

Anti-inflammatory effects of high-dose montelukast in an animal model of acute asthma

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Summary

Background Asthmatic inflammation is mediated by a network of cytokines, chemokines and adhesion molecules. Corticosteroids are the only effective agents available to control asthmatic inflammation. We investigated the effect of high-dose montelukast (MK), a selective cysteinyl leukotriene receptor 1 antagonist, on mediators of airway inflammation.

Objective The aim of this study was to determine the effect of a 3-day course of high-dose MK on mediators of airway inflammation induced by a single allergen challenge in sensitized mice.

Methods Ovalbumin (OVA)-sensitized BALB/c mice were treated with 25 mg/kg of MK or saline intravenously for 3 days. On the third day, a single inhalation challenge with OVA was given. Cellular infiltration was assessed in the bronchoalveolar lavage (BAL) and in the lung. Expression of IL-4, IL-5, IL-13 and eotaxin in the BAL, and the lung was determined. Serum IL-5 and total IgE was measured. IL-5 and eotaxin mRNA expression in the lung was determined. Finally, eotaxin and VCAM-1 expression in the lung was assessed by immunohistochemistry.

Results MK reduced the number of eosinophils in the BAL by > 90%. There was also significant reduction in IL-5 in the BAL, lung and the serum, and IL-5 mRNA expression in the lung. IL-4 level in the lung and BAL, and IL-13 level in the lung also significantly decreased. Serum IgE level and lung VCAM-1 expression was also significantly lower in treated animals, but eotaxin protein and mRNA expression in the lung remained unchanged.

Conclusion MK exerts its anti-inflammatory effect through the suppression of T helper type-2 (Th2) cytokines. The use of high-dose MK as an anti-inflammatory agent in acute asthma should be further explored.

Keywords eotaxin, IgE, interleukin-4, interleukin-5, interleukin-13, leukotrienes, VCAM-1

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Introduction

Airway inflammation is thought to play a major role in the pathogenesis of asthma. The pathophysiology of asthma is highly complex and involves a network of cytokines and effector cells [1, 2]. Initial sensitization to aeroallergens in susceptible individuals leads to a T helper type-2 (Th2) response [3] and the production of allergen-specific IgE [4, 5]. Further exposure to allergens leads to mast cell degranulation and T cell activation with the release of histamine and leukotrienes (LTs), which are potent constrictors of bronchial smooth muscles, and proinflammatory cytokines such as IL-4, IL-5 and IL-13 [6]. This leads to the upregulation of bone marrow eosinopoiesis [7] and the expression of chemokines and adhesion molecules on the airway epithelium [8], which result in the influx of eosinophils into the airway mucosa and submucosa. Activated eosinophils can induce airway hyper-responsiveness (AHR) and epithelial damage through the release of cationic proteins, lipid mediators, cytokines and chemokines [9]. The

specific roles of IgE, IL-4, IL-5 and IL-13 in the induction of AHR and epithelial damage are complex and interdependent (reviewed in [10]).

Cysteinyl-leukotrienes (cysLTs) are products of the 5-lipoxygenase (5-LO) pathway of arachidonic acid metabolism. They play an important role in both the early and the late asthmatic response, being 100–1000 times more potent than histamine in causing bronchospasm [11]. Furthermore, they have been shown to enhance mucus production [12], increase microvascular leakage [13] and recruit eosinophils [14] to the airways. Ovalbumin (OVA)-sensitized 5-LO knockout mice have attenuated methacholine responsiveness, airway eosinophilia and IgE response after OVA challenge when compared to wild-type controls [15]. Clinically, CysLT1 antagonists have been shown to improve asthma symptoms [16, 17] and reduce inflammatory cell infiltration in the bronchial mucosa [18]. The CysLT1 receptor antagonist, MK-571, has been shown to inhibit airway eosinophilia, hyper-responsiveness and microvascular leakage in mice after allergen challenge [19]. Recent evidence also suggests that montelukast (MK-0476), a CysLT1 receptor antagonist, can significantly reduce airway remodeling in a mouse model of chronic asthma [20]. Pre-treatment of a rat model of asthma with IL-2 increases airway responsiveness to LTD4 and

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allergen challenge, which is inhibited by MK [21]. This evidence suggests that leukotrienes play a major role in bronchial hyper-responsiveness, airway eosinophilia and airway remodeling. In this study, we employed an OVA mouse model of asthma to determine the acute effects of MK on cytokine, chemokine and adhesion molecule expression. We found high-dose MK given intravenously for 3 days to be highly effective in inhibiting eosinophil infiltration into the airway by suppressing IL-4, IL-5, IL-13, eotaxin and vascular cell adhesion molecule-1 (VCAM-1) expression. Furthermore, MK very effectively inhibited the rise in serum IgE in sensitized animals after allergen challenge. The use of high-dose MK as an anti-inflammatory agent in the treatment of acute asthma should therefore be further evaluated.

Materials and methods

Reagents and antibodies

Montelukast was provided by Merck & Co., Inc. (Rahway, NJ, USA). The lyophilized powder was reconstituted with sterile water just before injection each day. Ovalbumin was purchased from Sigma (St Louis, MO, USA). Inject alum was purchased from Pierce (Rockford, IL, USA). Centricon YM-3 filter units were purchased from Millipore (Bedford, MA, USA). OptEIA mouse IL-4 and IL-5 ELISA kits and mouse IgE enzyme-linked immunosorbent assay (ELISA) kit were purchased from Pharmingen (San Diego, CA, USA). Qantikine mouse IL-13 and eotaxin ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). Polyclonal goat IgG antibodies against VCAM-1 and eotaxin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Biotin-SP-conjugated mouse anti-goat IgG and peroxidase-conjugated streptavidin were purchased from Jackson ImmunoResearch (West Grove, PA, USA). Micro RNA Isolation Kit was purchased from Stratagene (La Jolla, CA, USA). DC protein assay was purchased from Bio-Rad Laboratories (Hercules, CA, USA). ThermoScrip RT-PCR System was purchased from Gibco-BRL (Gaithersburg, MD, USA). AmpliTaq Gold DNA polymerase was purchased from Perkin-Elmer (Boston, MA, USA).

Experimental design

The experimental protocol has been approved by the University of Hong Kong Committee on the use of live animals in teaching and research.

Six- to 8-week-old BALB/c mice were separated into three treatment groups. The SAL (saline) and MK (montelukast) groups were immunized by intraperitoneal (i.p.) injections of 50 µg of OVA (Sigma) and 100 µL of alum (Pierce) on days 0, 7 and 14. On day 21, the mice were placed in a 10 × 18 × 25 cm polypropylene chamber and challenged once with a nebulized solution of OVA (10 mg/mL) for 30 min. In the negative control group, OVA with alum was injected on days 0 and 7, SAL was injected on day 14, and the animals challenged with nebulized SAL on day 21.

To determine the dose-response, 10, 25 or 62.5 mg/kg of MK or SAL were injected into the caudal tail vein daily from day 19–23 and the animals killed 72 h after OVA challenge.

Bronchoalveolar lavage (BAL) fluid differential cell counts and lung histology were performed on these animals.

For the rest of the study, animals were given 25 mg/kg of montelukast (MK group) or saline (SAL group) intravenously (i.v.) from days 19 to 21. Twenty-four hours after OVA challenge, the mice were killed by i.p. injection of 0.2 mL sodium pentobarbital (60 mg/kg). The lungs were lavaged through the trachea with 1.2 mL of SAL. A differential count of at least 200 cells was performed. Lungs from the animals used for BAL were not used for other parts of the experiment.

Before removing the lungs, mice were anaesthetized, exsanguinated and the right ventricle perfused with cold SAL. Then, 5-µm-thick paraffin sections were stained with haematoxylin and eosin (H&E) or Congo Red to assess the degree of inflammation. Microwave-modified paraffin sections were incubated in 20% normal mouse serum in phosphate-buffered saline (PBS), and then incubated in polyclonal antibodies specific for mouse VCAM-1 or eotaxin at a dilution of 1 : 200 or 1 : 100. This was followed by incubation in Biotin-SP-conjugated mouse anti-goat IgG (1 : 500) and then peroxidase-conjugated streptavidin (1 : 500) for 45 min each. 3, 3'-Diaminobenzidine tetrahydrochloride (DAB) was used as a peroxidase substrate and the slides were counterstained with methyl green. For negative control, the primary antibodies were replaced with goat IgG. VCAM-1 immunoreactivity was determined by counting the number of blood vessels stained positive per 1 mm² of tissue under 100 × magnification by two blinded independent observers. A total of five fields were counted per section. Ten sections from each of four MK-treated and four SAL-treated animals were examined.

To determine cytokine protein expression, whole lungs were snap-frozen in liquid nitrogen and homogenized. The levels of cytokines in lung extracts were expressed as pg/mg of total protein content. IL-4, IL-5, IL-13 and eotaxin in the lung extract, BAL and serum, and serum IgE were measured by commercial ELISA kits. Lung extract and BAL samples were concentrated two- to threefold with centricon YM-3 filter units (Millipore).

To determine mRNA expression, total RNA was isolated from frozen lungs. Semi-quantification of eotaxin and IL-5 mRNA was performed by multiplex RT-PCR using β-actin and TATA-binding protein (TBP) as internal controls. The number of cycles, annealing temperature and primer concentrations was adjusted to fall on the exponential phase of the amplification reaction for each product. The reactions were performed in a Perkin-Elmer 9700 thermocycler.

PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide. Band intensities of eotaxin and IL-5 reaction products were detected by Gel Doc 1000 image analysis system (Bio-Rad), and then normalized to their respective internal control.

To determine the pharmacokinetics of MK in mice, 6-week-old BALB/c mice were injected through the tail vein with MK at a dose of 25 mg/kg every 24 h for three doses. After the last injection, blood was taken at time 0, 5 and 30 min, 1, 4, 8 and 24 h. There were three animals per time point. Plasma MK level was determined by liquid chromatography with fluorescence detection at Merck Frosst Laboratories in Quebec, Canada, as previously described [22].

Statistical analysis

Data are expressed as mean $\pm$ SE. Statistical significance ($P < 0.05$) was determined by Student's two-tailed *t*-test for independent means using Microsoft Excel program.

Results

Effects of MK on inflammatory cell infiltration induced by OVA challenge

BAL cytospin preparations stained with Wright–Geimsa were assessed. In the negative control group, the BAL total leukocyte count was $31 \pm 13.15 \times 10^4/\text{mL}$, consisting almost entirely of alveolar macrophages.

Twenty-four hours after aerosolized OVA administration to SAL-treated mice ($n = 6$), total BAL leukocyte count increased to $78.46 \pm 12.0 \times 10^4/\text{mL}$. Neutrophils and eosinophils represented 56.12% and 8.42% of all leukocytes, respectively. Then, 25 mg/kg of MK ($n = 10$) significantly reduced the number of total leukocytes, neutrophils and eosinophils compared with SAL treatment (Fig. 1a). There was no significant difference in the number of macrophages and lymphocytes.

Seventy-two hours after aerosolized OVA administration to the SAL-treated mice ($n = 6$), total BAL leukocyte count was $37.93 \pm 11.35 \times 10^4/\text{mL}$. The number of neutrophils markedly decreased, and the number of eosinophils increased when compared to SAL-treated mice at 24 h after aerosolized OVA administration. In the group treated with 10 mg/kg of MK ($n = 6$), there was no statistically significant difference in the number of BAL total leukocytes, neutrophils, eosinophils and lymphocytes when compared to SAL-treated animals, though the mean number of eosinophils has decreased by 45.2%. In the groups treated with 25 mg/kg ($n = 5$) and 62.5 mg/kg ($n = 5$) of MK, the number of total leukocytes, neutrophils, eosinophils, macrophage and lymphocytes in BAL decreased significantly. Eosinophil counts decreased by 91.8% and 97.1%, respectively, when compared to SAL-treated animals (Fig. 1b).

The histology of mouse lungs was assessed by H&E and Congo red staining (Fig. 2). It was observed that 25 mg/kg of MK administered for 3 or 5 days markedly reduced inflammatory cell infiltration around airways and blood vessels at 24 or 72 h after aerosolized OVA challenge.

Effect of MK on cytokines and serum total IgE levels

BAL, serum and lung tissue were obtained from SAL- and MK-treated animals 24 h after aerosolized OVA challenge. OVA challenge resulted in significantly increased total lung and BAL IL-4 and IL-5, and BAL eotaxin levels. There was no significant difference in total lung IL-13 levels due to a spuriously high reading in one of the control animals. After treatment with 25 mg/kg of MK, total lung IL-4, IL-5 and IL-13 protein expression significantly decreased when compared with SAL-treatment (Figs 3a–c). The BAL IL-4, IL-5 and eotaxin levels also significantly decreased in the MK-treated group when compared with the SAL-treated group (Figs 4a–c). Likewise, serum IL-5 and total IgE levels increased after OVA challenge, and both were significantly suppressed after MK treatment when compared with SAL treatment (Figs 5a and b). The reduction in total lung eotaxin protein expression did

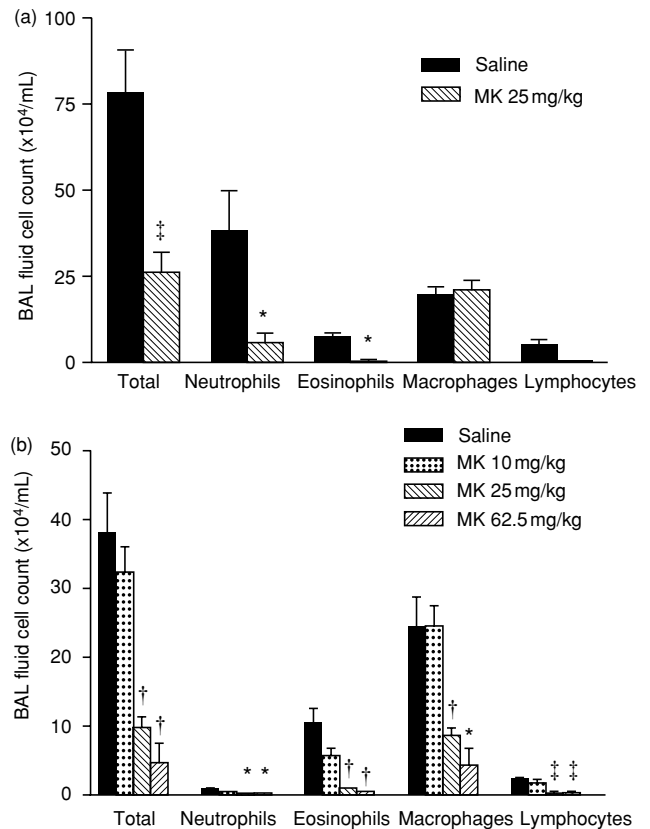


Fig. 1. Montelukast treatment reduced the number of inflammatory cells in the BAL fluid after OVA challenge. (a) OVA-sensitized BALB/c mice were given three daily treatments of saline ($n = 6$) or montelukast (25 mg/kg; $n = 10$) by tail vein injection. They were exposed to aerosolized OVA on day 3 of treatment and BAL fluid was obtained 24 h after challenge. (b) OVA-sensitized BALB/c mice were given five daily treatments of saline ($n = 6$) or montelukast at 10 mg/kg ($n = 6$), 25 mg/kg ($n = 5$) or 62.5 mg/kg ($n = 5$) by tail vein injection. They were exposed to aerosolized OVA on day 3 of treatment and BAL fluid was obtained 72 h after challenge. * $P \leq 0.05$, † $P \leq 0.01$, ‡ $P \leq 0.001$ versus saline.

not reach statistical significance (Fig. 3d). BAL IL-13 and serum IL-13 and IL-4 levels were undetectable.

Effect of MK on IL-5 and eotaxin mRNA expression

mRNA was extracted from the lungs 24 h after aerosolized OVA challenge. IL-5 and eotaxin mRNA expression was quantified by a semiquantitative multiplex RT–PCR assay. Results are expressed as the ratio of intensity of the product to that of the internal control. IL-5 mRNA expression in the OVA-challenged, SAL-treated group (1.31 ± 0.30 , $n = 8$) was significantly greater than that of the negative control group (0.2 ± 0.03 , $n = 3$; $P < 0.001$). Treatment with 25 mg/kg of MK significantly reduced IL-5 mRNA expression to 0.75 ± 0.05 ($n = 8$, $P < 0.05$). Eotaxin mRNA expression in the OVA-challenged, SAL-treated group (1.63 ± 0.14 , $n = 8$) was also significantly greater than in the negative control group (0.49 ± 0.13 , $n = 3$). However, there was no significant difference between the SAL-treated and MK-treated groups (1.67 ± 0.21 , $n = 8$).

Effect of MK on VCAM-1 and eotaxin expression in lung tissue

As VCAM-1 is the most important adhesion molecule in regulating eosinophil migration from peripheral blood to

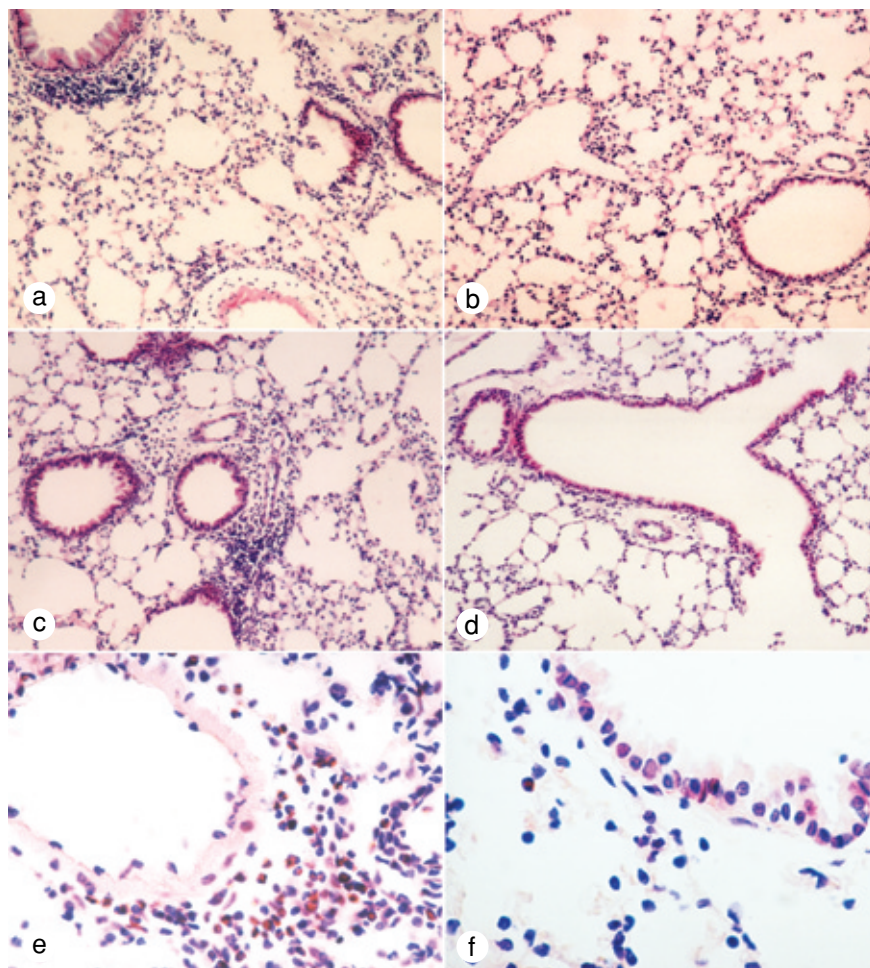


Fig. 2. Dramatic reduction in eosinophils and other inflammatory cells around blood vessels and airways in montelukast-treated mice (25 mg/kg) compared with saline-treated mice at 24 h and 72 h after aerosolized OVA challenge. (a) Saline-treated mice 24 h after OVA challenge. H&E $\times$ 100 magnification. (b) Montelukast-treated mice 24 h after OVA challenge. H&E $\times$ 100 magnification. (c) Saline-treated mice 72 h after OVA challenge. H&E $\times$ 100 magnification. (d) Montelukast-treated mice 72 h after OVA challenge. H&E $\times$ 100 magnification. (e) Saline-treated mice 24 h after OVA challenge. Congo red $\times$ 400 magnification. (f) Montelukast-treated mice 24 h after OVA challenge. Congo red $\times$ 400 magnification.

extravascular and alveolar spaces in the lung, we examined VCAM-1 expression in the lungs of animals treated with 25 mg/kg of MK or SAL 24 h after the last OVA challenge. VCAM-1 immunoreactivity on the airway vascular endothelium and the number of positive-staining vessels in MK-treated mice were markedly reduced compared with SAL-treated mice (Fig. 6). The mean number of positive-staining vessels per 5 mm² in the SAL-treated group was 33.94 ± 0.55 and that of the MK-treated group was 23.0 ± 1.53 ($P < 0.03$). In the negative control group, VCAM-1 staining was absent or faint (not shown).

Eotaxin immunoreactivity was mainly expressed on the airway epithelium and alveolar macrophages (not shown). No difference in staining was observed between the MK- and SAL-treated animals.

Pharmacokinetics of MK

As there are no published pharmacokinetics data on MK in mice, we measured plasma MK levels after the third dose. The data are presented in Fig. 7. At time 0, MK was undetectable, and 5 min after injection, the level rose to $58\,244 \pm 16\,691$ ng/mL. The level fell rapidly with a half-life of 21 min, reaching a mean level of 49 ng/mL at 24 h after injection. The area under the curve (AUC_{0–24}) was 52 634 ng/h/mL.

Discussion

In this study, we demonstrated that the CysLT₁ receptor antagonist MK reduced eosinophil recruitment into the airway by >90% after allergen challenge in sensitized mice. This is a significant finding as eosinophils play an important role in promoting the late asthmatic response. Eosinophil recruitment into the asthmatic airway following allergen exposure requires the co-ordinated expression of a complex set of cytokines, chemokines and adhesion molecules. IL-5 produced by Th2 cells is a key regulator in eosinophil development, migration and activation [23–25]. Allergen-challenge induces the expression of IL-5R α expression on CD34⁺ bone marrow cells, which differentiate into mature eosinophils in the presence of IL-5 [26]. Ihaku and colleagues showed that a single dose of MK given before allergen challenge reduced the number of IL-5 mRNA⁺ cells in the BAL fluid and lung tissue of Brown Norway rats [27]. In our model, MK given for 3 days prior to allergen inhalation challenge inhibited IL-5 mRNA expression in the lung and reduced IL-5 in total lung extract, BAL and serum. This was associated with a dramatic reduction in the number of neutrophils, eosinophils and lymphocytes in the BAL, whereas in the Ihaku study, only a reduction in mononuclear cells was found. This difference can be explained by the lower dose that was used in that study and the shorter interval

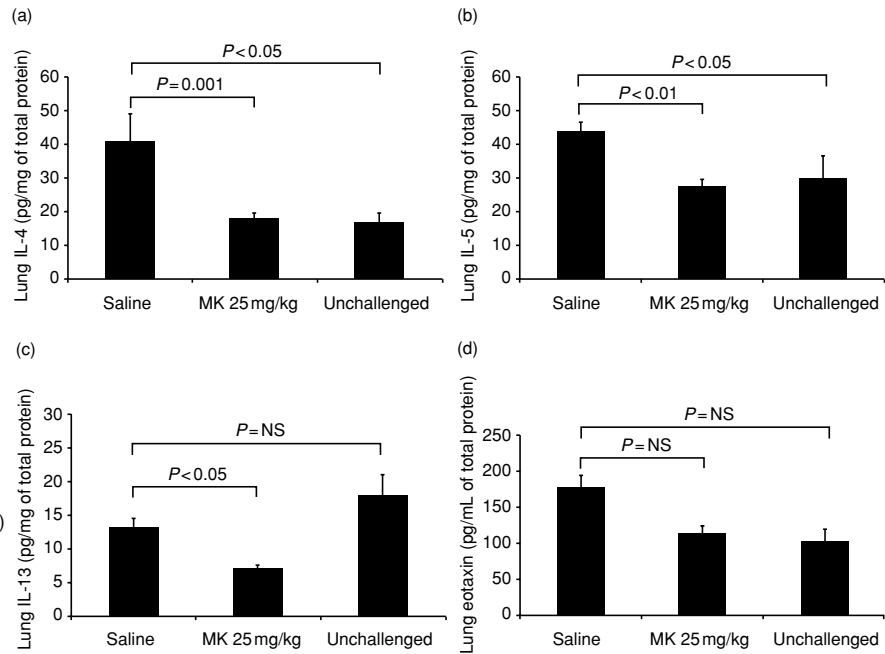


Fig. 3. Montelukast reduced IL-4, IL-5 and IL-13 protein expression in lung extracts 24 h after aerosolized OVA challenge. OVA-sensitized animals were given three daily treatments of montelukast (25 mg/kg; $n=9$) or saline ($n=9$) by tail vein injection. They were exposed to aerosolized OVA on day 3 of treatment. Negative control animals ($n=3$) received saline treatment and aerosolized saline challenge. The animals were killed 24 h after challenge and the levels of (a) IL-4, (b) IL-5, (c) IL-13 and (d) eotaxin in the lung homogenates were measured by ELISA and expressed as pg/mg of total protein content.

between allergen challenge and sample collection. Henderson and colleagues also showed that MK reduced lung IL-5 mRNA expression and BAL eosinophils in a chronic asthma model [20], but this was achieved using a low-dose continuous infusion over 76 days, and the steady-state blood level was not shown.

While IL-5 is important in mobilizing eosinophils from the bone marrow, tissue injection of IL-5 did not induce significant eosinophil accumulation [28], indicating that other chemotactic factors are required for tissue infiltration. Moreover, in a clinical trial, monoclonal antibody against IL-5 caused a dramatic reduction in peripheral blood and BAL eosinophils without any significant effect on the late asthmatic response or AHR [29], suggesting that other factors are also important. Eotaxin is a CC chemokine with potent and specific chemotactic activity for eosinophils [30], and its expression is increased in BAL and airways of asthmatics [31]. Eotaxin mRNA is constitutively expressed in the lungs of non-allergic animals, and expression can be localized to alveolar epithelial cells, macrophages, T cells and eosinophils after allergen challenge in sensitized animals. Elimination of either IL-5 or eotaxin greatly attenuates pulmonary eosinophilia after allergen challenge, without effecting AHR. However, elimination of both IL-5 and eotaxin abolishes AHR [10]. Our results did not show any significant difference in lung eotaxin protein and mRNA expression between SAL- and MK-treated animals. This is consistent with the result reported by Henderson and colleagues [20]. However, we did find a significant reduction in BAL eotaxin level in MK-treated animals. This is probably an indirect effect due to the reduction in lymphocytes and eosinophils in the airway of these animals, whereas airway epithelial cells, the main source of eotaxin in the lung parenchyma, were unaffected by treatment.

Other factors expressed by Th2 cells are also important in promoting airway inflammation. Th2 cells produce IL-4 and IL-13, and both of these cytokines activate STAT-6 through the IL-4R α chain. Stat6-deficient mice have defective development

of Th2 cells [32] and are protected from allergen-induced AHR and mucus production [33]. Moreover, neutralization of IL-13 can completely abolish allergen-induced AHR without affecting eosinophilia or serum IgE [34, 35]. In this study, we found a significant reduction in BAL IL-4, and total lung IL-4 and IL-13 expression in MK-treated animals. This result is consistent with the findings in Henderson's study using continuous MK infusion [20], although surprisingly, no reduction in AHR was found in that study. However, Blain and Sirois did find a reduction in bronchial hyperresponsiveness after high dose MK-571 treatment in mice [19].

OVA-sensitized animals showed an increase in total serum IgE after OVA challenge. This is analogous to the rise in pollen-specific IgE in hayfever sufferers during pollen season. MK inhibited this rise in serum total IgE, probably as a result of the reduction in IL-4. The role of IgE in asthma is still not entirely clear. Early studies showed that neutralization of IgE inhibited the recruitment of eosinophils into the airway and the production of IL-4 and IL-5 after allergen challenge [36]. Passive transfer of anti-OVA IgE resulted in airway eosinophilia and AHR after OVA challenge [37]. However, IgE-deficient mice sensitized to *Aspergillus fumigatus* were able to develop airway eosinophilia and AHR after allergen challenge [38]. IL-4 and IL-13 also promote adhesion of eosinophils and basophils to vascular endothelium through selective upregulation of the adhesion molecule VCAM-1 [39, 40], whose expression is increased on bronchial biopsies from asthmatic patients [41]. Selective blockade of IL-4 reduces VCAM-1 expression after allergen challenge [42]. Treatment with a monoclonal antibody against very late activation antigen-4 (VLA-4), the counterreceptor for VCAM-1, significantly inhibited airway eosinophilia and the late asthmatic response after allergen challenge in guinea pigs [43]. In our study, MK significantly suppressed VCAM-1 expression in the lung after allergen challenge. This effect is most likely mediated through the inhibition of IL-4 and

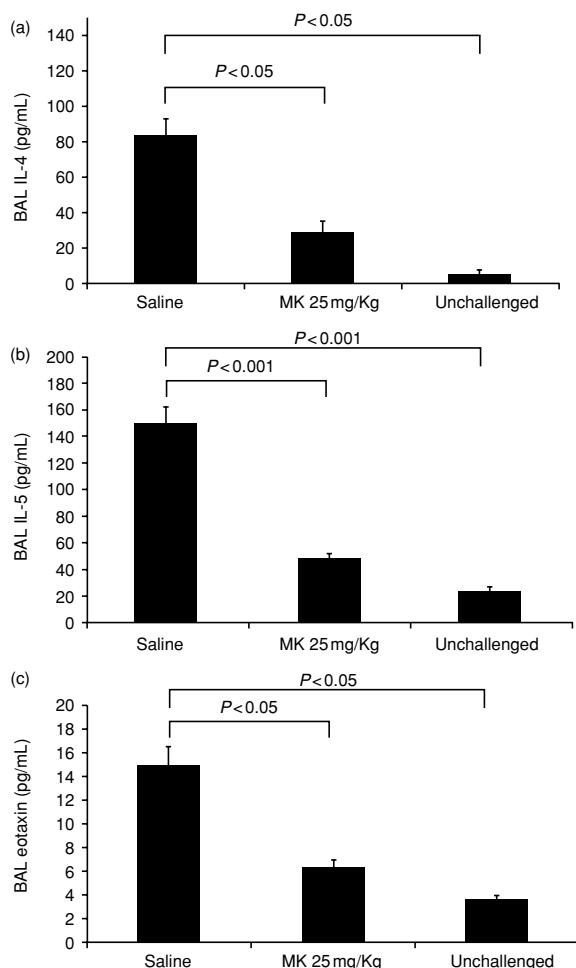


Fig. 4. Montelukast reduced IL-4, IL-5 and eotaxin in BAL fluid 24 h after aerosolized OVA challenge. OVA-sensitized animals were given three daily treatments of montelukast (25 mg/kg; $n = 9$) or saline ($n = 9$) by tail vein injection. They were exposed to aerosolized OVA on day 3 of treatment. Negative control animals ($n = 5$) received saline treatment and aerosolized saline challenge. The animals were killed 24 h after challenge and the levels of (a) IL-4, (b) IL-5 and (c) eotaxin in the BAL fluid were measured by ELISA.

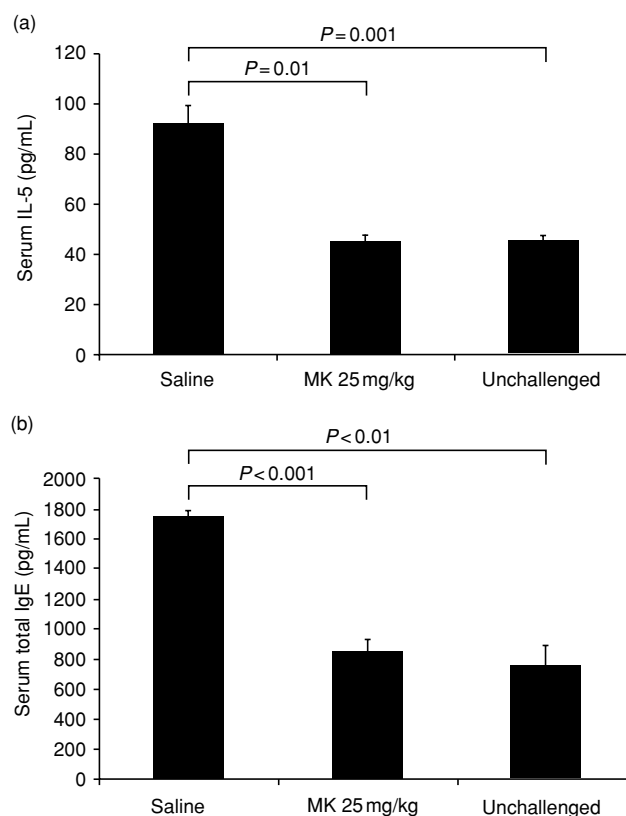


Fig. 5. Montelukast reduced serum IL-5 and total IgE levels 24 h after aerosolized OVA challenge. OVA-sensitized animals were given three daily treatments of montelukast (25 mg/kg; $n = 8$) or saline ($n = 8$) by tail vein injection. They were exposed to aerosolized OVA on day 3 of treatment. Negative control animals ($n = 6$) received saline treatment and aerosolized saline challenge. Blood was obtained 24 h after OVA challenge and serum (a) IL-5 and (b) IgE levels were measured by ELISA.

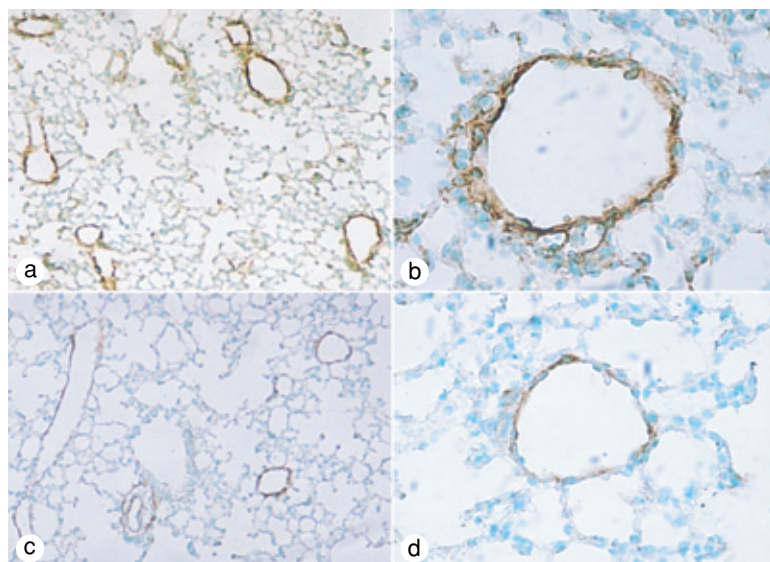


Fig. 6. Montelukast reduced VCAM-1 expression on pulmonary vascular endothelium 24 h after aerosolized OVA challenge. The number of VCAM-1-positive vessels is reduced in montelukast (25 mg/kg)-treated mice (c and d) compared with saline-treated mice (a and b) 24 h after aerosolized OVA challenge. Lung sections were photographed at $\times 100$ (a and c) and $\times 400$ (b and d) magnification.

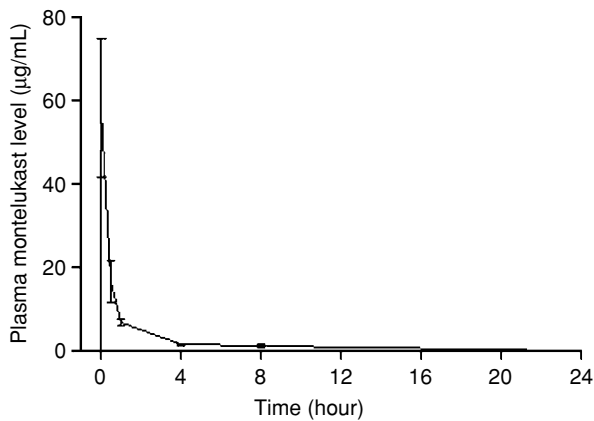


Fig. 7. Plasma montelukast level of 6-week-old BALB/c mice ($n = 3$ per time point) after three daily injections of montelukast (25 mg/kg) via the tail vein. Blood samples were obtained on day 3 after the third injection.

IL-13, and is partly responsible for the dramatic reduction in pulmonary eosinophilia seen after MK treatment.

We did not measure airway resistance in our study, but CysLT1 receptor antagonists have already been shown to inhibit the late asthmatic response in humans [44] and rodents [19, 27]. The dose of MK we chose for our experiments was based on its dose-response on BAL eosinophil count. This dose (25 mg/kg) is considerably higher than the recommended oral dose currently used in patients (0.2 mg/kg). In Blain and Sirois's study [19], maximum inhibition of airway eosinophilia was achieved with 100 mg/kg of MK-517. Our pharmacokinetics data showed that plasma clearance of MK is considerably more rapid in mice. After a 10-mg tablet of MK, the AUC in men with a mean weight of 76.5 kg was 2441 ng/h/mL [45], whereas we achieved an AUC of 52 634 ng/h/mL in our mouse model. Based on a published formula, an AUC of 52 634 ng/h/mL can be achieved at a dose of 1.62 mg/kg i.v. in humans [45]. The recommended dose of MK for patients was chosen based on its effect against the early asthmatic response. Because MK blocks the direct effect of CysLTs on bronchial smooth muscles during the early asthmatic response, the dose required to achieve an anti-inflammatory effect might be quite different. During preliminary pharmacokinetic studies in human subjects, single doses of up to 800 mg of MK have been given without adverse effects [46]. Based on our findings, high-dose MK might be effective as anti-inflammatory treatment for acute asthma exacerbation. The use of this drug as an adjunct to or a replacement for systemic corticosteroids under this clinical situation should therefore be further explored.

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References

- Bradley BL, Azzawi M, Jacobson M et al. Eosinophils, T-lymphocytes, mast cells, neutrophils, and macrophages in bronchial biopsy specimens from atopic subjects with asthma. comparison with biopsy specimens from atopic subjects without asthma and normal control subjects and relationship to bronchial hyper-responsiveness. *J Allergy Clin Immunol* 1991; 88:661–74.
- Broide DH, Lotz M, Cuomo AJ, Coburn DA, Federman EC, Wasserman SI. Cytokines in symptomatic asthma airways. *J Allergy Clin Immunol* 1992; 89:958–67.
- Robinson DS, Hamid Q, Ying S et al. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med* 1992; 326:298–304.
- Burrows B, Martinez FD, Halonen M, Barbee RA, Cline MG. Association of asthma with serum IgE levels and skin-test reactivity to allergens. *N Engl J Med* 1989; 320:271–7.
- Sears MR, Burrows B, Flannery EM, Herbison GP, Hewitt CJ, Holdaway MD. Relation between airway responsiveness and serum IgE in children with asthma and in apparently normal children. *N Engl J Med* 1991; 325:1067–71.
- Humbert M, Durham SR, Kimmitt P et al. Elevated expression of messenger ribonucleic acid encoding IL-13 in the bronchial mucosa of atopic and non-atopic subjects with asthma. *J Allergy Clin Immunol* 1997; 99:657–65.
- Inman MD, Ellis R, Wattie J, Denburg JA, O'Byrne PM. Allergen-induced increase in airway responsiveness, airway eosinophilia, and bone-marrow eosinophil progenitors in mice. *Am J Respir Cell Mol Biol* 1999; 21:473–9.
- Gonzalo JA, Lloyd CM, Kremer L, Finger E, Martinez AC, Siegelman MH. Eosinophil recruitment to the lung in a murine model of allergic inflammation. The role of T cells, chemokines, and adhesion receptors. *J Clin Invest* 1996; 98:2332–45.
- Coyle AJ, Uchida D, Ackerman SJ, Mitzner W, Irvin CG. Role of cationic proteins in the airway. Hyper-responsiveness due to airway inflammation. *Am J Respir Crit Care Med* 1994; 150:S63–71.
- Foster PS, Mould AW, Yang M et al. Elemental signals regulating eosinophil accumulation in the lung. *Immunol Rev* 2001; 179: 173–81.
- Barnes NC, Piper PJ, Costello JF. Comparative actions of inhaled leukotriene C4, leukotriene D4 and histamine in normal human subjects. *Thorax* 1984; 39:500–4.
- Marom Z, Shelhamer JH, Bach MK, Morton DR, Kaliner M. Slow-reacting substances, leukotriene C4 and D4 increase the release of mucus from human airways *in vitro*. *Am Rev Respir Dis* 1982; 126:449–51.
- Drazen JM, Austen KF, Lewis RA et al. Comparative airway and vascular activity of leukotriene C1 and D *in vivo* and *in vitro*. *Proc Natl Acad Sci USA* 1980; 77:4345–58.
- Nagy L, Lee TH, Goetzl EJ, Pickett WC, Kay AB. Complement receptor enhancement and chemotaxis of human neutrophils and eosinophils by leukotrienes and other lipoxygenase products. *Clin Exp Immunol* 1982; 47:541–7.
- Irvin CG, Tu YP, Sheller JR, Funk CD. 5-Lipoxygenase products are necessary for ovalbumin-induced airway responsiveness in mice. *Am J Physiol* 1997; 272:L1053–8.
- Suissa S, Dennis R, Ernst P, Sheehy O, Wood-Dauphinee S. Effectiveness of the leukotriene receptor antagonist zafirlukast for mild-to-moderate asthma. A randomized, double-blind, placebo-controlled trial. *Ann Intern Med* 1997; 126:177–83.
- Reiss TF, Altman LC, Chervinsky P et al. Effects of montelukast (MK-0476), a new potent cysteinyl leukotriene (LTD4) receptor antagonist, in patients with chronic asthma. *J Allergy Clin Immunol* 1996; 98:528–34.
- Nakamura Y, Hoshino M, Sim JJ, Ishii K, Hosaka K, Sakamoto T. Effect of the leukotriene receptor antagonist pranlukast on cellular infiltration in the bronchial mucosa of patients with asthma. *Thorax* 1998; 53:835–41.
- Blain JF, Sirois P. Involvement of LTD4 in allergic pulmonary inflammation in mice: modulation by cysLT1 antagonist MK-571. *Prostaglandins Leukot Essent Fatty Acids* 2000; 62:361–8.

- 20 Henderson WR, Tang LO, Chu SJ et al. A role for cysteinyl leukotrienes in airway remodeling in a mouse asthma model. *Am J Respir Crit Care Med* 2002; 165:108–16.
- 21 Nag S, Lamkhioed B, Renzi PM. Interleukin-2-induced increased airway responsiveness and lung Th2 cytokine expression occur after antigen challenge through the leukotriene pathway. *Am J Respir Crit Care Med* 2002; 165:1540–5.
- 22 Al-Rawithi S, Al-Gazlan S, Al-Ahmadi W, Alshowaier I-A, Yusuf A, Raines D-A. Expedient liquid chromatographic method with fluorescence detection for montelukast sodium in micro samples of plasma. *J Chromatogr B Biomed Sci Appl* 2001; 754:527–31.
- 23 Sanderson CJ. Interleukin-5, eosinophils, and disease. *Blood* 1992; 79:3101–9.
- 24 Lee JJ, McGarry MP, Farmer SC et al. Interleukin-5 expression in the lung epithelium of transgenic mice leads to pulmonary changes pathognomonic of asthma. *J Exp Med* 1997; 185:2143–56.
- 25 Hogan SP, Koskinen A, Matthaei KI, Young IG, Foster PS. Interleukin-5-producing CD4+ T cells play a pivotal role in aeroallergen-induced eosinophilia, bronchial hyper-reactivity, and lung damage in mice. *Am J Respir Crit Care Med* 1998; 157:210–8.
- 26 Sehmi R, Wood LJ, Watson R et al. Allergen-induced increases in IL-5 receptor α -subunit expression on bone marrow-derived CD34+ cells from asthmatic subjects. *J Clin Invest* 1997; 100:2466–75.
- 27 Ihaku D, Cameron L, Suzuki M, Molet S, Martin JMD, Hamid Q. Montelukast, a leukotriene receptor antagonist, inhibits the late airway response to antigen, airway eosinophilia, and IL-5-expressing cells in Brown Norway rats. *J Allergy Clin Immunol* 1999; 1147–54.
- 28 Dent LA, Strath M, Mellor AL, Sanderson CJ. Eosinophilia in transgenic mice expressing interleukin-5. *J Exp Med* 1990; 172:1425–31.
- 29 Leckie MJ, ten Brinke A, Khan J et al. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet* 2000; 356:2144–8.
- 30 Gonzalo JA, Jia GQ, Aguirre V, Friend D, Coyle AJ, Jenkins NA. Mouse eotaxin expression parallels eosinophil accumulation during lung allergic inflammation but it is not restricted to a Th2-type response. *Immunity* 1996; 4:1–14.
- 31 Lamkhioed B, Renzi PM, Abi-Younes S et al. Increased expression of eotaxin in bronchoalveolar lavage and airways of asthmatics contributes to the chemotaxis of eosinophils to the site of inflammation. *J Immunol* 1997; 159:4593–601.
- 32 Kaplan MH, Schindler U, Smiley ST, Grusby MJ. Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. *Immunity* 1996; 4:313–9.
- 33 Kuperman D, Schofield B, Wills-Karp M, Grusby MJ. Signal transducer and activator of transcription factor 6 (Stat6)-deficient mice are protected from antigen-induced airway hyper-responsiveness and mucus production. *J Exp Med* 1998; 187:939–48.
- 34 Wills-Carp M, Luyimbazi J, Xu X et al. Interleukin-13: central mediator of allergic asthma. *Science* 1998; 282:2258–61.
- 35 Grunig G, Warnock M, Wakil AE et al. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 1998; 282:2261–3.
- 36 Coyle AJ, Wagner K, Bertrand C, Tsuyuki S, Bews J, Heusser C. Central role of immunoglobulin (Ig) E in the induction of lung eosinophil infiltration and T helper 2 cell cytokine production: inhibition by a non-anaphylactogenic anti-IgE antibody. *J Exp Med* 1996; 183:1303–10.
- 37 Oshiba A, Hamelmann E, Takeda K et al. Passive transfer of immediate hypersensitivity and airway hyper-responsiveness by allergen-specific immunoglobulin (Ig)E and IgG1 in mice. *J Clin Invest* 1996; 97:1398–408.
- 38 Mehlhop PD, van de Rijn M, Goldberg AB et al. Allergen-induced bronchial hyper-reactivity and eosinophilic inflammation occur in the absence of IgE in a mouse model of asthma. *Proc Natl Acad Sci USA* 1997; 94:1344–9.
- 39 Schleimer RP, Sterbinsky SA, Kaiser J et al. IL-4 induces adherence of human eosinophils and basophils but not neutrophils to endothelium. Association with expression of VCAM-1. *J Immunol* 1992; 148:1086–92.
- 40 Bochner BS, Klunk DA, Sterbinsky SA, Coffman RL, Schleimer RP. IL-13 selectively induces vascular cell adhesion molecule-1 expression in human endothelial cells. *J Immunol* 1995; 154:799–803.
- 41 Gosset P, Tillie-Leblond I, Janin A et al. Increased expression of ELAM-1, ICAM-1, and VCAM-1 on bronchial biopsies from allergic asthmatic patients. *Ann NY Acad Sci* 1994; 725:163–72.
- 42 Henderson WR, Chi EY, Maliszewski CR. Soluble IL-4 receptor inhibits airway inflammation following allergen challenge in a mouse model of asthma. *J Immunol* 2000; 164:1086–95.
- 43 Sagara H, Matsuda H, Wada N et al. A monoclonal antibody against very late activation antigen-4 inhibits eosinophil accumulation and late asthmatic response in a guinea pig model of asthma. *Int Arch Allergy Immunol* 1997; 112:287–94.
- 44 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; 29:42–51.
- 45 Cheng H, Leff JA, Amin R et al. Pharmacokinetics, bioavailability, and safety of montelukast sodium (MK-0476) in healthy males and females. *Pharm Res* 1996; 13:445–8.
- 46 Schoors DF, De-Smet M, Reiss T et al. Single-dose pharmacokinetics, safety and tolerability of MK-0476, a new leukotriene D4-receptor antagonist, in healthy volunteers. *Br J Clin Pharmacol* 1995; 40:277–80.