

Provocation with adenosine 5'-monophosphate, but not methacholine, induces sputum eosinophilia

M. van den Berge*, H. A. M. Kerstjens*, D. M. de Reus†, G. H. Koëter*, H. F. Kauffman† and D. S. Postma*

Departments of *Pulmonology and †Allergology, University Hospital Groningen, Groningen, The Netherlands

Summary

Introduction Bronchial hyper-responsiveness is usually measured with direct stimuli such as methacholine (MCh) or histamine. Adenosine 5'-monophosphate (AMP), which acts indirectly via the secondary release of mediators, is another stimulus to measure bronchial hyper-responsiveness. **Aim** To investigate whether provocation with inhaled AMP itself initiates an inflammatory response resulting in an influx of eosinophils into the airway lumen.

Methods We have included 21 non-smoking atopic asthmatic subjects (mean FEV₁ 101% predicted, mean age 34 years). Each subject performed three sputum inductions on different days, at least seven days apart: one without previous provocation, one hour after PC₂₀ methacholine, and one hour after PC₂₀ AMP.

Results After provocation with AMP, but not methacholine, the percentage of sputum eosinophils increased significantly (from $1.9 \pm 0.5\%$ to $4.5 \pm 1\%$ ($P < 0.01$) and $1.9 \pm 0.5\%$ ($P = 0.89$)). No changes in the percentages of neutrophils, lymphocytes, macrophages, or bronchial epithelial cells were found.

Conclusion A provocation test with AMP leads to an increased percentage of sputum eosinophils. This observation cannot be explained by a non-specific response of the airways to a vigorous bronchoconstriction, since methacholine had no effect on inflammatory cells.

Keywords adenosine, adenosine 5'-monophosphate, hyper-responsiveness, inflammation, sputum

Submitted 9 January 2003; revised 3 May 2003; accepted 1 September 2003

Introduction

Bronchial hyper-responsiveness and airway inflammation, and in particular eosinophilic inflammation, are characteristic features of asthma. Bronchial hyper-responsiveness is usually measured with methacholine or histamine. These are both direct stimuli, since they exert their effect directly on airway smooth muscle. Another possible stimulus to measure bronchial hyper-responsiveness is adenosine 5'-monophosphate (AMP). Presently, there is an increasing interest in the role of AMP as a bronchoconstrictor stimulus in asthma, since there is suggestive evidence that the PC₂₀ AMP better reflects airway inflammation than the PC₂₀ methacholine [1–3].

Several lines of evidence indicate that AMP induces bronchoconstriction via the release of mast cell mediators through activation of A_{2B} receptors. Firstly, adenosine enhances the release of a variety of inflammatory mediators like histamine, prostaglandins, leukotrienes, and IL-8 from human mast cells *in vitro* [4, 5]. Secondly, endobronchial instillation of AMP results in an immediate increase in the

levels of both histamine and the (mast cell specific) protease tryptase in bronchoalveolar lavage fluid [6].

In recent years, there has been a tendency to attach less importance to the role of the mast cell as an effector cell in asthma. However, new findings have led to a resurgence of interest in the mast cell and support a reevaluation of its role. In particular, the discovery that mast cells are a source of T-helper 2 type cytokines suggests that mast cell activation can contribute to orchestration of the asthmatic inflammatory response [7–9]. The aim of this study was to investigate whether inhalation of AMP will initiate an inflammatory response resulting in eosinophilic influx in sputum. If so, it can be speculated that the AMP-induced increase in sputum eosinophils will be less pronounced in subjects who use inhaled corticosteroids, since inhaled corticosteroids have a general inhibitory effect on eosinophil recruitment, migration, and chemotaxis [10].

We have included 21 asthmatic patients. Eleven subjects used inhaled corticosteroids. Each subject performed three sputum inductions and blood collections on different days at least seven days apart: one without previous provocation, 1 h after PC₂₀ methacholine, and 1 h after PC₂₀ AMP. The study was designed in a crossover fashion such that each subject served as his or her own control. To exclude the possibility that our observations could be explained by a non-specific response of the airways to a vigorous bronchoconstriction, we used methacholine as a control challenge [11–14].

Correspondence: Professor D.S. Postma, Department of Pulmonary Diseases, University Hospital Groningen, Hanzplein 1, 9700 RB, Groningen, The Netherlands.
E-mail: D.S.Postma@int.azg.nl

Patients and methods

Patients

Twenty-one patients with a diagnosis of asthma, 18–65 years old, were included if they met the following criteria: a doctor's diagnosis of asthma, non-smoking, documented PC₂₀ methacholine ≤ 8 mg/mL, PC₂₀ AMP < 320 mg/mL at the first visit, at least one positive skin prick test out of 18 common aero-allergens, and the ability to produce sputum. All patients were required to have stable asthma for at least 1 month preceding and during the study. Long-acting beta-agonists were not allowed for 48 h before each visit. We have included both patients who regularly used inhaled corticosteroids and patients who had not used inhaled corticosteroids for at least 6 months.

Study design

Patients were asked to attend three visits to the hospital with an interval of 1–3 weeks. At the first visit, sputum induction and blood collection were performed 1 h after a PC₂₀ AMP (AMP visit). At the second and the third visits, sputum induction and blood collection were performed in random order either 1 h after a PC₂₀ methacholine (methacholine visit) or without previous provocation (control visit). Thus, the study was designed in a crossover fashion such that each subject served as his or her own control. The study protocol was approved by the local medical ethics committee and all participants gave their written informed consent.

Airway function

FEV₁ was measured with a calibrated dry wedge spirometer (Jaeger Masterscope, Hoechst, Germany) according to standardized guidelines [15, 16]. Provocation tests were performed using a 2 min tidal breathing method, adapted from Cockcroft and coworkers [17, 18]. After an initial nebulized saline challenge, subjects inhaled doubling concentrations, ranging from 0.04 to 320 mg/mL for AMP (Sigma, St Louis, USA) and 0.038 to 19.6 mg/mL for methacholine-bromide (Sigma, St Louis, USA), at 5 min intervals.

Sputum induction and sputum processing

Sputum was induced by inhalation of hypertonic saline aerosols as previously described [16]. Fifteen minutes after salbutamol (200 µg) inhalation, hypertonic saline (3%, 4%, and 5%) was nebulized for each concentration during 7 min. Whole samples were processed according to the method of Fahy et al. with some modifications [16, 19]. Samples with contamination of $> 80\%$ squamous cells were excluded from analyses. Subjects were excluded if they were unable to produce an evaluable sputum sample at one or more study visits. At least 600 non-squamous cells were counted by one investigator who was blinded to the type of challenge and the counts were expressed as percentages.

Biochemical assays

The concentration of eosinophilic cationic protein (ECP) in serum and sputum were measured using a fluoroenzyme assay, the ImmunoCAP ECP (provided by Pharmacia,

Uppsala, Sweden). The concentration of interleukin 8 (IL-8) in sputum was measured by ELISA (CLB, Amsterdam).

Statistical methods

All calculations of PC₂₀ were performed with the base-2 logarithm ($\log_2$) since this reflects doubling concentrations and normalizes the distribution. The normality of distributions was assessed using the Kolmogorov–Smirnov test. If this test resulted in a P -value < 0.05 , normalization by logarithmic transformation was attempted. All data were analysed using the Student's t -test for matched pairs in case of normal distribution and the Wilcoxon signed ranks test otherwise. Values are presented as mean $\pm$ standard error of the mean (SEM) unless stated otherwise. When analysing the subgroups of patients with and without inhaled corticosteroids, the Wilcoxon signed ranks test was used.

Results

Patient characteristics

Twenty-one patients were included into the study. Eleven patients used inhaled corticosteroids. The baseline characteristics are presented in Table 1. No significant differences in baseline characteristics were found between patients with and without the use of inhaled corticosteroids. Concentrations of sputum ECP and IL-8 were measured in 16 patients after a PC₂₀ AMP, in 17 patients after a PC₂₀ methacholine, and in 17 patients at the control visit. Blood cell numbers and differential counts were performed in 21 patients after a PC₂₀ AMP and in 19 patients after a PC₂₀ methacholine and at the control visit. The concentration of ECP in blood was assessed in all patients. We did not find any differences in baseline FEV₁% predicted between the AMP visit, the methacholine visit, and the control visit ($103 \pm 2.7\%$, $103 \pm 3\%$, and $103 \pm 3\%$, respectively, $P > 0.05$). Further, we found no difference in the maximum percentage fall in FEV₁ between an AMP challenge and a methacholine challenge (mean $\pm$ SEM $27 \pm 1.2\%$ vs. $27 \pm 1.6\%$, $P = 0.76$).

Changes in inflammatory parameters in induced sputum and blood one hour after a provocation test with either AMP or methacholine

For the group as a whole (both patients with and without inhaled corticosteroids), the percentage of sputum eosinophils increased significantly after a PC₂₀ AMP, but not after a PC₂₀ methacholine (from $1.9 \pm 0.5\%$ to $4.5 \pm 1\%$, $P < 0.01$ and $1.9 \pm 0.5\%$ to $1.9 \pm 0.5\%$ respectively, $P = 0.89$) (see Table 2 and Fig. 1). In addition, we found that the percentage of sputum eosinophils increased significantly after a PC₂₀ AMP within the subgroup of patients who did not use inhaled corticosteroids (from $2.0 \pm 0.73\%$ to $4.2 \pm 1.2\%$, $P = 0.04$), whereas a non-significant trend was found within the subgroup of patients who did use inhaled corticosteroids (from $1.9 \pm 0.7\%$ to $4.8 \pm 1.4\%$, $P = 0.09$) (see Fig. 1). When directly comparing the magnitude of the AMP-induced change in sputum eosinophils between the two groups (i.e. patients with and without inhaled corticosteroids), we did not find a significant difference (the change being $2.8 \pm 1.5\%$ vs.

Table 1. Characteristics and cell count data of the study population*

	All patients <i>n</i> = 21	Patients with ICS <i>n</i> = 11	Patients without ICS <i>n</i> = 10	
Age (years)	33 ± 2	35 ± 4	31 ± 7	NS
Gender M/F	7/14	5/6	2/8	NS
Smoking, <i>n</i>				
Current	0	0	0	NS
Never	17	7	10	NS
Dose of inhaled corticosteroids†		1072.7 ± 187.4		
FEV ₁ (%pred.)	103 ± 3	99 ± 3.5	107 ± 4.8	NS
PC ₂₀ meth (mg/mL)‡§	0.8 ± 1.4	1.1 ± 0.6	4.3 ± 1.8	NS
PC ₂₀ AMP (mg/mL)‡§	11.1 ± 1.4	8.6 ± 1.6	14.6 ± 1.8	NS
Blood eosinophils (10 ⁹ /L)	0.2 ± 0	0.2 ± 0.1	0.2 ± 0.1	NS
Sputum ECP (ng/mL)‡§	61.6 ± 1.4	67.5 ± 1.7	57.8 ± 1.7	NS
Sputum IL-8 (ng/mL)‡§	982.2 ± 1.3	790.5 ± 1.6	1118.7 ± 1.4	NS
Sputum				
Total cell count (10 ⁶ cells)‡,§	2.6 ± 1.3	2.1 ± 1.4	3.3 ± 1.4	NS
Macrophages, %	40.9 ± 6.3	42.3 ± 8.4	39.4 ± 10	NS
Lymphocytes, %	1 ± 0.2	1 ± 0.3	1 ± 0.3	NS
Neutrophils, %	55.7 ± 6.6	54.4 ± 8.7	57.2 ± 10.5	NS
Eosinophils, %	1.9 ± 0.5	1.9 ± 0.6	2 ± 0.7	NS
Bronchial epithelial cells, %	0.5 ± 0.2	0.5 ± 0.2	0.5 ± 0.3	NS

*Values are expressed as mean ± SE of the mean. †Expressed as µg beclomethasone or equivalent (100 µg beclomethasone = 100 µg budesonide = 50 µg fluticasone). ‡Geometric mean and geometric standard error of the mean. §Log transformed to obtain normal distribution.

Table 2. Blood and sputum inflammatory markers after a PC₂₀ with either AMP or methacholine and a control visit*

	PC ₂₀ AMP <i>n</i> = 21	PC ₂₀ methacholine <i>n</i> = 21	Control <i>n</i> = 21
Sputum			
Total Cell Count, (10 ⁶ cells)†‡	2 ± 0.1	1.2 ± 0.1	2.6 ± 0.1
Eosinophils, %	4.5 ± 1§	1.9 ± 0.5	1.9 ± 0.5
Lymphocytes, %	1.1 ± 0.3	0.8 ± 0.2	1 ± 0.2
Macrophages, %	34 ± 5.2	36.2 ± 5.3	40.9 ± 6.3
Neutrophils, %	59.4 ± 5.5	60.4 ± 5.5	55.7 ± 6.6
Bronchial epithelial cells, %	0.7 ± 0.2	0.8 ± 0.3	0.5 ± 0.3
ECP (ng /mL)†‡	55 ± 1.3	34.2 ± 1.3§	61.6 ± 1.4
IL-8 (ng/mL)†‡	1327 ± 1.2	1452.4 ± 1.3	982.2 ± 1.3
Blood			
Eosinophils (10 ⁹ /L)	0.22 ± 0	0.20 ± 0§	0.21 ± 0
Neutrophils (10 ⁹ /L)	4.3 ± 0.3	3.9 ± 0.3	4.1 ± 0.3
Lymphocytes (10 ⁹ /L)	2.0 ± 0.7	1.9 ± 0.2	2 ± 0.2
Monocytes (10 ⁹ /L)	0.5 ± 0.1	0.52 ± 0	0.5 ± 0
Basophils (10 ⁹ /L)	0.05 ± 0.03	0.04 ± 0.05	0.04 ± 0.01
ECP (ng/mL)	10.1 ± 1	10.5 ± 1.5	10.1 ± 1.2

*Values are expressed as mean ± standard error of the mean. †Geometric mean ± geometric standard error of the mean. ‡Log transformed to obtain normal distribution. §Significantly different compared with control.

2.2 ± 0.9%, *P* = 0.8). The concentration of sputum ECP and the number of eosinophils in blood decreased significantly after methacholine challenge (*P* = 0.04 and *P* = 0.01, respectively), but not after AMP challenge (see Table 2). No further changes in percentages of inflammatory cells in sputum and peripheral blood were observed after a provocation test with either AMP or methacholine. In addition, the concentration of IL-8 in sputum and ECP in blood did not significantly

change after a provocation test with AMP or methacholine. We have also analysed our data using total cell counts in sputum instead of percentages of cells. Similarly, the number of eosinophils in sputum tended to increase after a PC₂₀ AMP (*P* = 0.08), but not after a PC₂₀ methacholine (*P* = 0.43), whereas no changes in the numbers of lymphocytes, macrophages, neutrophils, or bronchial epithelial cells were observed.

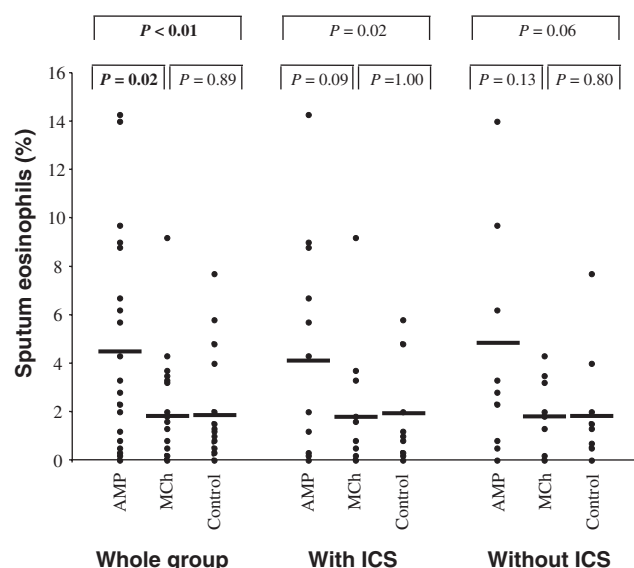


Fig. 1. Percentages of sputum eosinophils 1 h after provocation with either AMP or methacholine, and at the control visit. Data are presented for all patients (whole group), for patients who used inhaled corticosteroids (with ICS), and for patients who did not use inhaled corticosteroids (without ICS).

Discussion

This study demonstrates that a provocation test with AMP, but not methacholine, induces an increase in the percentage of sputum eosinophils in patients with asthma. These observations suggest that inhaled AMP has a rapid (within 1 h) effect on airway migration of eosinophils in asthmatic subjects.

Our findings of an increase in sputum eosinophils are compatible with the results of Spruntulis and colleagues [20] in an animal model. They observed a significant increase of inflammatory cells (both macrophages and eosinophils) in the bronchoalveolar lavage fluid (BAL) of guinea-pigs within 1 h after exposure to AMP. We have extended these findings in two ways. Firstly, we have demonstrated that an inflammatory response after inhalation of AMP also occurs in humans. However, we did not observe an increase in macrophages, but a selective increase of eosinophils. This could be due to differences in the airway response to AMP between humans and guinea-pigs. The difference in outcome with respect to macrophages may also be due to the use of different compartments of the airways (i.e. BAL and sputum), since sputum has been shown to contain less macrophages than BAL [21]. Secondly, we have also performed sputum induction 1 h after a PC₂₀ methacholine as a control challenge and no change in sputum cell differential was observed. This makes it unlikely that the observed increase in sputum eosinophils can be explained by a non-specific response to a vigorous bronchoconstriction of the airways, as has been suggested by Diamant et al. [12].

How can we interpret the findings of our study? Once inhaled, AMP is rapidly converted to adenosine by the ubiquitous enzyme 5'-nucleotidase. Adenosine can activate at least four different adenosine receptors, namely adenosine A₁, A_{2A}, A_{2B}, and A₃ receptors. The activation of A_{2B} receptors present on human mast cells is thought to mediate the

bronchoconstrictor response to AMP via the release of a variety of inflammatory mediators [22]. There are various suggestions in the literature that mast cell-derived mediators could account for the influx of eosinophils into the airway lumen. It has been demonstrated that the proteases tryptase and chymase provide a potent stimulus for eosinophil accumulation following an injection into the skin of guinea pigs [23–25]. Further, inhalation of LTD₄ and LTE₄, which are the major lipoxygenase products of mast cells and the cytokines TNF- α and IL-4, are associated with increases of eosinophils in sputum and bronchial biopsies [12, 13, 26–28]. It cannot be deduced from our data as to whether the increase in sputum eosinophils is due to an AMP-induced migration of eosinophils from peripheral blood to the airways or, alternatively, due to migration from the sub-epithelial compartment to the epithelium and hence sputum in response to the release of epithelial mast cell mediators.

Another possible mechanism for the AMP-induced increase in sputum eosinophils might be an effect of adenosine on A₁, A_{2A}, and A₃ receptors, which have been identified on neutrophils, eosinophils, and macrophages. Although the exact consequences of their activation are difficult to predict, various pro- and anti-inflammatory actions have been described; activation of adenosine A₁ receptors promotes chemotaxis of neutrophils and increases adherence of neutrophils to endothelial cells [29, 30]. In contrast, the activation of adenosine A_{2A} receptors reduces chemotaxis, activation, and degranulation of neutrophils and activation of A₃ receptors mediates the inhibition of eosinophil chemotaxis when activated [31, 32]. The exact consequence of activation of different adenosine receptors remains to be sorted out in future studies.

We did not find any changes in percentages of sputum eosinophils, macrophages, lymphocytes, neutrophils, or bronchial epithelial cells 1 h after provocation with methacholine. This is in agreement with other studies in which no changes in percentages of inflammatory cells in sputum or bronchoalveolar lavage fluid were found within 30 min, 1, 4, and 5 h after provocation with methacholine [11–14, 33]. However, we unexpectedly found a decrease in the concentration of sputum ECP and the number of peripheral blood eosinophils. This is in contrast to the findings of Spanevello and colleagues, who did not observe a change in the concentration of sputum ECP after methacholine challenge. A possible explanation for this discrepancy could be that Spanevello and colleagues have evaluated the effect of methacholine challenge on sputum ECP in only 9 patients and this small number may have limited the power to detect changes. Alternatively, the fall in sputum ECP and peripheral blood eosinophils after a PC₂₀ methacholine could be a chance finding, especially in view of the multiple parameters tested in this study.

In our study, all subjects performed a PC₂₀ AMP followed by sputum induction at the first visit, after which they were randomized to either methacholine provocation or baseline sputum induction at the second and the third visits. Thus, a point of criticism of our study design could be that an 'order of measurement bias' cannot be excluded. However, we are confident that this study design did not affect our results. Firstly, all study visits were separated by an interval of at least one week, because we felt it likely that the influence of either a

provocation test (with AMP or methacholine) or a sputum induction will have disappeared at the subsequent visit. In agreement with this, it has been demonstrated in a recent study that an interval of at least two days between subsequent sputum inductions gives reproducible cell differential counts [34]. In addition, no change in sputum cell differential counts could be observed in previous studies up to 4 h after a provocation test with methacholine [12, 35]. We are the first to demonstrate that a provocation test with AMP will increase the percentage of eosinophils in sputum, and it is unknown whether this increase will have normalized after 1 week. If not, our study design would have made it even more difficult to detect an AMP-induced increase in sputum eosinophils. Finally, it is unlikely that our results were influenced by seasonal effects, since patients were included into the study throughout the year and baseline FEV₁% predicted values were similar at all visits.

The observed AMP-induced increase in the percentage of sputum eosinophils appeared to be independent of the use of inhaled corticosteroids. This was an unexpected finding, since inhaled corticosteroids have a general inhibitory effect on eosinophil migration and chemotaxis [36, 37]. A possible explanation for this observation may be that the severity of asthma (i.e. the level of FEV₁, PC₂₀ methacholine, PC₂₀ AMP, and the extent and activation of airway inflammation) did not differ at baseline between patients with and without inhaled corticosteroids. In addition, airway insult was the same in both groups in terms of the achieved percentage drop in FEV₁ during the airway challenge test with AMP. It could be speculated that the pre-existing increased inflammatory activation state in those patients with inhaled corticosteroids was already counterbalanced by the well-known inhibitory effect of inhaled corticosteroids. An alternative explanation may be that AMP induces an increase in sputum eosinophils via a corticosteroid-insensitive mechanism.

In conclusion, we have demonstrated that a PC₂₀ AMP, but not PC₂₀ methacholine, increases sputum eosinophilia in asthmatic subjects. Our findings may indicate a role for adenosine and adenosine receptors in asthma, although the exact type of adenosine receptor is yet unresolved. Therefore, it is important to further elucidate the role of adenosine and adenosine receptors in asthma, as this may well have consequences for the treatment of asthma.

Acknowledgements

This study was supported by an unrestricted educational grant from GlaxoSmithKline. We thank M. van der Toorn and I. Sloots for their laboratory assistance.

References

- Van Den Berge M, Meijer RJ, Kerstjens HAM et al. PC₂₀ adenosine 5'-monophosphate is more closely associated with airway inflammation in asthma than PC₂₀ methacholine. *Am J Respir Crit Care Med* 2001; 163:1546–50.
- Van Den Berge M, Kerstjens HAM, Meijer RJ et al. Corticosteroid-induced improvement in the PC(20) of adenosine monophosphate is more closely associated with reduction in airway inflammation than improvement in the PC(20) of methacholine. *Am J Respir Crit Care Med* 2001; 164:1127–32.
- Polosa R, Ciamarra I, Mangano G et al. Bronchial hyperresponsiveness and airway inflammation markers in nonasthmatics with allergic rhinitis. *Eur Respir J* 2000; 15:30–5.
- Church MK, Holgate ST, Hughes PJ. Adenosine inhibits and potentiates IgE-dependent histamine release from human basophils by an A₂-receptor mediated mechanism. *Br J Pharmacol* 1983; 80:719–26.
- Hughes PJ, Holgate ST, Church MK. Adenosine inhibits and potentiates IgE-dependent histamine release from human lung mast cells by an A₂-purinoceptor mediated mechanism. *Biochem Pharmacol* 1984; 33:3847–52.
- Polosa R, Ng WH, Crimi N et al. Release of mast-cell-derived mediators after endobronchial adenosine challenge in asthma. *Am J Respir Crit Care Med* 1995; 151:624–9.
- Bradding P, Holgate ST. The mast cell as a source of cytokines in asthma. *Ann N Y Acad Sci* 1996; 796:272–81.
- Bradding P, Roberts JA, Britten KM et al. Interleukin-4, -5, and -6 and tumor necrosis factor- α in normal and asthmatic airways: evidence for the human mast cell as a source of these cytokines. *Am J Respir Cell Mol Biol* 1994; 10:471–80.
- Bradding P, Feather IH, Wilson SJ et al. Immunolocalization of cytokines in the nasal mucosa of normal and perennial rhinitic subjects. The mast cell as a source of IL-4, IL-5, and IL-6 in human allergic mucosal inflammation. *J Immunol* 1993; 151:3853–65.
- Barnes PJ. Scientific rationale for inhaled combination therapy with long-acting beta₂-agonists and corticosteroids. *Eur Respir J* 2002; 19:182–91.
- Spanevello A, Vignola AM, Bonanno A, Confalonieri M, Crimi E, Brusasco V. Effect of methacholine challenge on cellular composition of sputum induction. *Thorax* 1999; 54:37–9.
- Diamant Z, Hiltermann TJ, van Rensen EL et al. The effect of inhaled leukotriene D₄ and methacholine on sputum cell differentials in asthma. *Am J Respir Crit Care Med* 1997; 155:1247–53.
- Mulder A, Gauvreau GM, Watson RM, O'Byrne PM. Effect of inhaled leukotriene D₄ on airway eosinophilia and airway hyperresponsiveness in asthmatic subjects. *Am J Respir Crit Care Med* 1999; 159:1562–7.
- Kraft M, Bettinger CM, Wenzel SE, Irvin CG, Ackerman SJ, Martin RJ. Methacholine challenge does not affect bronchoalveolar fluid cell number and many indices of cell function in asthma. *Eur Respir J* 1995; 8:1966–71.
- Quanjer PH, Tammeling GJ, Cotes JE, Pedersen OF, Peslin R, Yernault JC. Lung volumes and forced ventilatory flows. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. *Eur Respir J Suppl* 1993; 16:5–40.
- Meijer RJ, Kerstjens HAM, Arends LR, Kauffman HF, Koeter GH, Postma DS. Effects of inhaled fluticasone and oral prednisolone on clinical and inflammatory parameters in patients with asthma. *Thorax* 1999; 54:894–9.
- Cockcroft DW, Killian DN, Mellon JJ, Hargreave FE. Bronchial reactivity to inhaled histamine: a method and clinical survey. *Clin Allergy* 1977; 7:235–43.
- Oosterhoff Y, Jansen MA, Postma DS, Koeter GH. Airway responsiveness to adenosine 5'-monophosphate in smokers and nonsmokers with atopic asthma. *J Allergy Clin Immunol* 1993; 92:773–6.
- Fahy JV, Liu JT, Wong H, Boushey HA. Cellular and biochemical analysis of induced sputum from asthmatic and from healthy subjects. *Am Rev Respir Dis* 1993; 147:1126–31.
- Okada S, Kita H, George TJ, Gleich GJ, Leiferman KM. Migration of eosinophils through basement membrane components *in vitro*: role of matrix metalloproteinase-9. *Am J Respir Cell Mol Biol* 1997; 17:519–28.

- 21 Pizzichini E, Pizzichini MM, Kidney JC et al. Induced sputum, bronchoalveolar lavage and blood from mild asthmatics: inflammatory cells, lymphocyte subsets and soluble markers compared. *Eur Respir J* 1998; 11:828–34.
- 22 Selroos O, Halme M. Effect of a volumatic spacer and mouth rinsing on systemic absorption of inhaled corticosteroids from a metered dose inhaler and dry powder inhaler. *Thorax* 1991; 46:891–4.
- 23 Walls AF, He S, Teran LM et al. Granulocyte recruitment by human mast cell tryptase. *Int Arch Allergy Immunol* 1995; 107:372–3.
- 24 He S, Walls AF. Human mast cell chymase induces the accumulation of neutrophils, eosinophils and other inflammatory cells *in vivo*. *Br J Pharmacol* 1998; 125:1491–500.
- 25 Compton SJ, Cairns JA, Holgate ST, Walls AF. Interaction of human mast cell tryptase with endothelial cells to stimulate inflammatory cell recruitment. *Int Arch Allergy Immunol* 1999; 118:204–5.
- 26 Gauvreau GM, Parameswaran KN, Watson RM, O'Byrne PM. Inhaled leukotriene E(4), but not leukotriene D(4), increased airway inflammatory cells in subjects with atopic asthma. *Am J Respir Crit Care Med* 2001; 164:1495–500.
- 27 Hirata N, Kohrogi H, Iwagoe H et al. Allergen exposure induces the expression of endothelial adhesion molecules in passively sensitized human bronchus: time course and the role of cytokines. *Am J Respir Cell Mol Biol* 1998; 18:12–20.
- 28 Blease K, Seybold J, Adcock IM, Hellewell PG, Burke-Gaffney A. Interleukin-4 and lipopolysaccharide synergize to induce vascular cell adhesion molecule-1 expression in human lung microvascular endothelial cells. *Am J Respir Cell Mol Biol* 1998; 18:620–30.
- 29 Cronstein BN, Daguma L, Nichols D, Hutchison AJ, Williams KM. The adenosine/neutrophil paradox resolved: human neutrophils possess both A1 and A2 receptors that promote chemotaxis and inhibit O₂ generation, respectively. *J Clin Invest* 1990; 85:1150–7.
- 30 Felsch A, Stocker K, Borchard U. Phorbol ester-stimulated adherence of neutrophils to endothelial cells is reduced by adenosine A₂ receptor agonists. *J Immunol* 1995; 155:333–8.
- 31 Suzuki H, Takei M, Nakahata T, Fukamachi H. Inhibitory effect of adenosine on degranulation of human cultured mast cells upon cross-linking of Fc epsilon RI. *Biochem Biophys Res Commun* 1998; 242:697–702.
- 32 Walker BA, Jacobson MA, Knight DA et al. Adenosine A₃ receptor expression and function in eosinophils. *Am J Respir Cell Mol Biol* 1997; 16:531–7.
- 33 Nowak D, Grimminger F, Jorres RA et al. Increased LTB₄ metabolites and PGD₂ in BAL fluid after methacholine challenge in asthmatic subjects. *Eur Respir J* 1993; 6:405–12.
- 34 Purokivi M, Randell J, Hirvonen MR, Tukiainen H. Reproducibility of measurements of exhaled NO, and cell count and cytokine concentrations in induced sputum. *Eur Respir J* 2000; 16:242–6.
- 35 Spanevello A, Migliori GB, Sharara A et al. Induced sputum to assess airway inflammation: a study of reproducibility. *Clin Exp Allergy* 1997; 27:1138–44.
- 36 Miyamasu M, Misaki Y, Izumi S et al. Glucocorticoids inhibit chemokine generation by human eosinophils. *J Allergy Clin Immunol* 1998; 101:75–83.
- 37 Lantero S, Sacco O, Scala C, Morelli MC, Rossi GA. Eosinophil locomotion and the release of IL-3 and IL-5 by allergen-stimulated mononuclear cells are effectively downregulated *in vitro* by budesonide. *Clin Exp Allergy* 1996; 26:656–64.