Induced sputum: Validity of fluid-phase IL-5 measurement

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Background: IL-5 measurement in the fluid phase of induced sputum is considered to be important in the assessment of asthma, but the validity of these measurements is uncertain.
Objective: We investigated the validity of sputum IL-5 measurements through a series of spiking experiments and examined the effect of dithiothreitol (DTT) on these measurements.
Methods: Induced sputum from 26 asthmatic subjects was spiked with IL-5 and processed, and the percentage of recovery was measured by means of immunoassay. In 6 of the 26 samples the effect of adding albumin to the processing fluids was studied. In 3 separate samples radiolabeled IL-5 was added, and the recovery measured by means of gamma counting and immunoassay were compared. In addition, the effect of DTT on the immunoassay was examined.
Results: The mean ± SD recovery of spiked IL-5 was 26.1% ± 14.6% measured by means of immunoassay; adding albumin increased the recovery to 47.7% ± 8.0% ($P < .001$). The mean recovery measured by means of gamma counting was 84.8% ± 5.7% ($P < .001$); adding albumin had no effect on recovery. DTT had no significant effect on IL-5 measurement.
Conclusion: The validity of IL-5 measurement by means of current methods is poor. The discrepancy in recovery as measured by gamma counting compared with immunoassay suggests that there is a problem with the recognition of IL-5 epitopes by immunoassay in induced sputum. This cannot be attributed to DTT but may be due to other interfering substances present in sputum, such as sputum proteases, soluble receptors, or autoantibodies. (J Allergy Clin Immunol 2000;105:1162-8.)

Key words: *Asthma, inflammation, sputum, IL-5*

Abbreviations used

BALF:	Bronchoalveolar lavage fluid
DTT:	Dithiothreitol
EIA:	Enzyme immunoassay
2-ME:	2-Mercaptoethanol

Analysis of induced sputum is a relatively noninvasive tool for investigation of airway inflammation. Measurements of cellular indices are valid, reliable, and responsive to change.[1-6] Increasingly, soluble mediators of inflammation are also being measured. One of these is IL-5, which is the predominant cytokine associated with eosinophilic inflammation and is of key importance in the pathogenesis of asthma.[7,8] IL-5 can be detected in the serum,[9-11] bronchoalveolar lavage fluid (BALF),[12-15] and sputum[3,16,17] of asthmatic patients.

Although it is difficult to compare cytokine measurements from different enzyme immunoassay (EIA) kits,[18] levels of IL-5 in sputum are generally lower than those reported in serum or BALF.[16,17] This is surprising, given that eosinophils are often proportionately higher in sputum than in serum during asthmatic exacerbations, that serum IL-5 is regarded as being spillover from the airways, and that BALF has a large dilutional component. Also, this observation is in contrast to other inflammatory mediators, such as eosinophil cationic protein, tryptase, and IL-8, in which levels are 10-fold higher in sputum compared with BALF.[19]

These anomalies in measuring IL-5 in sputum may relate to the steps required to disperse and extract the fluid phase from the viscid material. Dithiothreitol (DTT) is a commonly used mucolytic that reduces disulfide bonds present in mucus[20] and may affect the two disulfide bonds in the IL-5 molecule. Furthermore, the dispersed cell suspension is commonly filtered to remove clumps of mucus, and debris and IL-5 may be lost to nonspecific binding on the filter or on the sides of the tubes. Measurement of IL-5 is usually performed by using an EIA, which relies on the recognition of specific IL-5 epitopes. All assays for cytokines in biologic fluids have the potential to be affected by the presence of binding molecules, such as soluble receptors, carrier proteins, or autoantibodies.[21-24] Consideration of such possible interfering factors is necessary when interpreting results.[25]

The objective of this study was to identify why the reported levels of IL-5 in the fluid phase of induced sputum are low. This has relevance to establishing the mea-

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surement of IL-5 in induced sputum as a clinically useful biologic marker in the assessment of airway inflammation. The following questions were addressed. Is IL-5 measurement in induced sputum valid? If not, is IL-5 lost during processing to nonspecific binding sites on containers and filters by remaining bound to nonfiltered mucus particles or by denaturation by DTT? Alternatively, are the low levels of IL-5 a result of poor immunologic recognition by the antibodies in the assay, and if so, is this the result of an effect of DTT on the IL-5 epitopes?

The validity of a measurement is usually established by comparison to a valid gold standard of measurement, which in this case is absent. Therefore we performed a series of spiking experiments in which known amounts of IL-5 were added to sputum samples, and the recovery was then measured. Spiking with IL-5 radiolabeled with iodine 125 (^{125}I) was performed to identify potential sites of loss during processing. SDS-PAGE of ^{125}I IL-5 (alone, incubated with DTT, and processed with sputum) was performed to examine degradation. Other sputum samples were processed with added albumin to block nonspecific binding sites. IL-5 was also incubated with DTT under different conditions and subsequently assayed. Finally, the direct effects of DTT on the immunoassay were studied by incubation with the capture antibody and by assaying a mixture of DTT and IL-5 standard.

METHODS

Subjects

Sputum from 29 asthmatic subjects (27 of whom were atopic) was used in the spiking studies (Table I). The diagnosis of asthma was based on the American Thoracic Society criteria[26] and included variable airflow limitation and airway hyperresponsiveness. Subjects were further classified by means of spirometry as having no (FEV_1 >80% of predicted value), mild (FEV_1 60%-80% of predicted value), or moderate (FEV_1 40%-59% of predicted value) airflow limitation present.[27] The study was approved by the St Joseph's Hospital Ethics Committee.

Sputum induction

Sputum was induced as described by Pin et al[1] and modified by Pizzichini et al.[3] 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 $mL \cdot min^{-1}$) and a large particle size (5.58 μm aerodynamic mass, median diameter). Concentrations of 3%, 4%, and 5% saline were each inhaled for 7-minute 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 plastic container.

Sputum processing for cell counts

Sputum was processed as previously described.[3] Briefly, within 2 hours of collection, sputum was selected from the expectorate, and a weighed aliquot was dispersed with 4 volumes of freshly prepared 6.5 mmol/L DTT (Sputolysin; Calbiochem, La Jolla, Calif). The mixture was briefly vortically spun, and after being rocked at room temperature for 15 minutes, 4 volumes of Dulbecco's PBS (Gibco Diagnostics, Tucson, Ariz) were added, after which the dispersed sample was filtered through a 48-μm nylon mesh. Total cell count and cell viability of the filtrate were measured by using trypan blue exclusion, and cytospin preparations were made (Cytospin 3 Cytocentrifuge; Shandon Inc, Pittsburgh, Pa). Slides were stained with Wright's stain, and a differential cell count of 400 nonsquamous cells was performed.

Validity of IL-5 measurement: Sputum spiking

A total of 26 sputum samples were spiked with recombinant human IL-5 (Genzyme Diagnostics, Cambridge, Mass). Two different spiking methods were used, whereby IL-5 was added at different stages of processing (Fig 1). In the first method, 6 samples were divided in half (after removing a portion to be processed for cell counts), and IL-5 was added to one half (high spike). The amount of IL-5 added was such that if all spiked IL-5 was recovered, its concentration in the supernatant would be in the upper range of the assay. Both halves were then processed as described. The filtered suspensions were centrifuged at 290*g* for 4 minutes, and the supernatants were stored at –70°C. In a further 14 samples, IL-5 was added after the sputum had been dispersed with DTT, filtered, and divided into 2 or 3 aliquots. The number of aliquots depended on the volume available, with 11 of 14 samples being divided into 3 aliquots. One aliquot was not spiked to allow measurement of endogenous IL-5, and the second was spiked with a similar proportion of IL-5 as in the first method (high spike). If there was a third aliquot, it was spiked with a lower proportion of IL-5 (low spike). Further processing was as before. In a further 6 samples, BSA (1% wt/vol; Sigma, Oakville, Ontario, Canada) was added to the DTT and PBS, and the specimens were processed with these solutions.

Spiking with radiolabeled IL-5

Sputum from a group of 3 atopic asthmatic subjects was divided into 2 equal portions (Fig 2). One portion was dispersed with 6.5 mmol/L DTT alone, and the other was dispersed with 6.5 mmol/L DTT with 1% BSA. The suspensions were divided into 4 to 10 aliquots (depending on the volume available), and each was spiked with different concentrations of ^{125}I IL-5 (Allergic Diseases Research Laboratory, Mayo Clinic, Rochester, Minn). After filtration and centrifugation as described above, the supernatant was separated from the cell pellet. Radioactivity remaining in the tubes after decanting on the filter and in the centrifuged cell pellet was measured by using gamma counting (Crystal Multidetector Gamma System; United Technologies Packard, Mississauga, Ontario, Canada) and expressed as a fraction of the total radioactivity added, and the percentage of recovery was calculated.

Electrophoresis

Electrophoresis (Fig 3) was performed by using SDS-PAGE with a 10% polyacrylamide gel and a 5% stacking gel. Three conditions were studied: ^{125}I IL-5 alone, ^{125}I IL-5 incubated with DTT, and supernatant from sputum spiked with ^{125}I IL-5 and processed. The samples were run in both nonreduced and reduced forms with 2-mercaptoethanol (2-ME; 3.3% vol/vol). 2-ME, which cleaves disulfide bonds, is used to denature proteins and therefore allow the migration of polypeptides through the gel to be dependent on size and independent of charge. However, because DTT also reduces disulfide bonds and its effects on IL-5 may be masked by the stronger reducing effect of 2-ME (at the concentrations used), samples were also run without 2-ME pretreatment. The gel was run overnight, dried, and autoradiographed.

Effect of DTT on IL-5

Solutions of IL-5 were incubated with DTT at room temperature for 0, 5, 30, and 60 minutes before freezing at –70°C. The final con-

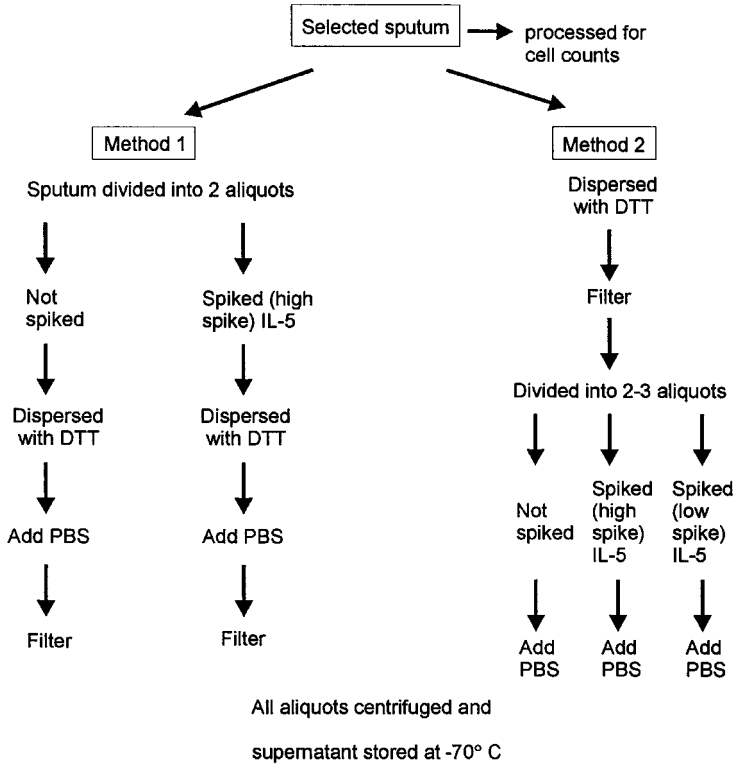

FIG 1. Flow diagram showing protocol for spiking of sputum with nonradiolabeled IL-5.

TABLE I. Subject characteristics

No. of patients	29
Age (y)	51.5 (24.5)
Sex (male)	16
Atopic	27
Airflow limitation	
None	9
Mild	10
Moderate	10
Sputum indices, median (IQR)	
Total cell count ($\times 10^6$/mL sputum)	4.9 (5.7)
% Viability	60.0 (27.5)
% Eosinophils	2.4 (4.9)
% Neutrophils	45.3 (44)
% Macrophages	49.4 (45.3)

IQR, Interquartile range.

centrations of IL-5 and DTT varied from 7.8 to 250 pg·mL^{-1} and 0.65 to 5.4 mmol/L, respectively. PBS was used as a negative control to examine the effect of dilution.

Effect of DTT on IL-5 immunoassay

To determine the possible effect of DTT on the capture antibody, it was preincubated with DTT (final concentration, 2.4 mmol/L) for 30 minutes in the wells of the assay plate. DTT was washed off before the blocking step. Measurements of IL-5 (7.8-250 pg·mL^{-1}) in DTT-treated and DTT-nontreated wells were compared. To assess the effects on the IL-5 epitopes and the detection antibody, DTT was also added to IL-5 standards (7.8-250 pg·mL^{-1}), which were assayed alongside the normal IL-5 standards, and the levels were compared.

IL-5 immunoassay

IL-5 was measured by using an EIA (Genzyme duoset, Genzyme Diagnostics) with a monoclonal capture antibody and a horseradish peroxidase–conjugated polyclonal detection antibody. All measurements were done in duplicate. The standard curve was generated with the same IL-5 (Genzyme Diagnostics) as was used in the spiking experiments. The lower limit of detection was 7.8 pg·mL^{-1}.

Statistical analysis

Data were analyzed by using the statistical software package SPSS for Windows, Release 8.0 (SPSS Inc, Chicago, Ill). Descriptive statistics were used to summarize the clinical and laboratory data, with qualitative variables expressed as percentages and normally distributed quantitative variables reported as the arithmetic mean and SD. Sputum cell counts were expressed as medians and interquartile ranges. Independent *t* tests were used for between-group comparisons, and paired *t* tests were used for within-group paired data comparisons. Correlations were performed with the Spearman rank correlation test. Two-tailed *P* values of less than .05 were considered significant.

RESULTS

Recovery of nonradiolabeled spiked IL-5

There was no significant difference in the recovery when spiking with IL-5 was carried out before or after dispersal with DTT, with the mean ± SD recovery being 27.7% ± 4.4% and 27.6% ± 3.1%, respectively. Therefore the results of the 20 high-spike aliquots were combined. The mean percentage recoveries of high- and low-spike aliquots were

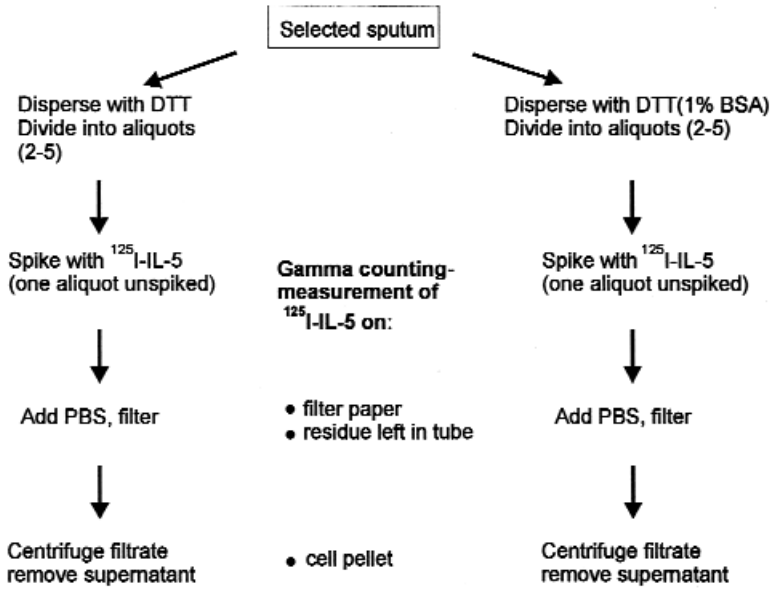

FIG 2. Flow diagram showing method for spiking of sputum with radiolabeled IL-5, with and without albumin.

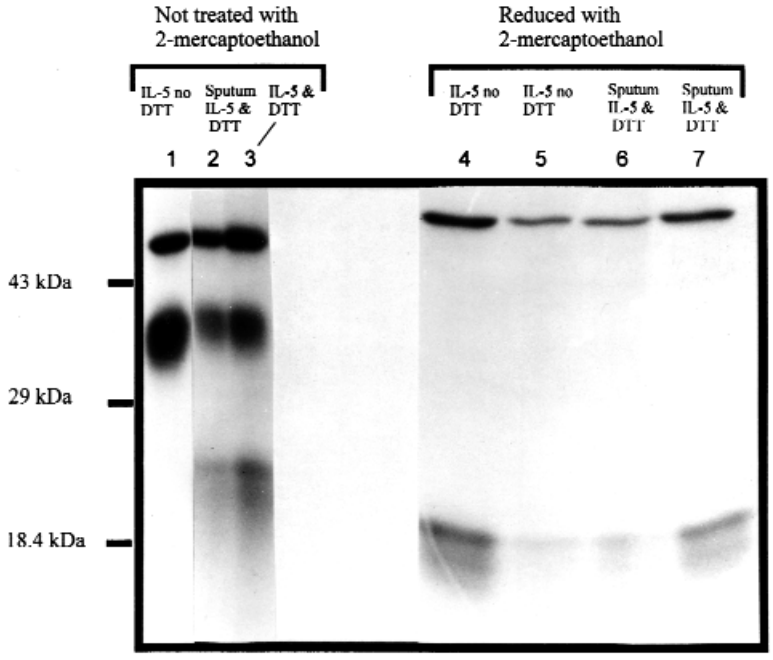

FIG 3. SDS-PAGE electrophoresis of radiolabeled IL-5: IL-5 with no DTT present, final concentration of 47 ng/mL *(lane 1)*; sputum processed with DTT and spiked with IL-5, final concentration of 31 ng/mL *(lane 2)*; IL-5 treated with DTT, final concentration of 47 ng/mL *(lane 3)*; IL-5 without DTT treated with 2-ME, final concentration of 47 ng/mL *(lane 4)* and 19 ng/mL *(lane 5)*; sputum processed with DTT and spiked with IL-5, final concentration of 19 ng/mL *(lane 6)* and 31 ng/mL *(lane 7)*.

TABLE II. Percentage recovery of spiked IL-5 as measured by immunoassay

	% Recovery of spiked IL-5 on the basis of airflow limitation			
	None (n = 6)	Mild (n = 10)	Moderate (n = 10)	% Recovery overall
High spike (n = 20)	32.7 ± 8.4	29.7 ± 5.5	21.6 ± 12.3	27.6 ± 9.8
Low spike (n = 11)	43.5 ± 12.3	11.7 ± 13.8	12.3 ± 21.3	23.4 ± 21.1
All spiked aliquots (n = 31)				26.1 ± 14.6

Values are presented as means ± SD.

TABLE III. Percentage recovery of spiked IL-5 by means of gamma counting and immunoassay: Effect of albumin

	Without albumin	With albumin
Gamma counting	85.4* ± 4.2	84.2 ± 7.2
Immunoassay	27.6* ± 9.8	47.7* ± 8.0

Values are presented as means ± SD.
*$P < .001$.

TABLE IV. Percentage radioactivity (^{125}I IL-5) lost during processing, as measured by gamma counting

	With albumin	Without albumin	Overall
Filter	6.1 ± 4.1	4.0 ± 1.3	5.1 ± 3.2
Tubes	6.1 ± 4.1	4.0 ± 1.3	5.0 ± 3.2
Cell pellet	3.6 ± 2.7	6.5 ± 4.5	5.1 ± 4.0
Total	15.8 ± 7.2	14.6 ± 4.2	15.2 ± 5.7

Values are presented as means ± SD.

TABLE V. Effect of DTT on IL-5: IL-5 incubated with PBS or DTT for 20 minutes and then frozen, thawed, and assayed

Standard IL-5	IL-5 in PBS	IL-5 (3.25 mmol/L DTT)
250	236	237
125	138	140
62.5	72	56.8
31.3	37	28.8
15.6	17.8	15.6
7.8	7.9	9.8

All values are in picograms per milliliter.

TABLE VI. Effect of DTT on immunoassay: Concentration of IL-5 standards

A	B	C
7.8	8.4	12
15.6	16.6	18.4
31.3	30.9	34.2
62.5	72	63.3
125	153.5	130.6
250	260.7	265

All values are in picograms per milliliter.
A, IL-5 without DTT; *B*, IL-5 with capture antibody pretreated with DTT; *C*, IL-5 assayed with DTT.

27.6% and 23.4%, respectively (Table II), giving a mean overall recovery of 26.1%. The recovery of spiked IL-5 was negatively correlated with FEV_1 ($r_s = –0.46$, $P < .05$ and $r_s = –0.65$, $P < .05$ for high and low spikes, respectively). There was no significant correlation with the sputum eosinophil count. Sputum processed with added albumin showed an improved but still incomplete percentage recovery of spiked IL-5, with a mean ± SD of 47.7% ± 8.0% (Table III) versus 27.6% ± 9.8% ($P < .001$ [high spike]).

Recovery of radiolabeled I^{125} IL-5

The percentage of radioactivity (assumed to be ^{125}I IL-5) that was lost at each step was assessed by means of gamma counting (Table IV). The addition of albumin to the processing fluids had no effect on the loss of radioactivity, and the mean overall loss was 15.2%. The mean recovery of ^{125}I IL-5 by using gamma counting was therefore 84.8%, which was significantly greater than that recovered by means of immunoassay (27.6%, $P < .001$, Table III).

Electrophoresis of ^{125}I IL-5

IL-5 alone (without added DTT or sputum), when unreduced, showed 2 bands, one with a relative molecular mass of approximately 50 kd and another broad band of 30 to 40 kd (Fig 3, *lane 1*). In the presence of DTT (Fig 3, *lanes 2* and *3*) another band appeared at about 18 to 25 kd that was consistent with some of the IL-5 being reduced to its monomeric form. Reduction by 2-ME (Fig 3, *lanes 4-7*) resulted in the disappearance of the band at 30 to 40 kd, probably representing complete reduction of the IL-5 in that band. Not all the IL-5 was reduced, however, because the band at 50 kd was still present. For ^{125}I IL-5 alone, treated with DTT, or processed in sputum with DTT, the electrophoretic pattern was similar after reduction with 2-ME. In Fig 3, bands in *lanes 1, 3, 4,* and *7* appeared denser than those in *lanes 2, 5,* and *6,* as a higher concentration of ^{125}I IL-5 was used.

Effect of DTT on IL-5 and the immunoassay

There was no effect on subsequent assay when IL-5 was incubated for various time periods and with different concentrations of DTT (Table V, all data not shown). Incubation of IL-5 with DTT for up to 60 minutes did not alter its measurement by immunoassay. Preincubation of capture antibody with DTT in the assay plate had no effect nor did the addition of DTT to the IL-5 standards used in the assay (Table VI).

DISCUSSION

This is the first study to examine the validity of IL-5 measurement in sputum systematically. The spiking studies showed that a mean ± SD of 26.1% ± 14.6% of IL-5 was recovered from processed sputum when measured by means of immunoassay. This indicates that the measurement of endogenous IL-5 in sputum has poor validity, and IL-5 is underestimated by using current methods. In contrast, a mean ± SD of 84.8% ± 5.7% of spiked radiolabeled IL-5 was recovered when measured by means of gamma counting. If the radiolabel was cleaved from IL-5 by sputum proteases, this could result in a falsely high recovery. However, this is unlikely because the radioelectrophoretic pattern (Fig 3) is similar if IL-5 (with DTT) is processed with or without sputum. Loss of IL-5 to the walls of containers and in the residue after filtering and centrifuging (total mean loss, 15.2%) cannot account for the poor recovery reported by immunoassay. Therefore the problem is at the level of the immunologic recognition of the IL-5 epitopes by using the immunoassay.

The increased recovery of IL-5 by immunoassay when albumin is added is unexplained. It is unlikely to be related to blocking nonspecific binding because the recovery measured by means of gamma counting is unaltered by albumin.

DTT did not interfere with measurement of spiked IL-5 in sputum either during processing or during immunoassay and is therefore unlikely to account for the poor immunologic recognition. There was no significant difference in recovery of IL-5 when it was added before or after processing with DTT, which supports this interpretation. The pattern on electrophoresis is similar to the observations of Takahashi et al,[28] who found 2 main broad bands under similar conditions. These bands are consistent with IL-5 being present in its dimeric form (relative molecular mass, 45-60 kd), with the multiple bands probably representing differential glycosylation of the dimer. The monomeric forms therefore also have different degrees of glycosylation, resulting in a broad band at 18 to 25 kd.

A potential cause of interference with immunologic recognition of IL-5 is denaturation of the epitopes by means of sputum proteases. Proteases have been detected in high levels in asthmatic sputum, and their level increases with increasing clinical severity of asthma.[29] The 10% gel used for electrophoresis would not retain molecules below 10 kd, and therefore small peptides split from the IL-5 molecule by proteases would not be visible. In addition, because disulfide bonds in IL-5 are only partially reduced by DTT at a concentration of 6.5 mmol/L, the presence of these bonds may mask cleavage of the molecule at other sites.[28]

The negative correlation of recovery of spiked IL-5 with the FEV_1 is interesting and suggests that a factor or factors in the sputum interfering with IL-5 measurement also increase with worsening clinical severity of asthma. A soluble form of the IL-5 receptor α-subunit has been described,[30,31] and there is evidence of its increased expression in asthmatic subjects and a direct correlation with FEV_1.[32] IL-5 complexed to its soluble receptor would not be recognized by neutralizing antibodies and therefore not be detected by the EIA we used because the detection body is a neutralizing antibody. However, because soluble receptor expression was directly correlated with FEV_1, it is unlikely to increase with worsening clinical severity. IL-5 complexed to the receptor would be biologically inactive and may not be clinically relevant.

We conclude that measurement of IL-5 in sputum is problematic, as shown by this series of spiking studies. Although we examined only selected sputum, similar effects on spiked IL-5 would be expected if unselected sputum was used. Measured levels of IL-5 in sputum should be interpreted with caution, and undetectable levels on immunoassay do not confirm that it is absent. DTT was shown to have no significant effect on recovery of spiked IL-5 and is therefore unlikely to affect measurement of endogenous IL-5. Added IL-5 was minimally lost during processing, and therefore we conclude that low measured levels of IL-5 are most likely to be related to poor recognition of IL-5 epitopes by immunoassay. Poor recognition may be due to degradation of the epitopes by proteases; to binding of free IL-5 in sputum by soluble IL-5 receptors, autoantibodies, or other binding proteins; or to various combinations of these and requires further investigation.

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