ORIGINAL ARTICLE

Validation of Total and Specific IgE Measurements in Induced Sputum

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Abstract

Background and objectives: Soluble components are increasingly analyzed in induced sputum supernatant. However, only a few studies have measured total or specific immunoglobulin (Ig) E in sputum and none have attempted to validate it. We aim to validate laboratory measurements of total and specific IgE in induced sputum supernatant and to evaluate the influence of sputum processing with dithiothreitol (DTT) on IgE measurements.
Methods: Total and specific IgE were measured by ImmunoCAP and the process was validated using sputum spiking experiments with total and specific IgE (to *Dermatophagoides pteronyssinus* and *Phleum pratense*) over a range of concentrations according to international recommendations. The Wilcoxon signed-ranks test was used for within-group comparisons and intraclass correlation coefficients were used to evaluate agreement between measurements. Two-tailed *P* values lower than .05 were considered significant.
Results: Samples from 18 patients (13 with interstitial lung disease, 2 with allergic asthma, and 3 healthy controls; 12 men; mean [SD] age, 45.6 [15.8] years) were evaluated. Median total IgE was 5.4 kU/L (interquartile range, 4.0-6.0 kU/L). Specific IgE levels to *D pteronyssinus* and *P pratense* were below 0.35 kUA/L in all samples. Recovery rates were above 80% for total and specific IgE over a wide range of values. No differences were found in total IgE measurements of sputum dispersed with DTT or phosphate-buffered saline, with a good intraclass correlation coefficient between both measurements (0.81, *P*=.01).
Conclusions: Total and specific IgE measurements performed in induced sputum with a commercially available immunoassay are valid over a wide range of IgE levels.

Key words: IgE. Induced sputum. Validation.

Resumen

Durante los últimos años, se están analizando de forma exhaustiva los componentes solubles del esputo inducido, no obstante pocos estudios analizan la IgE total y específica y ninguno de ellos ha intentado validar la técnica.
El objetivo de este estudio fue validar la cuantificación de IgE total y específica en el sobrenadante del esputo inducido y evaluar la influencia del procesamiento del mismo con dithiothreitol (DTT).
La IgE total y específica fueron determinadas mediante el fluoroenzymoinmunoensayo ImmunoCAP (Phadia ThermoFisher Scientific).
Para el proceso de validación utilizamos experimentos de adición de células del esputo con IgE total y específica (frente a Dermatophagoides pteronyssinus y Phleum pratense) en un rango de concentraciones de acuerdo a las recomendaciones de la ATS/ERS.
Para las comparaciones intragrupo se utilizó el test de Wilcoxon y el coeficiente de correlación intraclase para evaluar el grado de acuerdo entre ambas mediciones. Fueron considerados como significativos valores de $p < 0.05$.
En cuanto a los resultados obtenidos en las muestras de esputo procedentes de 18 pacientes (13 con enfermedad pulmonar intersticial, 2 con asma alérgica y 3 controles sanos), 12 varones, con una edad media de 45.6 ± SD 15.8 años, se obtuvo una media de IgE total de 5.4 (P25-754.0-6.0) kU/L. La IgE específica frente a *Dermatophagoides pteronyssinus* y *Phleum pratense* fueron inferiores a 0.35 kU_A/L en todas las muestras. Las cifras de recuperación de la IgE total y específica estaba por encima del 80% con un amplio rango de valores. No se hallaron diferencias significativas entre la cuantificación de IgE total y específica con PBS o con DTT, con un buen coeficiente de correlación intraclase (0.81; p=0.01).
En conclusión, la cuantificación de IgE total y específica en esputo inducido utilizando un imnunoensayo comercializado es válido y presenta una alta dispersión de rangos de niveles de IgE.

Palabras clave: IgE. Esputo inducido. Validación

Introduction

Sputum induction is a safe, well-tolerated noninvasive method for the investigation of inflammatory airway diseases such as asthma and chronic obstructive pulmonary disease. Its use for diagnostic and scientific purposes has increased dramatically in recent years, and it has even replaced the invasive technique of bronchoalveolar lavage in the monitoring of changes in airway inflammation [1,2].

Soluble components, such as eosinophil proteins, neutrophil proteins, mast cell products, plasma exudate markers, cytokines, chemokines, soluble cytokine receptors, proteases/inhibitors, and eicosanoids are increasingly analyzed in induced sputum supernatants [3-5].

Detection assays for many mediators have been validated, although the level of validation has varied from simple measurement to recovery rates after spiking experiments. Immunoglobulin (Ig) E is the antibody responsible for activation of allergic reactions and is particularly relevant for the development and persistence of allergic inflammation [6]. Only a few studies have measured either total or specific IgE in sputum but none have attempted to validate this method [7-9].

The objective of our study was to validate the laboratory measurements of total and specific IgE to *Dermatophagoides pteronyssinus* and *Phleum pratense* in induced sputum supernatant and to evaluate the influence of sputum processing with dithiothreitol (DTT) on IgE measurements.

Methods

All patients who underwent sputum induction in our department between July and September 2008 (including patients from a pilot study comparing sputum induction with bronchoalveolar lavage in interstitial lung diseases [10]) were invited to participate. The study was approved by the ethics committee at the Faculty of Medicine of Porto and patients gave their informed written consent.

Sputum Induction and Processing

Sputum induction was performed using inhalation of hypertonic saline (4.5%) through a mouthpiece connected to an ultrasonic nebulizer OMRON NE-U17 (Omron Healthcare Europe) with maximum output settings. Both induction and processing were performed according to the European Respiratory Society (ERS) task force recommendations [11,12]. Briefly, we used 4.5% saline inhalation for periods of 5 minutes. Peak expiratory flow (PEF) at baseline and after administration of 200 μg of salbutamol was registered using a Mini-Wright Peak-Flow Meter (Clement-Clarke International). Induction was stopped if any of the following criteria was met: *1)* the patient produced an adequate sputum sample, *2)* no sputum was produced after 20 minutes of induction, and *3)* PEF dropped below 80% of the baseline value. After induction and within 2 hours, all sputum samples that were macroscopically free of salivary contamination were selected and treated with dithiothreitol (DTT) (Sputolysin, Calbiochem Corporation) and phosphate-buffered saline (PBS). DTT has been shown to be more effective at dispersing cells than PBS, and has no adverse effects on cell counts, although it might affect some fluid-phase measurements [12]. The suspension was filtered and centrifuged, and the cell pellet resuspended and counted for viability and total cell numbers per gram of processed sputum. Coded cytospins were prepared and stained using the May-Grünwald Giemsa technique for differential cell counts of intact bronchial epithelial cells and leukocytes, up to a total of 500 nonsquamous cells. Sputum samples were considered adequate if they contained over 80% squamous epithelial cells from saliva.

Sputum Spiking

Sputum spiking experiments and all validation processes were performed according to American Thoracic Society/ERS recommendations [5]. For each case, the weighed aliquot was divided into 3 equal samples (Figure 1). Sample A was dispersed only with 4 volumes of PBS and sample B was routinely processed with 4 volumes of freshly prepared DTT (0.1% final concentration). The diluted suspensions A and B were subdivided into 2 aliquots. One—A1 and B1—was used for baseline IgE measurements and the other—A2 and B2—was spiked with known quantities of total and specific IgE. Comparison between samples A and B allows evaluation of the possible effect of DTT on the assay used, as DTT addition is recommended by international guidelines [12]. The third sample, C, was spiked before dispersion (to allow the detection of any interaction of added IgE to sputum components), and then diluted with 4 volumes of DTT. All samples were filtered through a 50-μm mesh and centrifuged (1500 RPM, 10 minutes) and the supernatant was frozen at −70ºC after separation from the cell pellet. For the spiking experiments, a pool of sera from 5 allergic patients (total IgE, 2268 kU/L), with high levels of specific IgE to *D pteronyssinus* (419 kU_A/L) and *P pratense* (14.3 kU_A/L). Spiking was performed with 400 kU/L of total IgE from this pool and the corresponding spiked value was calculated taking into account the final dilution factor associated with each experiment set. The percentage of recovery was calculated with the formula: (Measured mediator in the spiked sample/spiked value + mediator concentration in the native sample) × 100. Good spike recovery was defined as a recovery of over 80% [5,12].

Measurement of Total and Specific IgE Antibodies

Total and specific IgE in induced sputum supernatants were measured by the ImmunoCAP fluoroenzymeimmunoassay (Phadia ThermoFisher Scientific) following the manufacturer's instructions. The detection limit was set at less than 2 kU/L for total IgE and lower than 0.35 kUA/L for specific IgE.

Statistical Analysis

Statistical analyses were performed using the statistical package SPSS (version 16.0). Median and interquartile range (IQR) were used to describe data. The Wilcoxon signed-ranks test was used for within-group data comparisons. Intraclass correlation coefficients were used to measure agreement between samples processed with and without DTT. Two-tailed *P* values of less than .05 were considered significant.

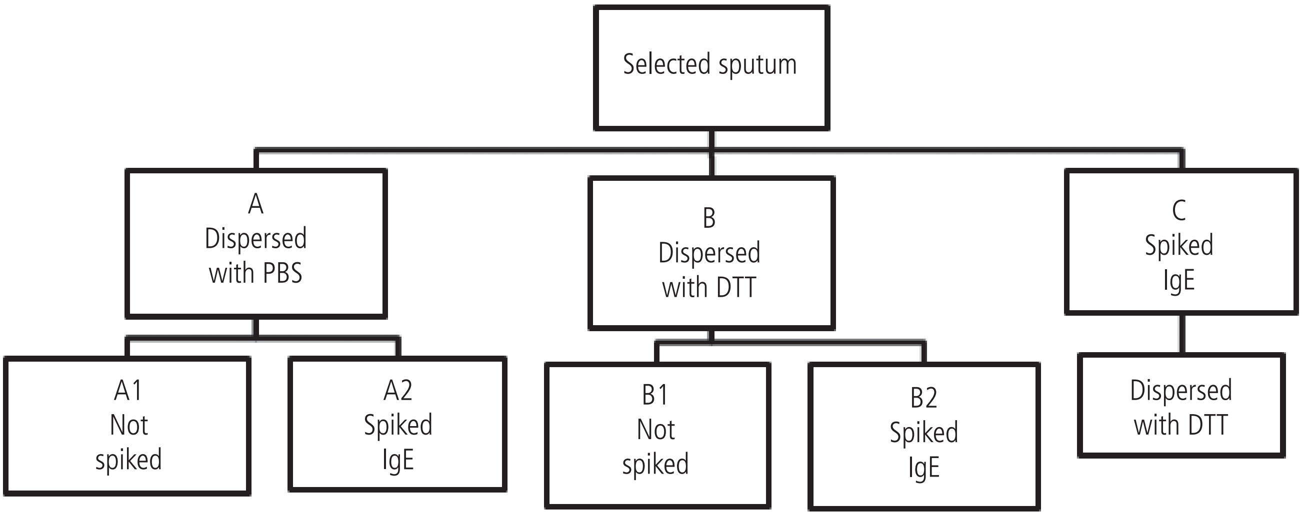

Figure 1. Flow diagram showing protocol for spiking of sputum with immunoglobulin (Ig) E. A, Dispersed with 4 × PBS; A1. Not Spiked; A2. Spiked with IgE; B, Dispersed with 4x DTT; B1, Not Spiked; B2. Spiked with IgE; C, Spiked and dispersed with 4 × DTT. DTT indicates dithiothreitol.

Results

Eighteen patients, 13 with interstitial lung disease (including 5 cases of hypersensitivity pneumonitis [3 bird fanciers disease and 2 suberosis]), 2 with allergic asthma, and 3 healthy controls were evaluated. Twelve of the patients were men and the mean (SD) age was 45.6 (15.8) years.

Low levels of total IgE were found in sputum samples (before spiking), with a median of 5.4 kU/L (IQR, 4.0-6.0 kU/L); there were no significant differences between samples for hypersensitivity pneumonitis or other interstitial lung diseases (data not shown). Specific IgE levels to *D pteronyssinus* and *P pratense* were below 0.35 kU_A/L in all sputum samples. (Table 1 – B1).

Recovery rates of total and specific IgE in sputum samples after the spiking experiments are presented in Table 2, which also shows the comparison between IgE spiking before (C) and after (B2) DTT addition. All recovery rates were above 80% for both total and specific IgE, and no significant differences were found before and after DTT addition, which makes the total and specific IgE measurements valid over a wide range of values (Figure 2).

No differences were found in total IgE measurements of sputum dispersed with DTT or PBS (Table 2), and there was a good intraclass correlation coefficient between both measurements (0.81; 95% CI, 0.25-0.95; *P*=.01). Also, no significant differences in total or specific IgE recovery rates were found between DTT and PBS-processed sputum.

Table 1. Immunoglobulin (Ig) E Levels and Recovery Rates in Sputum Supernatants[a]

Mediator	Sample Measurement (B1[b])	Recovery: Spiking Before DTT (C[b])	Recovery: Spiking After DTT (B2[b])	P[c]
Total IgE	5.4 (4-6)	84 (78-98)	107 (98-114)	.31
Samples tested, No.	18	11	13	9
Specific IgE Dp	<0.35	96 (87-106)	88 (75-102)	.21
Samples tested, No.	18	10	15	9
Specific IgE Ph	<0.35	112 (100-123)	103 (96-113)	.21
Samples tested, No.	18	8	12	8

Abbreviations: Dp, *Dermatophagoides pteronyssinus*; DTT, dithiothreitol; Ph, *Phleum pratense*.
[a]Total (kU/L) and specific IgE (kUA/L) levels in sputum supernatants and their recovery rates in samples spiked before and after sputum dispersion with DTT. Data presented as median (interquartile range).
[b]See Figure 1.
[c]Wilcoxon signed-ranks test comparing recovery rates with spiking before and after DTT addition.

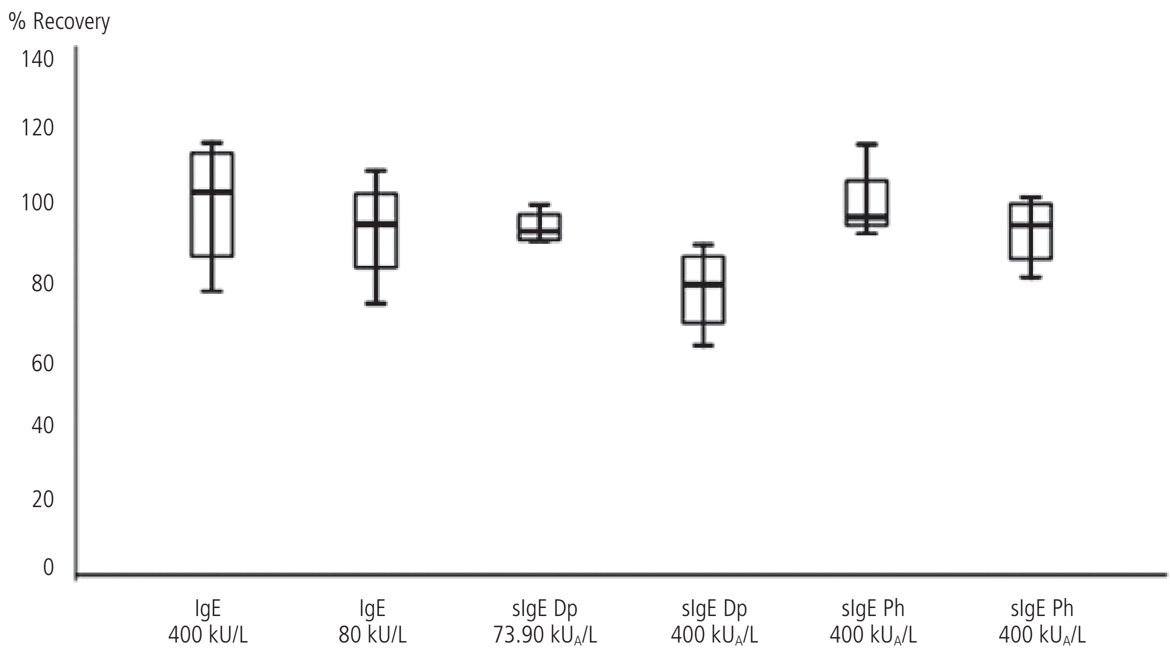

Figure 2. Boxplots showing median and interquartil range percentage of recovery after spiking experiments corresponding to different levels of total and specific immunoglobulin (Ig) E to *Dermatophagoides pteronyssinus* (Dp) and *Phleum pratense* (Ph). Spiking was performed with 400 kU/L of total IgE from a known pool and the corresponding spiked values were calculated taking in account the final dilution factor associated with each experiment set. Horizontal lines represent the median and interquartile range. Vertical lines represent the connection between minimum and maximum recovery rates values. sIgE indicates specific IgE.

Discussion

In this study we have shown that low levels of total IgE (mean, 5.4 kU/L) can be quantified in sputum supernatant using a widely available immunoassay. Moreover, spiked total and specific IgE both had recovery rates above 80%, validating these measurements over a wide range of total and specific IgE levels.

With a good recovery rate (>80%) it can be accepted that the assay is valid as proposed by international recommendations [5]. In some samples the calculated recovery was over 100%. This occurred in both PBS and DTT experiments and may be due to technical issues (pipetting technique, effects of freezing and thawing) or to unknown factors in individual sputum samples which may interfere with detection.

Our study has some limitations that might restrict the generalization of results. First, in some patients the volume of sputum collected was not sufficient to perform all the planned measurements and spiking experiments; therefore, PBS and spiking experiments were not possible in some cases. Second, and most important, we used samples mostly from patients with non-IgE-mediated lung diseases. The population studied allowed us to validate the laboratory methodology and technical interferences, but does not allow us to address the possible clinical significance of IgE measurement in sputum supernatant. International recommendations state that sputum from a range of individuals with different clinical conditions and disease severity should be used in spiking experiments during the validation of fluid-phase mediators [5]. Therefore, further studies including atopic and nonatopic asthmatics should be performed to evaluate the clinical implications of our findings.

Some previous studies have reported the measurement of IgE in sputum supernatant in asthmatics. Nahm and Park [8], for instance, demonstrated the presence of allergen-specific IgE antibodies to house dust mites in sputum samples from house dust mite–sensitive asthmatics using enzyme-linked immunosorbent assay (ELISA) and immunoblot analysis. They reported a significant association between sputum eosinophilia and allergen-specific IgE antibodies in induced sputum (but not with serum specific IgE) [7]. Margarit et al [9] measured total IgE in induced sputum in both asthmatics and controls using a commercial immunoassay (UNICAP, Pharmacia). They found a mean (SD) value of total IgE in sputum of 43.2 (23.0) kU/L in asthmatics (25.6 [3.0] in controls), suggesting that IgE in sputum might be produced locally. None of these studies, however, attempted to validate the laboratory quantification of total or specific IgE in induced sputum, as we have done using a commercially available assay.

There is also growing evidence supporting a local allergic-type immune response in some patients with negative systemic allergic markers (skin prick tests and/or serum specific IgE). The concept of localized mucosal allergen-specific IgE production in the absence of systemic atopy is not new. Indeed, as far back as 1975 [14] allergen-specific IgE was detected in the nasal secretions of nonatopic patients with rhinitis, and in

Table 2. Immunoglobulin (Ig) E Measurements and IgE Recovery Rate in Samples Processed With PBS and DTT[a]

Mediator	Sample Measurement			IgE Recovery, %		
	PBS (A1[b])	DTT (B1[b])	P[c]	PBS (A2[b])	DTT (B2[b])	P[c]
Total IgE	5.3 (4.1-6-6)	5.4 (4-6)	.767	102 (99-112)	107 (98-114)	.08
Samples tested, No.	11	18	10	5	13	5
Specific IgE Dp	<0.35	< 0.35	NA	83 (76-100)	88 (75-102)	.73
Samples tested, No.	13	18		8	15	7
Specific IgE Ph	<0.35	< 0.35	NA	117 (94-123)	103 (96-113)	.35
Samples tested, No.	10	18		5	12	5

Abbreviations: Dp, *Dermatophagoides pteronyssinus*; DTT, dithiothreitol; NA, nonapplicable; PBS, phosphate-buffered saline; Ph, *Phleum pratense*.
[a] Comparison of total IgE (kU/L), specific IgE (kU_A/L), and spiked IgE recovery rate (%) between samples processed only with PBS and processed normally with DTT. Data presented as median (interquartile range).
[b] See Figure 1.
[c] Wilcoxon signed-ranks test.

1983 it was reported that a subgroup of nonallergic patients with rhinitis presented a similar reaction after allergen nasal provocation to that seen in atopic patients [15]. More recently, Powe et al [16] showed that up to 42% of patients with nonallergic rhinitis have mucosal features suggestive of allergic disease (IgE$^+$ cells, mast cells, and eosinophils), and that up to 30% of these patients present mucosal allergen-capture [17]. Local IgE production has also been reported in esophageal mucosa [18], gut [19], adenoid tissue [20], and also in the lower airways [21-26]. Comparisons of bronchial biopsies from atopic and nonatopic asthmatics have shown molecular features associated with IgE switching (elevated interleukin [IL] 4 and IL-13), expression of germline gene transcripts, and Fc epsilon RI molecular RNA in both groups [20-24]. More recently, Takhar et al [26] provided the first direct evidence that bronchial mucosal B cells undergo class switch recombination from IgM and IgG to IgE in situ, both in atopic and nonatopic asthmatics, suggesting that this phenomenon might be a feature of asthma and might occur irrespective of the atopic status of the asthmatic patients as assigned by skin prick tests or allergen-specific IgE.

Sputum induction is a safe, noninvasive method for the assessment of inflammatory airway diseases in both adults [27] and children [28]. Measurement of induced sputum cell counts is reliable, valid, and responsive to changes in disease activity [27]. Many inflammatory mediators have been measured in sputum supernatant, including cytokines, chemokines, granulocyte proteins, vascular leakage markers, eicosanoids, proteases and others [5]. The quantification of mediators in induced sputum can be influenced by multiple factors, including the induction protocol, the sputum processing method, and the use of different quantification assays [13]. However, the reproducibility, precision, and validity of many of these determinations in sputum have not been investigated and, therefore, their utility as research and clinical tools remains uncertain and requires further study [5].

DTT, an agent with low redox potential that can reduce and split glycoprotein disulphide bonds, has been used since 1978 [29] to disperse sputum before the processing of smears, as it was shown that treatment with DTT did not affect cell morphology. However, it was not until 1997 that Efthimiadis et al [30] published the first comparison of inflammatory cells and fluid-phase mediators between induced sputum treated with DTT and PBS. They showed that DTT treatment disperses cells more effectively, and leads to higher eosinophil cationic protein (ECP) measurements and lower cell viability, while no significant changes were found with respect to differential cell counts, IL-5, or IL-8 when compared with PBS-processed sputum. A summary of the effects of DTT on fluid-phase sputum mediators can be found elsewhere [5]. In our study, DTT did not affect IgE quantification, with a good intraclass correlation coefficient between measurements in sputum with and without DTT. We also found no differences in total and specific IgE recovery rates between DTT-dispersed and PBS-processed sputum. In their aforementioned study using an "in-house" ELISA assay, Nahm and Park [7] reported that the addition of DTT to sputum and serum samples decreased allergen-specific IgE bindings and increased nonspecific bindings. However, these effects were only significant with DTT concentrations above 0.5%, which is much higher than the recommended concentrations we used (0.1%). Moreover, no differences in IgE recovery rates between DTT-dispersed and PBS-processed sputum were seen in our study, further excluding the possibility of interference with detection antibodies and solid-phase binding in the ImmunoCAP system. We also have shown that IgE measurements are valid over a wide range of values and that the incubation and dispersion of samples during sputum processing does not affect IgE levels.

Our findings, which show that total and specific IgE measurements in induced sputum are valid over a wide range of levels using a commercially available immunoassay, warrant future study of induced sputum IgE quantification in both atopic and nonatopic patients with asthma, as well as further research to evaluate a local/mucosal component of the IgE response in allergic airway disorders.

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Conflicts of Interest

All authors deny any financial or personal relationship that could result in a conflict of interest with regard to the published article.

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