Effect of Physical and Chemical Methods of Homogenization on Inflammatory Mediators in Sputum of Asthma Patients*

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**Background:** Dithiothreitol (DTT), which is used for sputum homogenization, may split S-S bonds of the bronchial mucins as well as other proteins and, thus, may have a detrimental effect on inflammatory mediators that are present in sputum.

**Objective:** To evaluate the effects of physical sputum homogenization, using ultrasonic and chemical (i.e., DTT) means, on the concentrations of eosinophil cationic protein (ECP), eosinophil protein X (EPX), eosinophil peroxidase (EPO), and myeloperoxidase (MPO) in the sputum of patients with asthma.

**Methods:** The collection of sputum samples from nine patients with asthma was induced by their inhaling a sterile 3% saline solution for 10 min from an ultrasonic nebulizer. One half of the sputum sample was homogenized by ultrasound, and the other half was liquefied by DTT. The supernatant of the ultrasonically homogenized specimen was divided into the following three portions: (1) immediately frozen; (2) stored for 15 min at 37°C; and (3) additionally treated with DTT. The supernatant of the sputum sample that was liquefied by DTT was divided into the following two portions: (1) immediately frozen; and (2) additionally subjected to ultrasound. The concentrations of ECP, EPO, EPX, and MPO in the sputum samples were measured using immunoassays.

**Results:** Statistically significant differences were found between the ultrasonically homogenized specimens that had been either processed immediately or stored at 37°C and those treated by DTT, but only for concentrations of EPO and MPO (p < 0.005). No effect of temperature on the mediators in the ultrasonically homogenized specimens could be detected. Ultrasonic homogenization had no influence on the mediators in the samples liquefied by DTT. However, the addition of DTT to the cell-free supernatant of the ultrasonically homogenized sputum samples caused a significant fall in measured EPO and MPO concentrations.

**Conclusions:** The sputum processing by DTT caused a statistically significant fall in EPO and MPO concentrations but did not significantly influence the measured concentrations of ECP and EPX.

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**Key words:** dithiothreitol; homogenization methods; induced sputum; ultrasonication

**Abbreviations:** DTT = dithiothreitol; ECP = eosinophil cationic protein; EPO = eosinophil peroxidase; EPX = eosinophil protein X; MoAb = monoclonal antibody; MPO = myeloperoxidase

Eosinophilic inflammation is the hallmark of bronchial obstruction in patients with asthma.1 The inflammatory process is revealed by the presence of inflammatory cells as well as by the concentrations of cellular mediators that are present in bronchial secretions or sputum.2–5

When measuring cellular mediators in bronchial secretions or sputum, the specimens have to be liquefied. There are several methods for liquefying sputum including mechanical or chemical homogenization. Mechanical homogenization using VirTris homogenization, Ten Broeck glass homogenization, and Branson ultrasonic homogenization does not destroy proteins, peptides, or enzymes released from the inflammatory cells into the sputum.6–8 On the other hand, chemical agents used to liquefy sputum,
such as trypsin or chymotrypsin, are known to destroy proteins and peptides in the sputum by cleaving peptide bonds.\textsuperscript{9} Therefore, these agents are no longer used to liquefy sputum samples.

Currently, dithiothreitol (DTT), another sputum-liquefying agent, is used widely for the homogenization of bronchial secretions, because it alters neither cellular morphology nor the microflora in cytologic specimens.\textsuperscript{10} However, this agent possesses free sulfhydryl groups that may split S-S bonds of the bronchial mucins and other proteins, including IgA.\textsuperscript{9} Given these properties, DTT may well have a detrimental effect on inflammatory mediators that are present in sputum.

Therefore, a trial was set up to study the effects of two methods for liquefying sputum (ie, ultrasonic homogenization and chemical homogenization using DTT) on the apparent concentrations of eosinophil cationic protein (ECP), eosinophil protein X (EPX), eosinophil peroxidase (EPO), and myeloperoxidase (MPO) in the sputum of asthma patients.

Materials and Methods

Subjects

Stable patients (seven men and two women; mean age, 54 years; age range, 38 to 68 years) with mild-to-moderate asthma, as defined by the American Thoracic Society,\textsuperscript{11} were chosen for the study. The mean FEV\textsubscript{1} was 77.3% predicted (range, 56 to 110% predicted); the FEV\textsubscript{1}/FVC ratio was 65% (range, 60 to 75%). The median dose of methacholine producing a 20% fall in FEV\textsubscript{1} was 460 µg with a range of 31 to 2,000 µg. Six patients were treated with inhaled budesonide therapy in a dose of 400 to 1,600 µg, and three patients were treated with β-agonist therapy only. None of the subjects had shown evidence of a respiratory infection or an exacerbation of asthma symptoms in the previous 4 weeks. All patients gave informed written consent.

Sputum Processing

To induce sputum formation, the patients inhaled a sterile 3% saline solution for 10 min from an ultrasonic nebulizer (Heyer Mono; Carl Heyer GmbH; Bad Ems, Germany) after pretreatment with inhaled salbutamol, 200 µg. The nebulizer generates particles with a mean diameter of 4.6 µm and has an output of 2 mL/min. The patients were encouraged to cough throughout the procedure and to expectorate all secretions that were raised (ie, sputum and saliva) into a plastic container.

The volume of the sample was measured. One gram of sputum was used to determine the total cell count with a standard hemocytometer. To that purpose, an equal volume of a 20% formalin solution was added to protect cells by fixation before staining, with some modifications, according to a previously described method.\textsuperscript{12} Two smears of the unfixed sputum were prepared and stained using crystal violet.\textsuperscript{13} A differential count of the cell types was performed, including all types of inflammatory and bronchial epithelial cells. The percentage of eosinophils as well as the total number of cells per milliliter of solution was determined.

The remaining sputum sample was divided into two portions. The first one was homogenized ultrasonically, and the second one was liquefied by DTT (Fig 1).

Inflammatory Markers

The EPO concentration was assessed using a prototype fluoroenzyme immunoassay (CAP system; Pharmacia & Upjohn Diagnostics AB; Uppsala, Sweden). Briefly, EPO that was purified, with some modifications, according to a previously described method was used as the standard and ranged in concentration from 0.5 to 200 µg/L.

Monoclonal antibodies (MoAbs) were produced by the hybridoma technique. One MoAb was covalently bound to the prototype fluoroenzyme, and the other MoAb was labeled with the enzyme β-galactosidase. In the assay, EPO in the sputum or the EPO standard reacts with anti-EPO coupled to the solid phase. Bound EPO then is detected by means of enzyme-labeled MoAbs and the substrate. The formed fluorescent product is directly proportional to the level of EPO in the sample.
The cross-reactivity with ECP and MPO in the EPO assay was < 0.3% and < 0.01%, respectively. The detection limit of the EPO assay was 0.5 μg/L, and the intra-assay and interassay coefficients of variation were < 10%.

ECP was measured using the ECP fluoroenzyme immunoassay (Pharmacia & Upjohn) according to the instructions of the manufacturer. The assay procedures were identical to those for EPO. The interassay and intra-assay coefficients of variation were < 12%, and the detection limit was < 2 μg/L.

EPX and MPO were measured using the EPX radioimmunoassay (Pharmacia) and the MPO radioimmunoassay (Pharmacia), according to the instructions of the manufacturer. Briefly, EPX in the sample competes with a fixed amount of 125I-labeled EPX for the binding sites of the specific antibodies that were raised in rabbit. EPX bound to the specific antibodies then is captured using antirabbit IgG coupled to Sepharose. The immune complex then is separated from free EPX by means of centrifugation. The radioactivity of the immune complex is inversely proportional to the level of EPX in the sample. The assay procedure for MPO is identical to that for EPX.

The interassay and intra-assay variations were < 15% and 13%, respectively, and the detection limits were < 3 μg/L and 8 μg/L, respectively.

Sputum samples were diluted at least five times in phosphate buffer (pH, 7.4) containing 0.15 mol/L NaCl, 0.1% bovine serum albumin, 10 mmol/L ethylenediaminetetraacetic acid, and 0.2% of the detergent N-cetyl-N,N,N-trimethyl ammonium bromide, and then they were assayed in duplicate.

Statistical Analysis

Results for the measurement of the inflammatory mediators are reported as median and interquartile range. Because their distribution is not normal, the concentrations of the mediators were log-transformed before analysis. A paired Wilcoxon signed rank test was used to compare the differences between homogenization methods. The global significance level was defined with α = 0.05 for two-sided tests.

RESULTS

The patients produced between 2.57 and 19.21 g sputum over a period of 10 min, the median total cell count was 2.96 × 10^6 mL, the median percentage of eosinophils was 6.6% (range, 0.4 to 54.1%), and the median percentage of neutrophils was 51.6% (range, 7.0 to 96.5%).

The individual values and medians for mediators present in sputum homogenized either by ultrasound or DTT are shown in Table 1. The concentrations for the four mediators in sputum homogenized by ultrasound are much higher than those for the concentrations measured in sputum treated by DTT. However, after log transformation, statistically significant differences among these four mediators in regard to the two homogenization methods were observed only for EPO and MPO (Fig 2).

Figure 2 shows the mean values of the log-transformed apparent concentrations of the mediators in the sputum samples homogenized either by ultrasound or DTT and processed as described earlier (Fig 1). Statistically significant differences were found between the samples homogenized by ultrasound that were either processed immediately or stored at 37°C and those that were homogenized by DTT, but only for EPO and MPO. Considering the effect of temperature at which the samples were homogenized by DTT, no statistically significant effect could be detected on the mediators of ultrasonically homogenized specimens. This was also the case regarding the effect of ultrasound on the mediators of samples liquefied by DTT. However, the addition of DTT to the supernatant of ultrasonically homogenized sputum (U + DTT) caused a significant fall in the measured concentrations of EPO and MPO.

Essentially, homogenization by DTT or the addition of DTT to ultrasonically liquefied sputum specimens caused a significant fall in the measured concentration of the peroxidases EPO and MPO in sputum samples. In contrast, none of the methods...
used to homogenize sputum seemed to have a statistically significant influence on the concentrations of ECP and EPX.

**DISCUSSION**

The effects of different methods for the homogenization of sputum on the inflammatory mediators have not yet been reported. Therefore, we evaluated the effects of physical sputum homogenization (ie, homogenization by ultrasound) and chemical homogenization using DTT on the concentrations of ECP, EPX, EPO, and MPO in sputum samples from patients with asthma. Statistically significant differences were detected between the ultrasonically homogenized samples that were either processed immediately or were stored at 37°C and those that were homogenized by DTT, but only for concentrations of EPO and MPO.

The concentrations of the four mediators in the sputum specimens homogenized by ultrasound were much higher than those measured in the sputum specimens homogenized by DTT. This finding was expected, because ultrasonically homogenized samples contain, apart from spontaneously released cationic proteins, proteins from eosinophils that are damaged during processing. DTT is not likely to be harmful to granulocytes, and, thus, one could only expect the presence of proteins released in vivo in samples homogenized with DTT. However, after the log transformation of the concentrations, statistically significant differences between the two homogenization methods were observed only for EPO and MPO.

In order to assess a possible effect of temperature on mediator concentrations in samples treated by DTT that had to be placed in a water bath at 37°C, the supernatant of the ultrasonically liquefied sputum was stored in a water bath at 37°C for 15 min as well. The measured concentrations of all four mediators of ultrasonically homogenized specimens did not decrease significantly after 15 min of storage at 37°C. This is in contrast to the findings of a previous study, in which a significant decrease in ECP concentration was seen when the specimen was stored at 25°C but only after 24 h of storage. Thus, the decrease of EPO and MPO concentrations in DTT-treated samples was caused by DTT alone.

Similarly, the apparent concentrations of the mediators in the DTT-treated specimens were not influenced when the cell-free supernatant was additionally ultrasonically homogenized. However, the addition of DTT to the supernatant of ultrasonically homogenized sputum caused a significant fall in EPO and MPO concentrations. This is in accordance with the findings of Ayre et al, who reported a significant reduction in the expression of intercellular adhesion molecule-1 in DTT-treated peripheral blood monocytes. Intercellular adhesion molecule-1 is known to be rich in disulphide bonds.

The reason why DTT caused a significant decrease of MPO and EPO may be the molecular structure of those peroxidases. The EPO molecule consists of at
At least two subunits, the MPO molecule of four subunits, two light and two heavy chains containing S-S bonds. These bonds are known to be susceptible to the lytic effects of DTT. On the contrary, ECP and EPX are single-chain proteins and, therefore, probably are more stable.

Another possibility could be the combined effect of DTT treatment and the freezing/thawing process. Although Holz et al. reported that the freezing and storing of sputum samples at $-20^\circ$C was not associated with a major alteration of differential cell counts, a negative effect of freezing and thawing on EPO and MPO cannot be excluded.

In conclusion, the homogenization of sputum specimens by DTT caused a statistically significant fall in EPO and MPO concentrations, but its influence on the measured concentrations of ECP and EPX was not significant.

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REFERENCES