

Effect of theophylline and specific phosphodiesterase IV inhibition on proliferation and apoptosis of progenitor cells in bronchial asthma

¹Chun-Hua Wang, ¹Horng-Chyuan Lin, ²Chien-Huang Lin, ¹Chih-Teng Yu, ¹Su-Ling Liu, ¹Kuo-Hsiung Huang, ³Kian Fan Chung & *¹Han-Pin Kuo

¹Department of Thoracic Medicine II, Chang Gung Memorial Hospital, Taipei, Taiwan; ²School of Respiratory Therapy, Taipei Medical University, Taipei, Taiwan and ³National Heart & Lung Institute, Imperial College School of Medicine & Royal Brompton Hospital, London

1 Theophylline possesses anti-inflammatory activities in asthma. We examined whether theophylline and agents that modulate cyclic AMP can determine the survival and proliferation of progenitor cells.

2 Progenitor cells from the blood of normal and asthmatic subjects were cultured for 14 days in methylcellulose with GM-CSF, stem cell factor, IL-3 and IL-5. Apoptosis was measured by flow cytometry of propidium-iodide-stained cells.

3 A greater number of colonies with a higher proportion of cells of eosinophil lineage from asthmatics compared to normal subjects were grown. Theophylline (at 5 and 20 $\mu\text{g ml}^{-1}$) significantly inhibited colony formation and increased apoptotic cells in asthmatics compared to control. Salbutamol (0.1, 1, 10 μM), dibutyryl-cAMP (0.1, 1 mM) and rolipram (0.1, 1 mM), a phosphodiesterase IV inhibitor, also dose-dependently decreased colony numbers and increased apoptosis of progenitor cells from asthmatics.

4 There was no significant effect of theophylline, db-cAMP, salbutamol or rolipram on colony formation or the survival of progenitor cells from normal subjects. AMP did not affect the colony formation and apoptosis. Expression of Bcl-2 protein on progenitor cells of asthma was downregulated by theophylline, salbutamol, db-cAMP and rolipram.

5 Theophylline and rolipram decreased colony formation committed to the eosinophil lineage, together with an increase in apoptosis through an inhibition of Bcl-2 expression effects that may occur through cAMP. The anti-inflammatory properties of theophylline include an inhibition of circulating progenitor cells.

British Journal of Pharmacology (2003) **138**, 1147–1155. doi:10.1038/sj.bjp.705131

Keywords: Phosphodiesterase inhibition; theophylline; asthma; CD34+ progenitor cells; Bcl-2 protein; apoptosis

Abbreviations: AMP, adenosine monophosphate; cAMP, adenosine 3',5'-cyclic monophosphate; CFU, colony-forming units; CFU-GM, granulocyte-macrophage CFU; db-cAMP, dibutyryl-cAMP; FEV1, forced expiratory volume in 1 s; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-3, interleukin-3; IL-5, interleukin-5; MFI, mean fluorescence intensity; PE, phycoerythrin; SCF, stem cell factor

Introduction

Asthma is characterized by chronic airway inflammation, cellular infiltration, and cytokine production (Laitinen *et al.*, 1985; Jeffery *et al.*, 1989; Laitinen *et al.*, 1993). Among the inflammatory cells, eosinophils may play an important role in bronchial asthma (Gleich, 1990). Increased numbers of progenitor cells expressing CD34+ committed to an eosinophilic lineage is found in the blood of patients with asthma (Chou *et al.*, 1999; Wang *et al.*, 1999). Furthermore, progenitor cells are increased during asthma exacerbation induced by controlled withdrawal of inhaled steroid therapy (Gibson *et al.*, 1990), and by experimental exposure to allergen (Gibson *et al.*, 1991). In addition to an increase found in peripheral blood, CD34+ cells expressing IL-5R α receptor have also been found in the airways of patients with asthma and have been correlated with asthma severity (Robinson *et al.*,

1999). Therefore, circulating CD34+ progenitor cells derived from bone marrow may migrate to airways and develop into mature cells under the effects of cytokines and haematopoietic factors, such as interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF) and interleukin-5 (IL-5).

Theophylline has been used in the treatment of asthma for several decades as a bronchodilator, but several lines of evidence indicate that it also has anti-inflammatory effects (Chung, 1996). Thus, theophylline reduces the numbers of eosinophils in the airway submucosa of patients with asthma (Sullivan *et al.*, 1994; Lim *et al.*, 2000). The mechanisms underlying this effect could be related to apoptosis or programmed cell death of eosinophils since theophylline increased apoptosis of IL-5-exposed eosinophils *in vitro* (Adachi *et al.*, 1996; Ohta & Yamashita, 1999). Since progenitor cells, particularly those committed to an eosinophil lineage, are increased in the circulation and in the mucosal

*Author for correspondence; E-mail: q8828@ms11.hinet.net

tissues of patients with asthma, we determined whether theophylline could also influence the survival and proliferation of progenitor CD34+ cells, similar to its effects on mature eosinophils. Apoptosis of haemopoietic and epithelial progenitor cells may be mediated by p53 and c-myc (Hockenbery *et al.*, 1991; Ryan *et al.*, 1994), and Bcl-2 acts as an inhibitor of apoptosis (Chao & Korsmeyer, 1998). We determined whether any potential effect of theophylline on apoptosis could be linked to modulation of Bcl-2 expression. Since the intracellular effects of theophylline could be secondary to phosphodiesterase (PDE) inhibition, we also examined the effects of a β -adrenergic agonist, salbutamol, of rolipram, a specific PDE type 4 inhibitor, and of a cell-permeant analog of cyclic adenosine monophosphate (cAMP).

Methods

Subjects and study design

A total of forty nonsmoking adults with mild intermittent asthma (17 males and 23 females) were recruited from outpatient clinics of Chang Gung Memorial Hospital (Table 1). All patients with asthma had a >15% improvement in forced expiratory volume in 1 s (FEV1) with inhaled albuterol (400 μ g). These patients had mild asthma symptoms with intermittent daytime asthma symptoms less than once per week, night time symptoms less than twice a month, and normal lung function between exacerbations were defined as mild intermittent asthma. Of these, thirty of the asthmatic volunteers were atopic as determined by history and by increased serum specific immunoglobulin E (IgE) to common aeroallergens (Phadiatop, Pharmacia, Sweden). All patients had been stable for at least 6 weeks before enrolment and were only taking inhaled short-acting β 2-agonists as a rescue medication. Inhaled β 2-agonists were withheld for 12 h before venepuncture and methacholine testing.

A total of thirty-five normal nonsmoking subjects (16 males and 19 females) without evidence of allergic rhinitis and asthma with normal lung function and no evidence of bronchial hyper-responsiveness to methacholine (as described and defined below), were recruited from our hospital staff or medical students. They had negative serum specific IgE (Phadiatop) and normal total IgE level as well as eosinophil counts. This study was approved by our Ethics Committee and all subjects gave informed consent.

Methacholine challenge testing

FEV1 was measured by a Spiroanalyzer ST-350R (Fukuda Sangyo Co. Ltd., Tokyo, Japan). The best of three reprodu-

cible values (with a difference within or less than 5%) was used for the calculation. Methacholine dissolved in phosphate-buffered saline (PBS) or PBS alone (10 mM, pH = 7.40) was delivered by a Rosenthal dosimeter (Model PF2A, Laboratory for Applied Immunology Inc., VA, U.S.A.) using 646 DeVilbiss nebulizer. PBS was inhaled first, followed by methacholine solution at increasing concentrations of 0.075, 0.15, 0.3, 0.6, 1.25, 2.50, 5.0, 10 and 25 mg ml⁻¹ at 5-min intervals. Patients received five inhalations for each concentration. FEV1 was measured 5 min after the inhalation, followed by inhalation of the next higher concentration until FEV1 has fallen by more than 20% of the starting FEV1. The log dose – response curves for methacholine was constructed as the percentage changes in FEV1 from the baseline (postbuffer) value. The dose of methacholine causing a 20% fall in FEV1 (PC₂₀) was measured by linear interpolation. Bronchial hyper-responsiveness was defined as a PC₂₀ of less than 8 mg ml⁻¹.

Preparation of progenitor cells

Heparinized venous blood (100 ml) was obtained from each subject. Our method for preparing progenitor cells has been described previously (Wang *et al.*, 1999). In brief, peripheral blood mononuclear cells were separated from the whole blood using Ficoll–Hypaque density gradient centrifugation at 400 \times g for 30 min. The mononuclear cells at the interface were harvested, washed twice, and resuspended in Iscove modified Dulbecco medium (IMDM). The nonadherent mononuclear cell fraction was separated by adherence technique (Shih & Lee, 1994). Resuspended cell pellets were incubated in 25 cm² flask at 37°C and 5% CO₂ for 2 h. The nonadherent mononuclear cells were separated by centrifugation at 4°C and 1500 r.p.m. for 5 min and resuspended in IMDM. These nonadherent mononuclear cells were mixed well with 100 μ l sheep red blood cells, then incubated at 4°C for 1 h, and depleted of E-rosette-forming cells by a second Ficoll–Hypaque centrifugation. Cells depleted of T-lymphocytes were obtained at the interface and these cells constitute the progenitor cell fraction we studied in this paper.

Granulocyte-macrophage progenitor (CFU-GM) assay

The progenitor cells (see above) were then cultured at a concentration of 2×10^5 cells in 1 ml of IMDM containing 0.9% (v/v⁻¹) of methylcellulose, 2-mercaptoethanol (50 μ M), 1% bovine serum albumin (BSA), and 30% fetal calf serum, with growth factors, GM-CSF (500 U ml⁻¹), stem cell factor (50 ng ml⁻¹), IL-3 (100 U ml⁻¹), and IL-5 (10 ng ml⁻¹) in 35-mm dishes (Shih & Lee, 1994; Wang *et al.*, 1999). Progenitor cells were treated with a range of concentrations of theophylline (0, 1, 5, 20, and 100 μ g ml⁻¹; Sigma, St Louis, MO, U.S.A.), salbutamol (0, 0.1, 1, 10 μ M; Sigma, St Louis, MO, U.S.A.), dibutyl adenosine 3',5'-cyclic monophosphate (db-cAMP, 0, 0.01, 0.1, and 1 mM; Sigma, St Louis, MO, U.S.A.) or adenosine monophosphate (AMP, 0, 0.01, 0.1, and 1 mM; Sigma, St Louis, MO, U.S.A.). In order to study the effect of PDE on colony formation of progenitor cells, rolipram (Sigma, St Louis, MO, U.S.A.) was added at the concentrations of 0, 0.01, 0.1 or 1 mM. Duplicate cultures were prepared for each sample and incubated for 14 days in a humidified atmosphere of 5% CO₂ in air. Colonies were scored 14 days after plating using an inverted light microscope. A colony was

Table 1 Demographic features of patients

	Normal subjects	Asthmatic patients
Age (years)	29.8 \pm 1.7	30.5 \pm 1.5
Sex (male/female)	16/19	17/23
FEV1 (% predicted)	85.9 \pm 3.6	80.3 \pm 2.2
FEV1/FVC (%)	84.3 \pm 2.7	80.7 \pm 1.4
PC ₂₀ (mg ml ⁻¹)	> 25	3.6 \pm 0.7

PC₂₀: concentration of methacholine to induce 20% decrease in FEV1. Data are mean \pm s.e.m.

defined as a group of 40 or more cells. Cyto centrifuge preparation smears of the cultured colonies were made for morphological identification and differential cell count determination.

All experimental procedures were performed in endotoxin-free plastic wares; according to the manufacturer's information, the levels of endotoxin contamination in the cytokine preparations were three endotoxin units mg^{-1} by the limulus assay, and the antibodies used in all experiments contained $<1 \text{ ng ml}^{-1}$ of endotoxin.

Assessment of apoptosis

Progenitor cells ($2 \times 10^5 \text{ cells ml}^{-1}$) were treated with or without theophylline, salbutamol, cAMP, AMP and rolipram at various concentrations in the IMDM containing 1% BSA, 30% fetal calf serum, with growth factors, GM-CSF (500 U ml^{-1}), stem cell factor (50 ng ml^{-1}), IL-3 (100 U ml^{-1}), and IL-5 (10 ng ml^{-1}), and incubated in 5% CO_2 in air at 37°C for 3 days. To measure apoptosis of progenitor cells, the cells were first fixed and permeabilized by adding $500 \mu\text{l}$ iced 70% alcohol to the cell pellet and incubating at room temperature for 10 min. The cells were washed twice and resuspended in 0.5 ml PBS. The cells were treated with $10 \mu\text{l}$ RNase A ($100 \mu\text{g ml}^{-1}$) and $10 \mu\text{l}$ propidium iodide (PI, $40 \mu\text{g ml}^{-1}$) at the same time, then incubated in a water bath at 37°C for 30 min. The proportion of cells within the hypodiploid DNA region was assessed by flow cytometry (Nicoletti *et al.*, 1991).

Measurement of Bcl-2 expression in CD34+ cells

To measure the expression of intracellular Bcl-2 in CD34+ progenitor cells, cells were initially stained with anti-CD34-conjugated phycoerythrin (PE) monoclonal antibody (Becton Dickinson, Mountain View, CA, U.S.A.) for 30 min at 4°C . Then, the cells were washed with PBS and subsequently permeabilized with FACS™ permeabilizing solution (Becton Dickinson, Mountain View, CA, U.S.A.) for 20 min at room temperature. The cells were washed with PBS twice, then followed by staining with anti-Bcl-2 monoclonal antibody-conjugated FITC (Ansell Co., Bayport, MN, U.S.A.) for 30 min at 4°C . Subsequently, the cells were washed with ice-cold PBS twice and analyzed using a FACScan® (Becton Dickinson & Co., Mountain View, CA, U.S.A.). Unrelated FITC-labelled anti-IgG1 (Ansell Co., Bayport, MN, U.S.A.) and PE-conjugated IgG (Becton Dickinson, Mountain View, CA, U.S.A.) monoclonal antibodies were used as negative control.

Materials

Theophylline, salbutamol, db-cAMP, AMP, rolipram, 2-mercaptoethanol, methylcellulose, BSA, RNase A, and PI were purchased from Sigma (St Louis, MO, U.S.A.). Growth factors of GM-CSF, IL-3, IL-5, and SCF were obtained from R&D systems Inc. (Minneapolis, MN, U.S.A.). IMDM, PBS, and fetal calf serum were purchased from Life Technologies (Grand Island, NY, U.S.A.). FACS™ permeabilizing solution, anti-CD34-conjugated PE and antimouse PE-conjugated IgG monoclonal antibodies were purchased from Becton Dickinson (Mountain View, CA, U.S.A.). Anti-Bcl-2 monoclonal anti-

body-conjugated-FITC and FITC-labelled anti-IgG1 were obtained from Ansell Co. (Bayport, MN, U.S.A.).

Statistical analysis

Data were analyzed for normal distribution and all data except for the data regarding the effect of rolipram on apoptosis were normally distributed. Data are presented as mean \pm s.e.m. Parametric statistical analyses were therefore employed and analysis of variance was used to analyze the overall effects of various *in vitro* treatments. Subsequent paired *t*-tests were used to assess the significance of differences between groups, and Bonferonni's correction was used to minimize the possibility of chance significance as a result of multiple comparisons. For the effects of rolipram on apoptosis, nonparametric analysis was used with Kruskal–Wallis test, followed by the Wilcoxon rank test (two-tailed). To assess the differences between responses of cells from normal or asthmatic individuals, unpaired *t*-tests were used. A *P* value of less than 0.05 was considered to be significant.

Results

Effect on progenitor cell proliferation and differentiation

Colony formation by progenitor cells was assessed by culturing these cells from normal subjects ($n = 35$) and patients with mild intermittent asthma ($n = 40$). There was a significantly higher number of CD34+ cells expressed as a percent of progenitor cells from asthmatic patients ($3.62 \pm 0.79\%$, $n = 40$) than that of normal subjects ($0.71 \pm 0.12\%$, $n = 35$, $P < 0.01$). There were significantly greater number of colonies grown from progenitor cells in asthmatics, cultured in methylcellulose in the presence of growth factors, GM-CSF, stem cell factor, IL-3 and IL-5 in asthmatics (146 ± 11 colonies, $n = 40$, $P < 0.0001$) than from normal subjects (60 ± 6 colonies, $n = 35$). Histochemical staining of cytospin preparations of the cultured progenitor cells revealed a significantly higher percentage of cells of eosinophil lineage in asthmatics ($13.4 \pm 0.8\%$, $n = 40$; $P < 0.001$) than that from normal subjects ($2.8 \pm 0.3\%$, $n = 35$).

The effects of theophylline, salbutamol, db-cAMP, AMP and rolipram on the colony formation of progenitor cells are shown in Figure 1. Colony formation of progenitor cells from patients with asthma was suppressed by theophylline in a dose-dependent manner in patients with asthma with significant effects at 5 and $20 \mu\text{g ml}^{-1}$ (Figure 1a). In contrast, there was no significant effect of theophylline (5 – $20 \mu\text{g ml}^{-1}$) on the colony formation from progenitor cells of normal subjects (Figure 1a). In a smaller study, a high concentration of theophylline ($100 \mu\text{g ml}^{-1}$) decreased colony numbers in both normal (28 ± 7 colonies, $P < 0.001$) and asthmatic (43 ± 5 , $P < 0.001$) cell cultures ($n = 4$ for both groups). Salbutamol, db-cAMP and rolipram also dose-dependently inhibited colony formation of progenitor cells from asthmatics (Figure 1b, c and e). There was no significant effect of salbutamol or db-cAMP or rolipram on the colony formation in normal subjects (Figure 1b, c and e). In contrast, addition of various concentrations of AMP did not affect colony growth either in asthmatics or in normal subjects (Figure 1d).

There was no significant change in the percentage of eosinophil or other colony-forming units with theophylline,

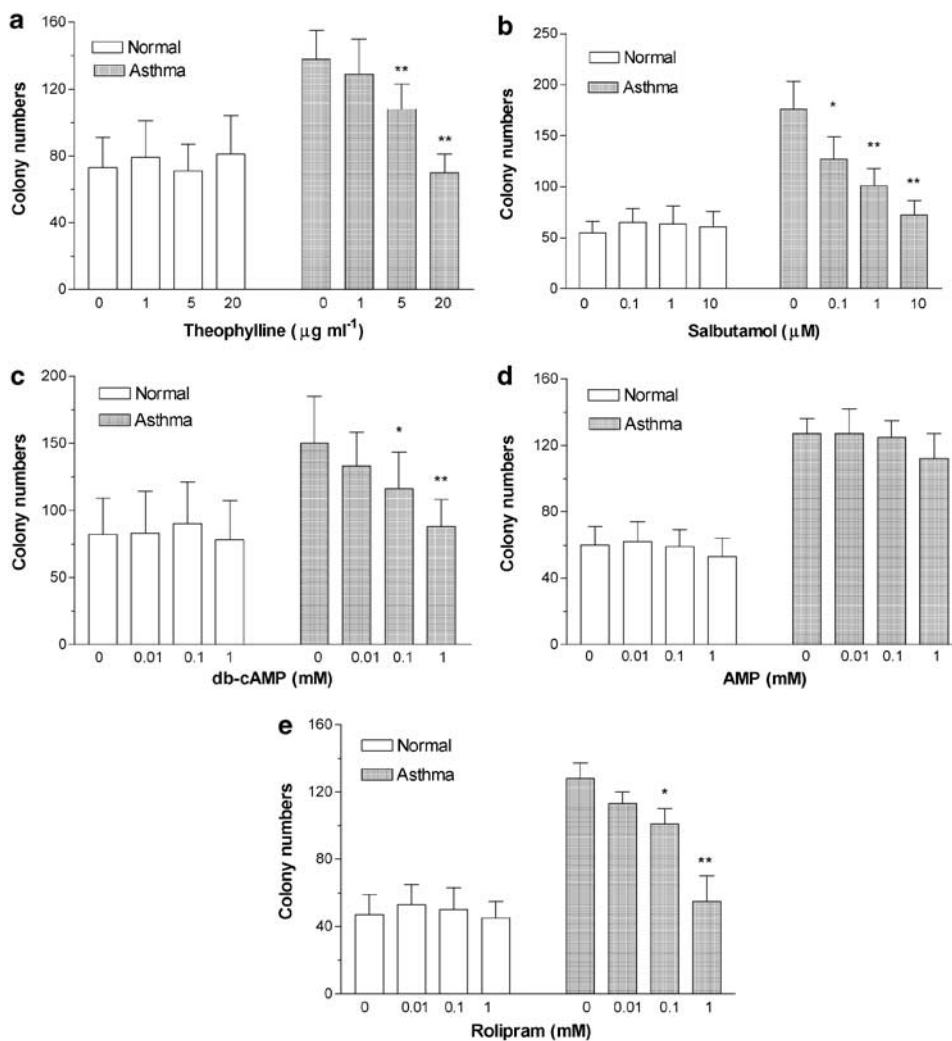


Figure 1 Effects of theophylline, salbutamol, db-cAMP, rolipram and AMP on the colony formation of progenitor cells cultured for 14 days from normal subjects ($n=7$, in each group) and patients with mild intermittent asthma ($n=8$, in each group). Data shown as mean \pm s.e.m. * $P<0.05$, ** $P<0.01$ compared with unstimulated controls in patients with asthma.

salbutamol, db-cAMP, rolipram or AMP in progenitor cells either in asthmatics or in normal subjects. The percentage (median and range) of various precursors and cells were in asthmatics: blast 55.4% (50.2–62.5), promyelocytes 1.8% (0–3.9), myelocytes 7.0% (2.6–10), metamyelocytes 2.7% (0–7.2), band 2.3% (0–6.6), segment 0.7% (0–1.5), eosinophils 11.9% (5.7–18.1), basophils 0.7% (0–3.6) and macrophages 17.6% (10.7–30.3) and in normal subjects: blast 71.6% (64.5–74.8), promyelocytes 0.5% (0–3.3), myelocytes 5.6% (1.3–7.4), metamyelocytes 1.2% (0–2.7), band 0.4% (0–1.0), segment 0.6% (0–2.3), eosinophils 2.7% (0.7–5.7), basophils 1.0% (0–3.6) and macrophages 14.4% (8.4–19.6), respectively.

Effects on progenitor cell apoptosis

Cellular apoptosis with DNA fragmentation induced by theophylline was confirmed by PI staining and analyzed by flow cytometry (Figure 2). In the presence of haematopoietic factors, such as SCF, GM-CSF, IL-3, and IL-5, addition of theophylline (5 and 20 $\mu\text{g ml}^{-1}$) induced apoptosis of progenitor cells in a dose-dependent manner (Figure 3a). In

asthmatics, salbutamol (Figure 3b), db-cAMP (Figure 3c) and rolipram (Figure 3e) also dose-dependently increased progenitor cell apoptosis. Salbutamol (1 and 10 μM), db-cAMP (0.1 and 1 mM) and rolipram (0.1 and 1 mM) induced a significant increase in the proportion of apoptotic progenitor cells in asthmatic patients when compared to those from normal subjects. AMP had no significant effect on progenitor cell apoptosis (Figure 3d). Theophylline, rolipram, db-cAMP, salbutamol or AMP did not affect apoptosis in progenitor cells from normal subjects (Figure 3).

Bcl-2 expression in CD4+ progenitor cells

Bcl-2 protein expression in progenitor cells was analyzed by flow cytometry, and its expression was found to be higher in cells from asthmatics compared to normals. Theophylline at the concentration of 5 $\mu\text{g ml}^{-1}$ significantly reduced the expression of Bcl-2 compared to control in CD34+ cells of asthmatics (Figure 5). Either db-cAMP (0.1 mM) or rolipram (0.1 mM) or salbutamol (1 μM) also inhibited the expression of Bcl-2 in the CD34+ progenitor cells of asthma compared to

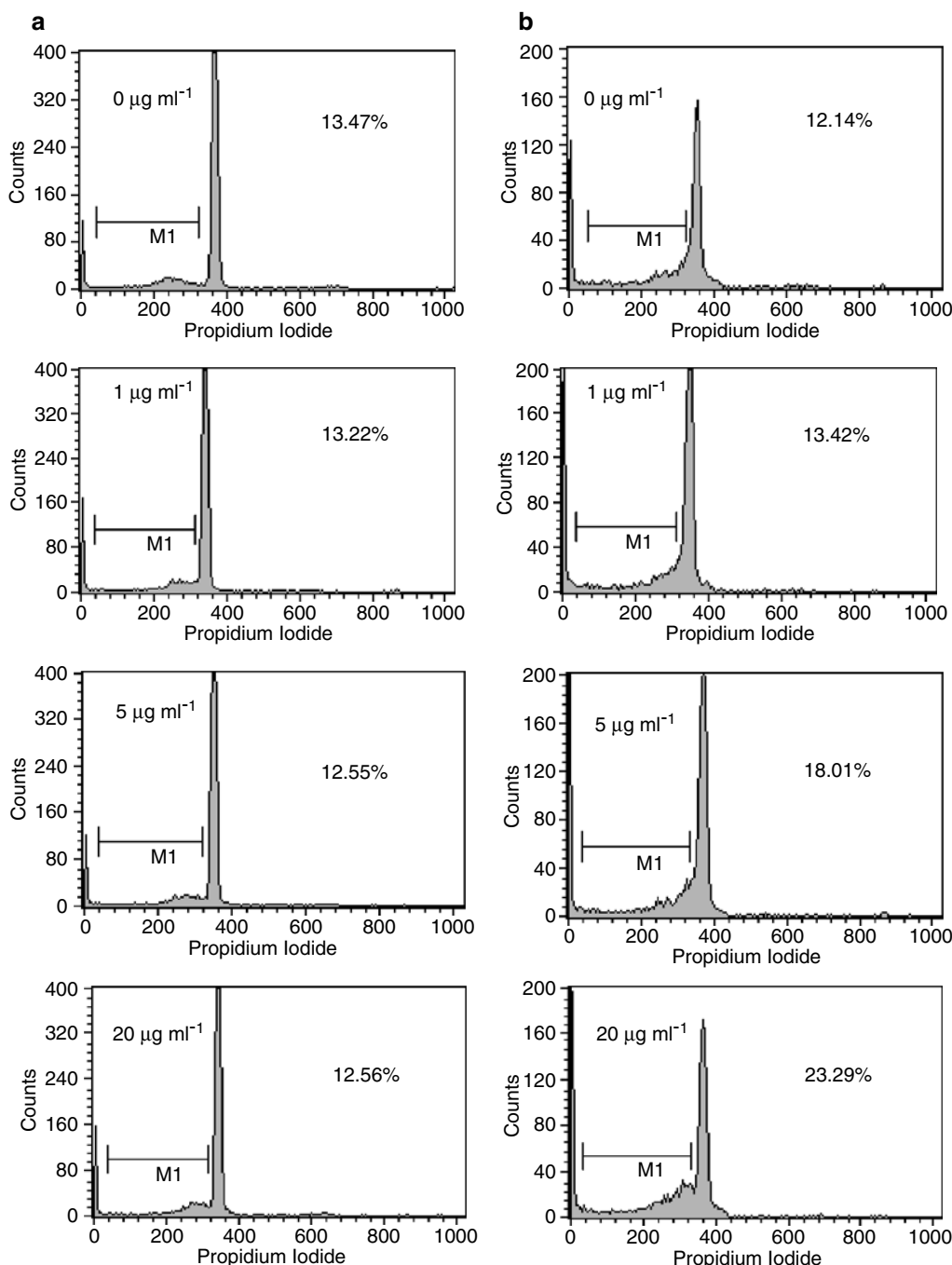


Figure 2 Detection of apoptosis of progenitor cells induced by theophylline plus growth factors (GM-CSF, IL-3, SCF and IL-5) using PI staining by flow cytometric analysis. Progenitor cells from normal subjects (a) or patients with asthma (b) were treated with various concentrations of theophylline, collected after 72-h culture period and stained with PI to detect DNA fragmentation by flow cytometry. Percentages indicate the percentage of apoptotic cell nuclei/total cell nuclei.

the controls (Figure 5). In contrast, neither the theophylline nor rolipram affected the expression of Bcl-2 in normal subjects (Figures 4 and 5).

Discussion

In the present study, we have demonstrated that theophylline inhibits colony formation by increasing apoptosis of progeni-

tor cells obtained from peripheral blood of patients with asthma. This effect is partly attributable to an increase in intracellular cAMP since the PDE type IV inhibitor, rolipram, the β_2 receptor adrenoceptor agonist salbutamol and db-cAMP significantly inhibited colony growth and induced apoptosis of progenitor cells in asthmatics in a dose-dependent manner. AMP did not affect cell apoptosis or growth. In contrast, neither theophylline nor salbutamol nor db-cAMP, nor rolipram significantly induced apoptosis or affected colony

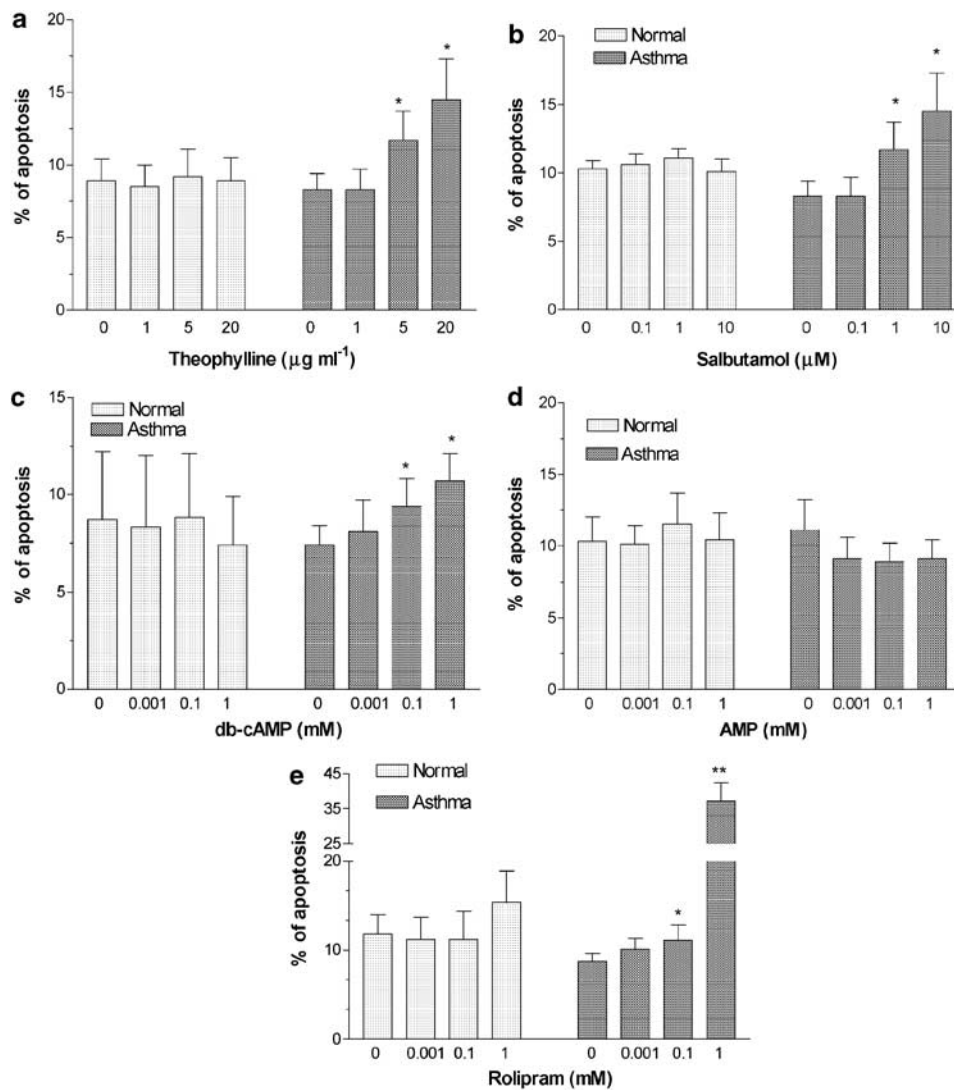


Figure 3 Effect of theophylline, salbutamol, db-cAMP, rolipram and AMP on the apoptosis of progenitor cells from normal subjects ($n = 7$, in each group) and patients with mild intermittent asthma ($n = 8$, in each group). The progenitor cells were incubated for 72 h in the presence of SCF, GM-CSF, IL-3 and IL-5. Data are shown as mean \pm s.e.m. * $P < 0.05$, ** $P < 0.01$ compared to controls in patients with asthma.

formation of progenitor cells from normal subjects. The inhibitory effect of theophylline, salbutamol, db-cAMP and rolipram on colony growth and development is not limited to the eosinophil lineage alone.

Haematopoietic cells appear to be more vulnerable to cAMP-mediated signalling than mature granulocytes. Theophylline induces apoptosis of chronic lymphocytic leukemia cells via increased levels of intracellular cAMP by the type IV PDE inhibitor, rolipram (Mentz *et al.*, 1995a; Kim & Lerner, 1998). Myeloid cell colony formation mediated by M-CSF can be inhibited by increasing intracellular cAMP levels (Kurland, 1978). Theophylline-induced mature granulocytes including eosinophil apoptosis is not mediated through the elevation of intracellular cAMP levels (Yasui *et al.*, 1997), but mediated via adenosine A_{2A} receptor antagonism rather than a cAMP-dependent pathway (Yasui *et al.*, 2000). In contrast, cAMP is a ubiquitous secondary messenger that inhibits B and T lymphocyte proliferation (Kizaki *et al.*, 1990) and induces the death of thymocytes through internucleosomal DNA

cleavage (McConkey *et al.*, 1990; Mentz *et al.*, 1995b). cAMP has been shown to induce G1 cell arrest by inducing the cyclin-dependent kinase inhibitor p27Kip1 (Kato *et al.*, 1994). The important role of the cAMP pathway in regulating large numbers of genes through cAMP-responsive element binding protein (CREB) (Lalli & Sassone-Corsi, 1994) may explain the effects of theophylline- or rolipram-induced apoptosis in progenitor cells from patients with asthma.

cAMP can induce cell cycle arrest at G1 phase in a human eosinophilic leukemia cell line (Uenoyama *et al.*, 1991), an effect associated with a decrease in Bcl-2 expression (Tai *et al.*, 1996). Bcl-2 plays an important role in cell growth/death and differentiation. Over-expression of Bcl-2 decreases cAMP-induced inhibition of cell proliferation and induction of apoptosis in SH-SY5Y human neuroblastoma cells (Kim *et al.*, 2000). In contrast, cAMP does not modulate the expression of Bcl-2 expression in mature cells (Wilson *et al.*, 1996). Thus, the resistance to cAMP-mediated apoptosis in mature granulocytes as shown in previous reports may result

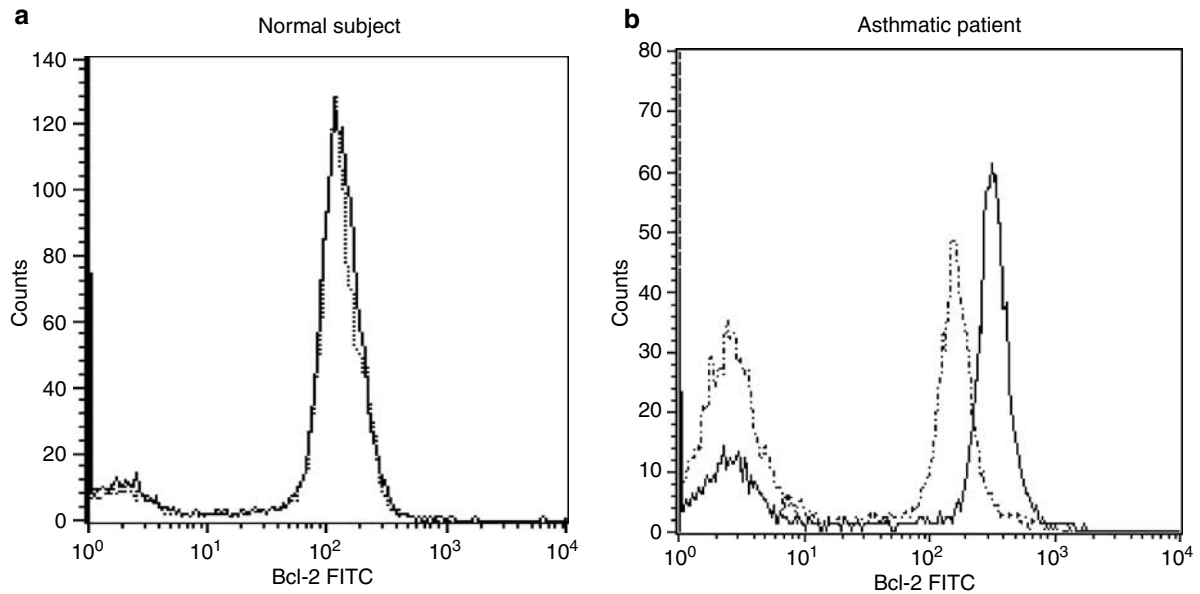


Figure 4 Direct immunofluorescence flow cytometry was performed to detect intracellular expression of Bcl-2 on CD34⁺ cells. Cells from normal subjects and asthmatic patients were incubated with or without $5 \mu\text{g ml}^{-1}$ theophylline for 24 h. The graph shows data expressed in the form of histogram depicting specific Bcl-2 staining with (dashed line) or without (solid line) theophylline treatment for a normal subject (a) and for an asthmatic (b).

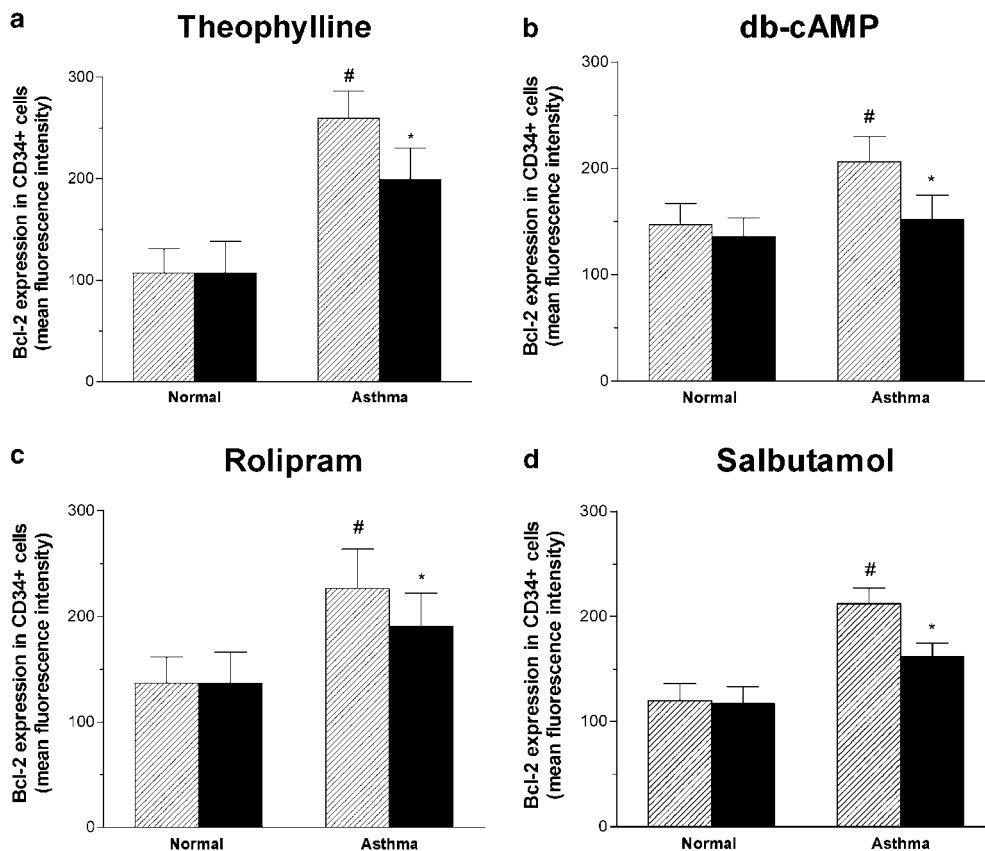


Figure 5 Direct immunofluorescence flow cytometry was performed to detect intracellular expression of Bcl-2 on CD34⁺ cells. The mean fluorescence intensity (MFI) of Bcl-2 expression in cells treated with (solid bars) or without (hatched bars) $5 \mu\text{g ml}^{-1}$ theophylline (a) or 0.1 mM db-cAMP (b) or 0.1 mM rolipram (c) or $1 \mu\text{M}$ salbutamol (d) for 24 h in normal subjects (normal, $n = 7$) or patients with mild intermittent asthma (asthma, $n = 8$). * $P < 0.05$ compared with the progenitor cells from normal subject in the absence of treatment. ** $P < 0.05$ compared with the progenitor cells in the absence of treatment. Data are means \pm s.e.m.

from alterations in the ability of cAMP to activate apoptotic signalling pathways between proliferating and mature cells. Our present study reveals that theophylline, salbutamol and db-cAMP downregulate the expression of Bcl-2 on progenitor cells, suggesting that theophylline induces progenitor cell apoptosis via a cAMP-dependent pathway, which may be different from mature eosinophils.

The reasons why these cAMP-elevating agents do not affect progenitor cells from normal subjects but only asthmatic cells are not clear from our study but several possibilities exist. PDE activity, which inactivates cAMP, is significantly increased in monocytes and eosinophils from asthmatics compared to those from healthy control subjects (Landells *et al.*, 2000; Takeuchi *et al.*, 2002). Either a heightened response of apoptotic pathways to cAMP or a greater increase in cAMP level in response to cAMP-elevating agents in asthmatic progenitor cells than that in normal cells may underlie the increased apoptosis observed in asthmatic cells. Secondly, cAMP-elevating agents inhibit the expression of several pro-inflammatory cytokines, such as GM-CSF (Seldon & Giembycz, 2001), IL-5, and IL-4 (Crocker *et al.*, 1996), that promote proliferation of progenitor cells. We have recently shown that, in progenitor cells from asthmatics but not in those from normals, IL-5 autoregulates the proliferation and differentiation of eosinophilic colony formation from progenitor cells which express greater levels of IL-5R α receptor (Kuo *et al.*, 2001). Therefore, asthmatic progenitor cells may be more sensitive to regulation by proliferative cytokines. Haematopoietic cells with high proliferation capacity appear to be more vulnerable to cAMP-mediated signalling pathway (Myklebust *et al.*, 1999). Augmentation of intracellular cAMP by cAMP-elevating agents significantly contributes to downregulation of Bcl-2 expression and acceleration of cell apoptosis in asthmatics, but not in normal subjects. Further studies are required to resolve the differences between progenitor cells from normal and asthmatic subjects.

Theophylline has been used worldwide in the treatment of bronchial asthma for several decades mainly as a bronchodilator (Weinberger, 1984), but has been reevaluated as an anti-inflammatory agent for the treatment of asthma (Chung, 1996). Previous studies have shown that doses of theophylline that achieve low serum therapeutic levels (5–10 $\mu\text{g ml}^{-1}$) attenuates eosinophil cell numbers in the peripheral blood

(Sullivan *et al.*, 1994). The immunomodulatory activities of theophylline in bronchial asthma include an induction of eosinophil apoptosis (Adachi *et al.*, 1996; Lim *et al.*, 2000) and an inhibition of T-cell proliferation and activation (Kidney *et al.*, 1995; Rabe & Dent, 1998). The present study extends the scope of the anti-inflammatory effects of theophylline. At a concentration as low as 5 $\mu\text{g ml}^{-1}$, theophylline induces a significant apoptosis of bone marrow precursor cells and inhibits the colony formation of progenitor cells from patients with asthma. Therefore, the suppressing effect of theophylline on peripheral blood eosinophilia and airway submucosal eosinophil infiltration (Sullivan *et al.*, 1994; Lim *et al.*, 2000) may occur at the level of circulating progenitor cells. However, it is not known whether the degree of increased apoptosis and of decreased colony formation in incubations with theophylline or rolipram *in vitro*, is of importance when patients are treated with clinical doses of these agents.

The inhibitory effect of either theophylline or the phosphodiesterase IV inhibitor on the colony-forming assay was not selective towards eosinophils since there was also inhibition of basophil and macrophage lineage. The effect on cells of neutrophilic lineage could not be determined from these experiments since the cytokine milieu of the study did not encourage colony-forming units of neutrophils. However, in chronic obstructive pulmonary diseases, neutrophils recovered in induced sputum from patients were reduced by treatment with theophylline (Culpitt *et al.*, 2002).

In conclusion, we have demonstrated that theophylline inhibits the proliferation of progenitor cells obtained from the peripheral blood of patients with asthma; this is associated with an increase of apoptosis of progenitor cells. We suggest that this could have resulted from an elevation of intracellular cAMP and a downregulation of Bcl-2 protein expression. However, further studies are needed to establish a definitive causal link. Our study is the first report to indicate that circulating progenitor cells could be a therapeutic target of theophylline in the treatment of bronchial asthma.

This study was supported by the Chang Gung Medical Research Project, CMRP-985, and the ROC National Science Council, NSC-89-2314-B-182A-028.

References

- ADACHI, T., MOTOJIMA, S., HIRATA, A., FUKUDA, T., KIHARA, N., KOSAKU, A., OHTAKE, H. & MAKINO, S. (1996). Eosinophil apoptosis caused by theophylline, glucorticoids, and macrolides after stimulation with IL-5. *J. Allergy Clin. Immunol.*, **98**, S207–S215.
- CHAO, D.T. & KORSMEYER, S.J. (1998). Bcl-2 family: regulators of cell death. *Annu. Rev. Immunol.*, **16**, 395–419.
- CHOU, C.L., WANG, C.H. & KUO, H.P. (1999). Upregulation of IL-5 receptor expression on bone marrow-derived CD34+ cells from patients with asthma. *Chang Gung Med. J.*, **22**, 416–422.
- CHUNG, K.F. (1996). Theophylline in chronic asthma: evidence for disease-modifying properties. *Clin. Exp. Allergy*, **26**, 22–27.
- CROCKER, I.C., TOWNLEY, R.G., & KHAN N.M. (1996). Phosphodiesterase inhibitors suppress proliferation of peripheral blood mononuclear cells and interleukin-4 and interleukin-5 secretion by human T-helper type-2 cells. *Immunopharmacology*, **31**, 223–235.
- CULPITT, S.V., DE MATOS, C., RUSSELL, R.E., DONNELLY, L.E., ROGERS, D.F., & BARNES, P.J. (2002). Effect of theophylline on induced sputum inflammatory indices and neutrophil chemotaxis in chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.*, **165**, 1371–1376.
- GIBSON, P.G., DOLOVICH, J., GIRGIS-GABARDO, A., MORRIS, M.M., ANDERSON, M., HARGREAVE, F.E. & DENBERG, J.A. (1990). The inflammatory response in asthma exacerbation: changes in circulating eosinophils, basophils and their progenitors. *Clin. Exp. Allergy*, **20**, 661–668.
- GIBSON, P.G., MANNING, P.J., O'BYRNE, P.M., GIRGIS-GABARDO, A., DOLOVICH, J., DENBURG, J.A. & HARGREAVE, F.E. (1991). Allergen-induced asthmatic responses: relationship between increases in airway responsiveness and increases in circulating eosinophils, basophils and their progenitors. *Am. Rev. Respir. Dis.*, **143**, 331–335.
- GLEICH, G.J. (1990). The eosinophil and bronchial asthma: current understanding. *J. Allergy Clin. Immunol.*, **85**, 422–436.
- HOCKENBERRY, D.M., ZUTTER, M., HICKEY, W., NAHAM, M. & KORSMEYER, S.J. (1991). Bcl-2 protein is topographically re-

- stricted in tissues characterized by apoptotic cell death. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 6961–6965.
- JEFFERY, P.K., WARDLAW, A.J., NELSON, F.C., COLLINS, J.V. & KAY, A.B. (1989). Bronchial biopsies in asthma. An ultrastructural, qualitative study and correlation with hyperreactivity. *Am. Rev. Respir. Dis.*, **140**, 1745–1753.
- KATO, J.Y., MATSUOKA, M., POLYAK, K., MASSAGUE, J. & SHERR, C.J. (1994). Cyclic AMP-induced G1-phase arrest mediated by an inhibitor (p27Kip1) of cyclin-dependent kinase 4 activation. *Cell*, **79**, 487–496.
- KIDNEY, J., DOMIGUEZ, M., TAYLOR, P.M., ROSE, M., CHUNG, K.F. & BARNES, P.J. (1995). Immunomodulation by theophylline in asthma: demonstration by withdrawal of therapy. *Am. J. Respir. Crit. Care Med.*, **151**, 1907–1914.
- KIM, D.H. & LERNER, A. (1998). Type 4 cyclic adenosine monophosphate phosphodiesterase as therapeutic target in chronic lymphocytic leukemia. *Blood*, **92**, 2484–2494.
- KIM, S.N., KIM, S.G., PARK, J.H., LEE, M.A., PARK, S.D., CHO-CHUNG, Y.S. & HONG, S.H. (2000). Dual anticancer activity of 8-Cl-cAMP: inhibition of cell proliferation and induction of apoptotic cell death. *Biochem. Biophys. Res. Commun.*, **273**, 404–410.
- KIZAKI, H., SUZUKI, K., TADAKUMA, T. & ISHIMURA, Y. (1990). Adenosine receptor-mediated accumulation of cyclic AMP-induced T-lymphocyte death through internucleosomal DNA cleavage. *J. Biol. Chem.*, **265**, 5280–5284.
- KUO, H.P., WANG, C.H., LIN, H.C., HWANG, K.S., LIU, S.L. & CHUNG, K.F. (2001). Interleukin-5 in growth and differentiation of blood eosinophil progenitors in asthma: effect of glucocorticoids. *Br. J. Pharmacol.*, **134**, 1539–1547.
- KURLAND, J.I. (1978). Role for monocyte-macrophage-derived colony-stimulating factor and prostaglandin E in the positive and negative feedback control of myeloid stem cell proliferation. *Blood*, **52**, 388–407.
- LAITINEN, L.A., HEINO, M., LAITINEN, A., KAVA, T. & HAAHTELA, T. (1985). Damage of the airway epithelium and bronchial reactivity in patients with asthma. *Am. Rev. Respir. Dis.*, **131**, 599–606.
- LAITINEN, L.A., LEITINEN, A. & HAAHTELA, T. (1993). Airway mucosal inflammation even in patients with newly diagnosed asthma. *Am. Rev. Respir. Dis.*, **147**, 697–704.
- LALLI, E. & SASSONE-CORSI, P. (1994). Signal transduction and gene regulation. The nuclear response to cAMP. *J. Biol. Chem.*, **269**, 17359–17362.
- LANDELLS, L.J., SPINA, D., SOUNESS, J.E., O'CONNOR, B.J., & PAGE, C.P. (2000). A biochemical functional assessment of monocyte phosphodiesterase activity in healthy and asthmatic subjects. *Pulmonary Pharmacol. Therap.*, **13**, 231–239.
- LIM, S., TOMITA, K., CARAMORI, G., JATAKANON, A., OLIVER, B., KELLER, A., ADCOCK, I., CHUNG, K.F. & BARNES, P.J. (2000). Low dose theophylline reduces eosinophilic inflammation but not exhaled nitric oxide in mild asthma. *Am. J. Respir. Crit. Care Med.*, **162**, 1912–1918.
- MCCONKEY, D.J., ORRENIUS, S. & JONDAL, M. (1990). Agents that elevate cAMP stimulate DNA fragmentation in thymocytes. *J. Immunol.*, **145**, 1227–1230.
- MENTZ, F., MERLE-BERAL, H., OUAZ, E. & BINET, J.L. (1995a). Theophylline, a new inducer of apoptosis in B-CLL: role of cyclic nucleotides. *Br. J. Hematol.*, **90**, 957–959.
- MENTZ, F., MOSSALAYI, M.D., OUAZ, F. & DEBRE, P. (1995b). Involvement of cAMP in CD3/T cell receptor complex- and Cd2-mediated apoptosis of human thymocytes. *Eur. J. Immunol.*, **25**, 1798–1801.
- MYKLEBUST, J.H., JOSEFSEN, D., BLOMHOF, H.K., LEVY, F.O., NADERI, S., REED, J.C. & SMELAND, E.B. (1999). Activation of the cAMP signaling pathway increases apoptosis in human B-precursor cells and is associated with downregulation of Mcl-1 expression. *J. Cell Physiol.*, **180**, 71–80.
- NICOLETTI, I., MIGLIORATI, G., PAGLIACCI, M.C., GRIGNANI, F. & RICCARDI, C. (1991). A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Methods*, **139**, 271–279.
- OHTA, K. & YAMASHITA, N. (1999). Apoptosis of eosinophils and lymphocytes in allergic inflammation. *J. Allergy Clin. Immunol.*, **104**, 14–21.
- RABE, K.F. & DENT, G. (1998). Theophylline and airway inflammation. *Clin. Exp. Allergy*, **28**, 35–41.
- ROBINSON, D.S., DAMIA, R., ZEIBECOGLOU, K., MOLET, S., NORTH, J., YAMADA, T., KAY, A.B. & HAMID, Q. (1999). CD34+ interleukin-5R messenger RNA+ cells in the bronchial mucosa in asthma: potential airway eosinophil progenitor cells. *Am. J. Respir. Cell Mol. Biol.*, **20**, 9–13.
- RYAN, J.J., PROCHOWNIK, E., GOTTLIEB, C.A., APEL, I.J., MERINO, R., NUNEZ, G. & CLARKE, M.F. (1994). C-myc and *bcl-2* modulate function by altering p53 subcellular trafficking during the cell cycle. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 5878–5882.
- SELDON, P.M. & GIEMBYCZ, A.M. (2001). Suppression of granulocyte/macrophage colony-stimulating factor release from human monocytes by cyclic AMP-elevating drugs: role of interleukin-10. *Br. J. Pharmacol.*, **134**, 58–67.
- SHIH, L.Y. & LEE, C.T. (1994). Identification of masked polycythemia vera from patients with idiopathic marked thrombocytosis by endogenous erythroid colony assay. *Blood*, **83**, 744–748.
- SULLIVAN, P., BEKIR, S., JAFFAR, Z., PAGE, C., JEFFERY, P. & COSTELLO, J. (1994). Anti-inflammatory effects of low-dose oral theophylline in atopic asthma. *Lancet*, **343**, 1006–1008.
- TAI, G., EUN-YOUNG, J., YUJI, H., MASAHIKO, K., TOSHIO, H., KENJI, K., KENSHI, F. & MITSUFUMI, M. (1996). Different effects of cyclic AMP and butyrate on eosinophilic differentiation, apoptosis and *bcl-2* expression of human eosinophilic leukemia cell line, EoL-1. *Hematol. Oncol.*, **14**, 181–192.
- TAKEUCHI, M., TATSUMI, Y., KITAICHI, K., BABA, K., SUZUKI, R., SHIBATA, E., TAKAGI, K., MIYAMOTO, K., HASEGAWA, T. & TAKAGI, K. (2002). Selective phosphodiesterase type 4 inhibitors reduce the prolonged survival of eosinophils stimulated by granulocyte-macrophage colony-stimulating factor. *Biol. Pharm. Bull.*, **25**, 184–187.
- UENOYAMA, Y., OHSHIMA, Y., MORITA, M., AKUTAGAWA, H., NAMBU, M., KIM, K.M., MAYUMI, M. & MIKAWA, H. (1991). Dibutyl cyclic AMP induces formyl peptide receptor expression and chemotactic response in a human eosinophilic cell line EoL-1. *Exp. Hematol.*, **19**, 823–828.
- WANG, C.H., SHEIH, W.Y., SHIH, L.Y., LIN, H.C., LIU, C.Y., CHUNG, K.F. & KUO, H.P. (1999). Increased progenitor cell proliferation in peripheral blood of patients with bronchial asthma: role of nitric oxide. *J. Allergy Clin. Immunol.*, **104**, 803–810.
- WEINBERGER, M. (1984). The pharmacology and therapeutic use of theophylline. *J. Allergy Clin. Immunol.*, **73**, 525–540.
- WILSON, B.E., MOCHON, E. & BOXER, L.M. (1996). Induction of *bcl-2* expression by phosphorylated CREB proteins during B-cell activation and rescue from apoptosis. *Mol. Cell Biol.*, **16**, 5546–5556.
- YASUI, K., AGEMATSU, K., SHINOZATCI, K., HOKIBARA, S., NAGUMO, H., NAKAZAWA, T. & KOMIYAMA, A. (2000). Theophylline induces neutrophil apoptosis through adenosine A2A receptor antagonism. *J. Leukoc. Biol.*, **67**, 529–535.
- YASUI, K., HU, B., NAKAZAWA, T., AGEMATSU, K. & KOMIYAMA, A. (1997). Theophylline accelerates human granulocyte apoptosis not via phosphodiesterase inhibition. *J. Clin. Invest.*, **100**, 1677–1684.

(Received September 11, 2002
Revised November 11, 2002
Accepted December 4, 2002)