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Novel Method to Process Cystic Fibrosis Sputum for Determination of Oxidative State

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Cystic fibrosis · Dithiothreitol · Glutathione · Induced sputum

Abstract
Background: Induced sputum is the most commonly used method to analyze airway inflammation in cystic fibrosis (CF) patients ex vivo. Due to the complex matrix of the sample material, precise and reliable analysis of sputum constituents depends critically on preanalytical issues. ***Objectives:*** Here we compared the commonly used method for sputum processing by dithiothreitol (DTT) with a novel mechanical method in regard to basal cellular parameters, neutrophil markers and glutathione (GSH) levels. ***Methods:*** Sputum samples from CF patients were processed in parallel with or without the use of DTT. The key improvement of the mechanical method was the processing in many very small aliquots. Cellular and humoral markers were assessed and compared according to Bland-Altman. ***Results:*** Total cell count, cell viability, differential cell count, neutrophil elastase levels and flow cytometrically analyzed neutrophil markers (CD63, CD11b, DHR) did not differ between the two methods. Intracellular and extracellular GSH levels were significantly higher in DTT-treated samples ($p = 0.002$). ***Conclusion:*** The mechanical sputum-processing method presented had a similar yield of cells and fluids as the conventional DTT method and the advantage of omitting the introduction of reducing agents. This method allows a more reliable analysis of redox-dependent airway inflammation in sputum cells and fluid from CF patients than methods utilizing DTT.

Background

Cystic fibrosis (CF) is the most frequent hereditary disease in Caucasians [1] caused by mutations in the CFTR (cystic fibrosis transmembrane conductance regulator protein) gene [2]. Although CF is a multiorgan disease, morbidity and mortality result mainly from progressive pulmonary disease [3]. The lung involvement in CF is characterized by an excessive neutrophilic inflammation in the airways associated with inflammatory markers, e.g. high levels of IL-8, elevated neutrophil elastase (NE) activity [4], and increased oxidative damage [5]. Past studies found that CFTR not only regulates the efflux of chloride but also of other organic anions such as glutathione (GSH) [6]. Previous data report that GSH

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concentrations are low in epithelial lining fluid of CF patients [7, 8]. Elevation of GSH levels in type II alveolar epithelial cells downregulated the proinflammatory cytokines IL-1β, IL-8 and tumor necrosis factor-α [9]. In clinical studies elevation of GSH levels by oral administration of the GSH prodrug N-acetylcysteine in CF patients over 4 weeks decreased levels of IL-8 in induced sputum samples [10] and a 2-week inhalation of GSH in CF patients decreased levels of the proinflammatory mediator PGE_2 [11], while GSH depletion was associated with elevated oxidative stress-mediated proinflammatory signaling [12]. Further studies suggest that GSH downregulates inflammatory response by inhibiting transcription factors such as NF-κB or AP-1 [13, 14]. Thus, in addition to its antioxidant property GSH plays a key role in immune responses [14].

In the past years, induced sputum samples are increasingly used, as they are a useful and minimal invasive tool to monitor pulmonary diseases [15–22]. Because of the high viscosity induced sputum samples of CF patients need to be dispersed before analysis. Among several methods evaluated, the method utilizing dithiothreitol (DTT) is widely used because of its ability to split disulfide bonds and disperse sputum samples [15]. However, due to its reducing and denaturing effect DTT has been shown to preanalytically affect levels of various mediators [23–28] and it is most likely that DTT also influences oxidant-dependent variables like GSH.

Several studies have assessed GSH levels in induced sputum samples of patients with various pulmonary diseases including CF [29], asthma [25, 30], and COPD [31, 32] without the use of DTT for sample processing. Though one study demonstrated an effect of DTT on the assay used for GSH measurements [30], none of these studies systematically compared methods with and without the use of DTT to assess changes in intra- and extracellular GSH levels and to analyze the impact on other relevant cellular parameters.

Our laboratory established a mechanical method without the use of DTT (hereinafter referred to as 'D-PBS method') for sputum processing to investigate oxidative state and inflammation in CF-induced sputum samples. Here we compared the new method with the conventional DTT method and the results show equivalence for basal cellular variables and superiority for extracellular and intracellular GSH measurements, which are artificially elevated by the DTT method. These results are clinically relevant and very important for the increasing number of studies analyzing airway inflammation in CF patients via sputum induction.

Material and Methods

Study Design

The study was approved by the institutional review board of the University of Munich and informed consent from all CF subjects was obtained prior to the study. Induced sputum samples from 10 CF patients were used in this study. Following the application of 200 μg of the β_2-sympathomimetic salbutamol, subjects inhaled hypertonic saline solution (5.85%) for 15 min using a Pari Boy SX combined with an LC Sprint jet nebulizer (Pari, Munich, Germany). To avoid contamination, saliva was expectorated into a separate container. Patients were encouraged to produce sputum samples at 5-min intervals. The first portion was used for microbiological analyses. Subsequent portions were transported to the laboratories on ice. Plugs were selected with forceps and the sample was divided into two approximately equal aliquots and processed within 30 min side by side in two ways. One aliquot was homogenized with 4 times the volume of D-PBS and 5 times the volume of 10% DTT (Calbiochem, Darmstadt, Germany) in a shaking water bath with 37°C for 15 min as routinely used and previously described [33]. The other aliquot was divided with two sterile blunt forceps into small portions of approximately 0.2 g and 9 times the volume of D-PBS was added. Samples were homogenized by gentle passage repeated 20 times through an 18-gauge needle. For some experiments, a further aliquot was divided and homogenized with 4 times the volume of D-PBS and 5 times the volume of 10% DTT on ice for 15 min.

The following steps were carried out in parallel for both methods. Homogenized samples were consecutively filtered through a 100- and 40-μm cell strainer and centrifuged at 300 *g* and 4°C for 10 min.

The supernatant was carefully transferred into other vials and centrifuged again at 10,000 *g* and 4°C for 5 min. The supernatant was pooled and divided into Eppendorf cups. One part was immediately frozen at –80°C until analysis. The other part was used for determination of thiols. To assess free GSH levels, samples were precipitated with trichloroacetate (Sigma-Aldrich, Munich, Germany) and centrifuged at 1,500 *g* and 4°C for 5 min. The supernatant was frozen immediately at –80°C until analysis. To measure total GSH levels, tributylphosphine (Sigma-Aldrich) buffer was added, and samples were incubated for 30 min at 4°C. Then the samples were precipitated and centrifuged at 1,500 *g* and 4°C for 5 min. The supernatant was frozen immediately at –80°C until analysis.

Cell suspension was resuspended with D-PBS, total cell count was determined in a Neubauer chamber and cell viability was assessed with the trypan blue exclusion method. Cytospin slides for differential cell counts with 30,000 cells per slide were prepared, fixed and stained according to May-Grünwald-Giemsa. The remaining cell suspension was diluted to 10^6 cells/ml and was used for FACS analysis.

Measurement of GSH Levels in Sputum Supernatant

Thawed samples were derivatized with 7-fluorobenzofurazan-4-sulfonic acid ammonium salt (SBD-F; Fluka, Munich, Germany) in boric acid (Merck, Darmstadt, Germany) buffer and were incubated at 60°C for 1 h as described previously [34]. Samples were stored at 4°C until analyzed by means of reversed-phase HPLC (RP-HPLC) within 24 h. Kuhn et al. [34] gave a detailed description of the running conditions. Standard measurements

Table 1. Comparison of methods of processing sputum samples

	Methods	
	D-PBS	DTT
Used weight, g	0.70 (0.35–0.86)	0.71 (0.3–0.93)
Total cell count		
10^6 cells/ml	6.47 (2.15–10.76)	6.51 (3.06–11.56)
Differential cell counts, %		
Neutrophils	96.89 (92.5–99.3)	97.06 (87.5–99.7)
Eosinophils	0.26 (0.0–2.3)	0.21 (0.0–1.8)
Basophils	0.19 (0.0–1.0)	0.31 (0.0–1.5)
Macrophages	0.94 (0.0–2.0)	1.18 (0.0–6.0)
Lymphocytes	1.35 (0.0–4.5)	1.18 (0.0–5.0)
Cell viability, %	83.7 (77–92)	83.5 (72–88)

All data are given as means (with the range in parentheses) from 10 different subjects. No significant differences were found for investigated variables.

were performed after analysis of ten samples. All runs were performed in duplicate. If deviation of duplicates was greater than 5% a third run was carried out. The mean deviation of the duplicates was 3.2% within a range of 0.2–7.1%. GSH concentrations were calculated with respect to GSH standard measurements which were carried out after every tenth sample. Intra-assay and inter-assay variability of the GSH standards were tested by linear regression ($r^2 = 0.997$ and $r^2 = 0.97$, respectively). In previous tests recovery experiments were performed by spiking and the mean recovery rate was 94% within a range of 87.6–99.6%.

FACS Analyses

Monoclonal fluorochrome-labeled antibodies were purchased from BD Pharmingen (Heidelberg, Germany) unless otherwise specified. Markers of interests were CD63, CXCR1, CD11b, CD15s, Cam5.2, CD3 and CD4. To assess intracellular respiratory burst we used the dihydrorhodamine (DHR) 123 (AnaSpec/MoBiTec, Göttingen, Germany) method [29]. Intracellular GSH levels were measured with monochlorobimane (Fluka) [10, 35]. Samples were measured with an FACSCalibur (Becton Dickinson, Heidelberg, Germany) and an FACS Canto II (Becton Dickinson). Results were analyzed with Cell Quest software version 1.3 (Becton Dickinson) and BD FACS Diva (Becton Dickinson) software, respectively. The results are given in mean fluorescence intensity. We used annexin V and propidium iodide to exclude apoptotic (annexin V+/propidium iodide–) and necrotic (annexin V+/propidium iodide+) cells from analysis as well as isotype controls to exclude nonspecific bindings. For DHR, monochlorobimane, annexin V, and propidium iodide negative controls were applied.

Measurement of NE

NE was analyzed in sputum supernatant by a chromogenic spectrophotometric assay as described elsewhere [36].

Statistical Analysis

Agreement between the two methods was analyzed by the Bland-Altman method [37]. The difference of the two methods (bias) is analyzed as a function of the mean of the two measurements of each sample. If the two methods produce the same result, the average of the bias will be close to zero. Agreement was considered to be acceptable if differences of means were approximately 5% or less.

Means and standard error of means or ranges are given for each variable. The sputum processing protocols were compared for differences with the nonparametric Wilcoxon-signed rank test for paired values. $p < 0.05$ was considered statistically significant.

Results

Patient Characteristics

Ten patients with CF were included in this study, 4 of whom were males (mean age 31.1 years, ranging from 22 to 41 years). Nine patients were infected with *Pseudomonas aeruginosa*. Mean FEV_1 (% of predicted) was 54.8% (range: 24.8–87.4%). All patients were stable and none had pulmonary exacerbations as defined by Fuchs et al. [38] within the preceding 4 weeks. No adverse events, i.e. bronchoconstriction, occurred due to the induced sputum procedure.

Cell Count and Cell Viability

The amount of sputum obtained was sufficient in all subjects and the average weight was 1.6 g (range: 0.679–2.23 g). There was no statistically significant difference for total cell count, differential cell count, and cell viability for the D-PBS and DTT method (table 1). Agreement of the two methods was high for neutrophils, but relatively low for other cellular subpopulations (table 2).

Neutrophil Markers

In the set of neutrophil surface markers used in this study and respiratory burst in neutrophils no significant difference was found for both methods (fig. 1). The agreement of the two methods was acceptable for CD63, CD11b, and DHR123, whereas it was relatively low for CXCR1 (table 2). NE levels in samples treated with DTT were comparable to those treated with PBS and the agreement of the two methods was good (fig. 2; table 2).

Intracellular GSH Levels

Cell type-specific intracellular levels of GSH were analyzed by flow cytometry. All samples were sufficient for measurement of neutrophils and epithelial cells. In neutrophils and epithelial cells intracellular GSH levels were significantly higher in DTT-treated samples compared to

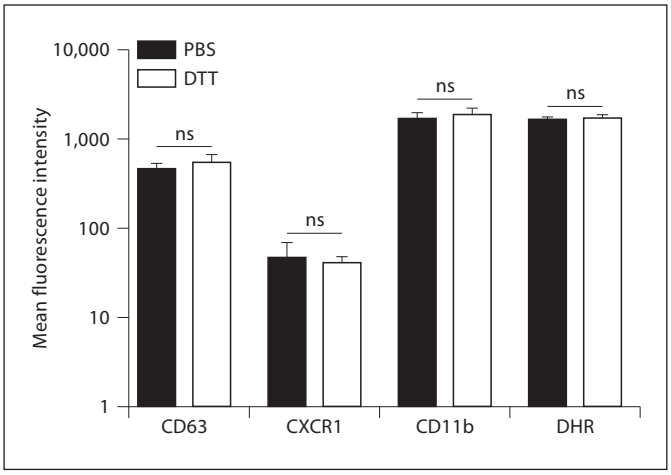

Fig. 1. FACS analysis of neutrophil markers and respiratory burst. Mean fluorescence intensity of CD63, CXCR1, CD11b, and DHR on neutrophilic granulocytes in IS samples processed with either D-PBS or DTT (n = 10) is shown. CD63 is a specific marker for intracellular primary granules containing active substances like myeloperoxidase and NE and is expressed on the surface by fusion of the granules with the cell membrane [10]. CXCR1 is a well-known receptor for the proinflammatory chemokine IL-8, which is abundant in airway fluids of CF patients. CD11b is expressed on neutrophils after diapedesis into the airways and is also thought to play a role in the phagocytosis activity of neutrophils [4]. None of the markers differed significantly between the two processing methods. Agreement was good for CD63 and DHR and acceptable for CD11b, whereas for CXCR1 it was poor.

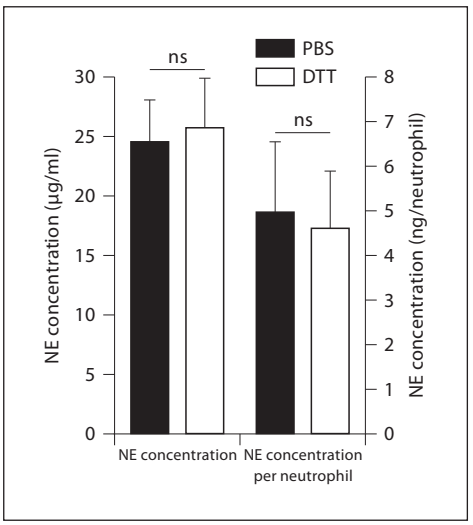

Fig. 2. NE levels. NE concentrations (left y-axis) in sputum supernatant and NE concentrations normalized to neutrophil counts (right y-axis) are given. There was no significant difference between the two methods. In one sample pair NE was not detectable (n = 10).

Table 2. Bland-Altman agreement of methods of processing sputum samples

	Bias, % difference of the two methods compared
Basal cellular parameters	
Total cell count	5.5 ± 5.5
Cell viability	–0.9 ± 1.6
Differential cell count	
Neutrophils	0.1 ± 0.7
Basophils	–12.2 ± 12.2
Eosinophils	29.5 ± 66.6
Lymphocytes	–21.4 ± 38.8
Monocytes	–41.4 ± 25.2
Neutrophil elastase	
Concentration in supernatant	2.3 ± 4.6
Concentration per neutrophil	–3.6 ± 7.0
Neutrophil markers in flow cytometry	
CD63	–0.6 ± 13.9
CXCR1	34.7 ± 32.3
CD11b	7.3 ± 5.5
DHR	–0.9 ± 7.1
Intracellular GSH levels	
Neutrophils	138.3 ± 14.5
Lymphocytes	150.3 ± 16.7
Epithelial cells	86.9 ± 15.0
Extracellular GSH levels	
Free GSH	105.9 ± 7.2
Total GSH	4.7 ± 5.1
Ratio free/total GSH	102.4 ± 7.3

Summary of the comparison according to Bland-Altman of the two methods is given, i.e. the novel mechanical sputum processing method and the conventional DTT method. For easy comparison the % of bias and their standard error of mean are given. A total of 10 pairs were analyzed except for intracellular GSH levels in lymphocytes, where only in 5 sample pairs the numbers of events were appropriate to perform FACS analysis.

D-PBS-treated samples (p = 0.002). In line with these data, the agreement between the two methods was not acceptable, suggesting that the thiol-independent method of sample preparation should be used if intracellular GSH is assessed (table 2). In lymphocytes the number of events detected was too low in 3 samples to assess the D-PBS method and in 4 samples to assess the DTT method. These samples were not included in the analyses and the numbers were too low to reach the significance level set before (fig. 3).

Independent of the processing method, intracellular GSH levels were lower in neutrophils than in lympho-

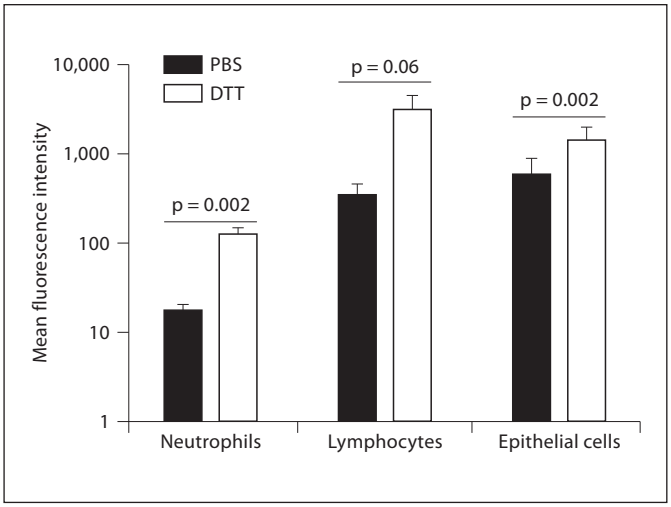

Fig. 3. Intracellular GSH levels. Comparison of GSH levels intracellularly assessed by FACS is shown. Intracellular GSH levels in samples processed with the DTT method were significantly higher in neutrophil granulocytes and epithelial cells ($p = 0.002$; $n = 10$). In lymphocytes, counted events in 3 samples processed with D-PBS and 5 samples processed with DTT were too low and were taken out of analysis. Statistically, there was only a tendency of higher intracellular GSH levels in lymphocytes in DTT samples compared to D-PBS samples ($p = 0.06$). Cam5.2 is a pan-epithelial marker [46], CD15s is expressed on granulocytes, while CD3 and CD4 are well-known markers for T cells. These markers were used for classification of the cell populations.

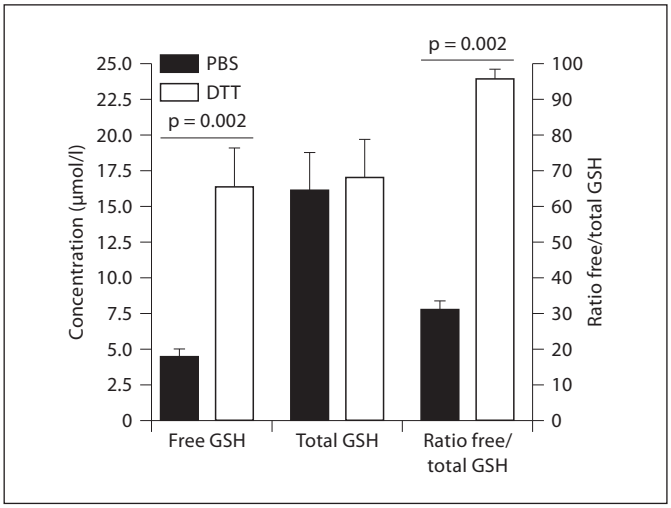

Fig. 4. Extracellular GSH levels. Extracellular levels for free and total GSH (left y-axis) and the ratio of free/total GSH (right y-axis) measured in induced sputum supernatant by RP-HPLC are given. Free GSH levels and the ratio of free/total GSH were significantly higher with the DTT method compared to the D-PBS method ($p = 0.002$; $n = 10$).

cytes and epithelial cells ($p < 0.05$ and $p = 0.002$, respectively). The latter two cell types had comparable intracellular GSH levels (fig. 3).

Assessment of Extracellular GSH by RP-HPLC

Extracellular free GSH levels were significantly higher in samples treated with DTT compared to those processed with D-PBS ($p = 0.002$). The levels of free GSH in samples treated with DTT were comparable with total GSH levels in samples treated with both methods. Mean ratio of free/total GSH was 31% (range: 15.8–41.5%) and 95.6% (range: 82.7–108.6%) in samples treated with D-PBS and DTT, respectively (fig. 4). In concordance with these results, the agreement of the two methods was poor (table 2), clearly showing an about 100% significant increase in free extracellular GSH and intracellular GSH (see above) after treatment with DTT. As expected, total GSH measured after complete reduction of the sample, which leads to availability of all thiol groups in the samples, showed comparable levels and good agreement of the two methods (table 2).

There Are No Temperature-Driven Effects on Determination of GSH

To exclude temperature-driven effects of DTT on GSH measurements, 4 induced sputum samples of CF patients were processed as mentioned above (1) by the presented method using D-PBS, (2) with DTT at 37°C, and (3) additionally with DTT on ice. The effect of DTT on GSH measurements was similar for both temperature conditions. These results indicate that the determination of GSH was not dependent on the temperature at which the preparation of sputum was done in the presence of DTT. Thus, elevated GSH levels were due to the addition of DTT (for all online supplementary data, see www.karger.com/doi/10.1159/000271607.

Discussion

Induced sputum is the most commonly used noninvasive method to analyze airway inflammation in CF patients ex vivo, but owing to the complex matrix of the sample material, precise and reliable analysis of sputum constituents depends critically on preanalytical issues. Here, we systematically compared the conventional method used for sputum processing by DTT with a novel method developed in our laboratories and demonstrat-

ed that the presented sputum processing method is superior to the conventional DTT method, in particular for measuring redox-dependent parameters, such as GSH, in sputum samples. These results are novel and clinically relevant for the increasing number of studies analyzing airway inflammation in CF patients via sputum induction.

DTT is a strong reductant which disrupts the sputum matrix that is stabilized by disulfide bonds and thus allows the liberation and analysis of cells and solutes. However, several studies pointed out effects of DTT on various markers [23, 25–28]. In this study we systematically analyzed the effects of the standard DTT sputum processing method on various cellular variables as well as on extra- and intracellular GSH levels in comparison to a novel method using mechanical disruption of very small aliquots of the samples. Viability, number of total cells recovered and cell differential were not significantly different between the two methods. The Bland-Altman analysis revealed that the agreement between the DTT method and the mechanical method was appropriate for total cell count, cell viability and differential cell count for neutrophils while it was low for differentials of other cell types. This was most likely due to the small numbers of the other cell types in investigated induced sputum samples (tables 1, 2). However, free extracellular and intracellular GSH levels were significantly higher in samples treated with DTT. Agreement analyses also revealed significant differences in extracellular and intracellular GSH measurements except for measurements of extracellular total GSH (table 2). This result may be expected because of the reducing capacity of DTT in releasing GSH from disulfide bonds in the same manner as tributylphosphine which we used to detect total GSH levels in our method. This altering effect of DTT was already shown in a previous study for extracellular GSH concentrations in induced sputum samples of patients with asthma [25], but to the authors' knowledge an alteration of intracellular GSH levels, i.e. the intracellular redox potential, has not been shown until now.

The alteration of GSH levels by DTT shown in this study could also be due to interference of DTT with the method for GSH measurements. Previous studies have shown significant effects of DTT on GSH measurements by a spectrophotometric method using the reagent DTNB which was – at least in part – due to the reduction of DTNB itself by DTT [25, 29, 30]. As for the RP-HPLC method we used for the present study, Kuhn et al. [34] previously reported that the reaction of DTT and SBD-F yields fluorescent by-products. To exclude possible interference, blanks with or without the addition of DTT were analyzed. Latter measurements revealed unspecific peaks after retention times of approximately 6.5–6.8 and 7.8–8.5 min in the presence of DTT. As the peak for GSH appears at approximately 8.6–8.9 min, the by-products should not or only minimally interfere with our GSH measurements (online suppl. data). This is also supported by our results on total GSH levels which were comparable with samples processed with and without DTT, indicating that there was no significant interference of DTT with the RP-HPLC assay we used in this study (fig. 4). Additionally, monochlorobimane – which was used for detection of intracellular GSH in the present study – is a non-fluorescent probe which is able to permeate living cells and specifically links to GSH via glutathione-S-transferase resulting in the fluorescent adduct glutathione-S-bimane [10]. The specificity of the intracellular enzyme system makes it unlikely that other DTT by-products are detected by this method. The results we obtained with the RP-HPLC method and with the flow cytometry method with monochlorobimane were consistent. Taken together, our results indicate that higher levels of GSH in DTT-treated samples result from the ability of DTT to break up disulfide bonds of protein disulfide (RS-SG).

In a previous comparative study using DTT or D-PBS for the preparation of sputum samples from patients with asthma, cell viability was higher with the D-PBS method while total cell count was higher when samples were processed with DTT [15]. The use of an optical isomer of DTT, dithioerythritol, also yielded a higher recovery of total cells compared to D-PBS [23]. However, in our study there was no significant difference and agreement was acceptable between the two methods regarding the means of cell count, cell differential and cell viability. The critical step responsible for the improved recovery by our method was the homogenization step. In pilot experiments we found that it is critical to divide the sputum aliquots into further small portions not larger than approximately 0.2 g and to homogenize these by gentle aspiration and dispersion in a 9-fold volume of D-PBS. Such a procedure was omitted in earlier studies. Previously, the authors treated the samples with PBS without any further homogenizing step [15]. Louis et al. [23] reported that sputum samples were agitated for 10 s and rocked for a further 30 min for either method. In case only PBS is used it is questionable whether this is enough to disperse cells in highly viscous sputum samples like those of CF patients.

The effect of DTT on the measurement of NE levels was tested in studies with a cohort of patients with CF [39] and chronic obstructive pulmonary disease [40]. No signifi-

cant changes were reported, which is in accordance with our findings. NE levels and levels normalized to neutrophil numbers in supernatant as assessed by Bland-Altman analysis were comparable for both methods (table 2).

As for the effect of DTT on surface markers and respiratory burst in neutrophils as assessed by flow cytometry in this study, to the authors' knowledge there have so far been no comparative studies. However, in this study DTT had no effect on CD63 and DHR, but a small effect on CD11b and a significant effect on CXCR1 measurements when compared to the D-PBS method. A possible explanation for the increased CXCR1 expression found on the neutrophil surface after DTT treatment could be the higher accessibility of anti-CXCR1 antibodies to the binding site. Within CXCR1, an extracellular disulfide bond exists between the N-terminus and the first extracellular loop [41]. DTT could break down this disulfide bond and change the conformation and antibody accessibility of the receptor.

In a previous study, our laboratory showed that DTT had no effect on measurements of the cytokines IL-1β and tumor necrosis factor-α, the leukotriene LTB_4, and the chemokine IL-8 in sputum samples from patients with CF (online suppl. data) [42]. Similarly, other studies did not find altered levels of IL-8 in sputum samples treated with either DTT or dithioerythritol compared to PBS in patients with asthma [15, 23]. In contrast, other studies did find DTT-dependent changes in measured levels of IL-8 as assessed by ELISA [40, 43] and in a range of cytokines and chemokines as analyzed by a multiplex bead array immunoanalysis [26]. However, in an extensive review of Kelly et al. [28] most of these markers were shown to be unaffected by DTT. Neverthelss, the ratio of the redox pair GSH/GSH disulfide (GSSG) is known to play an important role in the activation of the proinflammatory transcription factor NF-κB [44]. Elevation of intracellular GSH levels and GSH/GSSG ratio resulted in the inhibition of NF-κB activation [45]. Although it seems unlikely that the short incubation time (i.e. 15 min) of sputum samples with DTT exerts effects on the NF-κB-driven inflammatory response, to the authors' knowledge short-term effects of DTT on inflammatory pathways have never been investigated until now.

Conclusions

We developed a novel mechanical method processing many small portions of induced sputum samples. We show that this method is equivalent to the commonly used DTT method for measurement of many cellular and fluid phase variables in sputum samples. Additionally, the D-PBS method – in contrast to the DTT method – does not affect redox-dependent variables such as GSH. These results are novel and clinically relevant for the increasing number of studies analyzing airway inflammation in CF patients via induced sputum.

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