

Measurement of eotaxin (CCL11) in induced sputum supernatants: Validation and detection in asthma

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Background: Induced sputum is widely used in asthma research; however, for many mediators, the detection methods have not been validated.

Objective: We sought to optimize the method of detection of eotaxin, an important chemokine acting through the CCR3 receptor on eosinophils, basophils, and T_H2 cells.

Methods: Induced sputum from normal and asthmatic subjects was processed with dithioerythritol (DTE) or PBS; recovery of eotaxin was assessed by means of ELISA before and after spiking with recombinant eotaxin. Furthermore, the effects of removing DTE by means of ultrafiltration or the addition of protease inhibitors and high-speed centrifugation on endogenous levels and spiking recovery of eotaxin were assessed.

Results: Endogenous eotaxin was undetectable in DTE-processed samples, with a mean of only 30% (SD, 13%) spike recovery. DTE had no effect on the immunoassay capture antibody but dramatically reduced the detection of recombinant eotaxin. Removal of DTE from sputum before immunoassay did not improve detection, although it restored the recovery of a subsequent eotaxin spike. In contrast, PBS-processed sputum resulted in an eotaxin spike recovery of 101% (SD, 20%). Addition of protease inhibitors or high-speed centrifugation had no effect on eotaxin detection. By using this optimized protocol, eotaxin levels in PBS-processed sputum samples were found to be significantly increased in asthmatic sputum ($P < .05$).

Conclusion: Measurement of eotaxin by means of immunoassay is adversely affected by DTE, possibly through irreversible denaturation of epitopes, which makes eotaxin undetectable by using the immunoassay antibody. Sputum samples should be processed into PBS for assessment of eotaxin, which is present at increased levels in asthmatic sputum. (*J Allergy Clin Immunol* 2004;113:657-62.)

Key words: Eotaxin (CCL11), induced sputum, asthma, ELISA, dithioerythritol

Abbreviations used

DTE: Dithioerythritol

DTT: Dithiothreitol

ECP: Eosinophil cationic protein

PI: Protease inhibitor

Sputum induction is widely used as a safe and noninvasive research tool that allows many mediators to be measured by using immunoassays or enzyme activity assays.¹⁻³ Detection assays for many, but not all, mediators have been validated, although the level of validation has varied from simple measurement of recovery in spiking experiments, as has been the case for tryptase and eosinophil cationic protein (ECP),⁴ to an in-depth assessment of the potential pitfalls when detecting cytokines such as IL-5.⁵ Because the use of induced sputum extends toward study of other mediators that might interact physically with other components of sputum or be susceptible to the processing conditions, it is important to improve the validation methods and provide standards for the validation procedures.

Among the issues that are important in relation to the effect of various processing methods is that of interference by sulfhydryl-reducing reagents, such as dithioerythritol (DTE) or its optical isomer, dithiothreitol (DTT). These have been widely used to solubilize high-molecular-weight mucins to improve the quality of cytocentrifuge preparations and facilitate cell counting, in addition to improving recovery of some soluble mediators.^{4,6} However, the reducing potential of these agents is likely to have a deleterious effect on many mediators, particularly those with structures constrained by disulfide bonds. Further interference might arise from the effect of these agents on the antibodies of the immunoassay used. For the majority of asthma mediators, there has been no investigation into the effect of DTT on the validity of their current assays.⁵

The objective of this study was to optimize and validate the detection of eotaxin, a CC chemokine that has attracted much interest in allergic disease, including asthma, and to report on levels that can be measured in the sputum of asthmatic individuals. Unlike other CC chemokines, eotaxin (CCL11) selectively recruits eosinophils and other CCR3 receptor-bearing cells, which potentially makes it a central chemokine in asthma pathogenesis and a target for antiasthma drugs.⁷⁻¹¹ Because the secondary structure

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TABLE I. Subject and sputum characteristics

	Asthmatic subjects	Control subjects
No. of patients	21	17
Age, y*	36 (11)	28 (5)
Atopic	21	2
Taking inhaled bronchodilator	20	—
Taking inhaled glucocorticoids	8	—
Taking oral glucocorticoids	2	—
FEV ₁ , % predicted*	84 (18)	106 (12)
PC ₂₀ histamine, mg/mL†	1.2 (ND-8.0)	>16
Sputum indices*		
Total cell count, ×10 ⁶ /g	1.1 (0.8)	1.3 (0.5)
Squamous cell contamination, %	21 (17)	30 (15)
Viability, %	64 (17)	58 (18)
Eosinophils, %	1.9 (2.0)	0.4 (0.4)
Neutrophils, %	38 (22)	21 (10)
Macrophages, %	39 (16)	56 (13)

ND, Not detected.

*Values are shown as means (SDs).

†Values are shown as geometric means (ranges).

of eotaxin is maintained by 2 disulfide bonds,¹² we have speculated that disruption of the disulfide bridges by a reducing agent might pose a problem with immunologic detection of this chemokine. We have investigated whether and how DTE might interfere with the detection of eotaxin in sputum when using a commercial immunoassay. As a further step in validating the method, and after a report that proteases present in the airway lining fluid might have an effect on the stability and therefore detection of mediators such as IL-5,¹³ we have addressed the question of whether there would be any benefit from adding protease inhibitors (PIs) to sputum processed with PBS. Finally, we have examined the effect of high-speed centrifugation during processing with respect to effects on cell contamination and eotaxin levels.

METHODS

Subjects

Twenty-one asthmatic and 17 healthy control subjects participated in the study (Table I). All asthmatic subjects were atopic and had asthma of variable severity, ranging from mild asthma treated with inhaled β_2 -agonists alone to severe asthma requiring oral corticosteroids. The diagnosis of asthma was based on established criteria.¹⁴ All healthy and asthmatic subjects were nonsmokers, and none had had a respiratory infection for at least 6 weeks before the study. The study was approved by the Southampton and South West Hampshire Local Research Ethics Committee, and all subjects provided written informed consent.

Sputum induction

Sputum was induced as described previously⁴ and in keeping with recent guidelines of the European Respiratory Society Task Force on Induced Sputum.³ Sputum was collected into a plastic petri dish, placed on ice, and processed within 1 hour.

Sputum processing for cell counts

Aliquots of sputum were taken for cell counting from all samples before processing with DTE, PBS, or PBS plus PIs (PBS+PI). For cell counts, sputum was processed with DTE, and assessment of total

cell counts and cell viability was performed by using standard protocols.¹⁵ Cytospin preparations were prepared and stained with May-Grünwald/Giemsa, and 600 cells were counted per slide.

Sputum processing for analysis

Processing with DTE. Sputum was mixed with an equal volume of 10 mmol/L DTE and incubated on a rotor mixer for 30 minutes before the remaining mucus was removed by means of filtration, as recommended in standard protocols.¹⁶ Cells were pelleted by means of centrifugation at 400g for 10 minutes, and the supernatant was stored at -80°C .

Processing with PBS or PBS+PI. An equal weight of PBS was added to disperse the sputum, and the mixture was divided into 2 (or more if required) aliquots. These were further solubilized in an equal volume of PBS with or without 22.5 $\mu\text{L/mL}$ PI cocktail (protease and phosphatase inhibitor cocktail; Sigma, Dorset, United Kingdom) and mixed on a rotor mixer for 30 minutes on ice. Mucus was then removed by means of filtration through a 100- μm -pore nylon mesh, and the filtrate was centrifuged at 400g for 10 minutes to precipitate any cells present. The fluid phase was stored at -80°C until analysis. Eight sputum samples processed with PBS or PBS+PI were also further centrifuged at 13,000g for 30 minutes at 4°C . These supernatants were stored at -80°C and analyzed alongside the corresponding samples centrifuged at lower speed.

Sputum spiking with eotaxin

A total of 29 sputum supernatants (18 from asthmatic patients and 11 from control subjects) were spiked with 200 pg/mL recombinant human eotaxin (R&D Systems, Abingdon, United Kingdom) to validate the measurement of eotaxin. Fifteen PBS-, 3 PBS+PI-, and 11 DTE-processed samples were used in 3 different procedures.

Spiking of PBS- or DTE-processed samples. In the first experiments, 5 PBS, 3 PBS+PI, and 5 DTE samples were spiked with eotaxin, and recovery was immediately measured. Recovery of spiked eotaxin in sputum was calculated on the basis of a control eotaxin spike prepared in the assay calibrator diluent.

Spiking after removal of DTE by means of ultrafiltration. DTE-processed samples were processed by means of ultrafiltration through a 3000-Da cut-off membrane (Centricon YM-3, Millipore, Watford, United Kingdom) and reconstituted in fresh sterile PBS to examine the effect of removing DTE from sputum supernatant. After ultrafiltration and reconstitution, approximately 85% of the DTE was calculated to have been removed from the DTE-processed samples. Samples were then spiked with eotaxin and analyzed. One aliquot of each sample was not spiked to allow measurement of endogenous eotaxin. PBS-processed samples were processed alongside for comparison.

Spiking before removal of DTE by means of ultrafiltration. Finally, the reversibility of the effect of DTE on eotaxin was investigated by spiking DTE- and PBS-processed samples with eotaxin before ultrafiltration. Standard eotaxin either in PBS or DTE was processed alongside spiked sputum samples for comparison. Spiking, ultrafiltration, and assaying were performed on the same day.

Spike parallelism of sputum supernatant. Samples from the same asthmatic individual were spiked with eotaxin (200 pg/mL) and serially diluted to 100, 50, and 25 pg/mL before measurement to investigate the parallelism of DTE-processed sputum before and after buffer exchange and for comparison with PBS-processed sputum. The measurements for these dilutions were plotted against the amount of spiked eotaxin (endogenous eotaxin was subtracted) to determine whether sputum dilutions are parallel to the standard curve.

Effect of DTE on eotaxin and the immunoassay for eotaxin. The effect of DTE on eotaxin epitopes was studied by incubating standard solutions of eotaxin (31-500 pg/mL) with DTE (at a final

TABLE II. Endogenous eotaxin concentrations in DTE- and PBS-processed sputum

Subject group	Sputum in DTE		Sputum in PBS		%
	pg/mL	N	pg/mL	N	
Healthy	ND	4	35.7 (7.80-62.8)	10	60
Asthmatic	ND	8	87.7 (64.1-204)*	12	100

Values are shown as medians (interquartile ranges).

ND, Not detected (ie, value <7.8 pg/mL); %, percentage of sample in which eotaxin was detectable (ie, >7.8 pg/mL).

*Included 2 subjects receiving oral glucocorticoid treatment. These were the 2 lowest values: 29.5 and 46.8 pg/mL.

concentration of 5 mmol/L) at room temperature for 30 minutes before the assay. The effect on the capture antibody of the immunoassay was examined by preincubating the antibody-coated microplate wells with DTE (at a final concentration of 5 mmol/L) for 30 minutes and removing it by means of decanting and gentle washing before assaying the standards across the same range of concentrations. Solutions of eotaxin in either DTE-treated wells or in the presence of DTE were assayed alongside the normal eotaxin standards, and levels were compared.

Effect of PIs and high-speed centrifugation on eotaxin recovery. The effect of PIs on the detection of eotaxin was investigated by the addition of PI cocktail in 8 samples (collected from 4 different donors). An aliquot of each sample was centrifuged at high speed (13,000g) and then assayed for endogenous eotaxin to test whether the speed of centrifugation would have any effect.

The potential effect of PIs and high-speed centrifugation on eotaxin spiking recovery was studied in a total of 24 samples. Specifically, PBS- and PBS+PI-processed samples were collected at 400g, whereas others were also centrifuged at 13,000g. All samples were spiked with 200 pg/mL eotaxin, and recovery was measured with ELISA.

Eotaxin immunoassay

Eotaxin was measured by means of ELISA (Quantikine human eotaxin immunoassay; R&D Systems). The eotaxin standard from the kit was used to spike sputum and control samples and to generate the standard curve. The lower detection limit of the assay was 7.8 pg/mL.

RESULTS

Detection of endogenous eotaxin

Initial assays showed no immunoreactive eotaxin in supernatants of DTE-processed sputum from either healthy or asthmatic donors. In contrast, eotaxin was detected in samples obtained from the same donors but processed with PBS alone (Table II). Levels of eotaxin in the asthmatic patients were significantly higher when compared with those in the control subjects ($P = .025$, Table II), despite the fact that 2 asthmatic subjects were receiving oral glucocorticoid treatment.

Recovery of spiked eotaxin in DTE- or PBS-processed sputum

There was a striking difference in the mean recovery of spiked eotaxin from samples processed with PBS (101% [SD, 11.4%]) when compared with DTE-processed samples (30.0% [SD, 12.7%], Fig 1). By using a pool of

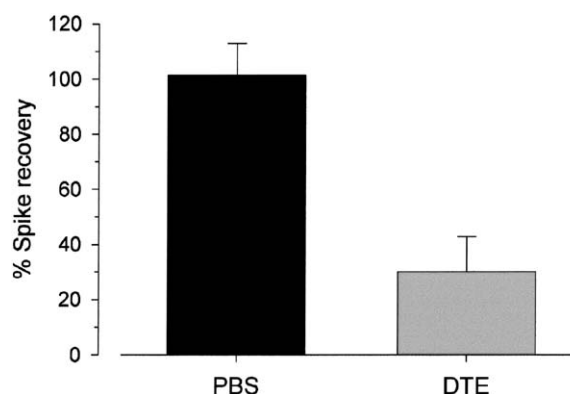


FIG 1. Spike recovery of eotaxin in DTE-processed (shaded bar) and PBS-processed (filled bar) sputum samples. Samples were assayed with and without the addition of 200 pg/mL eotaxin. Data are presented as means ± SDs by using samples from 5 subjects.

sputum prepared from 10 DTE-processed samples, it was observed that spike recovery increased with sputum dilution (with PBS), from 40% recovery at 1:2 dilution to 70% recovery at 1:10 dilution. Samples processed with DTE failed to show parallelism to the eotaxin standard curve (Fig 2, A). This and the establishment of good parallelism when DTE was removed before spiking indicated that DTE was interfering with eotaxin detection. In contrast to DTE-processed samples, good parallelism was achieved when assaying PBS-treated samples (Fig 2, B).

Samples spiked with eotaxin after buffer exchange showed high recovery with a mean of 101% (SD, 6.6%), which falls within the acceptable range (80%-120%) for a validated sample (Fig 3). At the same time, the spike parallelism of the DTE sample was restored (Fig 2, A). Further evidence of the problems arising with the use of DTE came from samples in which eotaxin was added before ultrafiltration. In this case the mean recovery of eotaxin was very low (5.2% [SD, 7.3%]). Similarly, less than 5% of eotaxin was recovered from a control eotaxin spike in DTE solution (data not shown). On the other hand, spiking before or after buffer exchange did not affect the recovery of eotaxin in PBS-processed sputum (112% [SD, 10%] and 97.5% [SD, 15.7%], respectively; Fig 3).

Effect of DTE on eotaxin and the immunoassay

No effect on levels of detected eotaxin was seen when the capture antibody was preincubated with 5 mmol/L DTE. Recovery of eotaxin was greater than 83% for all standards. In contrast, 30 minutes of incubation of eotaxin standards with DTE had a detrimental effect on the measured levels (Table III).

Effect of PIs on eotaxin

Addition of PIs during processing of sputum with PBS did not increase the detection of endogenous eotaxin levels (Table IV). Spike recovery was similarly unaffected

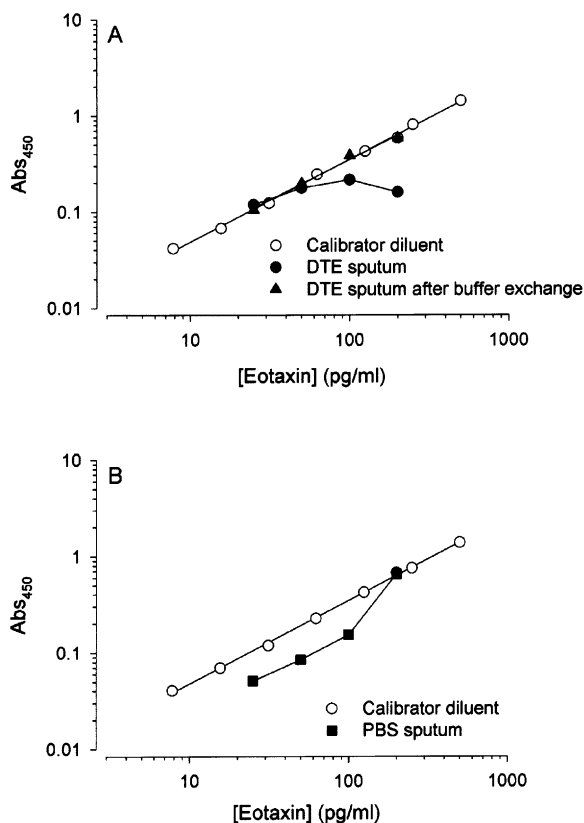


FIG 2. Parallelism of sputum supernatants processed in DTE (**A**) and PBS (**B**) with increasing concentrations of eotaxin. Buffer exchange to remove DTE from sputum conducted before the addition of eotaxin restored the parallelism (Fig 2, **A**).

(101% [SD, 20.2%] vs 102% [SD, 11.4%] recovery in the presence and absence, respectively, of PIs). In addition, cell counts were not affected by PIs (data not shown) and correlated with cell counts performed in DTE-processed samples.

Effect of centrifugation during processing on eotaxin

Routinely, sputum is processed at 400g, but occasionally, higher speeds have been reported.⁵ For very highly mucoid sputum samples, centrifugation at 400g was insufficient for complete removal of cells and, occasionally, did not yield a clear solution (unpublished observations). Therefore sputum supernatants in PBS were further centrifuged at 13,000g, yielding a faint pellet in most cases. The supernatants of such samples had endogenous eotaxin levels similar to those of samples centrifuged at 400g (Table IV). Spike recovery was similarly unaffected (99.1% [SD, 16.7%] vs 102% [SD, 11.4%] recovery after centrifugation at 13,000g and 400g, respectively).

DISCUSSION

In this study we have optimized and validated the detection of eotaxin in sputum and have shown that

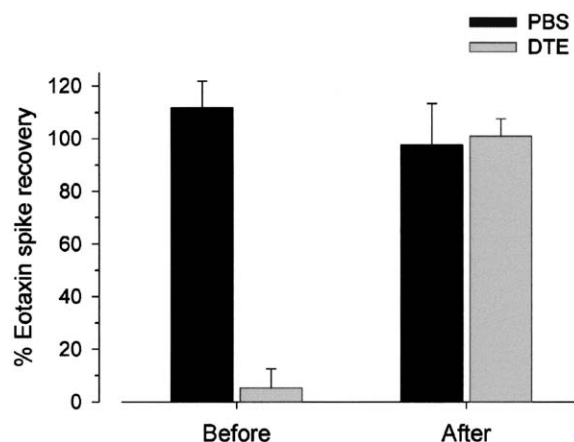


FIG 3. Effect of DTE (shaded bars) and PBS (filled bars) on the recovery of spiked eotaxin. Samples were spiked with eotaxin (200 pg/mL) before or after ultrafiltration (exchange of DTE with PBS buffer). Five PBS and 3 DTE samples were used in each experiment, and eotaxin was measured by using ELISA. Recoveries were compared with a control spike (in ELISA assay calibrator diluent) defined as 100%. Histograms show means $\pm$ SDs.

levels of this chemokine are clearly higher in asthmatic individuals. As could be predicted from the presence of disulfide bonds maintaining the 3-stranded β -sheet of eotaxin, the sulfhydryl-reducing agent DTE was found to interfere with the immunologic detection of this chemokine. In a series of experiments in which sputum was spiked with recombinant eotaxin, and additional experiments in which eotaxin standards were exposed to DTE, we found that DTE caused sufficient changes to the antigenic sites of eotaxin to interfere with its detection in the immunoassay. Importantly, removal of DTE by means of ultrafiltration before the immunoassay could not reverse this effect. In contrast to DTE, PBS was able to solubilize sputum without adversely affecting the recovery of eotaxin. As a further step in validating the method of processing sputum with PBS, we have shown that the addition of PIs or application of higher centrifugal speed does not affect the ability to detect eotaxin.

Previous studies reporting on levels of soluble mediators in sputum have not raised any concerns about the effects of DTE on mediator detection. In fact, levels of some mediators, such as ECP, tryptase, and IgA, have been shown to be higher in samples processed with DTE when compared with those processed with PBS.⁴ Levels of other mediators, such as fibrinogen, IL-5, and IL-8 (CXCL8), have been reported as being similar in DTE- and PBS-processed samples,⁶ whereas DTT had no effect on detection of IL-1 β , IL-6, IL-8, secretory leukoprotease inhibitor, or neutrophil elastase but significantly reduced detected levels of TNF- α , leukotriene B₄, myeloperoxidase, and eosinophil peroxidase.^{17,18} It can be presumed that levels of many mediators are higher in DTE-processed sputum because they are released from mucus as mucolytic agents break it down. Spike recovery of ECP and tryptase is similar in PBS- and DTE-processed

TABLE III. Effect of DTE on immunoassay

Eotaxin standard	Immunoreactive eotaxin standard with DTE*	Immunoreactive eotaxin standard with capture antibody pretreated with DTE*
31.3	ND	26.3
62.5	ND	54.9
125	ND	105
250	ND	224
500	ND	440

Immunoreactive eotaxin was measured in the presence or absence of DTE. For the effect on capture antibody, DTE was added into plate wells for 30 minutes and then washed off before the addition of eotaxin standards. All values are presented in picograms per milliliter.

ND, Not detected (value <7.8 pg/mL).

*5 mmol/L DTE/75 mmol/L NaCl.

samples, indicating that the immunoreactivity of these proteins is not altered by DTE.⁴ In the case of IL-8, another chemokine with disulfide bonds, detection is similar in PBS- and DTE-processed samples, regardless of whether the immunoassay that is used detects free IL-8 or IL-8 bound to IgA.⁴ Spiking experiments in which recombinant IL-8 has been added to DTE- or PBS-processed samples showed poor recovery, however, with only approximately 5% recovery of spiked IL-8 in PBS-processed samples and approximately 40% recovery in samples processed with DTE.⁴ This suggests that binding of IL-8 to macromolecules of sputum might mask the antigenic sites of IL-8 and that part of this effect might be inhibited by DTE.

Because the use of reducing agents is beneficial in other respects, we removed DTE through ultrafiltration with the view of reversing any interference with the detection of eotaxin or, less likely, reversing any deleterious effects DTE might have had on the eotaxin molecule itself. However, this had no effect on the detection of endogenous eotaxin. Recovery of spiked eotaxin was restored only when spiking was performed after, and not before, exchange of DTE with PBS buffer. These results indicated that, shortly (30 minutes) after contact with DTE, eotaxin was rendered undetectable. The most likely cause for this is denaturation of the eotaxin protein, resulting in disruption of the antigenic site or sites recognized by the antibodies used in the ELISA. Because this action probably involves reduction of disulfide bridges in the C-C region, it is also likely to render the chemokine biologically inactive. We speculate that other chemokines with a similar structure (eg, RANTES and macrophage inflammatory protein 1 α) might be affected by DTE, something that must be taken into account when performing immunoassays for these mediators.

We have sought to optimize further the method by evaluating whether detection of endogenous eotaxin in PBS-processed sputum is adversely affected by proteases, which might degrade eotaxin and alter its epitopes. It has been reported that measurement of IL-5 in sputum is improved in the presence of PIs.¹³ The effect of proteases

TABLE IV. Lack of effect of PIs and high-speed centrifugation on endogenous eotaxin recovery in PBS-processed sputum

Sample no.	Endogenous immunoreactive eotaxin (pg/mL)			
	Sputum in PBS		Sputum in PBS+PI	
	400g	13,000g	400g	13,000g
1	147	159	172	194
2	334	365	293	336
3	78.7	65.5	73.7	80.1
4	47.8	53.8	45.2	48.0

PBS or PBS+PI samples were collected at 400g and either analyzed directly or centrifuged at 13,000g before analysis.

on most asthma mediators is currently unknown. In this study we found no evidence to support significant loss of endogenous or spiked eotaxin caused by proteolytic degradation.

Finally, we have sought to investigate the effect of high-speed centrifugation, which might, conceivably, remove any cell debris or mucus that could interfere with mediator detection. However, this had no effect on eotaxin detection: endogenous eotaxin remained in the supernatant, and exogenous eotaxin, added before high-speed centrifugation, was also completely (99%) recovered from the same fraction. Taken together, this suggests that eotaxin in sputum supernatants is more likely to be free in the soluble phase rather than bound to particulate material in the sputum, and high-speed centrifugation has no effect on its recovery.

In summary, ELISA-based measurement of eotaxin in DTE-processed sputum was found to be invalid, which suggests that other mediators that contain disulfide bonds might be affected by processing with reducing agents. Therefore we recommend that all immunoassays and bioassays be validated in respect of DTE effects and that processing with PBS be considered as an alternative by using protocols similar to those developed for eotaxin. Although eotaxin was found to be unaffected by either PIs or high-speed centrifugation in the current study, we suggest that this might not be the case for other mediators and that this needs to be formally assessed such that optimal recovery can be achieved. Finally, this study suggests that eotaxin does not interact with other components of mucus in a way that interferes with its detection, but whether there are interactions that modulate its function remains to be determined.

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