Glutathione aerosol suppresses lung epithelial surface inflammatory cell-derived oxidants in cystic fibrosis

James H. Roum, Zea Borok, Noel G. McElvaney, George J. Grimes, Allan D. Bokser, Roland Buhl, and Ronald G. Crystal. Glutathione aerosol suppresses lung epithelial surface inflammatory cell-derived oxidants in cystic fibrosis. J. Appl. Physiol. 87(1): 438–443, 1999.—Cystic fibrosis (CF) is characterized by accumulation of activated neutrophils and macrophages on the respiratory epithelial surface (RES); these cells release toxic oxidants, which contribute to the marked epithelial derangements seen in CF. These deleterious consequences are magnified, since reduced glutathione (GSH), an antioxidant present in high concentrations in normal respiratory epithelial lining fluid (ELF), is deficient in CF ELF. To evaluate the feasibility of increasing ELF GSH levels and enhancing RES antioxidant protection, GSH aerosol was delivered (600 mg twice daily for 3 days) to seven patients with CF. ELF total, reduced, and oxidized GSH increased (P < 0.05, all compared with before GSH therapy), suggesting adequate RES delivery and utilization of GSH. Phorbol 12-myristate 13-acetate-stimulated superoxide anion (O$_2^-$) release by ELF inflammatory cells decreased after GSH therapy (P < 0.002). This paralleled observations that GSH added in vitro to CF ELF inflammatory cells suppressed O$_2^-$ release (P < 0.001). No adverse effects were noted during treatment. Together, these observations demonstrate the feasibility of using GSH aerosol to restore RES antioxidant balance in CF and support the rationale for further clinical evaluation.

Cystic fibrosis (CF), the most common lethal hereditary disorder of Caucasians, is caused by mutations in the 250-kb CF transmembrane conductance regulator (CFTR) gene on chromosome 7 (16, 30, 31, 43). The major clinical problems associated with CF are manifested on the respiratory epithelial surface, with the accumulation of thick, tenacious mucus, colonization with Pseudomonas bacterial species, and chronic inflammation (1, 12, 17, 20, 35, 43). The inflammation is characterized by an overabundance of activated neutrophils and macrophages on the respiratory epithelial surface. On activation, these cells release superoxide anion (O$_2^-$) generated via the cell membrane-associated NADPH oxidase system. Other oxidants are then produced in a series of reactions mediated by enzymes and/or metal ions (8, 14, 41, 42), leading to epithelial cell damage and alteration of host defenses and resulting in progressive derangements of the lung parenchyma (1, 20, 35, 42, 43).

There is increasing evidence to suggest that the release of oxidants by these inflammatory cells is a potential mechanism by which the epithelium is damaged in CF (8, 12, 14, 35, 37, 38, 41, 42). The release of oxidants is exaggerated in CF, partially because of the increased accumulation of inflammatory cells, particularly neutrophils, on the epithelial surface, as well as chronic activation of these cells in response to the epithelial colonization by bacteria (1, 6, 12, 14, 17, 20, 35, 37, 43).

In normal individuals, the first line of defense against oxidants released on the respiratory epithelial surface consists of the extracellular antioxidant defenses present on the respiratory epithelium (9, 11). An important component of the lung antioxidant defenses is reduced glutathione ([L-$\gamma$-glutamyl-$L$-cysteinyl-glycine (GSH)], a ubiquitous sulfhydryl-containing tripeptide found in very high concentrations in normal respiratory epithelial lining fluid (ELF) (9, 21). The GSH system efficiently scavenges oxidants, thereby protecting cells and tissues from damage by oxidants released by inflammatory cells or delivered from other exogenous sources (4, 7, 9, 11, 27). Interestingly, in a number of pulmonary disorders characterized by an excessive inflammatory cell-derived oxidant burden on the respiratory epithelial surface [including CF (35), idiopathic pulmonary fibrosis (IPF) (7), and acute respiratory distress syndrome (5, 24)], ELF GSH levels are greatly diminished, suggesting that a deficiency in ELF antioxidant protection may result from the excessive oxidant burden. Moreover, a number of in vitro models have demonstrated the damaging effects of oxidants on lung cells (8, 18, 19, 27, 41, 42) as well as the ability of GSH to protect against this oxidant-mediated damage (8, 18, 19, 27, 41, 42). With this background, the present study examines the feasibility of augmenting respiratory epithelial surface antioxidant defenses in CF patients with use of GSH. Because prior studies demonstrate that systemic administration of GSH will not be useful as a means of augmenting ELF GSH levels because of its rapid clearance and short plasma half-life (4, 44), we evaluated in vivo aerosol delivery of GSH to the CF respiratory epithelial surface.
**METHODS**

GSH aerosol therapy. The study population that received GSH aerosol therapy consisted of seven patients (5 men and 2 women, 25 ± 1 (SE) yr of age) with a diagnosis of CF as defined by standard criteria (43), including a positive sweat chloride test, Pseudomonas colonization of the lower respiratory tract, a history of frequent respiratory infections, and a chest roentgenogram revealing the characteristic features of CF (33). Pulmonary function tests (including vital capacity, total lung capacity, forced expiratory volume in 1 s, and diffusion capacity for carbon monoxide) were typical for moderate disease (Table 1). Each patient in the study underwent bronchoscopy with bronchoalveolar lavage, as described previously (20, 35), and venous phlebotomy before GSH aerosol administration (see below).

GSH (600 mg) was administered in six doses to each patient by aerosol every 12 h by means of an aerosol generator (Ultravent, Mallinckrodt) driven by compressed air (40 psi). Under these conditions, the mass median aerodynamic diameter of the droplets containing GSH was 2.8 µm (4). When GSH is aerosolized in this fashion, it remains in its reduced form (4). Aerosol delivery of each dose (administered by mouthpiece with the nostrils occluded to spontaneously breathing patients) required 20–30 min. To assess the effect of aerosol-delivered GSH on ELF and plasma GSH levels, bronchoscopy with bronchoalveolar lavage and phlebotomy were carried out 1 h after the last (6th) aerosol. All GSH determinations were performed in the fasting state. The protocols used for this study were approved by the institutional review board for human studies, and informed written consent was obtained from each patient.

Safety evaluation. To evaluate the safety of aerosol delivery of GSH, physical examination and pulmonary function testing were done daily during the study, before and 4 h after each morning GSH aerosol dose, and 24 h after the final dose. Blood and urine were collected before, during, and after the study for determination of values for routine clinical cellular, chemical, and coagulation parameters. A repeat chest roentgenogram was performed on completion of the study.

**Table 1. Clinical and lavage characteristics of the study population**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>Gender, M/F</td>
<td>5/2</td>
</tr>
<tr>
<td>Sweat Cl− positive</td>
<td>7/7</td>
</tr>
<tr>
<td>Lung function, %predicted</td>
<td></td>
</tr>
<tr>
<td>VC</td>
<td>67 ± 4</td>
</tr>
<tr>
<td>TLC</td>
<td>83 ± 6</td>
</tr>
<tr>
<td>FEV1</td>
<td>47 ± 4</td>
</tr>
<tr>
<td>FEV1/FVC</td>
<td>73 ± 4</td>
</tr>
<tr>
<td>DLCO</td>
<td>77 ± 7</td>
</tr>
<tr>
<td>Bronchoalveolar lavage</td>
<td></td>
</tr>
<tr>
<td>Lavage fluid recovered, %</td>
<td>52 ± 5</td>
</tr>
<tr>
<td>Total cells recovered, x106/ml lavage fluid recovered</td>
<td>171 ± 43</td>
</tr>
<tr>
<td>Total cells recovered, x106/ml ELF</td>
<td>102 ± 47</td>
</tr>
<tr>
<td>Macrophages, %</td>
<td>26 ± 8</td>
</tr>
<tr>
<td>Lymphocytes, %</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>Neutrophils, %</td>
<td>70 ± 10</td>
</tr>
<tr>
<td>Eosinophils, %</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

Values are means ± SE. M, male; F, female; VC, vital capacity; TLC, total lung capacity; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; DLCO, diffusion capacity of the lung for carbon monoxide, corrected for Hb; ELF, epithelial lining fluid. See Ref. 12 for lung function methods and predicted values.

GSH preparation. The reduced form of GSH for aerosol therapy was obtained as a free acid (Sigma F & D, St. Louis, MO). The purity of the material as assayed against a reference standard (Atomergic Chemetals, Farmingdale, NY) was 100 ± 1%. The preparation was filtered, freeze-dried in glass vials, and tested for sterility and pyrogenicity (Pharmaceutical Development Service, Clinical Center, National Institutes of Health, Bethesda, MD). After reconstitution in 4 ml of sterile 0.9% saline, the solution was stable at 4°C for 24 h, as determined by HPLC analysis. The percentage of GSH in the total GSH preparation after reconstitution was 97 ± 3%.

Sample preparation. Bronchoalveolar lavage cells were separated from the supernatant by cytocentrifugation and enumerated, and differential cell counts were determined in the standard manner (36). Cell viability was determined by trypan blue dye exclusion. Recovered cells were predominantly neutrophils (Table 1), similar to previous observations for adult CF patients in comparison to normal individuals (35). Lavage cells were placed in polypropylene tubes (12 x 75 mm; Falcon, Becton-Dickinson, Lincoln Park, NJ) at a concentration of 0.5 x 10⁶ cells/ml in DMEM (Biofluids, Rockville, MD), incubated for 1 h at 37°C, and then used for quantification of O₂− release (see below). Portions of the lavage fluid and plasma were used for GSH and urea assays (see below).

GSH levels and form. Total GSH levels in ELF and plasma were quantified as previously described (2, 4, 7, 9, 35). Briefly, each sample was mixed with an equal amount of 10 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; Sigma Chemical, St. Louis, MO), and the rate of reduction of DTNB in the presence of β-NADPH (Sigma Chemical) and GSH reductase (Sigma Chemical) was recorded spectrophotometrically at a wavelength of 412 nm (model DU-70, Beckman Instruments, Fullerton, CA). The concentration of total GSH was based on standard curves generated from known concentrations of oxidized GSH (GSSG; Sigma Chemical; 0.1–4.0 µM). To quantify the amount of GSSG, the sample was mixed with an equal volume of 10 mM N-ethylmaleimide (Sigma Chemical) and passed through a Sep-Pak C₁₈ cartridge (Waters Associates, Milford, MA), and the rate of reduction of DTNB was determined at 412 nm. Standard curves were derived from dilutions of known concentrations of GSSG (0.1–4.0 µM). The amount of GSH was obtained by subtracting the level of GSSG from the level of total GSH. All measurements were performed in duplicate. GSH concentrations in the lung were referenced to the volume of ELF recovered by bronchoalveolar lavage as assessed by the urea method (28).

Quantification of O₂− release by lung inflammatory cells. For quantification of O₂− release, the polypropylene tubes containing lavage cells were centrifuged (500 g, 15 min), and the supernatant was discarded. The cells were then re-incubated (30 min, 37°C) in the presence of 80 µM ferricytochrome c (Sigma Chemical) and 100 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma Chemical) suspended in Hank’s balanced salt solution (Biofluids). The amount of O₂− released by the cells was determined by absorbance at 550 nm with use of the DU-70 spectrophotometer. Additional parallel samples evaluated under identical conditions while in the presence of 2 ng/ml superoxide dismutase (Sigma Chemical) were analyzed for each patient to determine the total superoxide dismutase-inhibitable O₂− release by the cells. Data are presented as amount of O₂− released (nmol ferricytochrome c reduced·h⁻¹·10⁶ cells⁻¹), and all experiments were performed in duplicate.

Effect of GSH on O₂− release by lung inflammatory cells in vitro. Inflammatory cells were recovered by bronchoscopy with bronchoalveolar lavage in 17 CF patients who had characteristics similar to those in the GSH aerosol therapy...
study (P > 0.1 for all comparisons in Table 1). Samples were prepared in an identical manner. For evaluation of the effect of GSH on quantification of O\(_2\)\(_z\) release, lung inflammatory cells were resuspended and reincubated as described above (30 min, 37°C) in the presence of 80 \(\mu\)M ferriicytochrome c and 100 ng/ml PMA suspended in Hanks’ balanced salt solution diluted 1:1 with cell-free lavage fluid from the same patient without added GSH or with GSH added to a final concentration of 50 \(\mu\)M. The amount of O\(_2\)\(_z\) released by the cells was determined as described above.

Values are means ± SE. All statistical comparisons were made using the two-tailed Student’s t-test.

**RESULTS**

Effect of GSH aerosol therapy on ELF and plasma GSH levels. The pretherapy level of total GSH in CF ELF was 55.9 ± 12.4 \(\mu\)M, and GSH in ELF was 30.4 ± 6.5 \(\mu\)M; both were substantially less than in normal ELF (9, 35). The preaerosol ELF GSSG level was 25.5 ± 10.8 \(\mu\)M; i.e., 46% of the total GSH in ELF in these patients was oxidized, in contrast to <10% in normal ELF (9, 35). Total GSH in ELF increased 1 h after delivery of the last of six aerosol doses of 600 mg of GSH (174 ± 46 \(\mu\)M, P < 0.02; Fig. 1); ELF GSH (58.3 ± 11.8 \(\mu\)M, P < 0.03) and GSSG (115 ± 43 \(\mu\)M, P < 0.05; Fig. 1) increased. Because the process of aerosol generation and delivery itself does not alter the GSH molecule (4), this increase in GSSG suggests that the GSH encountered an oxidant burden in the lower respiratory tract and was utilized as an antioxidant.

Plasma GSH in CF patients before therapy was 2.3 ± 0.3 \(\mu\)M. Levels were unchanged after GSH aerosol delivery (plasma GSH after aerosol = 2.0 ± 0.1 \(\mu\)M, P > 0.2). This was also true for plasma GSSG (P > 0.1).

Safety of GSH aerosol therapy. No adverse effects were noted among any of the patients receiving GSH by aerosol. In this regard, physical examination, clinical blood and urine studies, chest roentgenogram, and pulmonary function tests were unchanged from baseline after GSH aerosol therapy. Bronchoalveolar lavage fluid and ELF total inflammatory and differential cell counts did not change after GSH aerosol delivery, and cell viability did not decrease (P > 0.1, all comparisons). Also there was no apparent “leak” of the alveolar-capillary barrier caused by GSH aerosol, with identical recovered ELF volumes obtained before and after aerosol delivery (P > 0.4).

Suppression of O\(_2\)\(_z\) release from lung inflammatory cells by GSH aerosol therapy. Consistent with its role as an antioxidant, GSH administered by aerosol to the respiratory epithelial surface of CF patients suppressed the release of oxidants by ELF inflammatory cells (Fig. 2). The PMA-stimulated release of O\(_2\)\(_z\) by ELF inflammatory cells from CF patients before GSH therapy was 39.8 ± 1.7 nmol·h\(^{-1}\)·10\(^6\) cells\(^{-1}\). After GSH aerosol therapy, PMA-stimulated O\(_2\)\(_z\) release decreased to 30.2 ± 2.2 nmol·h\(^{-1}\)·10\(^6\) cells\(^{-1}\) (P < 0.002).

Effect of GSH on release of O\(_2\)\(_z\) by lung inflammatory cells in vitro. Parallel to the findings with GSH administered by aerosol, GSH added in vitro to ELF inflammatory cells from CF patients suppressed the release of oxidants by these cells (Fig. 3). CF lung inflammatory cells suspended in their own bronchoalveolar lavage fluid and stimulated by PMA released 38 ± 3 nmol O\(_2\)\(_z\)·h\(^{-1}\)·10\(^6\) cells\(^{-1}\), a level comparable to that observed in the group of seven patients whose cells were studied before GSH aerosol (Fig. 2; P > 0.8). The addition of 50 \(\mu\)M GSH to the cells suppressed the release of O\(_2\)\(_z\) an average of 33%, to 29 ± 2 nmol·h\(^{-1}\)·10\(^6\) cells\(^{-1}\) (P < 0.001; Fig. 3).

**DISCUSSION**

CF is characterized by several pathological processes potentially capable of damaging the respiratory epithelium. First, there is a chronic accumulation of excessive numbers of activated inflammatory cells (1, 2, 17, 20, 35, 43). These cells, as part of their inflammatory armamentarium, release exaggerated levels of oxidants (14, 35, 42). In addition, CF ELF is deficient in GSH, one of the key components of the normal antioxidant defenses of the respiratory epithelium. Although the reasons for the low levels of ELF GSH are not completely understood, the significance of this deficiency is profound. Because GSH is a potent antioxidant capable of scavenging a variety of oxidant molecules, deficiency of GSH places the protein and lipid moieties of the respiratory epithelial cells, as well as extracellular molecules such as \(\alpha\)2-antitrypsin (\(\alpha\)2-AT), at increased risk for oxidative damage (15). When oxidized by exogenously or endogenously produced oxidants, \(\alpha\)2-AT becomes ineffective as an inhibitor of neutrophil elastase (NE), thus leaving the lung vulnerable to proteolytic degradation by NE (15). Conse-
quently, the exaggerated burden of oxidants on the respiratory epithelial surface in CF, along with a deficiency in one of the key antioxidant protective molecules, leaves the CF respiratory epithelium vulnerable to oxidative damage and NE-mediated proteolytic injury (42). These complex, interrelated pathological processes combine to play a central role in the progressive derangements that occur in the lung in CF.

In this context, we evaluated the feasibility of aerosol delivery of GSH to the lower respiratory tract in CF to augment the antioxidant barrier of the respiratory epithelium. We demonstrate that aerosol administration of GSH to CF patients effectively increases ELF GSH levels. Moreover, GSSG levels were also increased, suggesting that the delivered GSH was utilized within the lung as an antioxidant. Finally, as indicated by the decrease in $O_2^−$ release by inflammatory cells after GSH aerosol delivery, GSH acts at the level of the inflammatory cell to decrease the oxidant burden on the epithelial surface. Thus GSH aerosol administration is an effective method of augmenting the antioxidant protective barrier of the respiratory epithelium in CF.

The effects of GSH aerosol therapy appear to be lung specific; i.e., plasma levels of GSH and GSSG were not altered as a result of therapy. In addition, GSH aerosol therapy in CF is safe; no adverse clinical effects were noted in any of the patients. The lack of development of infectious symptoms or signs also argues that reduction of the oxidant burden in CF is not associated with inhibition of CF host defense against microorganisms, a potentially serious problem in the chronically colonized milieu in the CF lower respiratory tract. This is consistent with the finding that, in vitro, extracellular GSH concentrations of up to 300 µM do not inhibit bactericidal or phagocytic ability of neutrophils (26). Moreover, because deficiency of GSH has been associated with abnormal phagocytic cell function (10, 23, 32, 39) and, in vitro, increased extracellular GSH prevents exogenous oxidant-induced intracellular GSH depletion and decreased phagocytic capacity by neutrophils (27), augmentation of ELF GSH levels by aerosol therapy would be expected to improve antibacterial function on the respiratory epithelial surface in CF.

Fig. 2. Release of superoxide anion ($O_2^−$) by inflammatory cells recovered from respiratory epithelial surface from 7 patients with CF after administration of reduced GSH by aerosol. Cells were recovered by bronchoalveolar lavage before and 1 h after final (6th) dose of GSH (600 mg, twice daily for 3 days) and incubated with phorbol 12-myristate 13-acetate (PMA). $O_2^−$ release was quantified by reduction of ferricytochrome c. Data from each patient are indicated by a different symbol corresponding to those in Fig. 1. Each data point represents average of 2 determinations for each patient, and a line connects data points of same patient.

Fig. 3. Effect of reduced GSH on in vitro release of $O_2^−$ by inflammatory cells recovered from respiratory epithelial surface of 17 CF patients. Recovered cells were incubated with PMA alone or in presence of 50 µM GSH. Each data point represents mean of duplicate determinations; lines connect observations from same patient. Results are expressed as reduction of ferricytochrome c.
Concomitantly, evidence suggests that an increased oxidant burden actually promotes Pseudomonas virulence by inhibiting mucociliary clearance of the organism (37) and allowing for unopposed NE activity due to oxidant-induced antiprotease inhibition (1, 6, 15, 20). In addition to its direct antioxidant role, GSH may act to preserve antiprotease activity in these conditions, as suggested by several in vitro cell-free studies (3, 22, 25, 40). GSH inhibited myeloperoxidase-mediated inactivation of α1-AT (3). GSH in combination with GSH peroxidase inhibited loss of lipid peroxidation-induced α1-AT activity (22). Catalase-suppressible inhibition of α1-AT by gas-phase cigarette smoke was also reduced by GSH (25). GSH may also help maintain α1-AT activity by allowing reduction of the inactivated mixed-disulfide form of this molecule (40).

The effects of GSH aerosol therapy in CF are comparable to those observed after GSH aerosol administration in IPF (2), another disease characterized by exaggerated levels of oxidants on the respiratory epithelial surface. As in CF, GSH delivery by aerosol in IPF results in significant increase in ELF levels of total GSH and GSSG, consistent with aerosol-delivered GSH being utilized as an antioxidant in vivo. Although this has not been demonstrated directly, a previous study indicates that the process of aerosol generation alone does not result in oxidation of the GSH molecule (4). Furthermore, in healthy sheep, ELF recovered 1 h after a single dose of GSH aerosol showed only a small increase (10%) in the proportion of total GSH that was GSSG compared with before aerosol (4). These results support the concept that antioxidant augmentation therapy with GSH would be a generally effective method of antioxidant therapy and, thus, may be effective in other diseases characterized by an excessive oxidant burden on the respiratory epithelial surface, such as acute respiratory distress syndrome (5, 24).

Our study demonstrated a significant effect of six doses of GSH (600 mg) administered by aerosol every 12 h on ELF GSH levels and oxidant release by ELF inflammatory cells 1 h after the last delivered dose. A kinetic study of the effect of aerosolized GSH for >1 h would help determine optimal dosing amounts and intervals, but serial bronchoscopies with bronchoalveolar lavage would have been extremely difficult to perform in this relatively small CF patient population. In otherwise normal anesthetized and mechanically ventilated sheep undergoing serial bronchoscopies with bronchoalveolar lavage, ELF GSH remained elevated for >2 h after a single 600-mg aerosolized dose of GSH, with a half-life of ~1.5 h (4). Although comparisons between the animal model and CF patients require many assumptions, the rationale for multiple dosing of GSH aerosol within a 24-h period was evident. With regard to the inflammatory cell-derived oxidant burden, we observed a significant decrease in inflammatory cell oxidant release after GSH aerosol in CF but no decrease in the number of recovered inflammatory cells. Although the differential cell count of the ELF inflammatory cells remained the same after GSH aerosol, we cannot exclude the possibility that inflammatory cells with less potential for oxidant release were sequestered as a result of GSH aerosol. It also remains to be seen whether a more prolonged course of GSH aerosol administration to these patients would lead to changes in the number or types of inflammatory cells in CF ELF. Although the effects of GSH on acute and chronic inflammation in the lung may certainly be multifactorial [e.g., antioxidant (21), modulator of enzyme activity (3, 22, 25, 40), or modulator of inflammatory cell activity (27)], demonstration of persistence of effects demonstrated here or other potential long-term effects of aerosolized GSH on the oxidant burden and chronic inflammation in CF, of course, requires a longer-term study of aerosolized GSH.

Patients with CF are born with normal pulmonary microanatomy (43). The basic genetic defect of the CFTR gene causes pathophysiological changes in epithelial surface secretions (29, 43). These changes, in turn, are believed to be responsible for the “cascade” of pathophysiological changes that leads to the cycle of chronic infection and inflammation, resulting in irreversible lung and airway damage (1, 6, 12, 17, 20, 29, 35, 43). GSH aerosol delivery is not designed to “correct” the basic genetic defect of CF. It is hoped, however, that it will assist in arresting the relentless cycle of lung destruction due to unopposed oxidant and protease activity during any point in the progression of the disease (1, 20, 35). In turn, it may also improve the milieu at the respiratory epithelial surface, such that other forms of definitive therapy for the pulmonary manifestations of CF, such as gene therapy directed at the respiratory epithelial surface (34), may be delivered more effectively.

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