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Lymphocyte Glutathione Levels in Children With Cystic Fibrosis*

Larry C. Lands, MD, PhD; Vijaylaxmi Grey, PhD;
Argyrios A. Smountas, BSc, RRT; Violeta G. Kramer, BSPharm; and
Danielle McKenna, DEC

Objective: Lung disease in cystic fibrosis (CF) is characterized by a neutrophilic inflammatory response. This can lead to the production of oxidants, and to oxidative stress in the lungs. Glutathione (GSH) represents the primary intracellular antioxidant, and provides an important defense in the epithelial lining fluid. Evidence suggests that lymphocyte GSH reflects lung GSH concentrations, and so could potentially serve as a peripheral marker of lung inflammation.
Methods: We assessed peripheral blood lymphocyte GSH concentrations in 20 children (13 boys) with CF who were in stable condition at the time of evaluation. Values were compared with nutritional status and lung function parameters.
Results: Patients were 11.7 ± 3.03 years old (mean $\pm$ SD). Their percentage of ideal body weight was $101.8 \pm 17.92\%$; FEV_1, $79.5 \pm 19.22\%$ predicted; FEV_1/FVC, $75.0 \pm 10.08\%$; and residual volume (RV)/total lung capacity (TLC), $31.3 \pm 10.47\%$. For the group, the GSH concentration was 1.31 ± 0.52 $\mu mol/10^6$ lymphocytes, which was not different from laboratory control values. GSH values were correlated with nutritional status (percentage of ideal body weight: $r = 0.49$, $p < 0.03$) and the degree of gas trapping (RV/TLC: $r = 0.50$, $p < 0.03$), and were correlated inversely with airflow limitation (FEV_1, percent predicted: $r = -0.45$, $p < 0.05$; FEV_1/FVC: $r = -0.48$, $p < 0.04$), but not with age, height, or weight ($p > 0.1$).
Conclusions: We interpret the inverse correlation between lymphocyte GSH concentration and lung function as a reflection of upregulation of GSH production by lung epithelial tissue in response to oxidative stress. We interpret the correlation between lymphocyte GSH concentration and nutritional status as a reflection of the role of cysteine in hepatic glutamine metabolism. Peripheral blood lymphocyte GSH concentration may potentially serve as a convenient marker of lung inflammation. Furthermore, the increased demand for GSH production in the face of ongoing inflammation suggests a potential role for supplementation with cysteine donors.
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Key words: antioxidants; inflammation; oxidative stress

Abbreviations: CF = cystic fibrosis; GSH = glutathione; GSSG = glutathione disulfide; rpm = revolutions per minute; SSA = 5-sulphosalicylic acid; TLC = total lung capacity; TNF-α = tumor necrosis factor-α

The lung disease of cystic fibrosis (CF) is characterized by a chronic inflammatory reaction.[1–5] Central to this reaction is an intense neutrophil response with release of inflammatory agents, including elastase and oxidants.[1,2,6,7] A major front-line defense of the respiratory epithelium to oxidants is the production and release of glutathione (GSH) (γ-L-Glutamyl-L-cysteinylglycine).[8] However, while oxidative stress promotes production of GSH in respiratory epithelial cells, by

upregulating the expression of the rate-limiting enzyme γ-glutamylcysteine synthetase,[9] there is also upregulation of γ-glutamyl transpeptidase.[10] The upregulation of this latter enzyme can serve to recover GSH, and so preserve intracellular GSH.[11] This may account for the relatively low levels of GSH in the lung lining fluid of CF patients.[12,13]

Attempts have been made to assess oxidative stress in the lungs using peripheral blood markers. Plasma GSH levels do not relate to lung function,[12] but this may reflect the fact that plasma levels are dependent on a balance between hepatic production[14] and peripheral clearance, especially by the kidney[15] and lung.[16] Erythrocyte GSH concentrations correlate inversely with lung function,[17] suggesting that the upregulation of GSH production in respiratory epithelium could be mirrored in erythrocyte levels. Consistent with this, erythrocyte GSH concentrations are elevated in smokers.[18] However, because erythrocyte turnover of GSH is relatively slow[19] compared with lymphocytes,[20] it may not reflect acute changes. Studies have demonstrated that increases in lung GSH levels in response to treatment with L-2-oxothiazolidine-4-carboxylate, a cysteine precursor, are paralleled by increases in lymphocyte GSH levels,[20] but not by whole blood GSH levels.[21] Additionally, the decreases in lung GSH in response to buthionine sulfoximine, an inhibitor of the first step of GSH synthesis, γ-glutamylcysteine synthetase, are paralleled by decreases in peripheral blood lymphocyte concentrations.[16]

We hypothesized that measurement of peripheral lymphocyte GSH concentrations would correlate closely with clinical status in CF patients. We evaluated this measure in a cross-sectional study and examined the relationships between GSH concentrations and pulmonary and nutritional status in children with CF.

Materials and Methods

Twenty children (13 boys) who regularly attended the CF clinic at the hospital were recruited for the study. The diagnosis was based on two positive sweat chloride tests (> 60 mEq/L) and a compatible clinical history. All patients were studied when they were clinically stable, having been free of any acute increase in their respiratory symptoms for at least 2 months. The study had the approval of the hospital ethics committee, and signed informed consent was received before we proceeded with the study.

Height was measured with the patient in stocking feet on a stadiometer. Weight was measured with the patient lightly dressed on an electronic balance. Weight was expressed as a percentage of the predicted value for gender, age, and height.[22] Expiratory flows were assessed by spirometry, and lung volumes were assessed by whole body plethysmography (SensorMedics 6200 Autobox Dl; SensorMedics Corp; Yorba Linda, CA), with the results expressed as percentage of the predicted value.[23]

Preparation of Lymphocytes

Blood was collected after at least 4 h of fasting. Five mL of blood was collected in heparinized tubes. After dilution in an equal amount of RPMI 1640 media (Gibco BRL; Burlington, Ontario, Canada), the mixture was placed in a tube containing 4 mL of Ficoll-Hypaque and centrifuged at 400g (1,400 revolutions per min [rpm]; IEC-7 centrifuge; IEC & Labsystems; Needham,

MA) for 30 min. The cells at the interface (90% lymphocytes) were removed and resuspended in 10 mL of 4°C RPMI 1640, and kept on ice. The suspension was then centrifuged at 450g (1,800 rpm) in a 4°C centrifuge (IEC-PR6) for 10 min. After removal of the supernatant, the pellet was washed again in cold RPMI 1640. The pellet was resuspended in 4 mL of phosphate buffered saline 1X (pH, 7.40), and a 0.2-mL aliquot removed for automated cell counting (Coulter S-plus JR; Coulter Corp; Miami, FL). The cell count was used to calculate the suspension volume required for a 1×10^6-lymphocyte aliquot.

Aliquots of appropriate volume were then centrifuged in prechilled tubes at 500g (800 rpm Eppendorf 5402; Eppendorf; Hamburg, Germany) for 10 min at 4°C. The supernatant was removed, and the pellet was resuspended in 970 μL of cold, distilled water. To this, 30 μL of 30% 5-sulphosalicylic acid (SSA) was added to make a final concentration of 0.9% SSA, and the solution was incubated for 15 min on ice. The solution was then centrifuged at 5,000g (8,000 rpm Eppendorf 5402) for 10 min at 4°C. The supernatant was removed and stored at -70°C for later analysis of GSH.

GSH Analysis

Total GSH in the 0.9% SSA extract was determined by the GSH reductase recycling method of Tietze[24] adapted for the Cobas Mira spectrophotometer (Roche Diagnostic Systems; Somerville, NJ).[25] Briefly, the Cobas Mira spectrophotometer was used to pipette 210 μL of α-nicotinamide adenine dinucleotide phosphate (0.3 mmol/L); 30 μL of 5,5'-dithio-bis(2-nitrobenzoic acid) (6.0 mmol/L); and 95 μL of sample, standard, or 0.9% SSA into cuvettes. After a 4-min incubation at 37°C, 15 μL of GSH reductase (1.0 U/100 μL) was added, and the reaction was monitored every 24 s for 12 min. Under these conditions, the method is linear for GSH concentrations between 0.5 and 5 μmol/L. The instrument constructs a calibration curve by assaying known GSH standards, and from this the GSH concentration of the unknown is evaluated. Reproducibility for GSH at these concentrations is $< 2\%$ (intra-assay coefficient of variation). Laboratory control mean value (n = 7) is 1.31 μmol/10^6 lymphocytes, with a range of 0.69 to 2.18 μmol/10^6 lymphocytes.

Data Analysis

Statistical analysis was performed using Statistica 5.1 for Windows (Statsoft; Tulsa, OK). Data are expressed as mean ($\pm$ SD). Correlations between GSH concentrations and functional parameters were assessed by Pearson's correlation coefficient. A p value < 0.05 was considered significant.

Results

Patient characteristics are presented in Table 1. In general, the patients were well nourished and had mild to moderate lung disease. The average GSH concentration for the group was 1.31 ± 0.52 μmol/L for 10^6 lymphocytes. This value was not different from laboratory control values.

Lymphocyte GSH concentrations were correlated with nutritional status (percentage of ideal body weight: r = 0.49, p < 0.03; Fig 1) and the degree of gas trapping (residual volume/total lung capacity [TLC]: r = 0.50, p < 0.03). Lymphocyte GSH concentrations were inversely correlated with airflow

Characteristics	Mean ± SD
Age, yr	11.65 ± 3.03
Height, cm	148 ± 17.98
Weight, kg	42.1 ± 15.68
Ideal body weight, %	101.8 ± 17.92
FEV_1, % predicted	79.5 ± 19.21
FEV_1/FVC, %	75.0 ± 10.08
TLC, % predicted	109.8 ± 14.95
RV/TLC, %	31.3 ± 10.47

*RV = residual volume.

limitation (FEV_1 % predicted: r = −0.45, p < 0.05; FEV_1/FVC: r = −0.48, p < 0.04; Fig 2), but not with age, height, or weight (p > 0.1).

DISCUSSION

These data demonstrate that lymphocyte GSH concentrations in children with CF vary proportionately with nutritional status, and inversely with lung function. The pulmonary results are consistent with previous studies in CF patients[17] and healthy subjects,[26] using erythrocyte GSH concentrations.

The time course of change in lymphocyte GSH concentrations closely parallels that of the lungs in animal experiments. GSH synthesis is decreased in both lung and lymphocytes of rats given buthionine sulfoximine, an inhibitor of the enzyme of the first step of GSH synthesis, γ-glutamylcysteine synthetase,[16] and increased when rats were given the cysteine precursor, L-2-oxothiazolidine-4-carboxylate.[20] The higher peripheral blood lymphocyte concentrations may therefore reflect the higher intracellular stores of GSH in respiratory epithelium associated with more advanced lung disease.

Increased oxidative stress is associated with a proinflammatory state, and this places demands on the use of GSH. It is not surprising, then, that the respiratory epithelium, in response to oxidative stress, upregulates the enzymes responsible for GSH production and recuperation of extracellular GSH with a resultant increase in intracellular levels of GSH. The *de novo* synthesis of GSH demands an increased supply of precursors, most notably cysteine, which is generally considered to be rate limiting.[27] γ-Glutamyl transpeptidase, the enzyme that hydrolyzes extracellular GSH, providing precursors for GSH synthetase, is typically upregulated in response to oxidative stress. Recent work suggests that the activity of γ-glutamyl transpeptidase is increased in the lung lining fluid of CF patients experiencing inflammation associated with increased neutrophil counts and interleukin 8 concentrations.[13] In the lung-lining fluid of CF patients, total GSH is reduced.[12]

The correlation with nutritional status may reflect the role of cysteine in hepatic glutamine metabolism. Circulating cysteine is catabolized in the liver, providing hydrogen ions for formation of ammonium, which is then removed via glutamine synthesis in favor of urea production. Nitrogen is thus retained in the amino acid pool, potentially preventing tissue wasting.[28,29] In a proinflammatory situation, as occurs in CF, cysteine is preferentially used for enhanced GSH production at the expense of dysregulation of protein catabolism, and results in tissue wasting. It is interesting to note that when children with CF were treated with high-dose ibuprofen to reduce lung inflammation, it not only slowed their
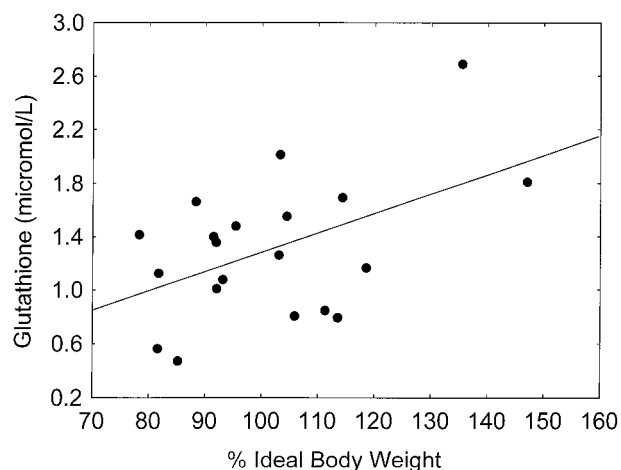

FIGURE 1. GSH vs the percentage of ideal body weight. Regression line represented by solid line (r = 0.49; p < 0.03).

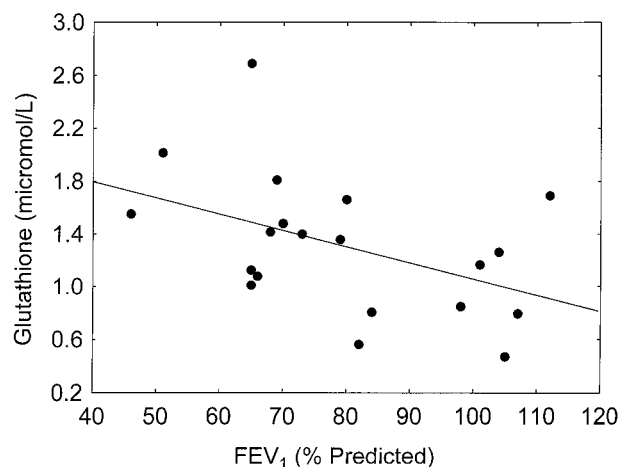FIGURE 2. Lymphocyte GSH concentrations vs percent predicted FEV_1. Regression line represented by solid line (r = −0.45; p < 0.05).

progression of lung disease, but maintained their percentage of ideal body weight.[30] Decreasing inflammation, and the requirement for augmented GSH production, may enable CF patients to utilize cysteine for muscle accretion.

Both interleukin 6 and tumor necrosis factor-α (TNF-α) are increased in CF sputum.[31] TNF-α is also elevated in the plasma of CF patients, correlating with resting energy expenditure. It was also seen to inversely correlate with arm muscle area, consistent with its effects on tissue wasting.[32] TNF-α also induces interleukin 6 production,[33] which promotes GSH synthesis in the liver, further contributing to lower levels of circulating cysteine.

TNF-α also promotes lactate production. Lactate, through its energy-wasteful conversion in the liver to glucose via the Cori cycle, will consume energy and protons. This utilization of protons diminishes the ability of hepatic cysteine catabolism to promote glutamine synthesis and to prevent wasting.[28,29] CF patients appear to have diminished skeletal muscle mitochondrial oxidative capacity, promoting lactate formation.[34] It is intriguing to speculate that in order to maintain circulating cysteine levels and lung GSH levels so that lung antioxidant defenses can be maintained, there is augmented skeletal catabolism and secretion of GSH.[35] A lack of GSH in skeletal muscle could, in turn, diminish mitochondrial function, and favor lactate formation.[36] An alternative possibility is that adenosine 5′-triphosphate is being consumed in muscle mitochondria for the production of glutamine, in order to support plasma levels. This could decrease the adenosine 5′-triphosphate available from aerobic processes for muscular contraction, and so promote glycolytic activity.[28]

In its role as a scavenger of reactive oxygen species, GSH is oxidized to GSH disulfide (GSSG) by the action of GSH peroxidase. The ratio of GSSG to total GSH (GSSG/GSH) is then often used as another marker of ongoing oxidative stress.[37] This ratio is normally < 10%, making an accurate determination of the small amount of GSSG difficult. Furthermore, GSH performs a variety of other tasks, such as regenerating vitamins C and E from their radical forms and maintaining protein sulfhydryl groups in their reduced state, all of which are independent of the oxidation of GSH by GSH peroxidase.[11,38,39] Nonetheless, the measurement of GSSG may add important information about the degree of oxidative stress, and how GSH is being utilized.

Our findings have two important implications. The first is that lymphocyte GSH concentrations have the potential to serve as a readily measurable, minimally invasive marker of ongoing lung inflammation. The second is that augmented GSH production in respiratory epithelial tissue and blood cells will require available precursors, of which cysteine is limiting.[27] A lack of cysteine would not only limit the production of GSH, but may also promote tissue catabolism. This suggests the potential beneficial role that cysteine donors could play.

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