activation and survival of human peripheral eosinophils by interferon-gamma (IFN- γ). *Clin Exp Immunol* 1999;**118**:340–343.
26. CORRIGAN CJ, HAMID Q, NORTH J, et al. Peripheral blood CD4 but not CD8 T-lymphocytes in patients with exacerbation of asthma transcribe and translate messenger RNA encoding cytokines which prolong eosinophil survival in the context of Th2-type pattern: effect of glucocorticoid therapy. *Am J Res Cell Mol Biol* 1995;**12**:567–578.
27. TSUKAHARA K, NAKAO A, HIRAGURI M, et al. Tumor necrosis factor- α mediates anti-apoptotic signals partially via p38 MAP kinase activation in human eosinophils. *Int Arch Allergy Immunol* 1999;**120**:54s–59s.
28. LEVI-SCHAEFFER F, TEMKIN V, MALAMUD V, FELD S, ZILBERMAN Y. Mast cells enhance eosinophil survival in vitro: role of TNF- α and granulocyte-macrophage colony-stimulating factor. *J Immunol* 1998;**160**:5554–5562.
29. DIAMANT Z, SAMPSON AP. Anti-inflammatory mechanisms of leukotriene modulators. *Clin Exp Allergy* 1999;**29**:1449–1453.
30. FRIERI M, THERATTIL J, THAN S, et al. Montelukast inhibits IL-5 expression and cysteinyl leukotriene (CysLT) production in ragweed and mite stimulated mononuclear cells (MNC) from asthmatics. *J Allergy Clin Immunol* 1999;**103**:203s.
31. LYNCH KR, O'NEILL GP, LIU Q, et al. Characterization of the human cysteinyl leukotriene CysLT₁ receptor. *Nature* 1999;**399**:789–793.
32. MITA H, HASEGAWA M, SAITO H, AKIYAMA K. Levels of cysteinyl leukotriene receptor mRNA in human peripheral leucocytes: significantly higher expression of cysteinyl leukotriene receptor 2 mRNA in eosinophils. *Clin Exp Allergy* 2001;**31**:1714–1723.
33. THIVIERGE M, STANKOVA J, ROLA-PLESZCZYNSKI M. IL-13 and IL-4 up-regulate cysteinyl leukotriene 1 receptor expression in human monocytes and macrophages. *J Immunol* 2001;**167**:2855–2860.
34. LARSSON S, LINDEN M. Effects of a corticosteroid, budesonide, on production of bioactive IL-12 by human monocytes. *Cytokine* 1998;**10**:786–789.
35. COTTER TP, HOOD PP, COSTELLO JF, SAMPSON AP. Exposure to systemic prednisolone for 4 hours reduces ex vivo synthesis of GM-CSF by bronchoalveolar lavage cells and blood mononuclear cells of mild allergic asthmatics. *Clin Exp Allergy* 1999;**29**:1655–1662.
36. LEE E, ROBERTSON T, SMITH J, KILFEATHER S. Leukotriene receptor antagonists and synthesis inhibitors reverse survival in eosinophils of asthmatic individuals. *Am J Respir Crit Care Med* 2000;**161**:1881–1886.
37. TAYLOR IK, O'SHAUGHNESSY KM, FULLER RW, DOLLERY CT. Effect of cysteinyl-leukotriene receptor antagonist ICI 204,219 on allergen-induced bronchoconstriction and airway hyperreactivity in atopic subjects. *Lancet* 1991;**337**:690–694.
38. WILSON AM, DEMPSEY OJ, SIMS EJ, LIPWORTH BJ. A comparison of topical budesonide and oral montelukast in seasonal allergic rhinitis and asthma. *Clin Exp Allergy* 2001;**31**:616–624.
39. REISS TF, CHERVINSKY P, DOCKHORN RJ, SHINGO S, SEIDENBERG B, EDWARDS TB. MONTELUKAST CLINICAL RESEARCH STUDY GROUP. Montelukast, a once-daily leukotriene receptor antagonist, in the treatment of chronic asthma: a multicenter, randomized, double-blind trial. *Arch Intern Med* 1998;**158**:1213–1220.