

Pulmonary function changes and immunomodulation of Th 2 cytokine expression induced by aminophylline after sensitization and allergen challenge in brown Norway rats

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Background and Objective: Evidence has shown that aminophylline has bronchoprotective, anti-inflammatory, and immunomodulatory effects. Our purpose was to evaluate the effect of different doses of aminophylline on the late-phase reaction, bronchial hyperresponsiveness (BHR) and T cell-related cytokine mRNA expression in brown Norway rats induced by ovalbumin (OA) sensitization.

Methods: Forty rats were equally divided into four groups. Groups I, II, and III animals were sensitized and subsequently provoked with OA. Aminophylline 25 mg/kg was given intraperitoneally to the group I animals and 5 mg/kg to group II animals. Group III animals received intraperitoneal normal saline. Group IV breathed aerosolized saline as a control. After OA provocation, the animals were anesthetized. Pulmonary function tests were performed at baseline and after varying doses of acetylcholine. Thereafter, bronchoalveolar lavage was performed and the lungs were examined histologically. Total RNA was extracted from lung tissue and reverse transcriptase-polymerase chain reaction was performed using primers for interleukin (IL)-2, IL-4, IL-5, IL-6, IL-10, interferon- γ , inducible nitric oxide synthase, and β -actin.

Results: Group III had worse pulmonary function tests, more severe BHR, and more severe lung inflammation, higher IL-4 and IL-10 cytokine levels in bronchoalveolar lavage fluid, and higher IL-4, IL-5, IL-6, IL-10, tumor necrosis factor- α and inducible nitric oxide synthase mRNA expression than the other three groups. Expression of IL-2 and interferon- γ was significantly reduced in group III.

Conclusions: Both low and high dose aminophylline are effective in preventing late-phase bronchoconstriction, BHR, and an inflammatory response. Aminophylline decreases T helper cell 2-related cytokine mRNA expression but increases T helper cell 1-related cytokines mRNA expression.

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INTRODUCTION

Bronchial asthma is a common disease. However, its prevalence and

mortality rate are increasing.^{1–4} Experimental models are needed to help investigate this disease. Asthma in brown Norway rats (BNR) has many similarities to human allergic asthma, as characterized by early- and late-phase reactions, bronchial hyperresponsiveness (BHR), and accumulation of eosinophils and activated T-lymphocytes in the airways after allergen challenge. The BNR has thus been widely used as an animal model for asthma.^{5–7}

Inflammation is reported to be related to bronchial asthma, its severity,

and bronchial hyperreactivity.^{8–12} T helper cell 2 (Th 2) but not Th 1 lymphocytes have been reported to have proinflammatory roles by expressing and generating cytokines that activate and attract effector cells and release other cytokines and mediators. There is also evidence demonstrating that Th 2 cytokines are increased, but Th 1 cytokines decreased in the lung tissue of ovalbumin (OA)-sensitized and -exposed BNR.⁸

In the past, bronchodilators played the most important role in the treatment of bronchial asthma.^{13–15} However, agents with anti-inflammatory action are now thought to be more important in the treatment of bronchial asthma.^{16–19} Theophylline was commonly used in the past as a bronchodilator to relieve acute and chronic symptoms of asthma.¹³ However, some recent evidence has demonstrated that the efficacy of theophylline seems to exceed that expected from its bronchodilator effect alone.^{18–19} It has been shown to have both bronchoprotective and anti-inflammatory, immunomodulatory effects, characterized by down-regulation of inflammatory and immune cell function in vitro and in vivo, and attenuation of allergen-induced inflammatory response in the asthmatic airway including: blunted late-phase increase in airway obstruction, decreased airway responsiveness to histamine, and decreased migration of activated eosinophils into the bronchial mucosa.^{16–19}

In renal transplant patients, theophylline also decreases the CD4/CD8

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T-cell ratio and increases T suppressor cell function that inhibits graft-versus-host response and reduces the dose of corticosteroids required to control rejection.²⁰ In asthmatic patients, theophylline can restore T-suppressor lymphocyte number and function toward normal.²¹ Theophylline inhibits eosinophil recruitment into the airway after bronchial challenge.²² It also reduces the number of eosinophil-granule-positive eosinophils in bronchial biopsies after bronchial challenge. Pauwels²³ demonstrated that a single intravenous dose of theophylline abolishes the late asthmatic response to allergen with little effect on the early asthmatic response. All these effects may be responsible for aminophylline's clinical efficacy in the treatment of chronic asthma.¹⁶⁻¹⁹

The purpose of this study was to evaluate the effect of aminophylline on the late-phase reaction and BHR in BNR after OA sensitization. We also examined its effect on Th 2- and Th 1-related cytokine mRNA expression patterns in BNR lung tissue.

MATERIALS AND METHODS

Forty BNR (weight ranging from 250 to 350 g) were divided into four groups. Each group consisted of 10 weight-matched male animals. The mean weight of group I was 290 ± 26 g; group II, 297 ± 24 g; group III, 294 ± 21 g; and group IV, 296 ± 18 g. Aerosol sensitization and challenge with OA were performed on groups I, II, and III, whereas group IV served as normal controls.

Each group I, II, and III BNR was put into a closed chamber ($30 \times 30 \times 16$ cm plastic box) with two small holes. One served as a gas inlet and the other as a gas outlet. Two milliliters of 1% OA was aerosolized by nebulizer and delivered continuously into the closed chamber at a flow of 8 L/minute. The animal was exposed to the OA in the chamber for 10 minutes.

A second sensitization was performed 7 days later using the same procedure. Another 7 days later, a provocation test was performed. Before provocation, aminophylline 25

mg/kg was given intraperitoneally to group I animals, aminophylline 5 mg/kg to group II animals, and normal saline to group III animals. Thirty minutes later, 3 mL of 4% aerosolized OA was delivered by nebulizer for 10 minutes into the closed chamber.

Control animals in group IV breathed aerosolized saline rather than OA under the same conditions as group I animals.

Study Protocol, Procedure, and Pulmonary Function Tests (PFT)

PFT and acetylcholine (Ach) provocation tests were carried out under general anesthesia (sodium pentobarbital, 50 mg/kg via intraperitoneal injection), using whole-body plethysmographic methods as previously described.^{24,25} For groups I, II, and III, this was done 24 hours after the OA provocation test, and for group IV, 24 hours after the final saline aerosolization. Animals were paralyzed with an intravenous injection of gallamine triethiodide (10 mg/kg) and artificially ventilated with a small animal ventilator at a tidal volume of 6 mL/kg and a frequency of 60 breaths/minute. All animals were stable without spontaneous breathing 5 minutes after gallamine was given. PFT including airway opening pressure (PaO) during ventilator breathing, total respiratory system compliance (Crs), total respiratory system resistance, and a maximal forced expiratory maneuver (MFEM) were measured at baseline. Thereafter, 25, 50, 75, and 100 μ g/kg of Ach were given intravenously at 30-minute inter-

vals. PFT were done 5 seconds after each dose of Ach.^{24,25} Before each successive dose of Ach, the flow volume (FV) loop returned to baseline. For the MFEM, the lungs were inflated to total lung capacity (TLC, lung volume at PaO = 30 cm H₂O) three times; the inflation was regulated by a solenoid valve. At peak volume during the third inflation, the inflation valve was shut off and another solenoid valve was opened immediately for deflation. The deflation valve was connected to a 20-L container, with a pressure of -30 cm H₂O (subatmospheric). The negative pressure of 30 cm H₂O produced maximal expiratory flow. The parameters of the FV loop, including peak flow (the maximal flow rate of the FV loop), MFEF_{75%} (the maximal forced expiratory flow rate at 75% TLC), MFEF_{50%} (the maximal forced expiratory flow rate at 50% TLC), and MFEF_{25%} (the maximal forced expiratory flow rate at 25% TLC) were recorded.^{24,25} PD₂₀MFEF_{50%}Ach was the dose of Ach required to produce a decrease in MFEF_{50%} of 20% from baseline.

Bronchoalveolar Lavage (BAL)

After completion of the PFT, BAL was performed using 10 mL normal saline twice (total 20 mL). The BAL fluid was collected into plastic flasks and then strained through one layer of surgical gauze and centrifuged at 500 *g* for 5 minutes. The cell pellet was washed three times with sterile saline solution and resuspended in RPMI-1640. A small portion was taken for evaluation of cell

Table 1. Comparison of Mean Peak Expiratory Flow Rate, MFEF_{75%}, MFEF_{50%}, MFEF_{25%}, Crs, PaO at Tidal Breathing, and TLC Among All Groups at Baseline

	Group I	Group II	Group III	Group IV
Peak expiratory flow rate, mL/sec	105.4 $\pm$ 4.6	101.9 $\pm$ 5.2	104.9 $\pm$ 5.9	103.2 $\pm$ 5.3
MFEF _{75%} , mL/sec	103.2 $\pm$ 4.8	100.8 $\pm$ 4.1	101.8 $\pm$ 4.6	101.2 $\pm$ 5.8
MFEF _{50%} , mL/sec	85.9 $\pm$ 4.2	82.7 $\pm$ 4.6	73.2 $\pm$ 5.1*	86.7 $\pm$ 4.6
MFEF _{25%} , mL/sec	45.8 $\pm$ 4.7	42.3 $\pm$ 4.5	32.3 $\pm$ 4.8*	46.3 $\pm$ 4.5
Crs, mL/cmH ₂ O	0.46 $\pm$ 0.05	0.45 $\pm$ 0.06	0.42 $\pm$ 0.05	0.48 $\pm$ 0.06
PaO, cm H ₂ O	3.5 $\pm$ 0.5	3.7 $\pm$ 0.6	3.9 $\pm$ 0.5	3.6 $\pm$ 0.5
TLC, mL	14.2 $\pm$ 0.5	14.7 $\pm$ 0.5	14.1 $\pm$ 0.5	14.3 $\pm$ 0.5

ANOVA test and Scheffe test were used.

* The comparison of MFEF_{25%} and MFEF_{50%} between groups I and III, groups II and III, and groups III and IV showed significant difference ($P < 0.05$).

number and viability, as evaluated by trypan blue exclusion.

Cytokine Levels in BAL Fluid

The concentrations of interleukin (IL)-2, IL-4, IL-10, and interferon (IFN)- γ in the BAL fluid were measured in duplicate, in a blinded fashion, using a commercially available rat-specific IL-2, IL-4, IL-10, and IFN- γ enzyme-linked immunosorbent assay kit (Biosource International, Camarillo, CA) with a detection limit of <10 pg/mL for IL-2, <5 pg/mL for IL-4, <10 pg/mL for IL-10, and <10 pg/mL for IFN- γ . A microplate reader (Spectra Reader, SLT Labinstruments, Grödig, Austria) was used to measure absorbance at 450 nm. Background absorbance of blank wells was subtracted from the standards and unknowns before determination of the sample concentrations. Total protein was measured using a commercial reagent (Bio-Rad, Hercules, CA) and concentration was calculated using a standard curve constructed with bovine serum albumin (Bio-Rad).

Histologic Examination

After BAL, each animal's chest was opened and the lungs were removed. Histologic specimens (six specimens per animal consisting of trachea and five lobes of the lung) were prepared. The trachea and each lobe were fixed in 10% formaldehyde, dehydrated by

different concentrations of alcohol, embedded in paraffin, cut into 4- μ m thicknesses, stained with hematoxylin and eosin (H&E), and examined by light microscope to evaluate the severity of the inflammation. Each section of trachea and lung parenchyma was blindly assigned a total inflammatory score by two pathologists: 0 = normal; 1 = inflammatory infiltrate of eosinophils, lymphocytes, and neutrophils in <20% of the whole section; 2 = inflammatory infiltrate of eosinophils,

lymphocytes, and neutrophils in 20 to 40% of the whole section; 3 = inflammatory infiltrate of eosinophils, lymphocytes, and neutrophils in >50% of the whole section. The total inflammation score for each animal was calculated as the mean of the scores for five lung sections and trachea.

Cytokine mRNA Analysis

Total RNA was extracted from lung tissue samples by a method described previously²⁶ and reverse transcriptase-

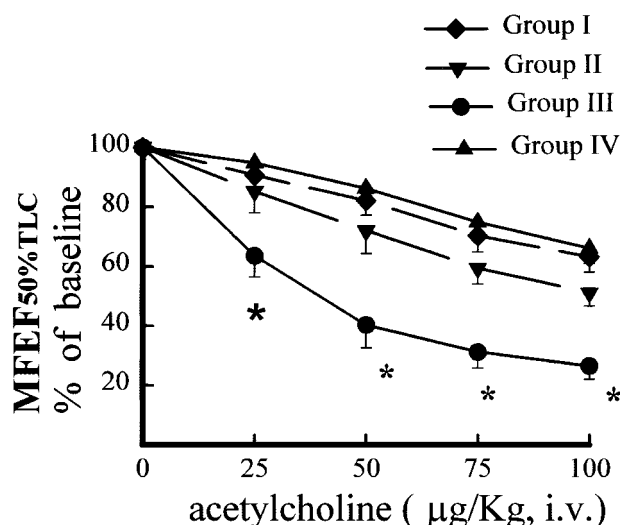


Figure 1. The dose-response curve of MFEF in groups I, II, III, and IV. The percentage change of MFEF_{50%} from baseline in group III was higher than in groups I, II, and IV when Ach doses higher than 25 μ g/kg were given.

Table 2. Total Differential Cell Counts in BAL Fluid ($\pm$ SD)

	Group I	Group II	Group III	Group IV
Total cell count ($\times 10^4$)/mL	3.9 $\pm$ 0.4‡	5.2 $\pm$ 0.5†	10.4 $\pm$ 1.6*	0.7 $\pm$ 0.2
Macrophages ($\times 10^4$)/mL	3 $\pm$ 0.3‡	3.65 $\pm$ 0.3†	5.8 $\pm$ 0.6*	0.60 $\pm$ 0.02
Percentage of total cell count (%)	77 $\pm$ 4.7‡	70.1 $\pm$ 4.1†	55.4 $\pm$ 5.7*	86.2 $\pm$ 2.8
Lymphocytes ($\times 10^4$)/mL	0.36 $\pm$ 0.08‡	0.64 $\pm$ 0.21†	1.9 $\pm$ 0.4*	0.05 $\pm$ 0.01
Percentage of total cell count (%)	9.3 $\pm$ 1.6‡	12.3 $\pm$ 2.6†	18.3 $\pm$ 3.7*	6.5 $\pm$ 1.8
Neutrophils ($\times 10^4$)/mL	0.06 $\pm$ 0.02‡	0.07 $\pm$ 0.03	0.19 $\pm$ 0.05*	0.01 $\pm$ 0.002
Percentage of total cell count (%)	1.5 $\pm$ 0.5	1.3 $\pm$ 0.5	1.8 $\pm$ 0.5	2.00 $\pm$ 0.7
Eosinophils ($\times 10^4$)/mL	0.48 $\pm$ 0.11‡	0.85 $\pm$ 0.21†	2.5 $\pm$ 0.43*	0.04 $\pm$ 0.01
Percentage of total cell count (%)	12.2 $\pm$ 2.2‡	16.3 $\pm$ 3.2†	24.5 $\pm$ 4.2*	5.3 $\pm$ 2.1

ANOVA test and Scheffe test were used.

* Differences between groups I and III, groups II and III, and groups III and IV were significant for all parameters ($P < 0.05$) except percentage of total neutrophil cell count to total cell count.

† Differences between groups I and II were significant for the parameters of total cell count, total macrophage count, total lymphocyte cell count, total eosinophil cell count and percentage of total macrophage count, total lymphocyte cell count, and total eosinophil cell count to total cell count ($P < 0.05$).

‡ Differences between groups I and IV were significant for all parameters ($P < 0.05$) except percentage of total neutrophil cell count to total cell count.

Table 3. Concentrations of IL-2, IL-4, IL-10, IFN- γ , and Total Protein in BAL Fluid ($\pm$ SD)

	Group I	Group II	Group III	Group IV
IL-2, pg/mL	113.4 $\pm$ 21.3‡	102.3 $\pm$ 15.2	61.8 $\pm$ 14.3*	144.3 $\pm$ 21.6
IL-4, pg/mL	96.2 $\pm$ 23.4‡	166.0 $\pm$ 15.7†	234.1 $\pm$ 24.7*	32.6 $\pm$ 10.2
IL-10, pg/mL	136.5 $\pm$ 18.2‡	173.1 $\pm$ 22.5†	230.3 $\pm$ 32.8*	67.2 $\pm$ 11.7
IFN- γ , pg/mL	10.2 $\pm$ 3.1‡	5.46 $\pm$ 1.2†	3.41 $\pm$ 1.1*	17.5 $\pm$ 5.8
Total protein, μ g/mL	18.6 $\pm$ 4.4‡	28.4 $\pm$ 5.5†	49.1 $\pm$ 5.7*	10.6 $\pm$ 2.9

ANOVA test and Scheffe test were used.

* Differences between groups I and III, groups II and III, and groups III and IV were significant for all parameters ($P < 0.05$).

† Differences between groups I and II were significant for IL-4, IL-10, IFN- γ , and total protein ($P < 0.05$).

‡ Differences between groups I and IV were significant for all parameters ($P < 0.05$).

polymerase chain reaction (RT-PCR) was performed using an RT-PCR kit (Clontech, Palo Alto, CA). The following T cell-related cytokines were evaluated by measuring their mRNA expression: IL-2, IFN- γ (Th 1-related), IL-4, IL-5, and IL-10 (all Th 2-related), and inducible nitric oxide synthase (iNOS) with a PCR kit (Kevin Science Technology, Brea, CA).

Preparation of cDNA and PCR Analysis

Briefly, 2 μ g RNA in a 25- μ L volume was first primed with 2 μ L oligo-(dT)₁₈ primer, 20 μ M, at 70° C for 2 minutes and then kept on ice for 5 minutes. After the reaction, a mixture containing 8 μ L of five times reaction buffer; 2 μ L, deoxyribonucleotide triphosphate mix 10 μ M (each); 1 μ L recombinant ribonuclease inhibitor, 40 units/ μ L; and 2 μ L Moloney murine leukemia virus RT, 200 units/ μ L was added to synthesize cDNA. cDNA was amplified by 40 PCR cycles in a thermal cycler (Hybaid Omnigene, Middlesex, UK), each cycle consisting of a denaturation step (94° C for 1 minute), an annealing step (65° to 55° C for 1 minute, 0.5° C/cycle touch down, 55° C for 19 cycles), and an extension step (72° C for 1 minute). During the last cycle, the 72° C step was extended to 5 minutes. The PCR product was analyzed with 2% agarose gel electrophoresis. The identity of each cytokine cDNA was determined by the PCR product obtained by electrophoresis, and confirmed by DNA sequencing of the PCR products. The quantities of target messages were determined

where the ratio of target band intensities were analyzed by equations of the β -actin line.²⁶

Serum Theophylline Level

Serum theophylline levels, determined from 1 mL of blood taken from lateral tail vein of the BNR were measured before and 30 minutes, 2 hours, and 24 hours after aminophylline was given in groups I and II.

Data Analysis

The Student's *t* test and the analysis of variance test were used for statistical analysis where appropriate. If the anal-

ysis of variance test showed statistical significance, the Scheffe test was also performed. All values were expressed as the mean $\pm$ standard deviation, with significance accepted when $P < 0.05$. Simple linear regression was used for correlation analysis.

RESULTS

PFT Data

The mean data at baseline of peak expiratory flow rate, MFEF_{75%}, MFEF_{50%}, and MFEF_{25%} of the FV loop for all four groups are shown in Table 1. There was no difference in peak expiratory flow rate, MFEF_{75%}, Crs, PaO, and TLC among the four groups. The MFEF_{50%} and MFEF_{25%} were significantly lower in group III than in the other three groups ($P < 0.05$), but did not differ significantly among groups I, II, and IV (Table 1).

In group III, the percentage change of MFEF_{50%} TLC (Fig 1) was significantly higher than the other three groups at doses of Ach higher than 25 μ g/kg ($P < 0.05$). The PD₂₀ Ach MFEF_{50%} TLC was significantly lower in group III than the other three groups

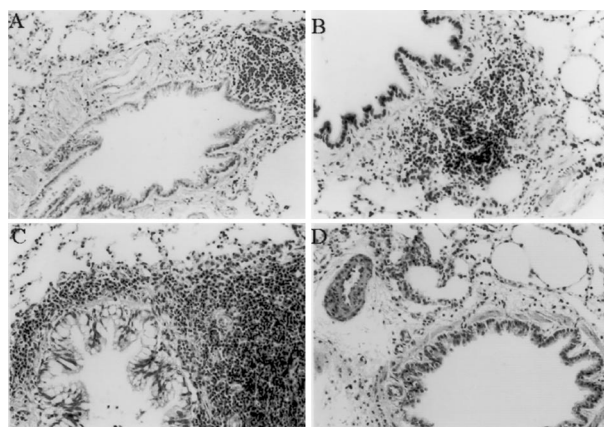


Figure 2. (A) The histology of lung tissue and bronchi from a representative group I animal (H&E stain 200 $\times$), demonstrating a mild inflammatory reaction, characterized by mild hyperemia, interstitial edema, and inflammatory cell infiltration (score 1) in the lung tissue and bronchi. (B) The histology of lung tissue and bronchi from a representative group II animal (H&E stain 200 $\times$), demonstrating a mild to moderate inflammatory reaction, characterized by mild hyperemia, interstitial edema and inflammatory cell infiltration (score 2) in the lung tissue and bronchi. (C) The histology of lung tissue and bronchi from a representative group III animal (H&E stain 200 $\times$), demonstrating a severe inflammatory reaction, characterized by hyperemia, interstitial edema, and inflammatory cell infiltration (score 3). (D) The histology of lung tissue and bronchi from a representative group IV animal (H&E stain 200 $\times$), demonstrating absence of hyperemia, interstitial edema, and inflammatory cells infiltration (score 0) in the lung tissue and bronchi.

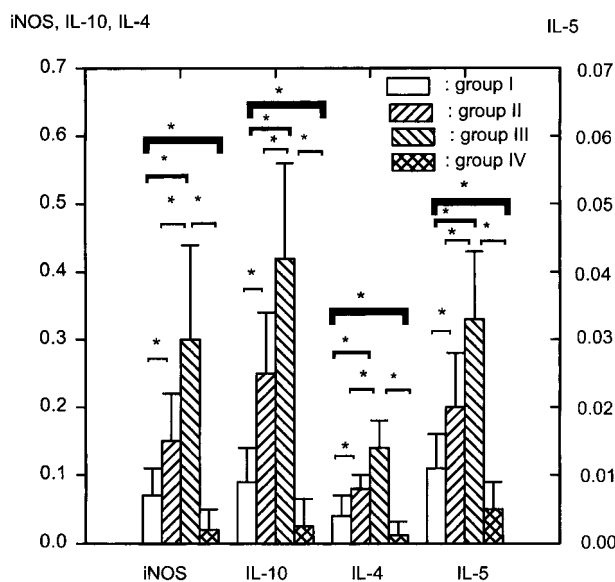


Figure 3. The mean ratio of IL-4, IL-5, IL-10, and iNOS mRNA levels to β -actin in groups I, II, and IV were lower than in group III as measured by densitometry.

(55 μ g vs 37 μ g vs 13 μ g vs 75 μ g; $P < 0.05$; Fig 1).

BAL

There was no difference in recovery rate of BAL fluid among the four groups ($84.3 \pm 2.3\%$ vs $85.8 \pm 2.7\%$ vs $85.5 \pm 3.2\%$ vs $84.9 \pm 3.4\%$) ($P > 0.05$). Group III had higher total cell counts and higher absolute counts of each cell type than any other group. The percentage of eosinophils and lymphocytes in group III was higher than in the other groups. In contrast, the percentage of macrophages was lower in group III. Group II had higher total cell counts, higher absolute counts of macrophages, eosinophils and lymphocytes, higher percentage of eosinophils and lymphocytes than group I, whereas the percentage of macrophages in group II was lower than in group I (Table 2). Group I had higher total cell counts and higher absolute counts of each cell type than group IV. The percentage of eosinophils and lymphocytes in group I was higher than in group IV, whereas the percentage of macrophages was lower in group I (Table 2). The group III BNR had higher IL-4, IL-10, and total protein, but lower IL-2 and IFN- γ cytokine levels in BAL fluid when com-

pared with the groups I and II aminophylline-pretreated BNR and group IV normal controls. Group II had higher IL-4, IL-10, and total protein, but lower IFN- γ cytokine levels in BAL fluid when compared with group I. Group I had higher IL-4, IL-10, and total protein, but lower IL-2 and IFN- γ

cytokine levels in BAL fluid when compared with group IV (Table 3).

Histology of Lung Tissue

There was only mild or mild-to-moderate hyperemia, interstitial edema, and inflammatory cell infiltration in the lung tissue and bronchi of the groups I and II (Fig 2, A and B). The lung tissue and bronchi of group III OA-sensitized BNR demonstrated a severe inflammatory reaction, characterized by hyperemia, interstitial edema, and inflammatory cell (especially lymphocytes and eosinophils) infiltration (Fig 2C). In contrast, there was absence of hyperemia, interstitial edema, and inflammatory cell infiltration in the lung tissue and bronchi of the group IV (Fig 2D). The mean total inflammatory scores of the group III were significantly higher than the other three groups ($P < 0.05$). The mean total inflammatory scores is group I, 1.1 ± 0.2 ; group II, 1.8 ± 0.2 ; group III, 2.5 ± 0.3 ; and group IV, 0.

Cytokine mRNA Profile Using RT-PCR

The mean ratios of IL-4, IL-5, IL-10, IL-6, TNF- α , and iNOS mRNA levels to β -actin in groups I, II, and IV were

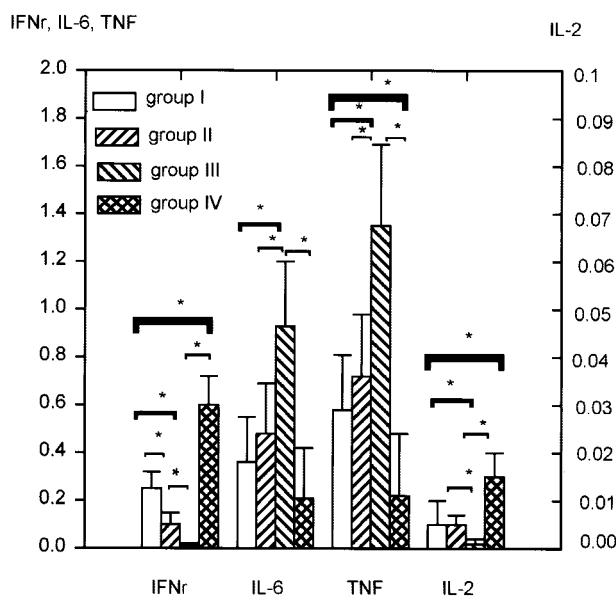


Figure 4. The expression of mean ratio of IFN- γ and IL-2 were significantly reduced in group III compared with groups I, II, and IV. The mean ratio of IL-6 and TNF- α mRNA levels to β -actin in groups I, II, and IV were lower than in group III as measured by densitometry.

lower than in group III (Figs 3 and 4). The mean ratios of IFN- γ and IL-2 mRNA levels to β -actin (Fig 4) were significantly lower in group III compared with groups I, II, and IV. The mean ratios of IL-4, IL-5, IL-10, and iNOS mRNA levels to β -actin in groups I were lower than in group II (Figs 3 and 4). The mean ratios of IFN- γ mRNA levels to β -actin were significantly lower in group II compared with group I (Fig 4). The mean ratios of IL-4, IL-5, IL-10, TNF- α , and iNOS mRNA levels to β -actin in group I were higher than in group IV (Figs 3 and 4). The mean ratios of IL-2 and IFN- γ mRNA levels to β -actin were significantly increased in group IV compared with group I (Fig 4). Figure 5 shows an example of these results. Group III had higher IL-4, IL-5, IL-6, IL-10, iNOS, and TNF- α mRNA expression, but lower IL-2 and IFN- γ mRNA expression when compared with the other three groups. Group I BNR with a higher dose aminophylline had lower IL-4, IL-5, IL-6, IL-10, iNOS, and TNF- α , but higher IL-2 and IFN- γ mRNA expression when compared with the group II animals treated with a lower dose of aminophylline.

Serum Theophylline Levels

The highest serum theophylline level was 30 minutes after aminophylline was given in both groups I and II animals. Group I had higher serum theophylline levels than group II at 30 minutes and 2 hours after aminophylline was given. The serum theophylline level was very low 24 hours after aminophylline was given for both groups I and II animals (Table 4).

DISCUSSION

Although several mechanisms have been proposed to explain the actions of theophylline, nonspecific inhibition of phosphodiesterase isoenzymes and nonselective antagonism of specific cell-surface receptors for adenosine are the only ones known to occur at clinically relevant drug concentrations. Its actions include inhibition of phosphodiesterase resulting in increased intracellular cyclic adenosine monophos-

phate; inhibition of types III and IV isoenzymes resulting in relaxation of smooth muscles in pulmonary arteries and airways; inhibition of type IV isoenzymes resulting in anti-inflamma-

tory and immunomodulatory effects; and inhibition of unknown types of isoenzymes resulting in central respiratory stimulation.^{27,28} In this study using MFEM, we demonstrated that pre-

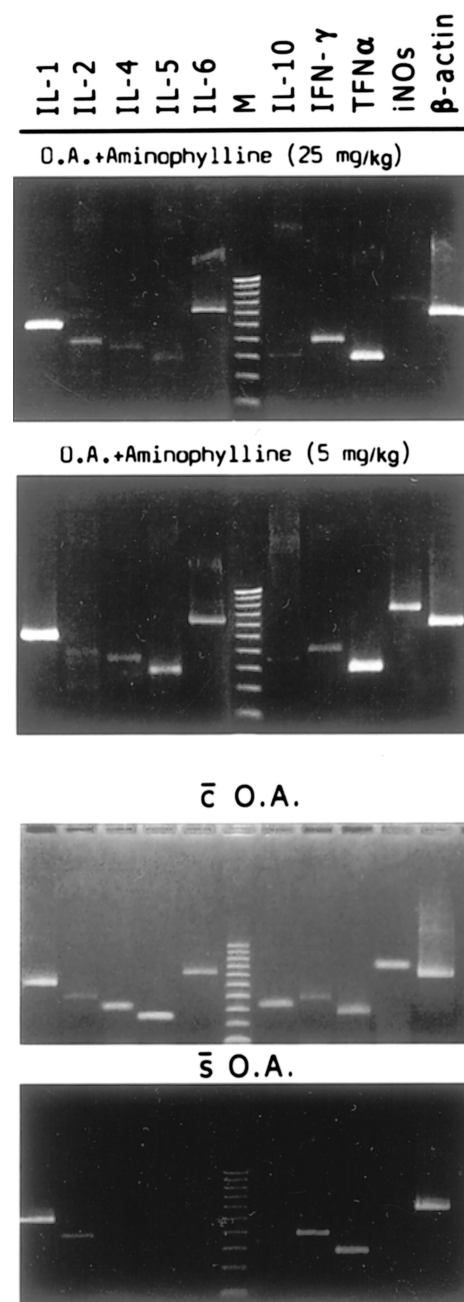


Figure 5. An example of cytokine results. Group III had increased IL-4, IL-5, IL-10, iNOS, and TNF- α mRNA expression, but decreased IL-2 and IFN- γ mRNA expression when compared with the other three groups. The group I animals treated with a higher dose aminophylline had lower IL-4, IL-5, IL-10, iNOS, and TNF- α , but higher IL-2 and IFN- γ mRNA expression when compared with group II animals treated with a lower dose of aminophylline.

Table 4. Mean Serum Theophylline Levels before and 30 Minutes, 2 Hours, and 24 Hours after Aminophylline Was Given to Groups I and II Animals

Mean serum theophylline level, $\mu\text{g/mL}$	Group I	Group II
Before aminophylline	undetectable	undetectable
30 minutes after aminophylline was given	$31.2 \pm 2.2^*$	7.1 ± 1.2
2 hours after aminophylline was given	$26.5 \pm 1.4^*$	5.0 ± 0.8
24 hours after aminophylline was given	0.2 ± 0.1	0.1 ± 0.1

Student's *t* test was used.

Data are presented as mean $\pm$ SD.

* The mean serum theophylline levels 30 minutes and 2 hours after aminophylline was given of the group I animals were significantly higher than the group II animals ($P < 0.05$).

treatment with either a single low or large dose of aminophylline prevented late-phase bronchoconstriction 24 hours after OA challenge in sensitized BNR. It also decreased the BHR induced by Ach. However, the serum aminophylline level was very low at 24 hours after aminophylline was given for both groups I and II animals. Therefore, the apparent bronchoprotective effect probably depended on some mechanism other than direct bronchodilation.

We also demonstrated that aminophylline pretreatment resulted in a much less severe inflammatory response after OA challenge than occurred in the untreated sensitized rats. The group III OA treated BNR had higher IL-4 and IL-10, but lower IL-2 and IFN- γ cytokine levels in BAL fluid when compared with the groups I and II aminophylline-pretreated BNR. We also found that increased lung tissue IL-4, IL-5, and IL-10 mRNA levels in non-aminophylline-treated sensitized animals. Expression of IFN- γ was significantly reduced in OA-sensitized but non-aminophylline-treated rats. TNF- α is considered an important proinflammatory cytokine that may play a role in the development of the late-phase asthmatic reaction. Gosset et al²⁹ showed that production of TNF- α by macrophages after an antigen challenge was increased only in patients who developed a late asthmatic response. Similar to TNF- α , IL-6 is a proinflammatory cytokine, and iNOS is considered an inflammatory marker. In this study, expression of TNF- α , IL-6, and iNOS mRNA were significantly reduced in OA-sensitized but aminophylline-treated rats. These results suggest that aminophylline had bronchoprotective,

anti-inflammatory, and immunomodulatory effects which were at least partially mediated by changes in Th 2- and Th 1-related cytokines.

An unexpected finding of our study is that the cytokine levels were much higher than in comparable human data. We do not know whether this represents a species difference. To our knowledge, no studies have been published of BAL cytokine levels in the BNR. Zangrilli et al³⁰ did sequential antigen challenge in 20 atopic asthmatic patients, finding that BAL fluid IL-4 increased from 51.7 ± 17.7 at baseline to 150.1 ± 58.8 pg/mL 24 hours after challenge. Of the 20 atopic asthmatic patients, 10 had a dual response, and their BAL fluid mean IL-4 level increased to >250 pg/mL 24 hours after challenge. Tomkinson et al³¹ evaluated cytokine kinetics in BAL fluid after a single OA challenge. An increase in the Th 2 cytokine IL-4 was observed at 8 hours, reaching a maximum at 24 hours (119 ± 29 pg/mL), but returning to baseline by 96 hours. Trifilieff et al³² also demonstrated that an increase in IL-4 was observed, reaching a maximum at 24 hours (>500 pg/mL) even higher than our data. Borish et al³³ demonstrated that the mean IL-10 concentration in BAL fluid of normal subjects was 130 ± 61 pg/mL. Different commercial reagents may also produce different results. BAL fluid assays are notoriously difficult to standardize because of the lipoproteins, mucoproteins, and other materials which can interfere with determination of low cytokine levels by enzyme-linked immunoadsorbent assay techniques. Some inves-

tigators control their assays using BAL fluid authentic protein of interest. This important step ensures that interfering substances are identified. In this study, the cytokines were measured using a commercial reagent; total protein was measured using a commercial reagent; and concentration was calculated using a standard curve constructed with bovine serum albumin.

Antigen-induced bronchoconstriction causing air-flow obstruction and acute airway inflammation is the most commonly used model to study the anti-inflammatory effect of theophylline in animals. Most studies have used only one dosage of theophylline, usually much higher than those used clinically. There is one reported study of theophylline in animal models using a dose similar to that administered in man.²⁷ The authors demonstrated that theophylline and the more specific phosphodiesterase inhibitor benazefrine caused inhibition of pulmonary eosinophil infiltration in the guinea pig airway after platelet activating factor and allergen challenge. In this study, we used 2 dosages of theophylline, one similar to that administered in man (5 mg/kg), and the other much higher than that used clinically (25 mg/kg). We found that either dose was effective in decreasing the severity of airway inflammation and decreasing the mRNA of Th 2-related cytokines (IL-4, IL-5), IL-6, TNF- α , and iNOS expression while preventing some of the reduction in the mRNA of Th 1-related cytokines (IL-2, IFN- γ) seen in the untreated group. This effect was most prominent with high-dose aminophylline.

CONCLUSION

We have demonstrated a protective effect of aminophylline at both low and high doses against late-phase bronchoconstriction induced by OA. Our results support the hypothesis that the drug exerts this effect at least in part by affecting T cell cytokine production.

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