

## Increased detection of interleukin-5 in sputum by addition of protease inhibitors

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**ABSTRACT:** The measurement of interleukin (IL)-5 in sputum is problematic, with interfering factors affecting immunoassay. The authors investigated whether sputum proteases could be acting as interfering factors by studying the effect of protease inhibitors (PI) on sputum IL-5 measurement.

Induced sputa from 20 subjects with asthma were divided into aliquots, processed with and without protease inhibitors (in low and high concentrations) and the levels of IL-5 (spiked and endogenous) measured by enzyme immunoassay were compared.

The concentration of sputum IL-5 was significantly increased by PI, with median (interquartile range) levels processed with no, low and high PI concentrations being 0 (0), 41.8 (75.6) and 66.1 (124.4) pg·mL<sup>-1</sup>, respectively. There was also a significant increase in percentage recovery of spiked IL-5. Although high concentrations of PI reduced cell viability, there was no effect on total or differential cell counts and low concentrations of PI had no effect on cell counts or viability.

Levels of endogenous interleukin-5 in sputum of asthmatic subjects can be significantly increased by the addition of protease inhibitors, and samples which would be regarded as negative for interleukin-5 without protease inhibitors may instead have considerable amounts of interleukin-5 detected.

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Examination of induced sputum is a minimally invasive method of studying airway inflammatory cells and mediators. Sputum induction is successful in >80% of subjects who do not produce sputum spontaneously, and is inexpensive, easy to perform, well-tolerated and safe [1–3]. Sputum cell counts are reliable, valid [4, 5], and responsive to treatment [6].

Interleukin (IL)-5 is a pro-inflammatory cytokine with a selective action on eosinophils, which are regarded as being of central importance in the pathophysiology of asthma [7]. Measurement of IL-5 is used to assess eosinophilic airway inflammation [8, 9] and has recently become a target of drug therapy for asthma [7, 8, 10]. However, the authors have previously shown that the validity of IL-5 measurements in sputum by current methods is poor, with a mean±SD recovery of spiked IL-5 of 26.1±14.6% [11]. The reduced detection was due to poor recognition of IL-5 epitopes by immunoassay. This may be due to interfering substances, such as soluble receptors to IL-5 or auto-antibodies, or due to alteration of the epitope by substances such as endogenous proteases.

Sputum from patients with inflammatory airways disease, including those with asthma, can have high

proteolytic activity [11–17], with the majority of sputum proteases originating from sputum neutrophils [12, 13]. These proteases are normally inhibited by complex formation with natural inhibitors present in sputum, such as  $\alpha$ -1 proteinase inhibitor, anti-leukocyte protease (secretory leukocyte protease inhibitor) and  $\alpha$ -2 macroglobulin [14, 15]. These complexes are bound to bronchial mucins, and mucolytic agents, such as dithiothreitol (DTT), may increase proteolytic activity by releasing proteases from such complexes [16]. In addition, neutrophils possess protease-activated receptors, which, when bound by proteases, produce activation and degranulation of neutrophils [17] with further release of proteases. The authors have demonstrated that the IL-5 molecule remains intact after sputum processing [11], suggesting that the effects of proteases may be more subtle, possibly by the alteration of epitopes. It has also been shown that DTT does not interfere with the measurement of IL-5 by enzyme-linked immunosorbent assay (ELISA) [11].

The objective of this prospective, cross-sectional study was to evaluate the effect of protease inhibitors (PI) on the measurement of IL-5 in induced sputum.

This included adding a known amount of IL-5 to the sputum (spiking the sputum) and assaying the sample before and after dilution in order to assess the presence of dilutable interfering factors. Since cell counts are an important part of sputum examination, the effect of PI on cellular indices was also investigated.

## Methods

### Subjects

Sputum from 20 subjects with asthma was processed with and without PI and IL-5 was measured. To determine the effect of PI on cell counts, sputum from a further 31 subjects (18 with asthma, five with chronic obstructive pulmonary disease (COPD), three with cystic fibrosis (CF) and five with chronic cough) was processed in a similar manner and the cell counts were compared (table 1).

The diagnosis of asthma was based on the American Thoracic Society (ATS) criteria [18], and included variable airflow limitation and airway hyperresponsiveness to a provocative concentration of methacholine causing a 20% fall in forced expiratory volume in one second (FEV<sub>1</sub>) (PC<sub>20</sub> <8 mg·mL<sup>-1</sup>) [19]. The patients with COPD had first experienced respiratory symptoms after the age of 40 yrs, had a >15 pack-yr smoking history and had evidence of chronic airflow limitation (FEV<sub>1</sub> <70% predicted, FEV<sub>1</sub>/vital capacity (VC) <70% and <10% improvement in FEV<sub>1</sub> after inhaled salbutamol) [20]. Patients were considered to have a chronic cough if they had a nonproductive cough of >3 weeks duration and the cause remained unknown despite careful evaluation, including chest radiography and spirometry [21]. The diagnosis of CF was based on clinical details, a positive sweat test and

Table 1. – Subject characteristics

PI condition	Subjects in whom sputum IL-5 measurements were made	Additional subjects in whom sputum cell counts were performed
Subjects n	20	31
Asthma	20	18
COPD		5
Cystic fibrosis		3
Chronic cough		5
Sex, male	11	17
Age yrs	52.2±15.9	53.4±33.3
FEV <sub>1</sub> % predicted	74.6±22.6	85.0±51.7
Sputum cell counts		
Total cell count×10 <sup>6</sup> ·g <sup>-1</sup>	4.2 (6.8)	8.4 (11.7)
Cell viability %	71.5 (20.0)	75.0 (35)
Eosinophils %	3.4 (10.5)	1.3 (1.7)
Neutrophils %	57.7 (38.3)	68.3 (45.3)
Macrophages %	40.6 (46.7)	28.9 (37.8)

Data are presented as mean±SD or median (interquartile range (IQR)). COPD: chronic obstructive pulmonary disease; FEV<sub>1</sub>: forced expiratory volume in one second; IL-5: interleukin-5; PI: protease inhibitor.

genetic analysis. The study was approved by the St. Joseph's Hospital Research Ethics Committee.

### Sputum induction

Sputum was induced as described by PIN *et al.* [1] and modified by PIZZICHINI *et al.* [5]. Briefly, after pretreatment with inhaled salbutamol, an aerosol of hypertonic saline was inhaled from a Medix ultrasonic nebulizer (Clement Clarke, Harlow Essex, UK) with a relatively low output (0.87 mL·min<sup>-1</sup>) and a large particle size (5.58 µm aerodynamic mass median diameter). Concentrations of 3, 4 and 5% saline were each inhaled for 7-min periods. At the end of each inhalation, subjects were asked to blow their nose, rinse their mouth with water and swallow to minimize contamination with postnasal drip and saliva. They were then asked to cough and expectorate into a polypropylene container.

### Sputum processing

Sputum was processed within 2 h of collection as described by PIZZICHINI *et al.* [22]. Sputum from each of the 20 subjects with asthma was selected from the expectorate and randomly divided into 3–4 (depending on the volume available) approximately equal aliquots and weighed (fig. 1). For all 20 samples, two aliquots were processed without PI, one to serve as the no-PI control for IL-5 measurements, while the other was processed for cell counts. Phosphate-buffered saline (PBS; Gibco Diagnostics, Tucson, AZ, USA) was added to the no-PI control (1 µL to 4 mg of sputum, 0.25 volumes). In 10 samples, four aliquots were available and 0.25 volumes of low concentration PI (low PI) and high concentration PI (high PI) were added to the third and fourth aliquots respectively. In the other 10 samples, only three aliquots were available and low PI was added to six samples and high PI to four.

The aliquots were dispersed with four volumes of freshly prepared 6.5 mM (0.1%) DTT (Sputolysin,

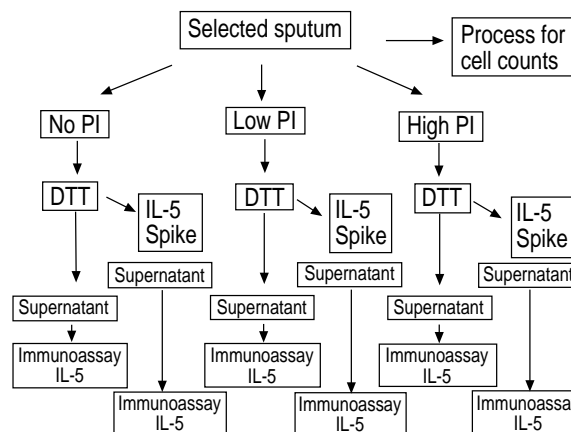


Fig. 1. – Diagrammatic representation of sputum processing. PI: protease inhibitor; DTT: dithiothreitol; IL-5: interleukin-5.

Calbiochem, La Jolla, CA, USA), after which, 3.75 volumes of PBS were added (relative to the original volume of sputum) and the sample was then filtered through a 48  $\mu\text{m}$  nylon mesh. Depending on the volume of filtrate available, a portion (250  $\mu\text{L}$ ) was "spiked" with a known amount of recombinant human IL-5 (Genzyme Diagnostics, Cambridge, MA, USA), such that the expected concentration of spiked IL-5, if there was 100% recovery, would be  $\sim 120 \text{ pg}\cdot\text{mL}^{-1}$ , which is on the lower part of the standard curve. Of 13 original samples, 13 (no PI), 10 (low PI) and nine (high PI) aliquots were spiked. To serve as a control for each spiked sample, 250  $\mu\text{L}$  of PBS with 1% bovine serum albumin (BSA) was spiked with the same amount of IL-5. The authors have previously shown that adding exogenous IL-5 before or after dispersal with DTT has no effect on the recovery [11].

In the aliquots processed for cell counts (not treated with PI), total cell count and viability were assessed by trypan blue exclusion in a haemocytometer, and cytopspins were prepared using a Shandon III cyto-centrifuge (Shandon Southern Instruments, Sewickley, PA, USA). Total cell count and viability were also measured in 12 aliquots processed with low PI, 10 aliquots processed with high PI and the corresponding no PI aliquots. Cytopspins were prepared from the high and no PI aliquots to enable differential cell counts to be made. The filtered suspensions were centrifuged ( $290\times g$ , 4 min) and the supernatants and the PBS controls stored at  $-70^\circ\text{C}$ .

Sputum from an additional 31 subjects was divided into three aliquots. One aliquot was processed with high PI and the other two with no PI, in order to evaluate the effect on cell counts and viability. The repeatability of cell counts with high PI *versus* no PI could then be compared to the within-sample repeatability with no PI.

#### *Protease inhibitors*

A combination of four PI was selected to have a broad spectrum of effect. The combination consisted of 4-(2-Aminoethyl)-benzenesulphonyl fluoride (AEBSF), pepstatin A, leupeptin and the disodium salt of ethylenediaminetetraacetic acid ( $\text{EDTA}\cdot\text{Na}_2$ ) (Sigma-Aldrich, Oakville, Ontario, Canada). AEBSF is a broad-spectrum serine-protease inhibitor; pepstatin A inhibits acid proteases including cathepsin D; leupeptin inhibits serine and thiol proteases including cathepsin A and B; and  $\text{EDTA}\cdot\text{Na}_2$  inhibits metalloproteases. The individual PI were reconstituted from powder and combined so as to obtain two mixtures of different concentrations. Low PI consisted of AEBSF,  $\text{EDTA}\cdot\text{Na}_2$ , pepstatin A, and leupeptin in concentrations of 41.75 mM, 26.86 mM,  $29.16\times 10^{-3}$  mM, and  $21.02\times 10^{-3}$  mM, respectively. High PI consisted of twice these concentrations, except for  $\text{EDTA}\cdot\text{Na}_2$ , which was left at the same concentration because of concerns of interference with protein stability.

The low or high PI was added to sputum in a ratio of 1  $\mu\text{L}$  of mixture to 4 mg of sputum. After adding

four volumes of DTT, the final concentrations of AEBSF,  $\text{EDTA}\cdot\text{Na}_2$ , pepstatin A, and leupeptin in the dispersed sputum were 2 mM, 1.29 mM,  $1.39\times 10^{-3}$  mM, and  $1.01\times 10^{-3}$  mM for low PI and 3.97 mM, 1.29 mM,  $2.78\times 10^{-3}$  mM, and  $2\times 10^{-3}$  mM for high PI, respectively. The final concentrations achieved with low PI were similar to those recommended by the supplier. Because the optimal working concentration in sputum is unknown, this concentration was doubled (except for the EDTA) for high PI conditions.

#### *Interleukin-5 immunoassay*

IL-5 in the supernatants and PBS controls was measured by enzyme immunoassay (EIA), using a monoclonal capture and a polyclonal detection antibody (Genzyme Diagnostics). All measurements were made in duplicate. The standard curve was generated with the same IL-5 used for spiking, and the lower limit of detection was regarded as being  $4.0 \text{ pg}\cdot\text{mL}^{-1}$ . The endogenous IL-5 concentration measured in the nonspiked portion was subtracted from that measured in the corresponding spiked portion. The value obtained (regarded as being the concentration of spiked IL-5 recovered) was expressed as a percentage of that measured in the spike control (PBS with 1% BSA). In addition, the spiked portions (six processed with high PI, seven with low PI and six with no PI) were diluted 1:2 with PBS and 1% BSA, and IL-5 was then measured. The recovery was then recalculated after correcting for dilution. To assess potential effects of PI on the assay itself, high PI was added to a set of standards (final concentration of PI similar to that in sputum supernatant) and assayed in parallel with the normal standards.

#### *Statistical analysis*

The Statistical Package for the Social Sciences was used to analyse the data. Clinical data that were normally distributed were expressed as mean values  $\pm$  sd. Sputum cell counts, IL-5 measurements and percentage recovery of spiked IL-5 were not normally distributed and were expressed as median values and interquartile range (IQR). For statistical purposes, a measured IL-5 concentration  $>0 \text{ pg}\cdot\text{mL}^{-1}$  and  $<4.0 \text{ pg}\cdot\text{mL}^{-1}$  was assigned the value of  $2 \text{ pg}\cdot\text{mL}^{-1}$  since, although IL-5 was detectable, the actual figure obtained could not be accurately measured below  $4.0 \text{ pg}\cdot\text{mL}^{-1}$ . All IL-5 values were corrected for nine-fold dilution during sputum processing.

The Wilcoxon signed-ranks test was used to compare IL-5 measurements with and without PI. Correlations between individual data were tested for significance with the Spearman rank correlation test. Two-tailed p-values  $<0.05$  were considered significant. Reproducibility of cell counts with and without PI was expressed by intraclass correlation coefficients (ICC) as the ratio of variance among subjects to total variance.

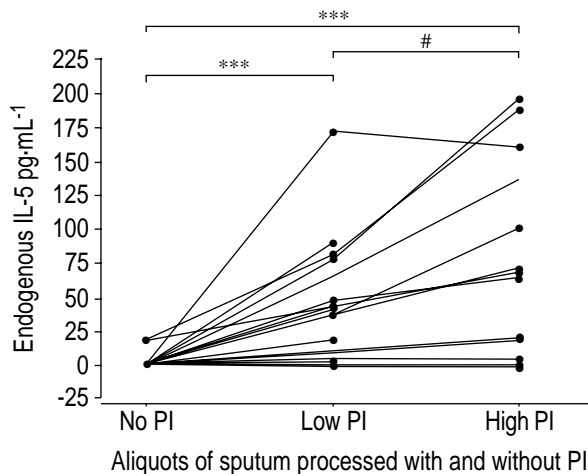


Fig. 2. – Concentration of endogenous interleukin (IL)-5 in sputum supernatant aliquots processed with no protease inhibitors (no PI), low concentration protease inhibitors (low PI) and high concentration protease inhibitors (high PI). \*\*\*:  $p=0.001$ ; #:  $p=0.017$ .

### Results

The addition of PI to the standards in the assay had no effect on IL-5 measurement. The median (IQR) levels of endogenous IL-5 measured in sputum processed with no, low and high PI, were 0 (0), 41.8 (75.6) and 66.1 (124.4)  $\text{pg}\cdot\text{mL}^{-1}$ , respectively, when all aliquots were taken into account (fig. 2). The addition of PI (low or high), increased detection and measured levels of endogenous IL-5, compared to paired aliquots with no PI added ( $p=0.001$ ). In addition, there was a further significant increase in measured endogenous IL-5 in aliquots processed with high PI compared to low PI ( $p=0.017$ ), indicating correlation of IL-5 levels with PI concentration. Total and percentage sputum eosinophils showed no correlation with IL-5 levels in sputum processed with or without PI.

There was a significant increase in spiked IL-5 percentage recovery in sputum processed with low ( $p=0.013$ ) and high PI ( $p=0.036$ ), compared to no PI, respectively, but no significant difference in percentage recovery between low and high PI (table 2).

The percentage recovery of no, low and high PI increased significantly ( $p=0.028$ ,  $p=0.018$  and  $p=0.027$ , respectively) after a two-fold dilution of the spiked samples, correcting for the dilution (table 3). The significant difference in the percentage recovery of spiked IL-5 between aliquots treated with low or high PI as opposed to no PI ( $p=0.016$  and  $p=0.045$ , respectively) persisted after the two-fold dilution (table 2).

Total and percentage sputum eosinophils showed no correlation with IL-5 levels in sputum processed with or without PI. There was a significant inverse correlation between the percentage of sputum neutrophils and endogenous IL-5 when processed with low PI ( $r=-0.61$ ,  $p=0.022$ ), but there was no relationship between sputum neutrophils and IL-5 levels when processed without PI or with high PI. In samples processed without PI, there was an inverse correlation between the recovery of spiked IL-5 and percentage and total sputum neutrophils ( $r=-0.66$ ,  $p=0.029$  and  $r=-0.65$ ,  $p=0.029$ , respectively). In sputum processed with low PI there was also an inverse relationship between recovery of spiked IL-5 and percentage sputum neutrophils ( $r=-0.79$ ,  $p=0.026$ ), but there was no correlation between sputum neutrophils and recovery of spiked IL-5 when processed with high PI. IL-5 levels with or without PI showed no correlation with sputum cell viability or with FEV<sub>1</sub> % predicted.

A total of 40 sputum samples had total cell count and viability assessed for each of three aliquots, two with no PI added and one with high PI added (table 4). Twelve pairs of aliquots of sputum were available to assess the effect of low PI on total cell count and viability. Cytospins were considered unsuitable for differential cell counts if there were too few cells present or the cell morphology was poor. The number of cytopins rejected was 21.2% for no PI and 22.7% for high PI, respectively. The ICC for total and differential cell counts showed good repeatability between no PI:low and no PI:high PI, which was comparable to the within-subject repeatability of no PI (no PI:no PI) (table 3). These results indicate there is no effect of PI on total or differential cell counts. However, the cell viability was significantly reduced with the addition of high PI, the median (IQR) being reduced from 70.0% (28.3) and 74.25% (33.1) (in pairs

Table 2. – Effect of protease inhibitors (PI): comparison of endogenous interleukin (IL)-5 and per cent recovery of spiked exogenous IL-5 with and without dilution between no, low and high PI

	Endogenous IL-5 $\text{pg}\cdot\text{mL}^{-1}$		Recovery spiked IL-5 %			
		p-value	Undiluted	p-value	Diluted 1:2	p-value
No PI versus low PI	0 (0) versus 41.8 (75.6)	0.001	26.5 (16) versus 35.1 (21.3)	0.013	36.3 (14.9) versus 59.9 (29.5)	0.016
No. of pairs	16		10		5	
No PI versus high PI	0 (0) versus 66.1 (124.4)	0.001	25.8 (12.6) versus 28.6 (17.9)	0.036	30.8 (8.9) versus 42.7 (37.4)	0.045
No. of pairs	14		9		4	
Low PI versus high PI	45 (64.8) versus 85.5 (119.3)	0.017	35.1 (28.7) versus 28.6 (25.6)	NS	69.6 (37.2) versus 59.2 (45.6)	NS
No. of pairs	10		6		4	

Results are presented as median (interquartile range). NS: not significant.

Table 3. – Percentage recovery of spiked interleukin (IL)-5 with no, low and high protease inhibitor (PI): effect of dilution

	Recovery spiked IL-5 %		p-value
	Undiluted <i>versus</i> diluted		
No PI	27.6 (15.7) <i>versus</i> 34.2 (11.5)		0.028
No. of pairs	6		
Low PI	35.1 (21.3) <i>versus</i> 62.2 (34.7)		0.018
No. of pairs	7		
High PI	28.6 (17.9) <i>versus</i> 47.3 (41)		0.027
No. of pairs	6		

Results expressed as median (interquartile range). PI: protease inhibitor.

processed without PI) to 52.6% (40.6) ( $p < 0.001$ ). In contrast to high PI, low PI did not significantly affect cell viability.

### Discussion

The addition of PI to sputum before processing resulted in significantly increased measurement of endogenous IL-5, with higher concentrations of PI resulting in correspondingly higher levels of IL-5 being measured. Inhibiting the protease activity may prevent alteration of IL-5 epitopes by proteases. PI may also block further release of proteases during sputum processing by preventing activation of protease-activated receptors on neutrophils [17].

The lack of correlation of sputum IL-5 processed without PI and sputum neutrophils is probably due to the fact that IL-5 levels were above the detection limit of the assay in only two of 20 cases. The significant inverse correlation of sputum neutrophils with sputum IL-5 processed with low PI and per cent recovery of spiked IL-5 when processed with and without low PI, supports the postulate that neutrophil proteases are at least partly responsible for low IL-5 levels in sputum. In sputum processed with high PI, levels (endogenous

or spiked) and the lack of correlation of IL-5 with sputum neutrophils could be explained by the presence of an adequate amount of added PI to neutralize neutrophil proteases.

Although the results of this study are reported using an IL-5 assay from Genzyme, the authors have previously found it difficult to detect IL-5 using assays from other manufacturers (R&D systems, Minneapolis, MN, USA, Pharmingen, San Diego, CA, USA and Amersham Pharmacia, Piscataway, NJ, USA, data not shown). This suggests that the addition of PI would be similarly applicable when using other immunoassays directed against IL-5.

The authors have previously shown that the recovery of spiked IL-5 was improved significantly ( $p < 0.001$ ) when albumin was added to the sputum processing fluids [11]. Since it was demonstrated that it was not acting as an IL-5 carrier agent, perhaps it was acting as an alternative substrate for sputum proteases.

The percentage recovery of spiked IL-5, although significantly increased by low and high PI, was less dramatic than the improvement in measured endogenous IL-5. Since the recovery was calculated after subtraction of the endogenous IL-5 level, the relatively high levels of endogenous IL-5 measured with added PI, compared to no PI, would have had a disproportionately greater effect on the calculated percentage recovery. This may also explain why the percentage recovery with low PI is greater than that for high PI, despite the addition of the high PI resulting in a higher median endogenous IL-5 level than with low PI.

The significant increase in percentage recovery when all aliquots were diluted is intriguing, and although the numbers are small, it suggests the presence of soluble dilutable factor(s) which may interfere with the recognition of IL-5 by the immunoassay. These interfering factors may be soluble receptors for IL-5 [23, 24] or carrier proteins such as  $\alpha$ -2 macroglobulin, which is known to bind other cytokines [25, 26]. IL-5 has also been reported to bind tightly to heparan sulphate proteoglycan [27], which may be present in

Table 4. – Effect of protease inhibitors (PI) on sputum cell counts

	n	No PI		High PI	Low PI	ICC		
		1st aliquot	2nd aliquot			No PI/ no PI	No PI/ high PI	No PI/ low PI
Total cell count $\times 10^6 \cdot g^{-1}$	12	5.1 (9.9)			5.8 (11.5)			0.99
	40	5.0 (10.7)	5.8 (6.8)	6.0 (5.6)		0.83	0.85	
Cell viability %	12	68.8 (17.9)			67.4 (12.8)			0.96
	40	70.0 (28.3)***	74.25 (33.1)	52.6 (40.6)***		0.93	0.77	
Eosinophils %	18	1.3 (1.9)	1.3 (1.5)	1.3 (1.8)	ND	0.97	0.78	ND
	21	1.3 (2.5)	1.5 (2.7)			0.80		
	30	1.3 (2.5)		1.3 (2.5)			0.96	
Neutrophils %	18	68.3 (50.6)	65.7 (47.9)	55.8 (64.8)	ND	0.93	0.96	ND
	21	60.4 (47.8)	62.7 (46.1)			0.96		
	30	60.8 (41.3)		60.2 (53.0)			0.94	
Macrophages %	18	27.5 (49.0)	26.5 (45.0)	27.5 (68.7)	ND	0.93	0.97	ND

Results are presented as median (interquartile range). n: the number of samples examined in duplicate or triplicate; ND: not determined; ICC: intra-class correlation coefficient. \*\*\*:  $p < 0.001$  (only significant p-values shown).

airway secretions [28, 29], as well as heparin [27], which may be released by mast cells [30, 31].

There appeared to be a dose-response effect of increasing PI on the measured levels of IL-5. However, the maximum effect of PI was not pursued, since the reduction in sputum cell viability seen with the high PI suggested that increased concentrations of PI may be detrimental to sputum cells.

In the present study, there was no correlation between IL-5 (with or without PI) and sputum eosinophils, either total or percentage. An apparent relationship between sputum IL-5 levels and eosinophils has been shown in some studies. Studies in which bronchoalveolar lavage fluid was examined before and after allergen challenge [32–35] found a high correlation of total eosinophils with IL-5. Studies have also shown a positive correlation between sputum eosinophils and IL-5 in subjects with asthma [5, 36], and PIZZICHINI *et al.* [37] found that sputum eosinophils and IL-5 decreased significantly after treatment of subjects with severe exacerbations of asthma with prednisone. However, although both were increased, there was no significant correlation between eosinophil counts and IL-5 before treatment. Similarly, KEATINGS *et al.* [8] examined IL-5 levels in sputum before and after allergen challenge and noted that although IL-5 levels were significantly increased after allergen challenge, there was no significant correlation between IL-5 and eosinophils.

A study examining phytohaemagglutinin (PHA)-induced IL-5 generation by sputum cells after allergen challenge [38] noted that sputum eosinophils were significantly increased 4 h after allergen challenge, whereas IL-5 was only significantly increased after 24 h. The relationship of IL-5 to eosinophils is probably not straightforward, as other cytokines, including regulated on activation, normal T-cell expressed and secreted (RANTES), eotaxin and IL-8, are also important in eosinophil chemotaxis [39, 40]. Thus, it appears that IL-5 levels increase (allergen challenge) or decrease (treatment with prednisone) along with eosinophils, but there is not necessarily a close relationship between their actual levels.

The lack of correlation between sputum IL-5 with or without PI and FEV<sub>1</sub> % pred may be due to the fact that most of the subjects had mild asthma, and a correlation may have been found if subjects with more severe asthma had been included.

It is suggested that the relationship of sputum interleukin-5 with eosinophil counts, as well as the responsiveness of interleukin-5 to various therapies can be more accurately determined with the addition of protease inhibitors to sputum. In addition, samples with interleukin-5 levels below the detection limit of the assay may have measurable levels after the addition of protease inhibitors. This method allows total and differential cell counts to be performed simultaneously, since low and high protease inhibitors did not interfere with total or differential cell counts, although high protease inhibitors did reduce sputum cell viability. It is believed that the addition of protease inhibitors in a concentration equivalent to at least low protease inhibitors would be of value in studies where the investigators plan to measure

interleukin-5. In addition, this method may be useful for the measurement of other cytokines in sputum, such as interleukins-4 and -13, and further spiking experiments with and without protease inhibitors are planned.

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