Increased Glutathione and Glutathione Peroxidase in Lungs of Individuals with Chronic Beryllium Disease

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Reactive oxygen species (ROS) are mediators of chronic tissue damage and fibrosis. Endogenous antioxidants may increase in response to oxidants and reduce tissue injury. We investigated the antioxidant response of the lungs to the chronic release of ROS, as occurs in the immune-specific granulomatous inflammation of chronic beryllium disease (CBD), and compared it with that in healthy controls and individuals exposed to cigarette smoke. The antioxidants superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione (GSH) were quantitated in lung epithelial lining fluid (ELF) and serum from control subjects (n = 10), cigarette smokers (n = 8), and individuals with CBD (n = 9). GPx activity and extracellular GPx (eGPx) protein were increased in the ELF of subjects with CBD in comparison with that of control subjects and smokers (eGPx in ELF: controls, 1.3 ± 0.2 μg/ml, smokers, 1.9 ± 0.3 μg/ml, CBD, 3.8 ± 0.8 μg/ml; $p = 0.002$; GPx U/ml ELF, controls 1.4 ± 0.3, smokers 1.8 ± 0.4, CBD, 4.5 ± 1, $p = 0.02$). Smokers' ELF had higher levels of GSH than that of controls, but CBD patients' ELF contained much more GSH than that of either controls or smokers ($p < 0.001$). Increases in GSH were correlated with eGPx, indicating similar inducing mechanisms for these antioxidants. Thus, coordinate augmentation of the glutathione antioxidant system occurs in granulomatous lung inflammation. **Comhair SAA, Lewis MJ, Bhathena PR, Hammel JP, Erzurum SC. Increased glutathione and glutathione peroxidase in lungs of individuals with chronic beryllium disease.** AM J RESPIR CRIT CARE MED 1999;159:1824–1829.

Occupational exposure to dusts or fumes of beryllium metal or salts can lead to lung inflammation and cell injury in individuals employed in the electronics, dental alloy preparation, nuclear weapons, metal extraction, and aerospace industries (1–6). In some instances, beryllium-induced inflammation is followed by epithelial repair, but in other circumstances a chronic cell-mediated immune response to beryllium leads to chronic beryllium disease (CBD), a granulomatous interstitial lung disorder occurring in up to 3% of beryllium workers (1–8). Although the mechanisms leading to granulomatous interstitial lung disease are not clear, chronic release of reactive oxygen species (ROS) from inflammatory lung cells is involved in the disease (9–11).

Endogenous antioxidants may increase in response to ROS and thus minimize tissue injury (12–17). For example, inhalation of tobacco smoke, which in addition to generating 10^{14} oxidant radicals per cigarette puff also activates phagocyte release of oxidants (14, 18–21), leads to increases in lung antioxidants (19–24). Lung antioxidant defenses are widely distributed and include both enzymatic and nonenzymatic systems. The major enzymatic antioxidants are superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) (15). SOD (EC 1.15.1.1), which degrades superoxide, exists in three forms, including the intracellular manganese SOD and CuZn SOD, and an extracellular SOD that is present in epithelial lining fluid (ELF) and blood vessels. Catalase [EC 1.11.1.6], found in the cell cytosol, removes hydrogen peroxide. GPx (EC 1.11.1.9) removes hydrogen peroxide and organic hydroperoxides by oxidizing glutathione, a water-soluble, low-molecular-weight tripeptide (L-γ-glutamyl-L-cysteinyl glycine) that is abundantly present in lung ELF (24). The lung contains both an extracellular GPx (eGPx), in the lung ELF, and an intracellular GPx (25).

Very little is known about the antioxidant response of the respiratory tract to chronic granulomatous inflammation in the human lung, as occurs in CBD. In sarcoidosis, a granulomatous inflammatory interstitial lung disease similar to CBD, alveolar macrophages (AM) release increased amounts of superoxide and hydrogen peroxide, which have been linked to the pathogenesis of this disease (9–11). Coincident with increased oxidant production, MnSOD is increased in AM in sarcoidosis (26). The present study was designed to investigate whether antioxidants are increased in response to chronic gran-

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ulomatous lung inflammation as occurs in CBD, in comparison with healthy controls and cigarette smoking individuals.

METHODS

Study Population

To evaluate antioxidants in the respiratory system *in vivo*, we included 27 subjects in the study population: 10 healthy, nonsmoking individuals, eight healthy smoking individuals, and nine individuals with CBD (Table 1). Nonsmoking volunteers with no history of pulmonary disease were enrolled as controls. Exclusion criteria for the three groups included age under 18 yr or over 65 yr, pregnancy, human immunodeficiency virus (HIV) infection, and a history of respiratory infection in the previous 6 wk. Additional exclusion criteria for control subjects included current tobacco use, prolonged exposure to secondhand smoke at home or at work, exposure to dusty environments or to known pulmonary disease-producing agents, or a history of recurrent episodes of breathlessness, chest tightness, cough, and/or sputum production. Smoking individuals were similar to healthy controls but had to have smoked a minimum of 5 pack-yr and be current smokers. Inclusion criteria for individuals with CBD were known exposure to beryllium; histologic evidence of disease, such as noncaseating granulomas and/or mononuclear cell infiltrates on lung biopsy specimen; and evidence of beryllium-specific, cell-mediated immunity in the lung as demonstrated by a positive lymphocyte transformation test (LPT) on blood or bronchoalveolar lavage fluid (BALF) (7, 8). Seven CBD patients were blood LPT positive, and five were BALF LPT positive.

Bronchoalveolar Lavage

Bronchoalveolar lavage (BAL) was performed on all subjects, using fiberoptic bronchoscopy as previously described (27). Briefly, after local anesthesia with 2% lidocaine, a bronchoscope was wedged in a segmental bronchus of the right middle lobe or lingula. Three 50-ml aliquots of warm physiologic saline were infused into the right middle lobe or lingula (total volume of 300 ml) and recovered with manual suction. The BALF from the right middle lobe and lingula were combined and filtered through a Y-type blood filter (Drip Chamber Pump; Allegiance Healthcare Corp., McGaw Park, IL) and cellular components were separated by centrifugation (700 $\times$ g for 10 min). Cells were washed once with Hanks' balanced salt solution (HBSS; GIBCO, Grand Island, NY) and counted with a hemacytometer. A cell differential count was done after Giemsa-type staining (Diff-Quick; American Scientific Products, Stone Mountain, CA). Peripheral blood was obtained from study subjects on the same day as BALF; serum was then extracted by centrifugation of the whole blood (1430 $\times$ g for 10 min).

TABLE 1

CHARACTERISTICS OF STUDY POPULATION

	Control (n = 10)	Smoking (n = 8)	CBD (n = 9)
Sex, M/F	5/5	3/5	9/0
Race			
Caucasian	8	8	9
African-American	2		
Age*	27 ± 1	32 ± 4	42 ± 3
Smoking			
Current smoker		8	
Ex-smoker			5
Nonsmoker	10		4
WBC ($\times 10^6$)/ml BALF*	14 ± 2	45 ± 10	27 ± 7
% Lymphocyte*	2.2 ± 0.5	1.4 ± 0.6	11 ± 2
% Macrophages*	96.4 ± 0.7	98.1 ± 0.5	88 ± 3
% Neutrophils	1.1 ± 0.7	0.5 ± 0.3	1.3 ± 0.2
% Eosinophils	0	0	0

Values are means ± SEM.

Definition of abbreviations: BALF = bronchoalveolar lavage fluid; CBD = chronic beryllium disease; WBC = white blood cells.

* $p < 0.05$.

The volume of ELF in BALF was determined with the urea method (27). Urea was measured in BALF and serum using the blood urea nitrogen (BUN ENDPOINT) reaction (Sigma Chemical Co., St. Louis, MO) as previously described. Relative levels of ELF were estimated by using simple dilution principles relating to the urea concentration in serum and BALF. Total protein was determined with a bicinchoninic (BCA) protein assay (Pierce, Rockford, IL).

SOD Activity

SOD activity was determined in BALF and serum from the rate of reduction of cytochrome c (15), with one unit (U) of SOD activity defined as the amount of SOD required to inhibit the rate of cytochrome c reduction by 50%. The final reaction volume was 3 ml, and included 50 mM potassium phosphate buffer, 2 mM cytochrome c, 0.05 mM xanthine, and a 0.1 mM ethylenediamine tetraacetic acid (EDTA) solution. Xanthine oxidase (Sigma) was added at a concentration sufficient to induce a 0.020 change in absorbance per minute at 550 nm.

GPx Activity

Total GPx activity was determined spectrophotometrically in BALF and serum through an indirect coupled assay (28). The BALF was incubated for 2 min at 37° C in the presence of 0.1 mM sodium azide, 1 U/ml glutathione reductase, 0.1 mM GSH, and 0.12 mM reduced β-nicotinamide adenine dinucleotide phosphate (β-NADPH), 0.016 mM dithiothreitol, 0.38 mM EDTA, and 50 mM sodium phosphate (pH 7.0). The reaction was initiated by the addition of 0.2 mM hydrogen peroxide. The decrease in absorbance at 340 nm over 3 min, as NADPH is converted to nicotinamide adenine dinucleotide phosphate (NADP) is proportional to the GPx activity. One unit of activity is defined as the activity that catalyzes the oxidation of 1 nmol NADPH/min, with a molar coefficient of extinction of 6.22×10^6 M^{-1} cm^{-1} used for NADPH.

Catalase Activity

Catalase activity was quantified with a method in which hydrogen peroxide is reacted with the components present in BALF (15). In this method the initial rate of disappearance of hydrogen peroxide (0 to 60s) is recorded spectrophotometrically at a wavelength of 240 nm; one unit of catalase activity was defined as the rate constant of the first-order reaction. The assay is specific for the detection of catalase activity (29).

Reduced Glutathione Levels

Quantification of GSH in BALF was done with a calorimetric assay (Glutathione assay kit; Calbiochem, La Jolla, CA) (21). This method takes advantage of a two-step chemical reaction. The first step leads to the formation of substitution products between a proprietary reagent and all mercaptans present in the sample. The second step is a β-elimination reaction under alkaline conditions that induces the transformation of GSH into a chromophore with maximal absorbance at 400 nm, which is compared with a known standard curve of GSH.

Extracellular GPx

Extracellular GPx (eGPx) was measured with an enzyme-linked immunosorbent assay (ELISA) (Calbiochem). This method is based on a sandwich-type immunoassay, and is specific for eGPx. The eGPx protein concentration present in BALF was based on four-parameter curve fit generated from known standard concentrations of eGPx.

Statistical Analysis

All data are expressed as the mean and SEM. The comparisons between the three groups were made through analysis of variance (ANOVA). A value of $p < 0.05$ was considered significant. Comparisons were also made with age- and gender-adjusted ANOVA models. The effect of previous smoking on antioxidants was also tested within the CBD group. Linear regression fitting of data was done with the Fastat statistical program (version 1.0; Systat Inc., Evanston, IL).

RESULTS

Patient Characteristics

Control, smoking, and CBD individuals were similar in terms of race and sex distribution (Table 1). Age was significantly greater

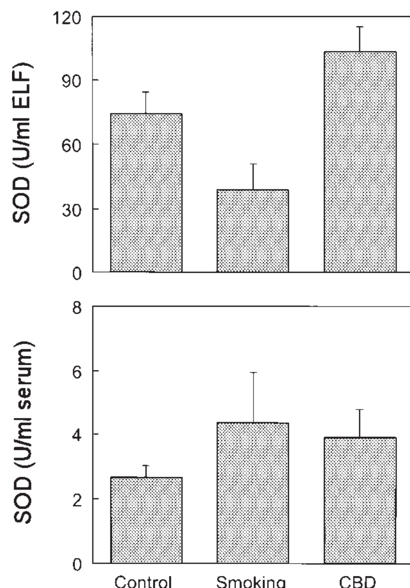

Figure 1. SOD activity in ELF and serum of controls, smokers, and CBD patients. Smokers had less SOD activity in ELF than controls or CBD patients (p = 0.008). The SOD activity in serum was not significantly different in the three groups. Values are mean ± SEM.

in the CBD group (p = 0.001). Bronchoscopy was well tolerated by all individuals. The total cell count in BALF was increased in smokers as compared with controls and CBD patients (p = 0.008). However, smokers' BALF cell differential counts were similar to those of controls, whereas CBD patients had higher percentages of lymphocytes than control or smoking individuals (p = 0.001). This increase in lymphocyte percentage was at the expense of a decreased percentage of macrophages (p = 0.001). The percentage recoveries of instilled saline and ELF were similar in the three groups (BALF volume: controls, 188 ± 7 ml; smokers, 165 ± 13 ml; CBD patients, 182 ± 8 ml; p = 0.229). The total protein in ELF was different in the three groups (total protein in ELF: controls, 11 ± 2 mg/ml; smokers, 13 ± 3 mg/ml; CBD, 24 ± 2 mg/ml; $p < 0.05$). In contrast, the urea in BALF was similar among the three groups (urea in BALF: controls, 1.5 ± 0.3 mg/ml; smokers, 1.7 ± 0.2 mg/ml; CBD, 1.4 ± 0.1 mg/ml; p = 0.533). There was no correlation of BALF cellularity with protein (R = 0.048, p = 0.813).

Antioxidant Activities in ELF and Serum

Smoking individuals had less SOD activity than controls or CBD patients (Figure 1). In contrast, individuals with CBD had SOD activity similar to that of controls (SOD in ELF: controls, 74 ± 12 U/ml; smokers, 39 ± 14 U/ml; CBD, 104 ± 13 U/ml; p = 0.008). The SOD activity in serum was not significantly different among the three groups (p = 0.517; Figure 1).

GPx activity in ELF was different among the three groups, with CBD patients having significantly higher levels of GPx activity than control subjects or smokers (GPx in ELF: controls, 1.4 ± 0.3 U/ml; smokers, 1.8 ± 0.4 U/ml; CBD, 4.5 ± 1 U/ml; p = 0.02; Figure 2). Serum GPx activity was also different among the groups, with elevated levels in smokers and CBD patients as compared with controls (GPx in serum: controls, 0.03 ± 0.01 U/ml; smokers, 0.13 ± 0.03 U/ml; CBD, 0.08 ± 0.02 U/ml; p = 0.02; Figure 2).

Catalase activity in ELF was similar in the three groups (catalase in ELF: controls, 55 ± 10 mU/ml; smokers, 75 ± 23 mU/ml; CBD, 78 ± 17 mU/ml; p ± 0.566).

eGPx Protein Levels in ELF and Serum

Normal ELF of the lung contains eGPx. Patients with CBD had higher levels of eGPx in ELF than did smokers or controls (eGPx in ELF: controls, 1.3 ± 0.2 μg/ml; smokers, 1.9 ± 0.3 μg/ml; CBD, 3.8 ± 0.8 μg/ml; p = 0.002; Figure 3). In contrast,

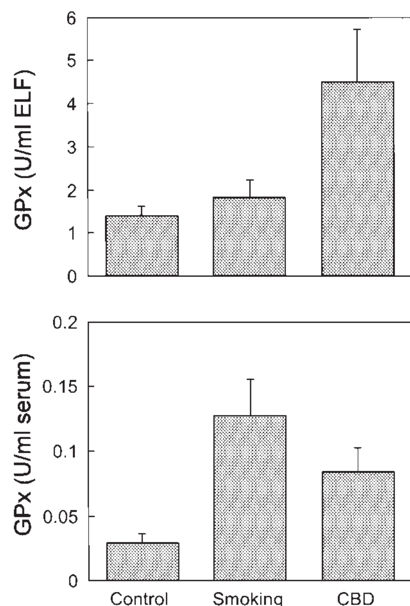

Figure 2. GPx activity in ELF and serum of controls, smokers, and CBD patients. GPx activity was greatest in ELF of CBD patients (p = 0.02). GPx activity in serum was also increased in smokers and CBD patients as compared with controls (p = 0.02).

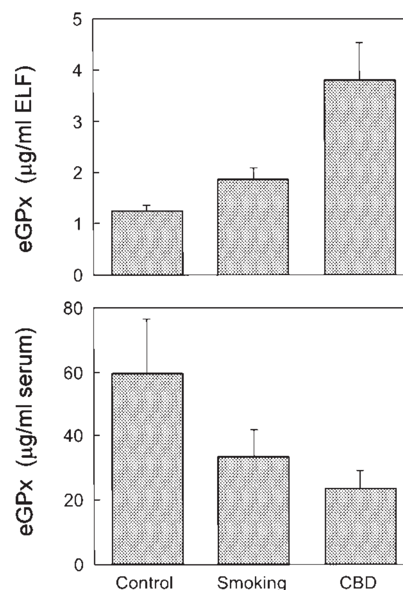

Figure 3. eGPx protein in ELF and serum of controls, smokers, and CBD patients. eGPx protein in ELF was higher in CBD patients than in smokers or controls (p = 0.002), whereas there was no significant difference in serum eGPx among the groups.

there was no significant difference in serum eGPx among the three groups ($p = 0.566$; Figure 3).

GSH Levels in ELF

Previously, GSH has been shown to compose over 95% of total glutathione in ELF (7). GSH levels were different among the three study groups. GSH levels in smokers were higher than in controls, but CBD patients had the highest levels in comparison with controls or smokers (GSH in ELF: controls, 0.60 ± 0.07 mM; smokers, 1.0 ± 0.1 mM; CBD, 2.0 ± 0.3 mM; $p < 0.001$; Figure 4A). Levels of GSH in the ELF were directly correlated with ELF eGPx in the three groups ($R = 0.863$, $p < 0.001$; Figure 4B). GSH also correlated with GPx activity ($R = 0.505$, $p = 0.007$), SOD activity ($R = 0.487$, $p = 0.01$), and total protein ($R = 0.79$, $p < 0.001$).

Age and Gender Adjusted Group Effects and Smoking Effect within CBD

The mean age in the CBD group was higher than that of controls, and the CBD patients were all men. Therefore, differences among groups were also tested with an ANOVA model that adjusted for age and gender. Despite the relationship of age to group, GSH, GPx, and SOD were still significantly different among the groups when adjusted for age and gender (all $p < 0.02$). Although eGPx failed to reach significance for difference among the groups when adjusted for age and gender, a trend toward a significant difference was still noted ($p < 0.07$). Effects of age or gender on antioxidants were not found within the groups.

Realizing that previous smoking may have an effect on antioxidants, we examined the smoking effect within the CBD group with the ANOVA model. Significant differences included higher ELF GSH and GPx activity in never-smoking as compared with ex-smoking CBD individuals (GSH in ELF, never-smoking CBD patients, 2.4 ± 0.5 mM (n = 4), ex-smoking CBD patients 1.69 ± 0.06 mM (n = 5); $p = 0.04$; GPx in ELF, never-smoking CBD patients, 8.4 ± 0.9 U/ml (n = 4); ex-smoking CBD patients, 1.4 ± 0.4 U/ml (n = 5); $p < 0.001$).

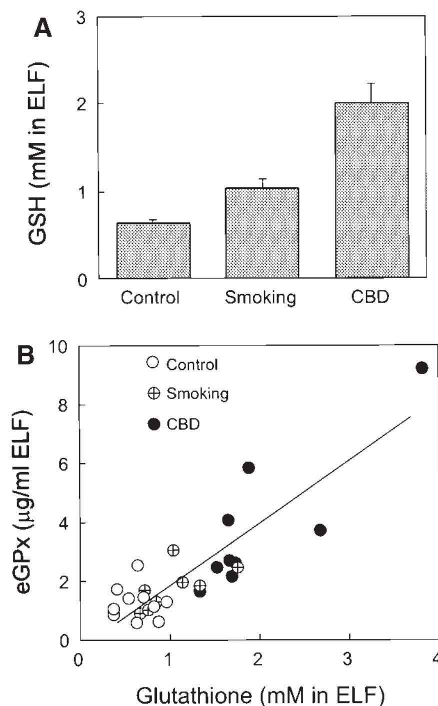

Figure 4. (*A*) GSH in ELF of controls, smokers, and CBD patients. GSH levels in ELF of smokers were higher than in controls, but CBD patients had even higher levels than smokers ($p = 0.002$). (*B*) Correlation of eGPx protein with GSH in ELF of controls, smokers, and CBD patients. Increases in GSH levels in ELF were directly correlated with increases in eGPx protein ($R = 0.863$, $p < 0.001$).

DISCUSSION

To our knowledge, this study is the first to demonstrate that the antioxidants GSH and GPx are increased in lungs of CBD patients as compared with healthy controls or smoking individuals. Other granulomatous inflammatory lung diseases, such as sarcoidosis and extrinsic allergic alveolitis, are accompanied by increased MnSOD in granulomas and BALF macrophages, which supports the concept that induction of antioxidants may be a nonspecific response to granulomatous lung inflammation (26). On the other hand, GSH is not increased in sarcoidosis (30). In this context, although increased antioxidants may not be unique to beryllium-induced granulomatous inflammation, neither can these results be generalized to all granulomatous lung diseases.

Evidence in the literature supports the hypothesis that upregulation of antioxidants occurs at both the transcriptional and translational levels (23, 31, 32). For example, induction of γ-glutamylcysteine synthetase, the rate-limiting enzyme in GSH synthesis, by oxidant stress from exposure to cigarette smoke, is due to increased transcription of the gene and is associated with activation of the redox-sensitive transcription factor activator protein 1 (AP-1) (23). Interestingly, increases in GSH and GPx were highly correlated in the present study, suggesting that induction of GPx may be mediated through redox mechanisms similar to GSH. Increases in GPx activity are most likely due to increases in eGPx protein in CBD patients' lungs. In the context that eGPx is synthesized and secreted by bronchial epithelial cells and AM (25), eGPx gene expression may be redox-mediated in the lung. In contrast, the increase in serum GPx activity in smokers and CBD patients is not related to levels of serum eGPx protein, indicating that other GPx enzymes are responsible for the systemic increase in GPx activity (25). The induction of the glutathione antioxidant system in CBD is especially striking in that the CBD patients in our study were older than the controls and smokers, and increased age reduces the ability to increase antioxidants in response to oxidant stress (20). Despite the confounding variable of age differences among the study groups, significant differences in antioxidants were still noted among the groups with the use of ANOVA models that took into account age and gender.

Alveolar–capillary permeability as determined by ELF-to-serum albumin ratios is increased in CBD as compared with control lungs (1). In the present study, total protein in ELF was twofold greater in CBD patients than in healthy controls. Leakage of serum proteins into ELF is unlikely to contribute to the increase in lung antioxidants in CBD, as opposed to the induction of antioxidants within the lung. Levels of GSH are 140-fold higher in ELF than in serum (24), whereas SOD and GPx are 25- and 50-fold higher in ELF than in serum from controls and CBD patients, respectively. Thus, increase of alveolar capillary permeability, with passive leakage of serum containing markedly lower antioxidant levels than in ELF, would more likely lead to dilution of than to increases in antioxidant levels.

Our results substantiate previous studies that have shown modifications in lung antioxidant status in smoking individuals

(19, 24). In general, levels of antioxidants reported in our study are similar to previously reported levels. Levels of GSH are within the range of but slightly higher than those in previous studies. Technical differences in method of lavage may account for these differences, e.g., all BALF in our study was collected for analysis, whereas previous studies discarded the first lavage aliquot return [21]. Despite these technical differences, our data confirm previous findings that smokers have higher levels of GSH in their ELF than do nonsmokers, but show no difference in ELF catalase (21, 23, 24). Some studies have shown increased GPx activity in the ELF of smokers as compared with nonsmokers (12), whereas others have shown decreased GPx in smokers (21). In the present study there was a trend toward higher ELF GPx levels in smokers than in nonsmokers. As in a previous study (21), ELF SOD was decreased in smokers as compared with nonsmokers in our study. Decreased SOD activity in the ELF of current smokers has been previously shown, and is attributed to decreased CuZnSOD activity (21). One possible mechanism for decreased SOD activity in smokers may be related to the oxidant-mediated inactivation of CuZnSOD (33, 34). Preservation of SOD activity in lung inflammation in CBD may relate to differences in the location and type of increased ROS in CBD patients as opposed to smoke-exposed lungs. Alternatively, the relative normality of ELF SOD activity in CBD may be due to increased expression of other SOD isoforms; e.g., MnSOD, such as occurs in sarcoidosis and extrinsic allergic alveolitis [26].

Five of the CBD patients in our study were ex-cigarette smokers. Analyses of ex-smoking in comparison to never-smoking CBD patients revealed higher levels of GSH and GPx activity in the never-smoking CBD patients. Thus, increases in antioxidant activity noted in the CBD group were not simply due to previous cigarette-smoke exposure. Further, although the cigarette-smoking group had alterations in antioxidants, as compared with healthy nonsmoking controls, antioxidants in the smoking group were also significantly different than in the CBD patients. Cigarette smoking induces a chronic inflammatory process in the airways, with an abundance of inflammatory cells, principally AM and neutrophils (19). The smokers in our study had marked increases in total numbers of inflammatory cells in their BALF, but cell differentials of smokers and healthy controls were similar. In contrast, CBD patients had an increased percentage of lymphocytes in their BALF, which have been previously shown to be beryllium-specific $CD4^+$ T-helper (Th) lymphocytes (3–8). Tumor necrosis factor-α, interleukin (IL)-6, and the lymphocyte-derived cytokine interferon-γ appear to play important roles in development of beryllium-induced granulomatous lung disease (35, 36). These cytokines increase ROS production, which may contribute in part to the chronic lung inflammation of CBD (9, 13, 35).

Interestingly, recent evidence shows that GSH levels in antigen-presenting cells determine whether Th lymphocytes become Th1 or Th2 effector cells (37). Th1 cells mediate inflammatory immune responses of delayed-type hypersensitivity, characterized by IFN-γ production, whereas Th2 cells mediate humoral/antibody responses and are characterized by IL-4 production (37). The Th1-cell–mediated immune response is favored by high GSH levels, whereas GSH depletion shifts the immune response to the Th2 type (37). Thus, increased GSH in CBD patients may contribute to the development and/or maintenance of a chronic Th1-cell–mediated immune response to beryllium. Importantly, CBD often reverses completely, suggesting that granulomatous inflammation and fibrosis are not essential consequences of the inflammatory response to beryllium (3–5). In this context, increased antioxidants likely are one defense mechanism for minimizing oxidant-induced lung injury in CBD, but may also influence the complex immune response to beryllium.

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