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Effects of theophylline, dexamethasone and salbutamol on cytokine gene expression in human peripheral blood CD4+ T-cells

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ABSTRACT: CD4+ T-cells are considered as pivotal in orchestrating the airway inflammation in asthma through the actions of their cytokines. Current hypothesis suggests that the anti-asthma effect of theophylline may be due to its anti-inflammatory actions, although the exact mechanisms remain unclear.

The *in vitro* effect of theophylline on cytokine gene expression in peripheral blood CD4+ T-cells in normal subjects was compared with that of dexamethasone and salbutamol. CD4+ T-cells were cultured with phytohaemagglutin and phorbol myristate acetate in the presence of different concentrations of theophylline (10^{-8}–10^{-3} M or 0.0018–180 $\mu g \cdot mL^{-1}$) in one group of subjects (n=8), dexamethasone (10^{-9}–10^{-6} M or 0.39–390 $ng \cdot mL^{-1}$) in a second group (n=8) and salbutamol (10^{-9}–10^{-4} M or 0.00058–58 $\mu g \cdot mL^{-1}$) in a third group (n=8). Gene expression of interleukin (IL)-3, IL-4, IL-5, granulocyte-macrophage colony-stimulating factor (GM-CSF) and interferon (IFN)-γ was semiquantified by reverse transcription-polymerase chain reaction.

Suppressed expression of IL-3 (36.9%), IL-4 (38.8%), GM-CSF (24.6%) and IFN-γ (37.7%), but not of IL-5, was only seen with theophylline at a concentration of 10^{-3} M (180 $\mu g \cdot mL^{-1}$) ($p<0.05$) and not at lower concentrations. In contrast, dexamethasone caused a dose-dependent suppression of transcription of all cytokines, with 39.5% for IL-3, 84.4% for IL-4, 40.6% for IL-5, 50.9% for GM-CSF and 31.8% for IFN-γ at 10^{-6} M (390 $ng \cdot mL^{-1}$) ($p<0.05$–0.001). Salbutamol did not suppress gene expression of any of the cytokines at the concentrations examined.

These data suggest that cytokine gene expression of CD4+ T-cells is not affected at therapeutic concentrations of theophylline and salbutamol, but its suppression is likely to be an important mechanism underlying the therapeutic effect of corticosteroids in asthma.

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It is now well recognized that asthma is closely associated with airway inflammation, even in patients with mild disease [1]. One of the most consistent inflammatory changes in the asthmatic airway is infiltration by activated lymphocytes, eosinophils and mast cells [1, 2]. Current evidence suggests that T-lymphocytes are pivotal in orchestrating this inflammatory response through the release of T-helper (Th)-2 cytokines from CD4+ cells [3].

Recognition of the inflammatory basis of asthma has led to the recommendation of early use of anti-inflammatory agents by a number of national and international guidelines of asthma management [4]. Amongst the anti-inflammatory agents, corticosteroids are by far the most effective in controlling asthma. Although the exact mechanisms underlying their therapeutic efficacy remain unclear, a study on bronchial biopsies in asthmatics have shown that corticosteroids may act by modulation of cytokine expression with consequent inhibition of the local bronchial inflammatory infiltrate and tissue eosinophilia [5]. T-lymphocytes, particularly the CD4+ cells, are a rich source of pro-inflammatory cytokines in asthma. Suppresion of the expression of these cytokines, *e.g.* interleukin (IL)-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF), by corticosteroids has been shown in asthmatics recovering from acute exacerbation of their disease [6–8].

Theophylline is a medication which has been used in the treatment of bronchial asthma for >50 yrs. Whilst traditionally theophylline has been regarded as a bronchodilator, there is now growing evidence to suggest it possesses anti-inflammatory properties even at subtherapeutic levels [9–11]. Indeed, several studies have demonstrated that xanthines may exert their anti-inflammatory and immunomodulatory effects by the inhibition of eosinophil degranulation [12], histamine release from mast cells and basophils [13] antigen- and mitogen-induced T-cell proliferation [14] and T-cell recruitment into the bronchial mucosa [10], and the generation of cytokines [15]. However, the current knowledge concerning its anti-inflammatory mechanisms remains nondefinitive. This study, aimed to further define the actions of theophylline by comparing their effects on cytokine gene expression of peripheral blood CD4+ T-cells with that of dexamethasone and salbutamol.

Methods

Subjects

Three groups of nonsmoking subjects were studied. All were normal healthy volunteers with no prior use of any

anti-asthmatic medications. Informed consent was obtained from each subject. Peripheral venous blood samples were taken once from all 24 subjects.

Isolation of CD4+ cell subset

Peripheral blood mononuclear cells (PBMCs) separated by Isopaque-Ficoll (Lymphoprep; Nycomed AS, Oslo, Norway) density gradient centrifugation were washed with phosphate buffered saline (PBS), and then resuspended in culture medium consisting of Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Chagrin Falls, NY, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS; Gibco), L-glutamine (2 mM), penicillin) 100 $U \cdot mL^{-1}$) and streptomycin (100 $\mu g \cdot mL^{-1}$). Magnetizable polystyrene beads coated with monoclonal antibody (MoAb) specific for CD4 (Dynal AS, Oslo, Norway) were used to select CD4+ T-cells from PBMCs as described previously [16]. CD4+ T-cells were 99.8± 0.02% (mean±SD) pure as assessed by flow cytometry (EPICS; Coulter Corporation, Miami, FL, USA) using fluorescin conjugated anti-human Leu-3A MoAb (Becton Dickinson, Mountain View, CA, USA). Immunofluorescence microscopy revealed that these CD4+ cells were also CD3 positive but were negative for CD11b. Cell viability was >98% as determined by the trypan blue dye exclusion test.

Culture of CD4+ cells

Isolated CD4+ cells, adjusted to 1 mL aliquots of 1×10^6 cells, were dispended into 48-well culture plates. The cells were incubated at 37°C for 6 h in a humidified atmosphere with 5% CO_2 in the presence of phytohaemagglutinin (10 $\mu g \cdot mL^{-1}$) and phorbol myristate acetate (0.5 $ng \cdot mL^{-1}$). It has been shown in previous studies that these concentrations induced maximal cytokine production with lowest toxicity [16, 17]. To study the effects of theophylline, dexamethasone and salbutamol on cytokine gene expression of these CD4+ T-cells, theophylline was added at concentrations of 10^{-8}–10^{-3} M (0.0018–180 $\mu g \cdot mL^{-1}$) to the cell cultures in the first group of subjects (n=8), dexamethasone at 10^{-9}–10^{-6} M (0.39–390 $ng \cdot mL^{-1}$) in the second group of subjects (n=8), and salbutamol at 10^{-9}–10^{-4} M (0.00058–58 $\mu g \cdot mL^{-1}$) in the third group of subjects (n=8). These particular concentrations were chosen to cover the normal therapeutic concentrations of these medications *in vivo*. Theophylline and dexamethasone were dissolved in water while salbutamol was dissolved in dimethylsulphoxide 0.005%. In a pilot study, solvents were used only as controls and no effect on cytokine gene expression was found.

Preparation of total ribonucleic acid

A microadaptation of the guanidinium thiocyanate/cesium chloride procedure was used to prepare total ribonucleic acid (RNA) from CD4+ cells [18]. All RNA samples prepared from 1×10^6 cells were dissolved in 20 µL diethylpyro-carbonate-treated (DEPC-dH_2O) and were stored at -70°C until assay. The quantity of RNA was checked by formaldehyde agarose gel electrophoresis and measured by spectrophotometry. Two microlitres of total RNA contained ~0.1 µg RNA.

Oligonucleotide primers

Specific primers for IL-3, IL-4, IL-5, GM-CSF, interferon (IFN)-γ and reduced glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtained from Clontech (Palo Alto, CA, USA). The sequences and related information are shown in table 1.

Detection of cytokine gene expression by polymerase chain reaction

Gene expression for the cytokines was studied in CD4+ cells using the GeneAmp RNA polymerase chain reaction (PCR) kit (Perkin Elmer Cetus, Norwalk, CT, USA). Messenger ribonucleic acid (mRNAs) of each sample was first reverse transcribed into complimentary deoxyribonucleic acid (cDNAs), which in turn was subjected to PCR amplification using specific primers. In brief, first-strand deoxyribonucleic acid (DNA) was synthesized in a final volume of 20 µL with the following components: 2 µL

Table 1. – Primers used in polymerase chain reaction

Cytokine	Primer sequences	PCR product bp	Concentration used $\mu M \cdot PCR^{-1}$
IL-3	5′ATGAGCCGCCTGCCCGTCCTG 5′GTTGTCTGCTGAAATCGGAGCG	449	0.15
IL-4	5′ATGGGTCTCACCTCCCAACTGCT 5′TACTCTCTCTCTTTATAAGTTTCACAAGC	456	0.5
IL-5	5′GCTTCTGCATTTGAGTTTGCTAGCT 5′GAATTATTTCTTTATGTAACTGCCGGT	293	0.5
GM-CSF	5′ATGTGGCTGCAGAGCCTGCTGC 5′TACGACAAAGCTCCAGCTTCTCGTAG	424	1
IFN-γ	5′ATGAAATATACAAGTTATATCTTGGCTTT 5′TACGACAAAGCTCCAGCTTCTCGTAG	494	1
GAPDH	5′TGAAGGTCGGAGTCAACGGATTTGGT 5′CACCACCTGGAGTACCGGGTGTAC	983	0.05

PCR: polymerase chain reaction; IL: interleukin; GM-CSF: granulocyte-macrophage colony-stimulating factor; IFN: interferon; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

total RNA in DEPC-dH_2O, 2.5 mM 16-oligomer of thymidine deoxynucleotide (Oligo d (T)), 5 mM $MgCl_2$, 10 nM Tris-HCL buffer, pH 8.3, 50 mM KCl, 1 mM dNTP, and 10 U of ribonuclease (RNase) inhibitor. The mixture was incubated in a Perkin-Elmer Cetus DNA Thermal Cycler at 42°C for 30 min followed by 5 min at 99°C and flash cooling to 4°C. The resulting cDNA (10 μL) was mixed separately either with 2 mM $MgCl_2$, 10 mM Tris-HCl buffer, 50 mM KCl, IL-3 IL-4 IL-5, GM-CSF, or (IFN)-γ specific PCR primers, GAPDH primer, 1.25 U AmpliTaq DNA polymerase, and 0.074 Bq [α-^{32}P]-deoxycytidine triphosphate (dCTP) in a final volume of 50 μL. Amplification was started with 2 min denaturation at 94°C followed by 30 PCR cycles. Each cycle consisted of 1 min at 94°C for denaturation, 1 min at 62°C for annealing, and 1 min at 72°C for extension. The final extension lasted 10 min at 72°C in all instances. The experimental condition and number of cycles of PCR were predetermined to ensure the amount of cytokine and "housekeeping" gene (GAPDH) fragments were in the linear range of amplification. The efficiency of cDNA synthesis was controlled using control cRNA (IL-1α RNA from plasmid pAW109 (pAW109 provided by Clontech) in every reaction.

Negative controls for PCR consisted of: samples in which the reverse transcriptase was omitted to detect any contamination by genomic cDNA; and a reagents control in which RNA was replaced by DEPC-dH_2O. Positive control DNAs were obtained from Clontech Laboratory.

Agarose gel electrophoresis and semiquantitation of polymerase chain reaction products

The PCR mixture (20 μL) was added to 5 μL of loading dye mixture and electrophoresed in a 90V constant voltagefield in 1.7% agarose (Sigma, St Louis, MO, USA) containing ethidium bromide until the bromophenol blue dye front had migrated for a distance of 6 cm. *Haemophilus aegyptius* (*Hae*)III-digested ΦX174 DNA (Gibco) was used to determine the size of the PCR products. After electrophoresis, gel slices corresponding to the radioactive bands of the PCR product were excised and counted by liquid scintillation. ^{32}P incorporated into the PCR products of each of the cytokines was normalized with that of the amplified GAPDH PCR products as described previously [19]. The results were expressed as a ratio calculated from the counts per minute (cpm) of amplified cytokine gene product over the cpm of amplified GAPDH PCR product.

Statistical analysis

All data are mean±SEM unless otherwise stated. The data (all nonparametric) were compared using Kruskal-Wallis followed by Mann-Whitney. All p-values of <0.05 were considered significant.

Results

The results of suppression of cytokine gene expression of CD4+ T-cells at various concentrations of theophylline, dexamethasone and salbutamol are presented in tables 2–4, respectively. The respective agarose gel electrophoreses are shown in figures 1–3. Suppressed expression of IL-3 (36.9%), IL-4 (38.8%), GM-CSF (24.6%) and IFN-γ (37.7%), but not of IL-5, was only seen with theophylline at a high concentration of 10^{-3} M (180 μg·mL^{-1}) ($p<0.05$–0.01) and not at lower concentrations. In contrast, dexamethasone caused a dose-dependent suppression of transcription of all cytokines, with 39.5% for IL-3, 84.4% for IL-4, 40.6% for IL-5, 50.9% for GM-CSF and 31.8% for IFN-γ at 10^{-6} M (390 ng·mL^{-1}) ($p<0.05$–0.001). Salbutamol did not produce any significant effects on cytokine gene expression at therapeutic concentrations.

Discussion

Clinical studies have shown that both corticosteroids [1] and xanthines [9–11] possess anti-inflammatory properties, although the exact mechanisms underlying their therapeutic efficacy remain unclear. In this study, the effects of these agents on the gene expression of various pro-inflammatory cytokines secreted by CD4+ T-cells has been examined. Previous studies have demonstrated the importance of CD4+ T-cells and their cytokines in the pathogenesis of asthma [7, 8]. *In vitro* studies indicate that IL-3, IL-5 and GM-CSF can influence the production, maturation and activation of eosinophils [20], whilst IL-4 is thought to be important in the promotion of isotype switching of B cells towards immunoglobulin

Table 2. – Percentage inhibition of cytokine messenger ribonucleic acid (mRNA) expression of CD4+ cells by theophylline

	Theophilline concentration M (μg·mL^{-1})					
	10^{-3} (180)	10^{-4} (18)	10^{-5} (1.8)	10^{-6} (0.18)	10^{-7} (0.018)	10^{-8} (0.0018)
IL-3/GAPDH	36.9±13.4*	23.2±7.0	-22.8±25.4	0.6±7.4	2.1±13.7	5.3±3.2
IL-4/GAPDH	38.8±5.1**	16.5±5.6	1.3±7.7	9.2±8.7	8.6±5.3	14.6±5.4
IL-5/GAPDH	9.5±6.5	-23.2±18.8	-15.1±6.3	-11.2±12.8	-1.1±11.7	7.1±6.4
GM-CSF/GAPDH	24.6±10.7*	20.5±6.4	6.3±7.2	12.8±10.4	11.7±11.9	6.4±8.6
IFN-α/GAPDH	37.7±7.4*	12.9±18.2	1.9±14.4	-7.0±14.8	-11.1±15.6	-1.0±13.5

Data are mean±SEM. IL: interleukin; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GM-CSF: granulocyte-macrophage colony-stimulating factor; IFN: interferon. *: $p<0.05$ *versus* phorbol myristate acetate (PMA)/phytohaemogglutinin (PMA) stimulation without theophylline; **: $p<0.01$ *versus* phorbol myristate acetate (PMA)/phytohaemogglutining (PMA) stimulation without theophylline (Kruskal-Wallis followed by Mann-Whitney analysis). Values are calculated as 100% - (cytokine expression with PHA/PMA and theophylline/cytokine expression with PHA/PMA only) × 100%.

Table 3. – Percentage inhibition of cytokine messenger ribonucleic acid (mRNA) expression of CD4+ cells by dexamethasone

	Dexamethasone concentration M ($ng \cdot mL^{-1}$)			
	10^{-6} (390)	10^{-7} (39)	10^{-8} (3.9)	10^{-9} (0.39)
IL-3/GAPDH	39.5±10.6*	38.0±8.5*	31.5±8.1*	8.4±7.4[#]
IL-4/GADPH	84.4±2.7***	80.5±4.0***	66.1±2.9***	16.7±11.9*[‡]
IL-5/GADPH	55.6±5.4***	54.5±5.3***	40.6±9.7***	21.2±9.6*[#]
GM-CSF/GAPDH	50.9±8.5**	54.2±6.4**	35.0±17.7*	11.9±11.1[$]
IFN-α/GAPDH	31.8±13.1*	24.0±12.0	25.5±10.3*	17.8±14.8

Data are mean±SEM. For definitions, see footnote to table 1. *: $p<0.05$ *versus* PHA/PMA stimulation without dexamethasone; **:$p<0.01$ *versus* PHA/PMA stimulation without dexamethasone; ***: $p<0.001$ *versus* PHA/PMA stimulation without dexamethasone; [#]: $p<0.05$ *versus* PHA/PMA stimulation with dexamethasone at concentrations of 10^{-6}–10^{-8} M; [‡]: $p<0.001$ *versus* PHA/PMA stimulation with dexamethasone at concentrations of 10^{-6}–10^{-8} M; [$]: $p<0.05$ *versus* PHA/PMA stimulation with dexamethasone at concentrations of 10^{-6}–10^{-7} M, Kruskal-Wallis followed by Mann-Whitney analysis. Values are calculated as 100 - (cytokine expression with PHA/PMA and dexamethasone/cytokine expression with PHA/PMA only) × 100.

(Ig)E-synthesis and the stimulation of proliferation of Th-2 cells [21, 22]. Indeed, clinical experiments suggest a sequential involvement of these cytokines, with IL-4 directing T-cells to a Th-2 phenotype, release IL-3, IL-5 and GM-CSF upon inhaled allergen stimulation, resulting in airway eosinophilia, and possibly airway hyperresponsiveness [23]. The data in the current study suggest that whilst the suppresion of these cytokines by CD4+ cells is likely to be an important mechanism underlying the therapeutic effect of corticosteroids in asthma, theophylline is likely to exert its anti-inflammatory actions *via* different mechanisms. These data also demonstrate that salbutamol has no significant effects on these cytokines.

In this study, it was decided to choose healthy subjects to avoid the effects of any previous drug treatment might have on the T-lymphocytes. In previous studies, no significant difference in the gene expression of the cytokines between normal control subjects and stable asthmatics was found, although the expression in acute severe asthmatics was increased [7, 8]. These studies also found that steroid treatment resulted in a fall in the level of gene expression in subjects with acute severe disease, to a level similar to the current findings amongst normal subjects. Hence, the authors feel that the use of normal subjects in this experimental study is valid and that the steroid arm of this current study is acting as a positive control in demonstrating the effects of theophylline and salbutamol on the gene expression of these cytokines. Criticism may arise from the use of peripheral T-cells instead of cells derived from the lung. However, previous finding of increased cytokine gene expression in peripheral T-cells in acute asthma has also been confirmed by other researchers who studied inflammatory cells within the bronchial mucosa [24]. Hence, it is likely that peripheral blood T-cell cytokine gene expression may reflect the situation in the bronchial mucosa.

The therapeutic range of theophylline is usually taken as 10–20 $\mu g \cdot mL^{-1}$ [25]. After 12 mg dexamethasone$\cdot day^{-1}$ is given intravenously or orally, plasma concentrations range 40–100 $ng \cdot mL^{-1}$ [26]. Hence, these *in vivo* concentrations are covered by the range of concentrations used in the present study. The results show that at therapeutic concentrations, theophylline does not exert any significant effects on the cytokine gene expression of CD4+ cells. On the other hand, suppression of cytokine gene expression occurs even at low concentrations of dexamethasone.

Several clinical studies have shown that theophylline may exert its immunomodulatory effects by regulating the trafficking of T-lymphocytes rather than their activation. KIDNEY *et al.* [10] demonstrated that in asthmatics treated with long-term theophylline and high dose inhaled corticosteroids, the withdrawal of theophylline was accompanied by a worsening of asthma symptoms and its control, together with a reduction of CD4+ lymphocytes in peripheral blood and an increase of these cells in bronchial biopsies. These findings were complemented by another double-blind, placebo controlled study which demonstrated that treatment of atopic asthmatics with theophylline was accompanied by a significant reduction in

Table 4. – Percentage inhibition of cytokine messenger ribonucleic acid (mRNA) expression of CD4+ cells by salbutamol

	Salbutamol concentration M ($\mu g \cdot mL^{-1}$)					
	10^{-4} (58)	10^{-5} (5.8)	10^{-6} (0.58)	10^{-7} (0.058)	10^{-8} (0.0058)	10^{-9} (0.00058)
IL-3/GAPDH	17.6±6.5	0.6±15.0	11.9±13.9	2.9±11.8	-14.8±18.9	10.7±9.9
IL-4/GADPH	13.4±14.3	-3.0±24.1	11.2±6.8	12.3±12.0	-5.1±25.1	10.3±17.1
IL-5/GADPH	-5.5±15.6	-14.4±26.8	13.3±10.8	-8.5±13.8	-20.7±27.8	13.9±12.1
GM-CSF/GAPDH	-1.9±20.9	-20.0±19.4	8.8±16.6	-10.6±18.3	14.6±12.0	-11.2±-11.1
IFN-α/GAPDH	-17.0±16.4	12.1±19.2	-11.5±12.3	-6.8±13.0	-14.7±14.5	-11.6±13.0

Data are mean±SEM. For definitions, see footnote to table 2. Salbutamol did not produce any significant suppression of gene expression of any of the cytokines at concentrations of 10^{-4}–10^{-9}. Statistical analysis was Kruskal-Wallis followed by Mann-Whitney tests. Values are calculated as 100% - (cytokine expression with PHA/PMA & salbutamol/cytokine expression with PHA/PMA only × 100%.

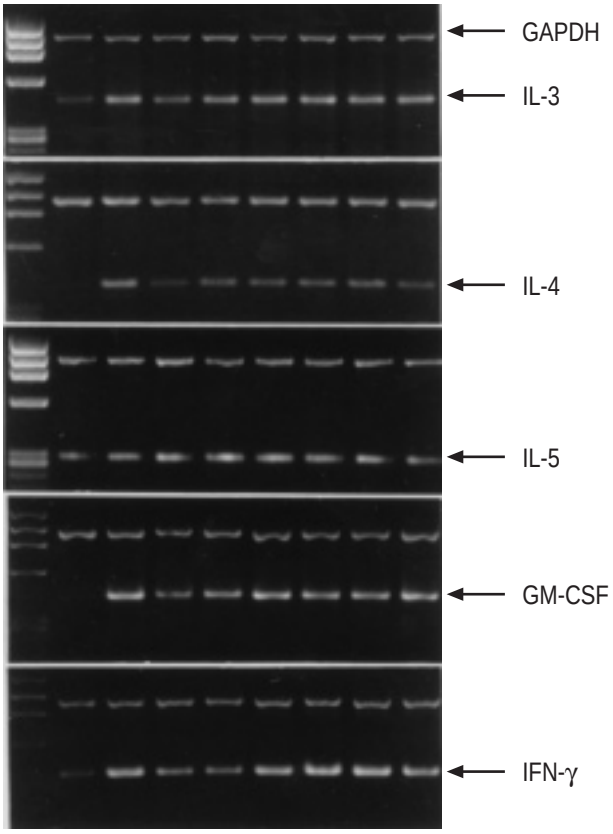

Fig. 1. – Agarose gel electrophoresis of polymerase chain reaction (PCR) products of reduced glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and cytokine (interleukin (IL)-3, IL-4, IL-5, granulocyte-macrophage colony-stimulating factor (GM-CSF) and interferon (IFN)-γ) messenger ribonucleic acid (mRNA) from CD4+ T-cells with decreasing concentrations of theophylline. Lane 1: *Haemophilus aegyptius* (Hae)III-digested ΦX174 deoxyribonucleic acid (DNA) size standards; 2: medium; 3: phorbol myristate acetate (PMA)/phytohaemogglutinin (PHA) alone; 4–9: PMA/PHA with 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M theophilline (180–0.0018 $\mu g \cdot mL^{-1}$), respectively.

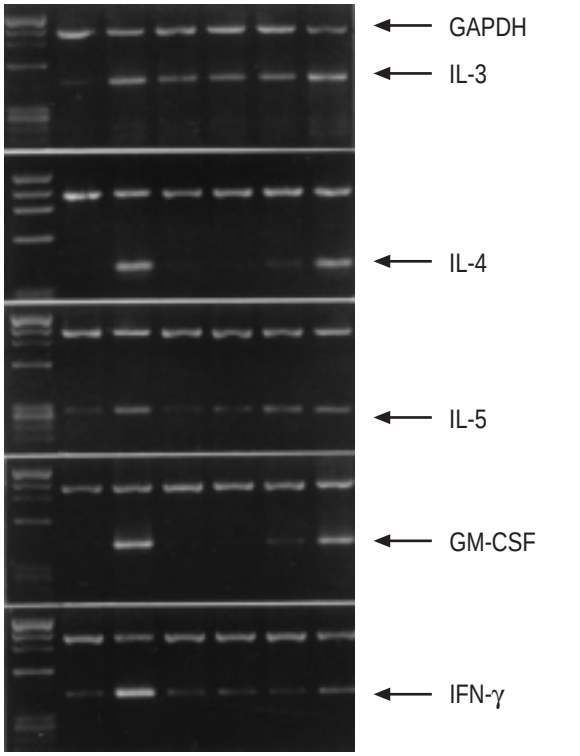

Fig. 2. – Agarose gel electrophoresis of polymerase chain reaction (PCR) products of GAPDH and cytokine (IL-3, IL-4, IL-5, GM-CSF and IFN-α) mRNA from CD4+ T-cells with decreasing concentrations of dexamethasone. Lane 1: *Hae*III-digested ΦX174 DNA size standards; 2: medium; 3: phorbol myristate acetate (PMA)/phytohaemogglutinin (PHA) alone; 4–7: PMA/PHA with 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} M dexamethasone (390–0.39 $ng \cdot mL^{-1}$), respectively. For definitions, see legend to figure 1.

the number of CD4+ lymphocytes in bronchoalveolar lavage (BAL) fluid and a corresponding elevation of these cells in peripheral blood [27]. Moreover, the authors in the latter study [27] found that theophylline therapy appeared to be associated with a reduction in the absolute numbers of BAL CD4+ T-cells expressing activation markers but not a decrease in the proportion of these cells. In other words, the drug may have modulated the movement both of activated and nonactivated lung CD4+ T-cells rather than acting directly to inhibit the activated status of CD4+ cells. This hypothesis is consistent with the results of the present study.

A previous study of the effects of theophylline on mucosal inflammation in asthmatic airways utilizing bronchial biopsy results found that in patients with mild to moderately severe atopic asthma, treatment with theophylline resulted in a significant reduction of the number of cells containing IL-5 [15]. However, the cellular origins of these cells are not known. Both mast cells and eosinophils are capable of synthesizing IL-5 [28, 29] and theophylline may exert its cytokine inhibitory effects on them. Furthermore, some of the subjects in that study were receiving inhaled corticosteroids, making the interpretation of results difficult. The present utilized normal subjects so that all subjects would be naïve to all anti-asthmatic medications. Based on these results, it would appear that theophylline does not have any inhibitory effects on cytokine production in CD4+ cells.

These studies together with the results of this study suggest that corticosteroids and theophylline probably act *via* different mechanisms to produce anti-inflammatory effects. This has important therapeutic implications as it suggests that these two agents would have additive effects. Studies have shown that high-dose inhaled corticosteroids may cause significant systemic effects, including the suppression of the pituitary-adrenal axis, decreased skin thickness, and enhanced bone absorption with reduced bone formation. Furthermore, even high doses of inhaled corticosteroids may not provide adequate asthma control, despite optimal compliance and technique [30, 31]. The addition of a second drug that would improve control and at the same time act as a steroid-sparing agent is therefore highly desirable. Indeed, there are now at least four randomized, double-blind, controlled trials confirming that theophylline adds substantial clinical benefit to asthmatics treated with inhaled or systemic corticosteroids [10, 30–32].

Recent studies have suggested that regular β-agonist administration may increase airway inflammation and hyperresponsiveness to histamine and methacholine in asthmatic subjects [33]. The mechanism whereby this occurs remains unknown. The results of the present study

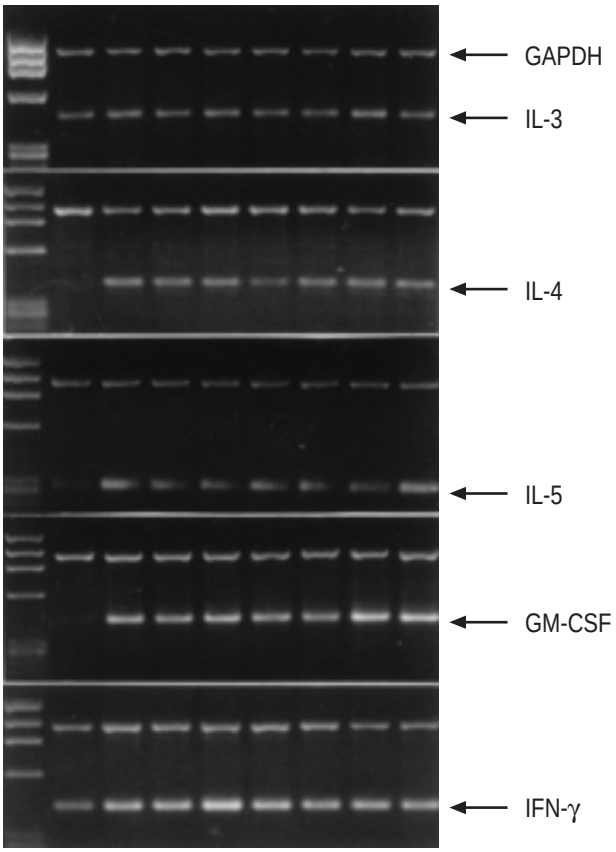

Fig. 3. – Agorose gel electrophoresis of polymerase chain reaction (PCR) products of GAPDH and cytokine (IL-3, IL-4, IL-5, GM-CSF and IFN-γ) mRNA from CD4+ T-cells with decreasing concentrations of salbutamol. Lane 1: *Hae*III-digested ΦX174 DNA size standards; 2: medium; 3: phorbol myristate acetate (PMA)/phytohaemogglutinin (PHA) alone; 4–9: PMA/PHA with 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} M salbutamol (58–0.00058 $\mu g \cdot mL^{-1}$), respectively. For definitions, see legend to figure 1.

demonstrate that if β-agonists do possess pro-inflammatory properties, these are unlikely to be related to their effects on cytokine production of CD4+ cells. Regular β-agonist administration may allow an increased allergen load to reach the airways by masking the allergen-induced early asthmatic response leading to the development of a late asthmatic response [34]. Alternatively, tachyphylaxis of β-receptors present on mast cells may result in destabilization causing these cells to release their inflammatory mediators more readily [35].

In conclusion, this study demonstrates that the suppression of cytokine gene expression in CD4+ T-cells does not occur at therapeutic concentrations of theophylline and salbutamol but is likely to be an important mechanism underlying the therapeutic effect of corticosteroids in asthma.

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