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RESEARCH ARTICLE

Expression of glutathione *S*-transferase variants in human airway wall after long-term response to sulfur mustard

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Abstract

Context: Sulfur mustard (SM) is an alkylating agent identified as a potent chemical warfare agent. More recently, SM was used in the Iraq conflict against Iranian troops and civilians. At present, there are many people suffering from chronic obstructive pulmonary disease (COPD) due to mustard gas in Iran. SM increases the endogenous production of reactive oxygen species (ROS). The oxidant/antioxidant imbalance present in the lungs of these patients also results from the impaired capacity of the antioxidant/detoxification enzymes to detoxify the harmful reactive oxygen metabolites. *Objective*: One of the major antioxidants in human airways is glutathione *S*-transferase. They facilitate the detoxification of various environmental of oxidative stress. In this study, we attempted to understand the significance different in expression of GSTs in airway wall of chemical patients and control. *Materials and methods*: Seven normal and 20 SM induced COPD individuals were studied. Bronchoscopy was performed in all subjects and two specimens were taken from the main bronchus for mRNA extraction, PCR analysis and immunohistochemistry. *Results*: SM-induced COPD individuals showed expression of GSTA1 2.51 ± 0.83-, GSTM1 2.84 ± 1.71- and GSTP1 5.61 ± 2.59-folds higher than those of controls that revealed. GSTP1-immunoreactivity was strongly expressed in luminal border of normal samples. SM patient samples immunoreactivity for GSTP1 in the same area were negative. *Discussion and conclusion*: According to these findings, we speculated that overexpression of GSTs mRNA in patients revealed that GSTs plays an important role in cellular protection against oxidative stress of MS in airway wall of patients.

Keywords

Airway, glutathione *S*-transferase, sulfur mustard

History

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Introduction

Sulfur mustard (SM) is a strong alkylating and vesicant agent which has been used in several military conflicts. It was first synthesized in 1822 by Despretz and modified in 1860 by Niemann and Guthrie. More recently, mustard was used in the Iraq–Iran conflict against Iranian troops and civilians. Nowadays, over 100 000 Iranians are suffering from SM late signs. This agent causes acute and chronic complications with special characteristics including inflammation, fibrosis and airway remodeling most similar to chronic obstructive pulmonary disease (COPD) (1–3). COPD is a progressive and irreversible airflow limitation which is usually associated with dysregulated inflammatory response of lungs to noxious particles or gases (4–6). Although COPD affects the lungs, it induces significant systemic consequences such as chronic inflammation and oxidative stress, inextricably linked together (7,8).

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Reports indicate that SM increases the endogenous production of reaction oxygen species (ROS) (9), which derives oxidative stress and inflammation (10). Accordingly, oxidative stress depletes cellular detoxifying thiol levels including glutathione. Provision and availability of scavengers of ROS and electrophilic compounds such as glutathione, sulfhydryl compounds, antioxidants and substances that increase the production of endogenous scavengers may be considered as appropriate protective strategy (11). Studies imply oxidant–antioxidant imbalance as an important argue in the pathogenesis of COPD (12). This event results from the impaired capacity of the antioxidant/ detoxification enzymes to detoxify the harmful reactive oxygen metabolites (13,14). Very little is known about specific changes in the major antioxidant defense mechanisms in mild or severe COPD. One of the major antioxidants in human airways is glutathione *S*-transferase. This large family of phase II enzymes facilitates the detoxification of various environmental toxins and oxidative stress products. However, the regulatory mechanisms controlling the intra- and extra-cellular concentrations of GST are not completely understood (15,16). But in mammals, there are currently five known families of soluble GSTs, Alpha, Mu, Pi, Sigma and Theta, each with distinct catalytic

and structural properties. The most abundant mammalian GSTs are the class alpha, mu and pi enzymes and their regulation has been studied in more details (17).

GSTA1 provide protection against oxidative stress by attenuating lipid peroxidation and modulating apoptosis signaling in small cell lung cancer (18). Moreover, the polymorphisms of GSTM1, and P1 have been associated with lung, bladder, breast and colon cancers (19). The GSTM1 null genotype was reported to be associated with emphysema (20). The GSTP1 105Ile allele has been associated with COPD in Japanese population (21). However, Yim and colleagues reported that none of the GSTM1 and GSTP1 polymorphisms were associated with COPD in a Korean population (22–24). Furthermore, glutathione *S*-transferase P1 (GSTP1), the abundant isoform of glutathione *S*-transferases (GSTs) in lung epithelium, plays important role in the cellular protection against oxidative stress and toxic foreign chemicals. Seemingly, GSTP1 roles in allergic asthma as well as non-allergic subtypes by modulation of ROS production (25).

To the best of our knowledge, there is no report of *GST* gene polymorphism and its interaction with SM gas on lung function in adults. In this study, we tried to find out the significant difference among GSTA1, M1 and P1 expression in COPD complications induced by SM gas.

Materials and methods

Subjects and selection

This study comprised 20 SM-exposed patients and 7 healthy participants. The exposed patients were injured by single dose of mustard gas individuals who had a documented encounter with SM within the Iran–Iraq war. The mean age of normal subjects was 41.3 ± 2.5 years, and SM-induced patients were male with a mean age of 43.2 ± 6.4 years summarized in Table 1.

Patients with other chronic pulmonary diseases (such as asthma), lung cancer, autoimmune diseases (such as rheumatoid arthritis), diabetes mellitus, pneumonia or acute infective bronchitis were excluded. In addition smokers, addicts, elders, organ transplant recipients or patients with occupational history of toxic fume exposure also were excluded. Finally, 7 normal and 20 SM-induced COPD individuals were selected in a protocol approved by ethics committee of Baghiyatallah University of Medical Sciences.

Bronchoscopy biopsy sampling and handling

Bronchoscopy was performed in all subjects using flexible fiber-optic bronchoscope 5 (Olympus Biopsy forceps FB-15 C-1, Tokyo, Japan), according to our previous study (26). Briefly, the upper respiratory tract was anesthetized with 2% lidocaine. Then, atropine (0.75 mg intramuscularly) was administered before the procedure. Also, supplemental oxygen was given throughout the procedure, and the oxygen saturation was monitored by continuous pulse oxymeter. Two specimens were taken from the sub-segmental carinae of lower lobe. One of them was immediately immersed into the Tripure isolation reagent (Roche Diagnostics GmbH, Foster City, CA) and stored at −80 °C until RNA extraction and another one was immersed into the 4% buffered paraformaldehyde for immunohistochemical analysis.

Table 1. Characteristics of SM-injured patients and control group.

Groups	*N*	Sex (M/F)	Age range	Age mean ± SD	*p*
Control group	7	6/1	39.0–44.0	41.3 ± 2.5	0.64
SM-injured group	20	20/0	36.0–58.0	43.2 ± 6.4	

RNA extraction

Total RNA of biopsy was extracted by Tripure isolation reagent (Roche Diagnostics GmbH), according to the manufacturer. Sample concentration and purity were determined by optical density (OD) measurement at 260 nm by NanoDrop spectroscopy (ND-1000, Wilmington, DE). Then, total RNA quality was checked on 1% agarose gel stained with ethidium bromide.

cDNA synthesis

Reverse transcription was performed by SuperScript III reverse transcription (Invitrogen, Carlsbad, CA) with 1 µg of total RNA followed by DNaseI (Invitrogen) treatment and heat inactivation in order to eliminate any chromosomal DNA contamination.

Assessment of GSTs expression

Semiquantitative PCR was performed using Taq DNA polymerase (Takara, Japan) in a PCR system (mastercycler ep, Ependorf, Germany), by initial denaturation (30 s at 95 °C), annealing (30 s at 55 °C for human GSTs and 59 °C for β-actin), extension (1 min at 72 °C) and terminal extension (5 min at 72 °C) in 30 cycles. Primer set for the human GSTs were: GSTA1, 5′-GTGCAGACCAGAGCCATTCTC-3′ (forward), 5′-GCAAGCTTGGCATCTTTTTCC-3′ (reverse); GSTM1, 5′-ATGCCCATGATACTGGGGTAC-3′(forward), 5′-GTGAGCCCCATCAATCAAGTAG-3′ (reverse); GSTP1, 5′-ACCTCCGCTGCAAATACATCTC-3′ (forward), 5′-GGC TAGGACCTCATGGATCAG-3′ (reverse). For the normalization, expression of β-actin was examined and the primer set as forward 5′-TTCTACAATGAGCTGCGTGTGG-3′ and reverse 5′-GTGTTGAAGGTCTCAAACATGAT-3′. PCR products were separated in 2% agarose gel and dyed with ethidium bromide and then detected under UV light. All results were normalized with β-actin expression to compensate for differences in the amount of cDNA. For quantitative measures and evaluation, PCR results on gel were

Table 2. Results of pulmonary function test in SM-injured patient and control group.

	Control group subjects, $n = 15$	SM-injured patients, $n = 24$	*p* Value
FVC (mean ± SD)	3.34 ± 0.79	2.87 ± 0.89	0.11
FEV1 (mean ± SD)	2.71 ± 0.81	1.92 ± 0.87	0.007*
FEV1/FVC (mean ± SD)	79.85 ± 6.15	67.88 ± 15.81	0.001*
RV (mean ± SD)	2.38 ± 0.97	3.75 ± 1.75	0.04*

FVC, forced vital capacity; FEV1, forced expiratory volume in 1 s; RV, residual volume; SD, standard deviation.
*$p < 0.05$.

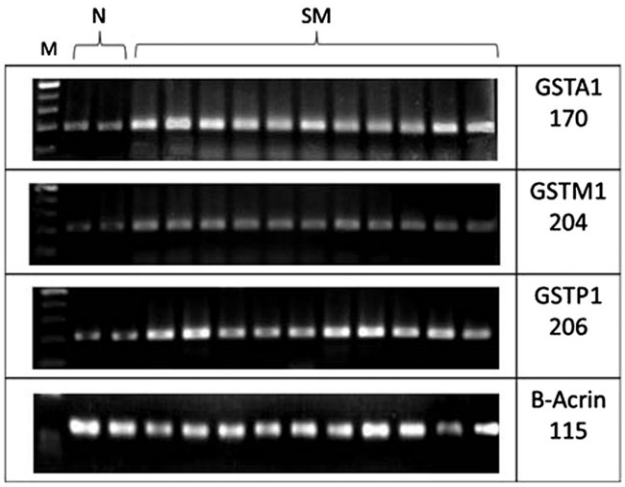


Figure 1. Expression levels of GSTA1, GSTM1 and GSTP1 in patients with long-term exposure to sulfur mustard. Total RNAs were purified and then were analyzed by semiquantitative RT-PCR. This panel shows gel bands in order to PCR amplification products of GSTA1 (170 bp), GSTM1 (204 bp), GSTP1 (206 bp) and β-actin (115 bp) transcripts. (A) Marked increase in GSTA1 expression levels of SM-exposed patients (lanes 3–13) was recognized in comparison to expression level of unexposed cases (lanes 1 and 2). (B) The density band of GSTM1 in SM-exposed patients also shows highlighted deference with mRNA of unexposed group. GSTP1 also shows the deference in mRNA expression level. (E) B-actin was used as internal control. Lane M shows DNA ladder (100 bp).

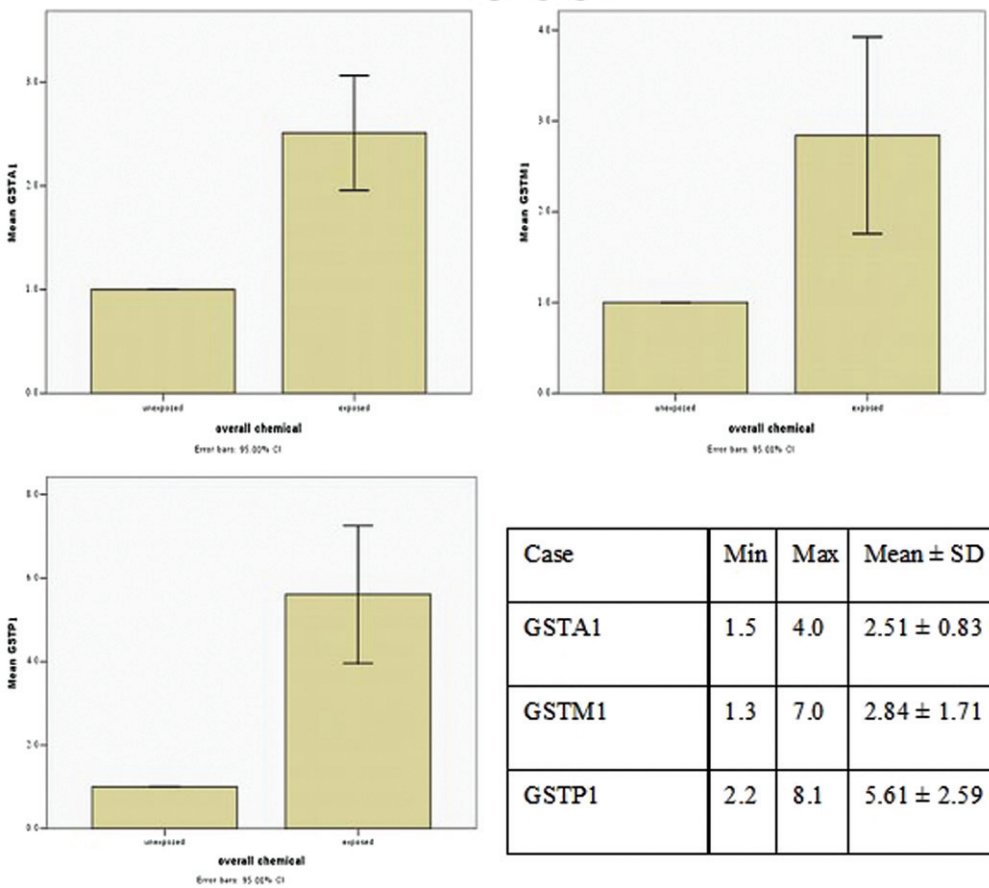
densitometered by using special Image Analysis software (Scion Corporation, Frederick, MD).

Immunohistochemistry analysis

For immunohistochemistry, details were already described elsewhere by us (43,44), briefly individual airway wall biopsies were fixed with 4% buffered paraformaldehyde for fixation for one day. The biopsies were cryoprotected by immersed overnight in a phosphate buffer containing 30% sucrose to prevent the formation of ice crystal artifact in frozen tissue section. Sections of 20 µm thickness were cut on a cryostat microtome (Histo line, Milano, Italy) and incubated with monoclonal anti mouse GSTP1-antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a dilution 1:200, for 12 h at 4 °C. After incubation with the primary antibody, the sections were incubated with biotinylated anti-mouse secondary antibody IgG (Santa Cruz Biotechnology Inc). The sections were subsequently visualized using ABC complex (avidinbiotinylated peroxidase complex) system (Vector Laboratory, Burlingame, CA) with DAB as a substrate.

Statistical analysis

Results are revealed as mean ± SD of fold changes of each gene were analyzed using the SPSS statistical package (version 13.0; SPSS Inc., Chicago, IL) and Mann-Whitney *U* test was used to compare the differences between groups ($p < 0.05$).



Case	Min	Max	Mean ± SD
GSTA1	1.5	4.0	2.51 ± 0.83
GSTM1	1.3	7.0	2.84 ± 1.71
GSTP1	2.2	8.1	5.61 ± 2.59

Figure 2. SM-induced COPD individuals showed expression of GSTA1 2.51 ± 0.83, GSTM1 2.84 ± 1.71 and GSTP1 5.61 ± 2.59 folds higher than those of controls.

Results

Subject clinical characteristics

Totally, 27 cases were participated in this study. There were 20 SM exposed patients and 7 normal controls. There was no statistically difference between the average ages of two groups (Table 1). Pulmonary function testing showed no difference in airflow limitation between groups, although SMs had positive methacholine challenge and/or evidence of spontaneous airway reactivity. Bronchoscopy was performed in all individuals without complications. However, the results of pulmonary function testing are shown in Table 2. Although forced vital capacity in the control group was higher than in SM-injured cases, but the difference was not statistically significant ($p = 0.11$). On the other hand, forced expiratory volume in 1 second (FEV1) in the SM group was significantly lower than in the controls ($p = 0.007$). Moreover, FEV1/forced vital capacity also differed between the two groups, being significantly higher in the controls ($p = 0.001$). Residual volume was significantly elevated in SM-injured patients in comparison with controls ($p = 0.04$).

Overexpression of GSTs mRNA

The expressions of GSTs were assessed by semi-quantitative RT-PCR on total RNA extracted from fresh airway biopsy of healthy controls and SM-induced COPD individuals. GSTA1, GSTM1 and GSTP1 mRNAs were present at expected sizes of 170, 204 and 206 bp, respectively. Specific primers were used to achieve full length human GSTs (Figure 1). SM-induced COPD individuals showed expression of GSTA1 2.51 ± 0.83, GSTM1 2.84 ± 1.71 and GSTP1 5.61 ± 2.59-folds higher than those of controls that reveal (Figure 2). According to these findings, we speculated that GSTs mRNA overexpression in airway wall biopsy was due to oxidative stress induction.

Immunohistochemistry analysis

To examine whether SM induces GSTP1 expression in protein level immunohistochemistry analysis was performed. GSTP1-immunoreactivity was strongly localized in luminal border of epithelial cells and numerous cells vicinity to basement membrane was also immunopositive to GSTP1 of normal samples. SM patient samples immunoreactivity for GSTP1 in

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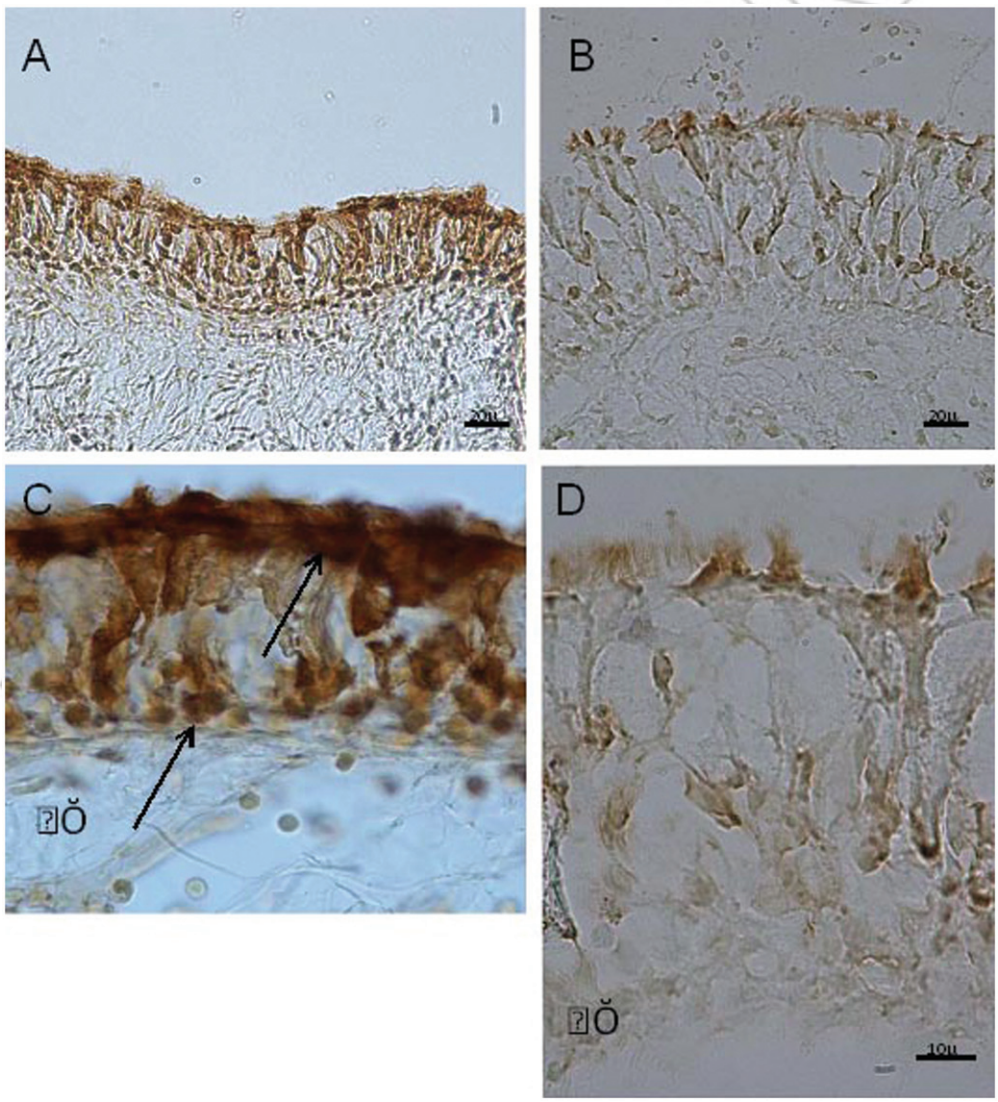

Figure 3. Light micrograph of GSTP1-immunoposive cells in the bronchial epithelium. (A) GSTP1-immunoreactivity in bronchial epithelial cell of control group. GSTP1-immunopositivity strongly demonstrated in luminal border of epithelium and substantial number of cells vicinity to basement membrane (BM). In epithelial cells of chemical injured patients no immunoreactions are seen throughout the section. Note that the thickness of epithelial cell layer in experimental group is higher than control group due to chemical injury. BM, basement membrane.

the same area and cells were negative, so in particular the epithelial cells reveal immunonegative reactivity for GSTP1 (Figure 3).

Discussion

An emerging body of evidence indicates that COPD, asthma and respiratory infections increase pulmonary and systemic levels of oxidative stress (27–31). The recent finding that SM increases the endogenous production of ROSs (9,45) has suggested that ROSs are likely involved in this toxicity (10,11). The imbalance between oxidant and antioxidant systems is suggested to be involved in the pathophysiology of SM-induced pulmonary lesions. Thus, several studies have shown the importance of oxidative stress in the pathogenesis of COPD whose signs and symptoms are similar to delayed pulmonary sequels observed in SM-injured patients (32–34,46).

This study provides an outstanding opportunity to investigate the effect of gene–gene interactions and interaction of genotype with SM exposure on severity of lung function impairment in patient with airflow obstruction. Almost, most of SM-induced COPD patients reveal a significant difference pulmonary function tests such as FEV_1, FVC and FEV_1/FVC with control group and this clinical finding confirmed that COPD is presented on SM exposed patients. Moreover, we found that the variants of GSTs were associated with low pulmonary function. There have been a variety of proposals that genetic variations are responsible for detoxifying harmful inhaled agents and there are important factors that affect this genetic predisposition of COPD. The most important of these enzymes are glutathione *S*-transferase family. GSTs have been associated with COPD and asthma as an important enzymatic antioxidant in the airflow limitation pathogenesis at older ages (20,21,35,36) which functions as an antioxidant in xenobiotic, peroxide and hydroperoxides metabolism pathways to reduce oxidative stress (29,30).

Our finding reveals that, GSTA1 genotype is expressed 2.51 ± 0.83, GSTM1 2.84 ± 1.71 and GSTP1 5.61 ± 2.59-fold higher than those of controls group. GSTA1 provide protection against oxidative stress by attenuating lipid peroxidation (18). In a cytotoxicity study (37), GSTA1 up-regulated against administration of environmental contaminants and in an immunohistochemistry study on smokers. The distribution of the class A enzymes was widest of all the GSTs studied, extending from the epithelium of the central bronchi to the type 1 and type 2 alveolar epithelium. GSTA1/A2 was also seen in bronchial and vascular smooth muscle and in alveolar macrophages (38). This could suggest that GSTA1 isoform has detoxification role against environmental contaminants.

Based on prior studies, we identified three candidate GSTs based on a hypothesis about pathways involved in lung function (17,35,39–42). The results of the present investigation extends our previous knowledge about GSTs role in protecting human pulmonary system against toxic metabolites (17).

Conclusion

We hypothesized that excess oxidative stress provides a mechanistic framework that unifies interrelationships between asthma and respiratory infections, and environmental exposures such as air pollution, tobacco smoke and SM inhalation, and factors such as diet and genetics (39). Our findings support the proposed mechanistic framework and the usefulness of studies focusing on genes involved in oxidative stress pathways.

Declaration of interest

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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