

Inflammatory changes associated with circadian variation in pulmonary function in subjects with mild asthma

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Summary

Background Nocturnal enhancement of airway inflammation has been demonstrated in patients with asthma who have a significant drop in pulmonary function at night.

Objective To investigate the circadian changes in airway inflammation and their relationship with variations in pulmonary function in subjects with mild atopic asthma.

Methods Twelve asthma subjects were admitted to the hospital for two separate 24-h visits. Bronchoalveolar lavage (BAL) was performed at 04:00 hours during one visit, and at 16:00 hours during another visit. BAL cells were analysed for lymphocyte phenotype and the capacity to secrete cytokines following *ex vivo* stimulation with phytohaemagglutinin (PHA).

Results The numbers of BAL lymphocytes and the percentage of CD4⁺ T cells were higher at 04:00 hours compared with 16:00 hours. At 04:00 hours, the forced expiratory volume in 1 s (FEV₁) was inversely correlated with BAL lymphocytes and CD4⁺ cells. PHA-induced generation of IL-5 by BAL cells correlated with BAL eosinophils and CD4⁺ cells. Moreover, there was a linear relationship between the relative change (16:00–04:00 hours) in IL-5 and circadian variation in FEV₁.

Conclusions These data suggest that the circadian variation in lung function in asthma is associated with increased airway CD4⁺ lymphocyte numbers and their capacity to generate IL-5. Furthermore, in mild asthma, these circadian changes appear to fall into a continuous range, suggesting that day/night variations in airway inflammation and lung function occur on a continuum, rather than as an all-or-none phenomenon.

Keywords airway lymphocytes, asthma, bronchoalveolar lavage, circadian rhythm, IL-5

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Introduction

Pulmonary function exhibits a circadian rhythm, with an afternoon maximum (16:00 hours) and an early morning minimum (04:00 hours) [1]. In asthmatic patients, this circadian variation is more pronounced, and is accompanied by circadian variation in bronchial responsiveness (BR) [2]. Endogenous factors, (e.g. circadian fluctuation in serum cortisol, histamine and epinephrine, responsiveness to steroids and β -agonists [3–7]) and exogenous factors (e.g. allergen exposure [8–10]) may contribute to day/night variation in lung function, but the factors that determine the magnitude of variation in pulmonary function are not well understood.

Day/night variation in lung function in asthmatics tends to be greater in those with more severe forms of the disease [11]. Moreover, airway inflammation may play a role in the increased airway hyper-responsiveness and obstruction observed at night and the magnitude of circadian variation may be related to the overall severity of inflammation [12–16]. Inflammatory changes in the distal alveolar spaces have been

reported to be more relevant to circadian variation in pulmonary function than proximal airway inflammation [17, 18].

It has been proposed that only those subjects with more severe asthma have inflammatory changes associated with circadian variation in lung function, since circadian inflammatory changes have not been well documented in subjects with milder disease [12, 14, 19, 20]. To further elucidate the relationship between airway inflammation and circadian variation in pulmonary function in subjects with less severe asthma, we performed bronchoalveolar lavage (BAL) on subjects with mild asthma. BAL cell phenotype and the capacity to produce cytokines were evaluated at 16:00 and 04:00 hours to determine if changes in pulmonary function are related to inflammatory changes in the airway.

Methods

Study design

During the screening phase, each subject underwent a medical history, physical examination, and pulmonary function testing by spirometry and response to inhaled β -agonist. Airway responsiveness to histamine was assessed and defined

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as the concentration of histamine that caused a 20 % drop in forced expiratory volume in 1 s (FEV₁). Peak expiratory flow rate (PEFR) was recorded by each subject nine times daily (including bedtime, upon awakening, every 4 h while awake, and during the night if subjects woke because of asthma symptoms) for 1 week. For the study, subjects were admitted to the General Clinical Research Center at the University of Wisconsin Hospital for two separate 24-h visits. Bronchoscopy with BAL was performed toward the end of each visit. During one visit, the subject was admitted at 20:00 hours, spirometry was performed at 20:00, 22:00, 04:00, 06:00, 08:00, 10:00, 12:00, 14:00, 15:45 hours, and BAL was performed at 16:00 hours. During the other visit, the subject was admitted at 08:00 hours, spirometry was performed at 08:00, 10:00, 12:00, 14:00, 16:00, 18:00, 20:00, 22:00, 03:45 hours, and BAL was performed at 04:00 hours. Subjects were monitored for a minimum of 2 h following each bronchoscopy, to ensure stability prior to discharge. The two hospital visits were separated by a minimum of 1 week and the order of the visits was random. The study was approved by the University of Wisconsin-Madison Center for Health Sciences Human Subjects Committee. Informed consent was obtained from each subject prior to participation.

Subjects

Twelve asthmatic subjects with a history of nocturnal worsening of asthma symptoms were recruited for the study. The subjects ranged in age from 20 to 37 years, and included nine males and three females. Asthma was defined as a history of recurrent wheezing, dyspnoea, and cough. In addition, each subject had increased airway responsiveness (AR) to histamine (PC₂₀ < 8 mg/mL) and/or reversibility with inhaled β_2 -agonist ($\geq 12\%$ improvement in FEV₁). Each subject had a positive skin test to one or more aeroallergens. We excluded smokers, subjects with FEV₁ less than 70%, a respiratory tract infection within the past month, immunotherapy within 3 years, pulmonary disease or other medical illnesses, and those who were using oral or inhaled steroids or leukotriene-

modifying agents. Theophylline and β -agonists were withheld for 24 h prior to each hospital visit.

Characterization of circadian variation

For each hospital visit, at least nine spirometry measurements were taken. The circadian variation in FEV₁ was expressed as ((FEV₁ peak – FEV₁ nadir)/FEV₁ mean) $\times$ 100. For each subject, the circadian variation was calculated as the average circadian change in FEV₁ for the two hospital visits. The subject characteristics are shown in Table 1.

Bronchoscopy and BAL

Bronchoscopy was performed as described previously [20]. Premedications included atropine (0.6 mg) and midazolam (1.0 mg), given by intramuscular injection, and albuterol 180 μ g, administered by a metered dose inhaler. BAL was performed in the right middle lobe for the first study visit and in the lingula for the second study visit. Six 40-mL aliquots of sterile 0.9% NaCl were injected through a flexible fiberoptic bronchoscope and recovered by gentle hand suction.

Analysis and culture of BAL cells

BAL cells were recovered from the lavage fluid by centrifugation at 400 g for 10 min at 4 °C. BAL fluids were stored at –70 °C until analysis. BAL cells were washed twice with Hanks' balanced salt solution containing 2% newborn calf serum. The total cell numbers were determined by a haemocytometer, using Turk's counting solution containing acetic acid and methylene blue. Cell viability was determined via trypan blue exclusion. Differential cell counts were determined on cytopsin preparations of BAL cells stained with the Giemsa-based Diff-Quik stain (Baxter Scientific Products, McGaw Park, IL, USA). To determine the *ex vivo* capacity of BAL cells for cytokine generation, BAL cells were cultured in the presence or absence of 10 μ g/mL phytohaemagglutinin (PHA) (Sigma L9132, St Louis, MO, USA) as previously described [21]. Briefly, cells were cultured in

Table 1. Subject characteristics

Subject (number)	Gender	Age (years)	FEV ₁ (% pred)*	Reversibility (%)†	HPC ₂₀ (mg/mL)‡	Δ PEFR (%)§	Δ FEV ₁ (%)¶
1	M	31	99	11	2.8	13.2	24.2
2	M	37	74	18	0.1	26.3	25.4
3	M	20	91	12	0.2	18.6	21.1
4	M	24	80	23	1.4	35.5	15.4
5	M	23	93	28	5.3	13.3	23.6
6	M	32	77	17	0.4	16.5	13.9
7	M	31	93	15	0.5	10.6	12.9
8	F	25	118	7	4.7	6.7	8.8
9	F	30	96	3	1.5	17.0	7.5
10	M	25	104	10	7.2	19.3	8.4
11	F	35	104	13	0.2	13.7	11.5
12	M	22	84	12	2.5	18.7	6.6
	9M/3F	28 $\pm$ 2	93 $\pm$ 4	14 $\pm$ 2	2.2 $\pm$ 0.7	17.5 $\pm$ 2.2	14.9 $\pm$ 2.0

M, male; F, female.

*Forced expiratory volume in 1 s (FEV₁) prior to 04:00 hour BAL. †Expressed as % change from baseline. ‡Histamine provocative concentration. §Average 24-h fluctuation in peak expiratory flow rate over 7 days. ¶Expressed as ((FEV₁ peak – FEV₁ nadir)/FEV₁ mean) $\times$ 100. Mean of two hospital stays. || Mean $\pm$ standard error of means.

triplicate at a concentration of 2×10^6 viable cells per millilitre for 48 h. Culture supernatant fluids were removed and stored at -20°C until analysis.

Cytokine ELISAs

A sensitive two-step sandwich ELISA was used as previously described [21]. Monoclonal cytokine-specific antibodies (unlabelled coating antibodies and biotinylated detection antibodies) were purchased from PharMingen (San Diego, CA, USA). To increase ELISA sensitivity, a streptavidin horseradish peroxidase polymer, POLY-HRP-40 (Research Diagnostics Inc., Flanders, NJ, USA), was used for the enzyme and a one-component 3,3',5,5'-tetramethylbenzidine (TMB) (Kirkegaard and Perry Labs, Gaithersburg, MD, USA) was used for the substrate. BAL fluids were concentrated by $20 \times$ with a low protein-binding concentrator (Centriprep[®], Amicon, Beverly, MA, USA) with a molecular weight cut-off limit of 3 kDa. Cell-culture supernatant fluids were diluted to optimal concentrations. The sensitivity for each cytokine assay was as follows: IL-4, 0.8 pg/mL; IL-5, 1.5 pg/mL; IFN- γ , 0.8 pg/mL; IL-10, 6.0 pg/mL; granulocyte macrophage colony-stimulating factor (GM-CSF), 1.5 pg/mL.

Measurement of eosinophil-derived neurotoxin (EDN)

EDN was measured in $1 \times$ BAL fluid by a double antibody competition radioimmunoassay (RIA), using radioiodinated EDN, rabbit anti-EDN antibody, and burro anti-rabbit IgG [22]. The threshold value was 10 ng/mL.

Flow cytometric analysis

Flow cytometric analysis was performed as previously described for lymphocytes [21] and eosinophils [23]. Briefly, unseparated BAL cells (1×10^5 cells) were stained through simultaneous addition of fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated antibodies specific for cell-surface markers (Becton–Dickinson Immunocytometry Systems, San Jose, CA, USA). For analysis, 10 000 photoelectric events were recorded with a Becton–Dickinson FACScan II, and data analyses were performed with the Lysys II software package.

Statistical analysis

Data are expressed as means $\pm$ standard error of means. To compare samples at 16:00 vs. 04:00 hours, a paired *t*-test (normally distributed data) or a Wilcoxon signed-rank test was used. Correlations were made with a Spearman's rank-order correlation, and the correlation coefficient is denoted as r_s . A *P*-value of <0.05 was considered significant. Statistical analysis was performed with a SigmaStat software package (Jandel Scientific Software, San Rafael, CA, USA).

Results

Subject characteristics

Twelve subjects completed the study. All subjects had a history of recurrent nocturnal asthma symptoms and showed some circadian variation in the pulmonary peak flow rate

measured over 1 week while at home (Table 1). Subjects' histories of nocturnal exacerbations did not predict the changes in lung function during the hospital stays; the fluctuation in FEV₁ recorded in the hospital did not correlate with the percent change in at-home PEFR measurements. FEV₁ at the time of the 04:00 hour bronchoscopy was, however, inversely correlated to β_2 -agonist reversibility ($r_s = -0.657$, $P = 0.02$). This relationship to asthma severity was not seen with FEV₁ at the time of the 16:00 hour bronchoscopy.

Cellular characteristics

At 04:00 hours there was a slight, but statistically significant decrease in the percentage of alveolar macrophages and an increase in the percentage of neutrophils (Table 2). When the total cell numbers were evaluated, the absolute number of lymphocytes and neutrophils was significantly greater at 04:00 hours compared with 16:00 hours (Table 2). At 04:00 hours, there was an inverse correlation between lymphocytes and FEV₁ ($r_s = -0.604$, $P = 0.042$, Fig. 1a), which was not seen at 16:00 hours. Although neutrophils increased at 04:00 hours, they did not correlate with lung function. Flow cytometric analysis of BAL lymphocyte subsets (Table 3) revealed that the percentage of BAL lymphocytes bearing CD4 was greater at 04:00 hours than at 16:00 hours. Furthermore, the percentage of CD4⁺ BAL cells was inversely correlated to FEV₁ at 04:00 hours ($r_s = -0.582$, $P = 0.045$, Fig. 1b), but not at 16:00 hours ($r_s = -0.035$, $P = 0.92$). Thus, at 04:00 hours, a higher percentage of lymphocytes, and in particular, CD4⁺ T cells, in the BAL fluid was associated with poorer lung function. The percent of CD4⁺ lymphocytes bearing the activation marker CD25 was also higher at 04:00 hours than at 16:00 hours (Table 3), but the percentage of CD25⁺ cells was not related to lung function. There were no significant differences in the activation status of BAL eosinophils as determined by the expression of cell-surface activation markers (Table 3) or levels of EDN in BAL fluid (EDN was below the level of detection, data not shown). Blood eosinophils increased by a mean of 20.7% between 16:00 and 04:00 hours (Table 2; $P < 0.001$), but did not correlate with lung function.

Table 2. Total cell counts and differential*

	Percentage		Total cell numbers ($\times 10^4$)	
	16:00 hours	04:00 hours	16:00 hours	04:00 hours
BAL				
Macrophages	87.7 ± 2.1	$83.0 \pm 2.2^\dagger$	1517 ± 133	1730 ± 188
Lymphocytes	9.9 ± 2.0	12.6 ± 1.6	169 ± 32	$254 \pm 40^\dagger$
Neutrophils	0.8 ± 0.2	$3.0 \pm 1.7^\dagger$	13 ± 3	$40 \pm 14^\dagger$
Eosinophils	1.5 ± 0.6	1.3 ± 0.4	27 ± 13	24 ± 6
Blood				
White blood cells ($\times 10^6/\text{mL}$)			5.9 ± 1.2	6.1 ± 1.6
Eosinophils/ mm^3			332 ± 167	$417 \pm 193^\dagger$

BAL, bronchoalveolar lavage.

*Mean $\pm$ standard error of means. $^\dagger P < 0.05$, 04:00 vs. 16:00 hours.

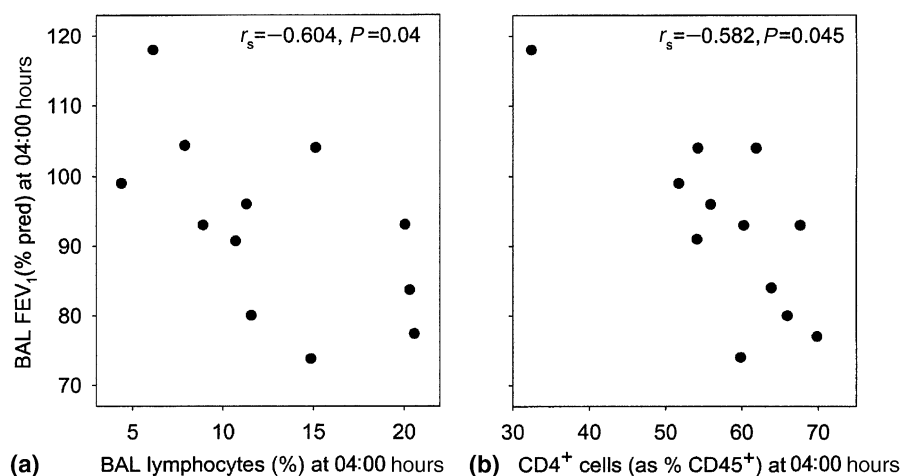


Fig. 1. Relationship between pulmonary function and lymphocytes present in bronchoalveolar lavage (BAL) at 04:00 hours. (a) At 04:00 hours, BAL lymphocytes (expressed as percent of BAL cells) were inversely correlated ($r_s = -0.604$, $P = 0.042$) with forced expiratory volume in 1 s (FEV₁) (expressed as percent predicted). (b) CD4⁺ cells (expressed as percent of CD45⁺ BAL cells) were also inversely correlated ($r_s = -0.582$, $P = 0.045$) with FEV₁.

Table 3. BAL lymphocyte and eosinophil subpopulations*

	16:00 hours	04:00 hours
CD3 (% of CD45 ⁺ leucocytes within the lymphocyte gate)	92.8 ± 1.1	94.4 ± 0.9
CD4 (% of CD45 ⁺ leucocytes within the lymphocyte gate)	52.5 ± 4.4	58.1 ± 2.9†
CD8 (% of CD45 ⁺ leucocytes within the lymphocyte gate)	36.5 ± 2.4	35.5 ± 3.4
CD19 (% of CD45 ⁺ leucocytes within the lymphocyte gate)	2.1 ± 0.6	1.8 ± 0.2
NK (% of CD45 ⁺ leucocytes within the lymphocyte gate)	5.1 ± 1.2	4.2 ± 0.8
CD4/CD25 (% of CD4 ⁺ lymphocytes)	18.7 ± 2.8	23.6 ± 3.0†
CD4/CD69 (% of CD4 ⁺ lymphocytes)	66.4 ± 3.2	62.7 ± 3.5
CD4/HLA-DR (% of CD4 ⁺ lymphocytes)	64.5 ± 4.7	66.7 ± 4.7
EOS/CD69 (% of eosinophils)	68.3 ± 7.6	69.6 ± 5.6
EOS/HLA-DR (% of eosinophils)	50.4 ± 6.0	37.4 ± 5.6

BAL, bronchoalveolar lavage.

*Mean ± standard error of means. † $P < 0.05$, 04:00 vs. 16:00 hours.

Cytokines

To evaluate the inflammatory potential of airway cells, we determined the capacity of BAL cells to generate cytokines following *ex vivo* stimulation with the T cell mitogen, PHA. The biological significance of *ex vivo* generated IL-5 is indicated by the close correlation between PHA-induced IL-5 and numbers of BAL eosinophils at 04:00 hours ($r_s = 0.724$, $P = 0.006$, Fig. 2). A circadian pattern was seen for IL-5 production such that the relative change (16:00–04:00 hours) in the capacity of BAL cells to secrete IL-5 was significantly correlated with the circadian variation in FEV₁ ($r_s = 0.773$, $P = 0.004$, Fig. 3a). The circadian change in CD4⁺ T lymphocytes also correlated with the circadian change in IL-5 production ($r = 0.645$, $P = 0.03$, Fig. 3b). PHA-induced IFN- γ production was similar at 04:00 hours (4439 ± 813 pg/mL) and 16:00 hours (4670 ± 798 pg/mL). PHA-induced production of IL-4, IL-10 and GM-CSF was low and did not have a clear circadian pattern.

Many BAL samples had cytokine levels below the assay detection limit, despite being concentrated 20-fold. No circadian differences were detected for BAL fluid IL-4, IFN- γ or IL-10. BAL fluid IL-5, however, showed a 26.1% increase ($P = 0.03$) from 16:00 hours (2.8 ± 1.2 pg/mL of $20 \times$ BAL fluid, three of 12 samples within the detection range) to 04:00 hours (4.4 ± 1.2 pg/mL of $20 \times$ BAL fluid, seven of 12 samples within detection range). BAL fluid GM-CSF increased 33.9% ($P = 0.04$) from 16:00 hours (12 ± 2 pg/mL of $20 \times$ BAL fluid, one of 12 samples below detection limit) to 04:00 hours (24 ± 6 pg/mL of $20 \times$ BAL fluid, one of 12 samples below detection limit). BAL fluid levels of IL-5 and GM-CSF did not correlate with changes in lung function.

Discussion

We evaluated the relationship between inflammatory changes in the airway and circadian variation in pulmonary function in subjects with mild atopic asthma. These subjects had a greater number of lymphocytes and a higher percentage of CD4⁺ T cells in the BAL fluid at 04:00 hours compared with 16:00 hours. Those subjects with poorer lung function at 04:00 hours (but not necessarily at 16:00 hours) had more lymphocytes and a higher percentage of CD4⁺ T cells in their BAL fluid. In addition, the capacity of BAL cells to produce IL-5 increased in relation to a greater day/night variation in FEV₁. Finally, the amount of IL-5 produced *in vitro* correlated with the number of BAL eosinophils and the percentage of CD4⁺ cells in the BAL fluid. Our data demonstrate that, in patients with mild atopic asthma, increased day/night variation in pulmonary function is related to increased numbers of airway lymphocytes and an increased capacity for IL-5 production at night.

Although circadian patterns have been demonstrated for several macrophage-derived cytokines, including IL-1 and GM-CSF [20, 24, 25], changes in T cell-associated cytokines have been more difficult to detect [20, 26]. By evaluating the *ex vivo* capacity of BAL cells to generate T cell-associated cytokines, we were able to demonstrate circadian variation of lymphocyte function in asthma. In a previous study, we

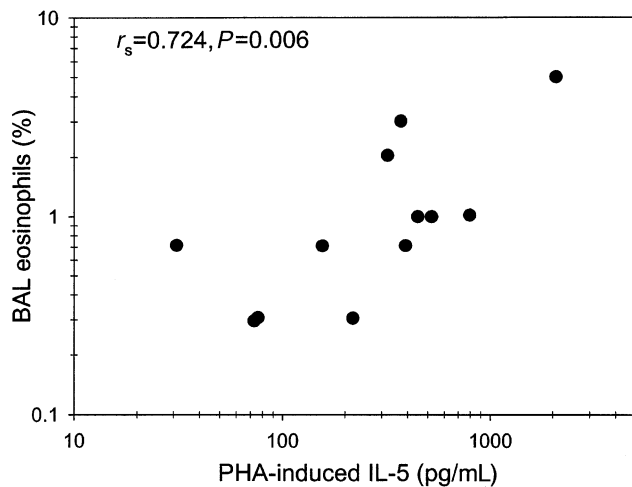


Fig. 2. Relationship between IL-5 production by phytohaemagglutinin (PHA)-stimulated bronchoalveolar lavage (BAL) cells and eosinophils present in BAL fluid. Data are from 04:00 hours. BAL eosinophils are expressed as percentage; PHA-induced IL-5 is expressed as pg/mL of culture supernatant fluid; $r_s = 0.724$, $P = 0.006$.

identified CD4⁺ T cells as a primary source of IL-5 in PHA-stimulated BAL cell cultures [21]. Eosinophils are also a potential source of IL-5 but, in our hands, neither purified blood nor BAL eosinophils secrete IL-5 following *ex vivo* stimulation with PHA (data not shown). Consistent with this observation, we found that circadian changes in IL-5 production were accompanied by changes in the number of CD4⁺ cells in the BAL fluid.

IL-5 is critical to eosinophil maturation, recruitment, survival, and activation [27], but the precise role of IL-5 in asthma pathogenesis is not entirely clear. We have recently demonstrated that BAL eosinophils exhibit a markedly reduced expression of IL-5 receptor and responsiveness to IL-5 (as measured by their capacity to degranulate) compared with peripheral blood eosinophils [28]. Furthermore, anti-IL-5 treatment decreases eosinophils in the circulation and airway lumen [29], but not in the tissues [30]. We have therefore

proposed that the role of IL-5 in asthma is limited to the recruitment of eosinophils to the airway [31]. In this regard, we have demonstrated increased numbers of circulating eosinophils at 04:00 hours compared with 16:00 hours (Table 2). This suggests that more eosinophils are available for recruitment to the airway at night.

Although we did not detect circadian changes in BAL eosinophil numbers in the present study, we have demonstrated elsewhere [32] that sputum eosinophils and EDN are increased in the early morning in subjects with mild asthma. Our observations may provide an important link to previously published observations demonstrating the recruitment of eosinophils to the lung parenchyma and/or distal airways in asthmatics with nocturnal asthma [18].

Asthmatics with large variations in pulmonary function have been shown to exhibit circadian patterns of inflammatory changes in the airway, including increased BAL eosinophils and lymphocytes at 04:00 hours [33, 34]. Reports linking inflammatory changes to circadian variation in lung function in subjects with less severe disease have been inconsistent. Some studies, including our own, have shown no relationship between day/night fluctuations in the numbers of eosinophils, lymphocytes, or macrophages and circadian changes in PEFR [12, 14, 20, 35, 36]. Others have demonstrated day/night changes in eosinophil and CD4⁺ lymphocyte numbers in the alveolar tissue, but not in the proximal airway, and showed a significant correlation between these inflammatory responses and circadian changes in pulmonary function [17, 18]. In the present study, we demonstrated a nocturnal increase in CD4⁺ BAL lymphocytes, which was associated with decreased FEV₁ at 04:00 hours. Variations in the study protocol, subject selection, or methods for cellular analysis may contribute to some of the differences in study results. Our findings indicate that small variations in lung function are accompanied by inflammatory changes, although they may be more subtle than those associated with more severe disease.

Our study is unique in that it allowed us to characterize the relationship between circadian variations in airway

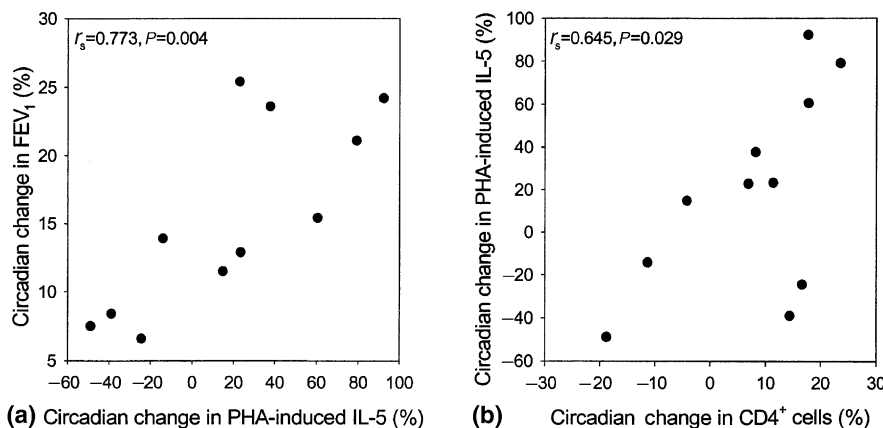


Fig. 3. (a) Relationship between circadian variation in IL-5 production by phytohaemagglutinin (PHA)-stimulated bronchoalveolar lavage (BAL) cells and circadian variation in pulmonary function. The percent change in PHA-induced IL-5 production at 04:00 vs. 16:00 hours is expressed as ((04:00 hour value – 16:00 hour value)/04:00 hour value) $\times$ 100. The percent fluctuation in forced expiratory volume in 1 s (FEV₁) is expressed as ((FEV₁ peak – FEV₁ nadir)/FEV₁ mean) $\times$ 100; $r_s = 0.773$, $P = 0.004$. (Note: the PHA-induced IL-5 values for one subject differed from the mean of the remaining subjects by more than nine standard deviations, so this outlier was excluded from data analyses, with subject included $r_s = 0.783$, $P < 0.001$). (b) Relationship between circadian variation in IL-5 production by PHA-stimulated BAL cells and CD4⁺ cells present in BAL fluid. Percent change at 04:00 vs. 16:00 hours is represented as ((04:00 hour value – 16:00 hour value)/04:00 hour value) $\times$ 100; $r_s = 0.645$, $P = 0.03$.

lymphocyte function and pulmonary function in subjects with mild asthma. There are some limitations to a study of this type. First, it could be argued that PHA is not a physiological stimulant. Response to PHA was used as a measure of lymphocyte function and amplified cytokine signals that were difficult to detect in unstimulated BAL cell cultures or in $20 \times$ concentrated BAL fluid (in this study and others [20, 26]). The strong correlation between PHA-stimulated IL-5 production and BAL eosinophils (Fig. 2 and previous publications [21, 23, 37]) provides evidence that *ex vivo* generation of IL-5 does, in fact, reflect *in vivo* production of IL-5. Second, we did not evaluate cells within the mucosa or in distal airways. The fact that we were unable to detect a significant increase in the number or activation status of BAL eosinophils at 04:00 hours may be reflective of this limitation. Using transbronchial vs. endobronchial biopsy, Kraft et al. [18] have demonstrated that at 04:00 hours, eosinophils accumulate to a greater extent in the distal alveolar tissues than the proximal airways. However, the cytokine profile within the alveolar tissues has not yet been reported. Third, bronchoscopy at 04:00 and 16:00 hours may not coincide with the actual peak and nadir for all parameters of inflammation in every patient, but were chosen based on published studies [5] and our own observations [3]. Ideally, circadian patterns for cytokine secretion and their temporal relationship to changes in BAL cell constituents and pulmonary function could be determined by analysis at 3–4-h intervals, but such studies would pose obvious practical and ethical problems and are unlikely to be performed. Finally, our analysis was restricted to asthma subjects with atopy. Whether circadian changes in lymphocyte function occur in non-atopic asthma subjects remains to be determined.

A further unique aspect of this study is that we focused on those subjects with mild asthma who had a range of circadian variation in FEV₁ from 6.6% to 25.4%. This is in contrast to other studies, including some of our own that used a circadian change in PEFR or FEV₁ of $\geq 15\%$ or 20% to divide subjects into separate groups [12, 14, 17, 18, 33, 35]. By studying the group as a whole, we were able to demonstrate a linear relationship between the circadian variation in FEV₁ and the circadian change in IL-5 production. Thus, the relationship between circadian variation in pulmonary function and airway IL-5 production appears to be a continuum, rather than an all-or-none phenomenon. The linear relationship between nocturnal changes in IL-5 production and circadian variation in FEV₁ implies that the change in the degree of airway obstruction at night coincides with changes in airway cell function. This spectrum of airway cell function has been observed not only for IL-5 production, as seen in the current study, but also for superoxide release, as reported in our previous study [38].

In summary, we have provided evidence that circadian variations in lung function in subjects with mild asthma are associated with circadian changes in airway T cell-related inflammation. These changes appear to fall into a continuous range, suggesting that day/night variations in pulmonary function may occur on a continuum, rather than as an all-or-none phenomenon. We hypothesize that incremental changes in airway inflammation, including accumulation of CD4⁺ lymphocytes and production of IL-5, lead to incremental changes in airway obstruction in asthma.

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