Modulation of Lymphocyte Proliferation by Antioxidants in Chronic Beryllium Disease

Dave R. Dobis^{1*}, Richard T. Sawyer^{1,2*}, May M. Gillespie¹, Jie Huang¹, Lee S. Newman^{2,3}, Lisa A. Maier¹⁻³, and Brian J. Day^{1,2,4}

¹Department of Medicine, Robert H. Hollis Laboratory of Environmental and Occupational Health Sciences, National Jewish Medical and Research Center, Denver, Colorado; and ²Department of Medicine, ³Department of Preventive Medicine and Biometrics, and ⁴Department of Immunology, University of Colorado Health Sciences Center, Denver, Colorado

Rationale: Occupational exposure to beryllium (Be) can result in chronic granulomatous inflammation characterized by the presence of Be-specific CD4⁺ T cells. Studies show that oxidative stress plays a role in the pathogenesis of chronic inflammatory disorders.

Objectives: We hypothesized that Be-induced oxidative stress modulates the proliferation of Be-specific CD4⁺ T cells.

Methods: Thirty-three subjects with chronic beryllium disease (CBD), 15 subjects with beryllium sensitization, and 28 healthy normal control subjects were consecutively enrolled from the Occupational and Environmental Health Clinic of the National Jewish Medical and Research Center.

Measurements and Main Results: All studies were performed with Ficoll-Hypaque-isolated peripheral blood mononuclear cells from subsets of the study subjects. Decreased intracellular levels of the thiol antioxidants, glutathione and cysteine, were observed in peripheral blood mononuclear cells from subjects with beryllium sensitization and CBD, as compared with healthy control subjects. Beryllium stimulation decreased intracellular thiol antioxidants by more than 40%, accompanied by increased reactive oxygen species levels and the proliferation of Be-specific blood CD4⁺ T cells from subjects with CBD. Be-induced T-cell proliferation was inhibited by treatment with the thiol antioxidant *N*-acetylcysteine or the catalytic antioxidant manganese(III) 5,10,15,20-tetrakis(4-benzoic acid)porphyrin (MnTBAP). MnTBAP treatment also inhibited T-cell proliferation in response to the unrelated, MHC class II-restricted antigen tetanus toxoid. Treatment of CBD blood lymphocytes, but not antigen-presenting cells, with MnTBAP decreased Be-induced T-cell proliferation by more than 40%.

Conclusions: Beryllium can mediate a thiol imbalance leading to oxidative stress that may modulate the proliferation and clonal expansion of Be-specific blood CD4⁺ T cells. These data suggest that Be-induced oxidative stress plays a role in the pathogenesis of granulomatous inflammation in CBD.

Keywords: T cells; reactive oxygen species; glutathione; *N*-acetylcysteine; oxidative stress

Beryllium (Be) is a lightweight metal that continues to be used in technology industries because of its unique chemical and physical properties. Since the 1970s in the United States, it is estimated that workplace exposure to Be has placed 800,000 individuals at

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Chronic beryllium disease is a chronic progressive lung disease characterized by the presence of granulomatous inflammation. Several studies suggest that oxidative stress plays a role in chronic inflammatory disorders.

What This Study Adds to the Field

Antioxidants inhibit beryllium antigen-stimulated CD4⁺ T-cell proliferation in peripheral blood mononuclear cells from subjects with chronic beryllium disease, suggesting a potential therapeutic utility of antioxidant molecules in this and similar granulomatous lung diseases.

risk for the development of chronic beryllium disease (CBD) (1). In genetically susceptible individuals, inhalation of Be particles causes beryllium sensitization (BeS), which is defined as the onset of a CD4⁺ T-cell-mediated immune response directed against the Be antigen (2). Progression from sensitization to CBD occurs at a rate of approximately 6–8%/year and is characterized by the proliferation and accumulation of Be-specific, helper T-cell type 1 (Th1) cytokine-secreting CD4⁺ T cells in the lung (3). These oligoclonal CD4⁺ T-cell populations recognize Be in an MHC class II-restricted manner that occurs predominantly through HLA-DP molecules possessing a negatively charged glutamic acid at position 69 of the β chain (reviewed in [2]).

Reactive oxygen species (ROS) modulate important cellular responses mediating cell proliferation, differentiation, migration, and death at relatively low concentrations (4). ROS control two pathways that determine whether T cells die by apoptosis, or survive apoptotic death to become memory T cells after antigen activation (5). One experimental approach to test the hypothesis that Be-induced ROS might regulate T-cell responses in CBD is to inhibit ROS-mediated T-cell activation and proliferation with antioxidants. To do this, we took advantage of the presence of Be-specific CD4⁺ T cells in the peripheral blood of subjects with CBD and BeS (2, 3). We also took advantage of the cell-permeable catalytic antioxidant manganese(III) 5,10,15,20-tetrakis(4-benzoic acid)porphyrin (MnTBAP). MnTBAP protects biological systems against superoxide- and hydrogen peroxide-mediated injury (6, 7). We also examined a thiol-based antioxidant, *N*-acetylcysteine (NAC), to replete cellular thiol levels depleted by Be. Previously, we found that activated T cells are protected from superoxide generation, caspase-dependent DNA loss, loss of mitochondrial transmembrane potential, and apoptotic cell death by MnTBAP (8). We also observed that Be stimulation up-regulates the production of ROS in mouse macrophages, resulting in the development of apoptosis that was ameliorated by treatment with MnTBAP (9).

(Received in original form July 11, 2007; accepted in final form January 24, 2008)

*These authors contributed equally to this work.

Supported by RO1 ES-012504, ES-06538, PO1 ES11810, RO1 HL62410, and MO1 RR00051 from the National Institutes of Health.

Correspondence and requests for reprints should be addressed to B. J. Day, Ph.D., Department of Medicine, National Jewish Medical and Research Center, 1400 Jackson Street, Denver, CO 80206. E-mail: dayb@njc.org

This article has an online supplement, which is available from the issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 177, pp 1002–1011, 2008

Originally Published in Press as DOI: 10.1164/rccm.200707-1021OC on January 24, 2008
Internet address: www.atsjournals.org

Oxidative stress is often defined as an imbalance between oxidant production and antioxidant and repair defenses. In the current study, Be induced oxidative stress through its ability to deplete endogenous thiol antioxidants and increase ROS levels. Moreover, proliferation of blood CD4⁺ T cells in response to Be stimulation corresponded with increased levels of oxidative stress. The treatment of Be-stimulated T cells from subjects with CBD with antioxidants, MnTBAP, or NAC inhibited Be-specific T-cell proliferation. These findings suggest that Be-induced oxidative stress may modulate the downstream activation and proliferation of Be-specific CD4⁺ T cells. ROS may play a key role in the immunopathogenesis of CBD and suggest a rationale for antioxidant therapy.

METHODS

Written, informed consent was obtained from each patient enrolled in this study and the protocol was approved by the Human Subjects Institutional Review Board at National Jewish Medical and Research Center (Denver, CO). Subjects with CBD (*n* = 33), subjects with BeS (*n* = 15), and non-Be-exposed, healthy control subjects without lung disease (*n* = 28) were consecutively enrolled on the basis of the availability of blood and bronchoalveolar lavage (BAL) samples. With access to a limited number of subjects with CBD and BeS enrolled in this study, individual experiments were of necessity performed with samples from subsets of study subjects as specified in RESULTS. Blood and BAL samples were obtained from subjects with CBD and BeS at bronchoscopy and blood was obtained from healthy control subjects by venipuncture. The Be lymphocyte proliferation test (BeLPT) was performed for the clinical evaluation of blood and BAL T-cell proliferation response to Be stimulation as shown in Table 1, where we report the mean ($\pm$ SEM) peak stimulation index (SI) for thymidine uptake in triplicate cultures as the ratio of the test sample counts per minute to the counts per minute in the unstimulated (medium alone) control subjects (10).

All studies were performed with Ficoll-Hypaque-isolated peripheral blood mononuclear cells (PBMCs) from subsets of study subjects. Unless indicated otherwise, isolated PBMCs and BAL cells were adjusted to 1×10^6 cells/ml of RPMI complete medium with 10% heat-inactivated calf serum (see the online supplement for more details). For all experiments, including the functional studies and the clinical BeLPT, 200- μ l aliquots were then cultured in triplicate wells per treatment condition, using 96-well round-bottom plates (Costar 3799; Corning, Inc., Corning, NY), to yield a final concentration of 0.2×10^6 cells per well. Cells were

cultivated at 37°C in a humidified atmosphere containing 5% CO₂. For the characterization of Be-specific T cells, PBMCs were labeled with monoclonal antibodies and corresponding isotype-matched controls directed against CD4 and CD3 (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. CD4⁺ PBMC T cells were identified on the basis of forward and 90° light scatter patterns and gating was performed to include CD3⁺CD4⁺ double-positive T cells and to exclude macrophages by flow cytometry, using a BD FACSCalibur cytometer (BD Biosciences).

Total glutathione and cysteine levels in CD4⁺ PBMC T cells were determined by HPLC with fluorometric detection of thiols derivatized with monobromobimane (Invitrogen Molecular Probes, Eugene, OR). Intracellular thiol levels were evaluated in CD4⁺ PBMC T cells with the fluorescent probe monochlorobimane (MCIB; Invitrogen Molecular Probes). Oxidative stress was determined in CD4⁺ PBMC T cells by evaluating changes in fluorescence caused by the intracellular oxidation of ROS-sensitive fluorescent probe 2',7'-dichlorofluorescein diacetate (Invitrogen Molecular Probes). These studies were performed after 120 hours, an interval that matches the time of peak BeLPT SI for the majority of CBD and BeS PBMCs.

Using a subset of PBMCs, antigen-presenting cells (APCs) were separated by adherence to plastic as previously described (11). Adherent cells served as APCs whereas nonadherent cells served as responding T lymphocytes. Separated APCs and T cells were incubated overnight in 96-well round-bottom plates at 37°C in a humidified 5% CO₂ atmosphere. Adherent APCs were washed three times with 200 μ l of complete culture medium per well, with the last 200- μ l aliquot being discarded. Wells containing the matched lymphocyte targets were gently suspended and transferred to wells containing the autologous, washed APCs at a 1:1 ratio to yield a final concentration of approximately 0.2×10^6 cells per well. Proliferation assays were performed in triplicate as described previously. The separated APCs or lymphocytes were exposed overnight to MnTBAP (50 and 100 μ M) before the addition of either lymphocytes or APCs, respectively. Assays were then performed 120 hours after Be stimulation. For additional details, see METHODS in the online supplement.

Statistical comparisons were done by repeated measures analysis of variance. After the data were checked for significant treatment differences, individual contrasts were calculated to compare treatment means of interest. Normalizing transformations were made when the data were non-Gaussian. All other comparisons were done by Wilcoxon rank sum test and paired Student *t* test. A *P* value less than 0.05 was used to determine statistical significance.

RESULTS

Subject Demographics

Clinical characteristics for the subjects with CBD (*n* = 33) and BeS (*n* = 15) and the healthy normal control subjects (*n* = 28) enrolled in this study are shown in Table 1. Eight subjects with CBD were currently receiving corticosteroids. All of the subjects with BeS had normal lung histology whereas 32 of the 33 subjects with CBD had noncaseating granulomas on transbronchial lung biopsy. Transbronchial biopsy was not performed on one subject with CBD for health reasons. BAL cells from subjects with CBD had a significantly greater percentage of lymphocytes, as compared with subjects with BeS, consistent with a lymphocytic alveolitis in the CBD lung (2, 3). The peak blood BeLPT stimulation index (SI) was threefold higher in the subjects with CBD compared with the subjects with BeS. Only BAL cells from subjects with CBD proliferated in the presence of Be, with a ninefold higher peak SI response compared with subjects with BeS. None of the healthy normal control subjects showed a significant BeLPT proliferative response (SI < 2.5) to stimulation with 10 or 100 μ M Be.

Thiol Levels in PBMCs from Patients with CBD and BeS

We measured intracellular thiol levels in PBMCs from subsets of study subjects with CBD (*n* = 8) and BeS (*n* = 9), and from

TABLE 1. CLINICAL CHARACTERISTICS OF STUDY POPULATION

	BeS (<i>n</i> = 15)	CBD (<i>n</i> = 33)	Control (<i>n</i> = 28)
Age, yr	57.3 $\pm$ 3.0	62.8 $\pm$ 1.3	34.6 $\pm$ 2.1*
Race, W/AF/H	12/0/3	29/1/3	24/2/2
Sex, F/M	4/11	8/25	19/9
Smoking status, CS/FS/NS	0/9/6	0/12/21	0/6/22
Corticosteroid treatment, yes/no	1/14	8/25	N/A
Granulomas, yes/no	0/15	32/ND	N/A
BeLPT, SI			
Peak blood BeLPT SI	2.9 $\pm$ 0.7	11.4 $\pm$ 3.0†	N/A
Peak BAL BeLPT SI	1.6 $\pm$ 0.1	15.5 $\pm$ 7.8†	N/A
BAL cells			
Total white blood cell count, $\times 10^6$	36.3 $\pm$ 9.9	41.9 $\pm$ 5.4	N/A
Macrophages, %	90.0 $\pm$ 1.4	75.1 $\pm$ 2.6†	N/A
Lymphocytes, %	9.3 $\pm$ 1.4	22.5 $\pm$ 2.6†	N/A

Definition of abbreviations: AF = African American; BAL = bronchoalveolar lavage; BeLPT = beryllium lymphocyte proliferation test; BeS = beryllium sensitization; CBD = chronic beryllium disease; CS = current smoker; FS = former smoker; H = Hispanic; N/A = not applicable; ND = biopsy not done for medical reasons; NS = never smoker; SI = stimulation index; W = white.

Data are expressed as means $\pm$ SEM.

* *P* < 0.05, healthy normal control subjects versus subjects with CBD and subjects with BeS.

† *P* < 0.05, subjects with CBD versus subjects with BeS.

healthy normal control subjects ($n = 12$) (Figure 1). HPLC with fluorometric detection was used to determine total intracellular glutathione and cysteine levels present within PBMCs. Intracellular glutathione levels in PBMCs from healthy normal control subjects were 1.3- to 2-fold higher than those found in subjects with CBD and BeS (Figure 1A). CBD PBMCs exhibited a higher but not significantly different level of total glutathione as compared with BeS PBMCs.

A similar trend was observed for intracellular cysteine levels (Figure 1B). PBMCs from a subset of the healthy normal control subjects had 1.2- to 1.6-fold higher cysteine levels as compared with the intracellular levels in PBMCs from subsets of study subjects with CBD and BeS. Although slightly higher cysteine levels were measured in CBD PBMCs as compared with BeS PBMCs, no significant differences were noted between CBD and BeS PBMC levels.

Thiol Depletion in Peripheral Blood CD4⁺ T Cells after Beryllium Stimulation

The observation that thiol levels were significantly reduced in subsets of BeS and CBD PBMCs, but not in a subset of healthy normal control PBMCs, led us to test the hypothesis that Be

stimulation could have an immediate effect on thiol levels in Be-specific CD4⁺ blood T cells. To evaluate intracellular thiol levels, flow cytometry was used to identify peripheral blood CD3⁺CD4⁺ T cells from a subset of CBD subjects ($n = 4$) that were colabeled with the fluorescent probe MCIB, a nonspecific marker used to identify cellular thiols. Beryllium stimulation induced thiol depletion in CD4⁺ T cells from CBD PBMCs in a dose-dependent manner (Figure 2A). The maximal decrease in thiol levels was observed after stimulation with Be ranging from 50 to 100 μ M. After 96 hours, 100 μ M Be stimulation of CD4⁺ T cells from subjects with CBD significantly lowered MCIB fluorescence by 44%, as compared with unstimulated control subjects. Beryllium stimulation also depleted endogenous thiol levels in CD4⁺ T cells from a subset of healthy normal control subject PBMCs ($n = 3$) by 48% after 96 hours of exposure to 100 μ M Be (Figure 2B).

Generation of ROS in Peripheral Blood CD4⁺ T Cells after Beryllium Stimulation

Our data show that thiol levels were reduced in PBMCs from subsets of subjects with CBD and BeS, and that Be stimulation decreased thiol levels in blood CD4⁺ T cells from both a subset of subjects with CBD and normal healthy control subjects. We

Figure 1. Intracellular (A) total glutathione (GSH) and (B) cysteine levels in peripheral blood mononuclear cells (PBMCs) from subjects with chronic beryllium disease (CBD; $n = 8$), beryllium-sensitized subjects (BeS; $n = 9$), and healthy normal control subjects (normal; $n = 12$), detected by fluorometric HPLC. * $P < 0.05$, BeS versus normal; ** $P < 0.05$, CBD versus normal. Values represent means (nmol/mg protein) and SEM.

Figure 2. Intracellular thiol levels from subsets of (A) subjects with chronic beryllium disease (CBD; $n = 4$) and (B) healthy normal control subjects ($n = 3$) after 96 hours of beryllium sulfate (BeSO_4 ; 25–100 μM) stimulation in CD4^+ T cells, using the fluorescent probe monochlorobimane (MCIB). Mean fluorescence values for each subject were normalized to 100, where 100 indicated the mean fluorescence in unstimulated T cells. * $P < 0.05$, BeSO_4 -stimulated versus unstimulated. Values are represented as mean MCIB fluorescence and SEM.

therefore tested the hypothesis that Be stimulation might up-regulate ROS in PBMC CD4^+ T cells from subsets of subjects with CBD and normal healthy control subjects (Figure 3). Flow cytometry was used to gate $\text{CD3}^+\text{CD4}^+$ PBMC T cells that were costained with the fluorescent probe 2',7'-dichlorofluorescein diacetate, which is converted to fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS (*see* References E12 and E13 in the online supplement). Beryllium stimulation at both 10 and 100 μM increased ROS generation in a subset of CBD CD4^+ PBMC T cells ($n = 3$) in a dose- and time-dependent manner (Figure 3A). DCF fluorescence was increased approximately threefold in CBD PBMC CD4^+ T cells after 120 hours of 100 μM Be stimulation in comparison with unstimulated control cells. After 120 hours of 100 μM Be stimulation there was a twofold increase in DCF fluorescence expressed by a subset of PBMCs from normal healthy control subjects ($n = 3$) (Figure 3B).

Inhibition of Lymphocyte Proliferation with Catalytic Antioxidant MnTBAP

The preceding data suggest that Be can deplete intracellular thiols and increase ROS levels in Be-specific CD4^+ T cells from subjects with CBD. We tested the hypothesis that a broad-spectrum catalytic antioxidant could inhibit Be-specific CBD CD4^+ T-cell proliferation. CD4^+ T cells from a subset of subjects

with CBD ($n = 6$) proliferate robustly after 120 hours of exposure to 10 μM Be (Figure 4). T-cell proliferation was not observed in control cultures that were untreated or treated with 10 μM aluminum sulfate, or up to 100 μM MnTBAP. By comparison, Be-specific CD4^+ T-cell proliferation was significantly reduced in cultures that were stimulated with 10 μM Be in the presence of increased levels of MnTBAP.

Next, we tested the hypothesis that MnTBAP might also reduce the proliferation of T cells that were specific for a different MHC class II-restricted peptide antigen, tetanus toxoid (Figure 5). Stimulation for 120 hours with 10 μM Be induced the proliferation of a subset of Be-specific CD4^+ T cells from subjects with CBD ($n = 5$) (mean SI $\pm$ SEM, 3.4 ± 0.7 ; $P < 0.05$). Similarly, tetanus toxoid (2.5 lymphocyte-flocculating units) induced the proliferation of antigen-specific T cells from a subset of healthy normal control PBMCs ($n = 4$) (mean SI $\pm$ SEM, 4.7 ± 0.3 ; $P < 0.0001$). To compare MnTBAP-induced inhibition of T-cell proliferation as a function of increasing MnTBAP concentration, Be- and tetanus toxoid-stimulated SI values observed in the absence of MnTBAP were normalized to a value of 100. MnTBAP treatment produced a dose-dependent inhibition of Be-stimulated lymphocyte proliferation, by 62% at the maximal concentration of MnTBAP (Figure 5). MnTBAP treatment also inhibited T-cell proliferation induced by tetanus toxoid, by 88%

Figure 3. Intracellular levels of reactive oxygen species (ROS) from (A) subjects with chronic beryllium disease (CBD; $n = 3$) and (B) healthy normal control subjects ($n = 3$) after BeSO₄ (10–100 μ M) stimulation in CD4⁺ T cells, using the ROS-sensitive fluorescent probe dichlorofluorescein (DCF). * $p < 0.05$, 10 μ M BeSO₄ versus unstimulated; ** $p < 0.05$, 100 μ M BeSO₄ versus unstimulated. Values represent means (DCF fluorescence) $\pm$ SEM.

at the maximal concentration of MnTBAP. Compared with the inhibition of Be-stimulated T-cell proliferation, MnTBAP was two times more effective at inhibiting tetanus toxoid-stimulated proliferation (Figure 5). These results may reflect the higher oxidative environment of CBD lymphocytes, as was previously demonstrated (*see above*), by decreased thiol content and increased ROS levels.

Inhibition of Lymphocyte Proliferation with Thiol Antioxidant NAC

Our data suggest that Be depletes endogenous thiol levels in lymphocytes and that subjects with BeS and CBD have lower endogenous thiol levels in their PBMCs. We therefore determined whether the thiol antioxidant NAC could modulate Be-stimulated proliferation of CD4⁺ CBD T cells. NAC treatment alone did not significantly alter PBMC viability ($92.2 \pm 1.4\%$ viable after treatment with 1 mM NAC and $89.2 \pm 1.6\%$ viable after treatment with 5 mM NAC). Beryllium-specific CD4⁺ CBD T cells ($n = 5$) proliferated after treatment with 10 μ M Be (Figure 6A). In comparison, there was a 63% decrease in the SI of Be-stimulated CBD T cells in the presence of 5 mM NAC. Proliferation of CD4⁺ CBD T cells was not observed in control cultures that were unstimulated or exposed to 5 mM NAC alone.

We also determined whether the thiol levels in Be-stimulated CD4⁺ CBD T cells were restored by NAC cotreatment (Figure 6B). Thiol levels were measured by flow cytometry, using the fluorescent probe MCIB in gated peripheral blood CD3⁺CD4⁺ CBD T cells ($n = 5$). We observed significantly decreased thiol

levels in CD4⁺ CBD T cells stimulated with 10 μ M Be alone. Treatment with 5 mM NAC significantly increased thiol levels by 42% in Be-stimulated CD4⁺ CBD T cells ($n = 5$). Thiol levels in CD4⁺ CBD T cells treated with 5 mM NAC alone were unchanged relative to thiol levels in the unstimulated control subjects.

Beryllium-specific CBD T Cells Are Targets for MnTBAP Inhibition of Beryllium-stimulated Proliferation

Treatment with antioxidant MnTBAP or NAC reduced Be-stimulated CD4⁺ T-cell proliferation. Our data also suggested that the catalytic antioxidant MnTBAP was able to reduce MHC class II-restricted, antigen-stimulated CD4⁺ T-cell proliferation in response to two independent MHC class II-restricted antigens. We next determined whether the inhibitory effect of MnTBAP was directed toward the APCs or Be-specific CD4⁺ T cells in the mixed PBMC cultures. We developed a crossover model in which the mixed PBMCs were separated by plastic adherence into adherent APCs and nonadherent T cells (11). To ensure that the separation procedure had no effect on Be-induced T-cell proliferation, we performed BeLPT proliferation assays with PBMCs from subsets of subjects with CBD ($n = 7$) and BeS ($n = 5$) and from normal healthy control subjects ($n = 9$) (Table 2). Similar BeLPT responses were observed when using the separated APCs and T cells as compared with mixed PBMCs. Beryllium-specific T cells proliferated in response to 10 μ M Be stimulation when isolated from subjects with CBD and subjects with BeS, but not control subjects. Proliferation was not observed

Figure 4. Proliferation (stimulation index [SI]) by chronic beryllium disease (CBD) peripheral blood mononuclear cells (PBMCs) ($n = 6$) that were unstimulated, or exposed to aluminum sulfate (10 μ M), manganese(III) 5,10,15,20-tetrakis (4-benzoic acid)porphyrin (MnTBAP; 50 and 100 μ M), BeSO₄ (10 μ M), or BeSO₄ (10 μ M) plus MnTBAP (50 and 100 μ M) for 120 hours. * $P < 0.05$, BeSO₄ alone versus unstimulated; ** $P < 0.05$, BeSO₄ plus MnTBAP versus BeSO₄ alone. Values represent mean SI and SEM.

in cultures treated with the control metal salt aluminum sulfate, and T-cell proliferation was observed in all cultures stimulated with the positive control phytohemagglutinin.

The crossover model was employed to determine whether the catalytic antioxidant MnTBAP would inhibit Be-induced CBD CD4⁺ T-cell proliferation, depending on which cell population was exposed to antioxidant (Figures 7A and 7B). CD4⁺ T cells from a subset of subjects with CBD ($n = 7$) (Figure 7A and 7B, *dark gray columns*) proliferated after exposure to 10 μ M Be. Proliferation of CBD T cells was not observed in cultures that were unstimulated or stimulated with 100 μ M MnTBAP. There was no significant difference in SI values between cultures in which APCs were exposed only to 10 μ M Be as compared with 10 μ M Be plus APC pretreatment with 100 μ M MnTBAP (Figure 7A). In contrast, there was a 40% decrease in the SI of CBD PBMCs after overnight 100 μ M MnTBAP pretreatment of the

separated lymphocyte population before 10 μ M Be exposure (Figure 7B). Similar results were obtained with MnTBAP pretreatment of T cells from a subset of subjects with BeS ($n = 6$) (Figures 7A and 7B, *light gray columns*).

DISCUSSION

In the present study, we examined the role of Be-induced oxidative stress in the proliferative capacity of Be-specific CD4⁺ T cells in the blood of subjects with CBD and BeS. We found that Be significantly increased levels of oxidative stress by inducing the depletion of thiol antioxidants and by elevating ROS production. Depletion of thiols with an increase in ROS production was associated with Be-specific proliferation of CBD CD4⁺ peripheral blood T cells. Importantly, MnTBAP and NAC significantly inhibited Be-specific lymphoproliferation and NAC treatment

Figure 5. Proliferation (stimulation index [SI]) by a subset of beryllium-stimulated (10 μ M BeSO₄) chronic beryllium disease (CBD) peripheral blood mononuclear cells (PBMCs) ($n = 5$) and tetanus toxoid-stimulated (2.5 lymphocyte-flocculating units) healthy normal control PBMCs ($n = 4$) in the absence and presence of MnTBAP (0–100 μ M) after 120 hours. To compare MnTBAP inhibition of T-cell proliferation as a function of increasing MnTBAP concentration, beryllium-stimulated and tetanus toxoid-stimulated SI values observed in the absence of MnTBAP were normalized to 100. * $P < 0.05$, MnTBAP plus BeSO₄ or plus tetanus toxoid versus unstimulated; + $P < 0.05$, MnTBAP (50 μ M) plus tetanus toxoid versus MnTBAP (50 μ M) plus BeSO₄; ++ $P < 0.05$, MnTBAP (100 μ M) plus tetanus toxoid versus MnTBAP (100 μ M) plus BeSO₄. Values represent mean normalized SI $\pm$ SEM.

Figure 6. (A) Proliferation (stimulation index [SI]) and (B) intracellular thiol levels (MCLB fluorescence) of a subset of chronic beryllium disease (CBD) ($n = 5$) peripheral blood mononuclear cells (PBMCs) that were unstimulated or exposed to *N*-acetylcysteine (NAC; 5 mM), BeSO₄ (10 μM), or BeSO₄ (10 μM) plus NAC (5 mM). * $P < 0.05$, BeSO₄ alone versus unstimulated; ** $P < 0.05$ BeSO₄ plus NAC versus BeSO₄ alone. Values represent means and SEM.

restored Be-depleted intracellular thiol levels in CD4⁺ CBD T cells. We conclude that the ability of Be-specific T cells to proliferate is partially modulated by oxidative stress. Moreover, in a cell crossover model we demonstrated that Be-induced T-cell proliferation was inhibited by pretreatment of lymphocytes, but not APCs, with the catalytic antioxidant MnTBAP. We conclude that in both CBD and BeS, Be exposure induces a direct effect on Be-specific CD4⁺ T cells to deplete thiol antioxidants, and to increase ROS generation that is related to the clonal expansion of Be-specific T cells.

The generation of ROS has been previously observed in the chronic inflammatory environment of granulomatous lung diseases, such as sarcoidosis (12, 13). Increased levels of glutathione and glutathione peroxidase have been measured in the epithelial lining fluid of patients with CBD, suggesting a role for the glutathione antioxidant system in granulomatous lung disease (14). PBMCs from subjects with CBD and subjects with BeS had decreased levels of thiol as compared with PBMCs from healthy control subjects, suggesting that Be disease has a profound effect on the oxidative status of this mixed cell population. This might be explained by age-related decreases in glutathione (GSH) levels between the normal control PBMCs and cells isolated from the older subjects with CBD and BeS. Except for the presence of Be

antigen-specific CD4⁺ T cells in their peripheral blood, demonstrated by a positive BeLPT, subjects with BeS are otherwise healthy and we did not anticipate observing decreased levels of GSH and cysteine in isolated PBMCs from subjects with BeS. This suggests the need for future experiments designed and powered to resolve this potentially confounding factor.

In addition to inhibiting Be-stimulated T-cell proliferation by CBD PBMCs, MnTBAP was more effective at inhibiting antigen-specific T-cell proliferation induced by tetanus toxoid. We speculate that this effect may be due to the higher oxidative stress exerted by the lower endogenous thiol levels found in PBMCs from subjects with CBD and the ability of Be to deplete thiols and increase ROS, whereas tetanus toxoid may be increasing ROS only through T-cell receptor (TCR) activation. Therefore MnTBAP will have greater effects at lower concentrations on tetanus toxoid proliferation responses than Be-stimulated proliferation responses. Our observation that Be, in the form of Be antigen, persists inside the CBD lung even a decade after the last Be exposure may have bearing on these observations (15). Precisely how TCR activation up-regulates ROS-mediated gene activation for T-cell proliferation is an area of intense study (5).

A growing body of evidence demonstrates that T cells are strongly regulated by ROS such as hydrogen peroxide and super-

TABLE 2. PROLIFERATIVE RESPONSE USING MIXED VERSUS SEPARATED PERIPHERAL BLOOD MONONUCLEAR CELLS

	BeSO ₄ (10 μ M)	Al ₂ (SO ₄) ₃ (100 μ M)	PHA (100 μ M)
Mixed PBMCs			
Control (n = 9)	1.1 $\pm$ 0.2	0.6 $\pm$ 0.2	13.7 $\pm$ 4*
BeS (n = 5)	2.3 $\pm$ 0.3*	0.8 $\pm$ 0.2	8.9 $\pm$ 3.9*
CBD (n = 7)	3.9 $\pm$ 0.9*	0.9 $\pm$ 0.2	22.8 $\pm$ 6.6*
Separated PBMCs			
Control (n = 9)	0.9 $\pm$ 0.1	0.3 $\pm$ 0.1	11.2 $\pm$ 3.4*
BeS (n = 5)	3.3 $\pm$ 0.9*	0.9 $\pm$ 0.3	21 $\pm$ 5.5*
CBD (n = 7)	3.5 $\pm$ 0.9*	0.5 $\pm$ 0.2	31 $\pm$ 12*

Definition of abbreviations: BeS = beryllium sensitization; CBD = chronic beryllium disease; PBMCs = peripheral blood mononuclear cells; PHA = phytohemagglutinin.

Stimulation index (SI) for CBD (n = 7), BeS (n = 5), and normal control (n = 9) PBMCs that were separated into beryllium-exposed antigen-presenting cells and lymphocyte cell populations, incubated overnight, and recombined. Thymidine incorporation uptake was measured after 72 hours.

Data are expressed as means $\pm$ SEM.

* $P < 0.05$, treatment stimulation index (SI) versus unstimulated SI (unstimulated SI = 1).

oxide anion. The generation of ROS has been shown to occur in response to activation through a variety of cellular receptors including those for transforming growth factor- β (16), insulin (17), and angiotensin II (18). In these instances, ROS function as

a second messenger, required for protein kinase activation, gene expression, and proliferation (19). The generation of ROS in T cells has also been detected after exposure to mitogens (20, 21), superantigens (8), and anti-TCR monoclonal antibodies (22). Previous studies found that ROS are generated within 2–4 minutes in murine and human blastocytic T cells after TCR engagement (22, 23). Others have demonstrated that mature primary human and mouse T-cell blasts express a functional phagocyte-type NADPH oxidase, the absence of which leads to a deficiency in TCR-stimulated ROS generation and subsequent alteration of T-cell responses (23). The rapid ROS production in T cells is consistent with their potential role to regulate T-cell activation (20). However, Tatla and coworkers (21) showed that much higher concentrations of antioxidants were required to inhibit proliferation and IL-2 production compared with blockade of ROS generation, suggesting that ROS are not necessary for optimal T-cell activation. Despite this controversy, it does appear that the addition of antioxidants to mitogen-stimulated T-cell cultures inhibits both proliferation and IL-2 production (24–28).

Traditionally, increased levels of ROS are associated with damage to a variety of host cell molecules including proteins, carbohydrates, lipids, and nucleic acids, and are also known to induce transient growth arrest, apoptosis, and necrosis. A growing body of literature shows that ROS are intimately involved in cell signaling including key molecules in phosphorylation and dephosphorylation pathways (29), in the organization of the

Figure 7. Proliferation (stimulation index [SI]) of a subset of chronic beryllium disease (CBD; n = 7) and beryllium-sensitized (BeS; n = 6) peripheral blood mononuclear cells (PBMCs) that were separated into (A) adherent antigen-presenting cells and (B) non-adherent responding T cells. PBMCs were unstimulated or exposed to MnTBAP (100 μ M), BeSO₄ (10 μ M), or BeSO₄ (10 μ M) plus MnTBAP (100 μ M) for 120 hours. * $P < 0.05$ BeSO₄ alone versus unstimulated; ** $P < 0.05$, BeSO₄ versus BeSO₄ plus MnTBAP. Values represent mean SI and SEM.

cytoskeleton (30), and in the regulation of cell cycle progression (31). These processes are thought to be due to effects of ROS on cyclins, cyclin-dependent kinases, and downstream signals that up-regulate the expression of transcription factors including nuclear factor (NF)- κ B and AP-1 family members (31, 32). Our study demonstrates Be-specific up-regulation of the nuclear levels of both NF- κ B and activator protein 1 (AP-1) transcription factors in CBD BAL CD4⁺ T cells (33). A role for ROS has been implicated in the formation of lipid raft membrane microdomains, which may serve as the structure for the formation of T-cell receptor activation complexes (34). Therefore, induction of TCR membrane raft formation and subsequent T-cell activation in Be-specific CD4⁺ T cells may be enhanced by Be-stimulated up-regulation of ROS and Be-induced depletion of intracellular thiol antioxidants.

It is increasingly clear that the antioxidant status of immune cells and their microenvironment can regulate T-cell responses. Studies have shown that levels of endogenous antioxidants regulate switching between Th1 and Th2 phenotypes (35). Lowering intracellular glutathione levels impairs T-cell functions and increases NF- κ B activation (36–38) and sensitivity to tumor necrosis factor (TNF)- α induced apoptosis (39). Antioxidants have also been shown to down-regulate inflammatory cytokine synthesis and activation (32, 40). Antioxidants can attenuate the levels of the inflammatory cytokines TNF- α , IL-1, IL-6, and IL-8 in PBMCs and biopsies of inflamed colonic mucosa from patients with inflammatory bowel disease (41). Malorni and coworkers showed that exposure to the glutathione precursor NAC can inhibit TNF- α -induced apoptosis of chronically HIV-infected cells *in vitro* (42). Our observations in this study suggest that the depletion of cellular antioxidant defenses, accompanied by Be-induced up-regulation of ROS, may result in increased production of Be-induced IFN- γ and TNF- α by Be-specific CD4⁺ T cells (2), and thereby promote the persistent and chronic inflammation characteristic of this granulomatous lung disorder.

It is also important to acknowledge the limitations in our study design. Throughout this study experiments were performed with PBMCs from subsets of enrolled subjects, principally because of the limited availability of samples. Limitations could have been introduced by our method of PBMC isolation or by performing our studies in complete medium that contained serum. We also make note of the significant age difference between the normal control subjects and the subjects with beryllium disease; however, we believe that this did not confound the results because in all cases the internal experimental control data using unstimulated cells was comparable and not significantly different between normal and disease cells. In this study, we attempted to control these limitations by comparing data obtained with isolated PBMCs from subjects with CBD and BeS with data for cells from normal healthy control subjects, taken as a baseline. Thus, the observed reductions in the levels of GSH and cysteine observed in freshly isolated PBMCs from subjects with BeS and CBD were compared with levels in fresh normal control PBMCs. We believe that the inclusion of control subjects was sufficient to limit possible pitfalls in our experimental design.

Another limitation of this study is that it did not determine the precise mechanism by which Be stimulation up-regulated CD4⁺ Be-specific T-cell ROS-mediated proliferation. Our data implicate peroxides as the Be-induced ROS, based on detection of increased levels of intracellular fluorescent DCF in CD4⁺ T cells, although we did not rule out other possible Be-induced ROS. Thiols are also reactive toward peroxides and it is possible that depletion of cellular thiols could increase peroxide steady state levels. This observation could also be due to the direct toxic effects of Be on CD4⁺ T cells including possible, but as yet unidentified, effects on mitochondria, cell membrane receptor

systems that could also form Be adducts. On the basis of our preliminary observations, we speculate that Be-induced peroxide production in T cells may alter the steady state balance between regulatory protein tyrosine phosphatases and protein tyrosine kinases (PTKs) (43). Hydrogen peroxide-mediated oxidation of an active site cysteine residue can inactivate protein tyrosine phosphatases, allowing PTKs to drive protein phosphorylation that in turn favors the activation of cell signaling pathways that promote proliferation. Thus, Be-stimulated CBD CD4⁺ T cells might proliferate and activate genes encoding proinflammatory cytokines that result in granulomatous inflammation (2, 10, 44). These hypotheses will be tested in future studies.

Our findings show for the first time that Be is unique in that it is both an antigen and a stimulus for increased oxidative stress in T cells. Our results suggest that both of these processes contribute to Be-specific CD4⁺ T-cell proliferation and Th1 cytokine secretion, two key processes in the pathogenesis of CBD. In conclusion, antioxidants can inhibit Be antigen-induced CD4⁺ T-cell proliferation, which suggests a potential therapeutic use of antioxidant molecules in Be-induced lung disease and similar granulomatous lung diseases.

Conflict of Interest Statement: D.R.D. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.T.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.M.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. L.S.N. reviewed beryllium lymphocyte proliferation test results for his former employer, National Jewish Medical and Research Center, until December 2005. He received no direct compensation for that activity. Dr. Newman has testified regarding the beryllium lymphocyte proliferation test and the health effects of beryllium in workers' compensation hearings and other legal proceedings. He received no direct compensation for these activities. He has provided consultation for governmental agencies, labor, and industry regarding beryllium medical monitoring programs that have included the use of beryllium immunologic assays. Dr. Newman receives funding from federal agencies (National Institutes of Health and Department of Energy) to conduct research, and medical monitoring for a variety of conditions that includes use of the BeLPT, and serves as director of a data coordinating center for a beryllium biorepository. L.A.M. has a grant from Centocor to investigate the effects of infliximab in the treatment of CBD. She has coauthored a review on CBD for *Up-to-Date* and receives royalties in the sum of \$250/year. B.J.D. is a consultant for Aeolus Pharmaceuticals, is a cofounder of the company, and holds equity in Aeolus Pharmaceuticals. He is an inventor on nine patents covering the use and composition of matter of metalloporphyrins. He received \$200,000 in research grants from Aeolus Pharmaceuticals from 2005 to 2007.

References

1. Henneberger PK, Goe SK, Miller WE, Doney B, Groce DW. Industries in the United States with airborne beryllium exposure and estimates of the number of current workers potentially exposed. *J Occup Environ Hyg* 2004;1:648–659.
2. Fontenot AP, Kotzin BL. Chronic beryllium disease: immune-mediated destruction with implications for organ-specific autoimmunity. *Tissue Antigens* 2003;62:449–458.
3. Newman LS, Mroz MM, Balkissoon R, Maier LA. Beryllium sensitization progresses to chronic beryllium disease: a longitudinal study of disease risk. *Am J Respir Crit Care Med* 2005;171:54–60.
4. Reth M. Hydrogen peroxide as a second messenger in lymphocyte activation. *Nat Immunol* 2002;3:1129–1134.
5. Hildeman DA, Mitchell T, Kappler J, Marrack PC. T cell apoptosis and reactive oxygen species. *J Clin Invest* 2003;111:575–581.
6. Day BJ, Fridovich IA, Crapo JD. Manganic porphyrins possess catalase activity and protect endothelial cells against hydrogen peroxide-mediated injury. *Arch Biochem Biophys* 1997;347:256–262.
7. Day BJ, Shawen S, Liochev SI, Crapo JD. A metalloporphyrin superoxide dismutase mimetic protects against Paraquat-induced endothelial cell injury, *in vitro*. *J Pharmacol Exp Ther* 1995;275:1227–1232.
8. Hildeman DA, Mitchell T, Teague TK, Henson P, Day BJ, Kappler J, Marrack PC. Reactive oxygen species regulate activation-induced T cell apoptosis. *Immunity* 1999;10:735–744.
9. Sawyer RT, Dobis DR, Goldstein M, Velsor L, Maier LA, Fontenot AP, Silveira L, Newman LS, Day BJ. Beryllium-stimulated reactive

- oxygen species and macrophage apoptosis. *Free Radic Biol Med* 2005; 38:928–937.
10. Mroz MM, Kreiss K, Lezotte DC, Campbell PA, Newman LS. Re-examination of the blood lymphocyte transformation test in the diagnosis of chronic beryllium disease. *J Allergy Clin Immunol* 1991;88: 54–60.
 11. Sawyer RT, Day BJ, Fadok VA, Chiarappa-Zucca M, Maier LA, Fontenot AP, Sliveira L, Newman LS. Beryllium-ferritin: lymphocyte proliferation and macrophage apoptosis in chronic beryllium disease. *Am J Respir Cell Mol Biol* 2004;31:470–477.
 12. Fels AO, Nathan CF, Cohn ZA. Hydrogen peroxide release by alveolar macrophages from sarcoid patients and by alveolar macrophages from normals after exposure to recombinant interferons α , β , and γ and 1,25-dihydroxyvitamin D₃. *J Clin Invest* 1987;80:381–386.
 13. Calhoun WJ, Salisbury SM. Heterogeneity in cell recovery and superoxide production in buoyant, density-defined subpopulations of human alveolar macrophages from healthy volunteers and sarcoidosis patients. *J Lab Clin Med* 1989;114:682–690.
 14. Comhair SA, Lewis MJ, Bhatena 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.
 15. Sawyer RT, Abraham JA, Daniloff E, Newman LS. Secondary ion mass spectroscopy demonstrates retention of beryllium in chronic beryllium disease granulomas. *J Occup Environ Med* 2005;47:1218–1226.
 16. Thannickal VJ, Day RM, Klinz SG, Bastien MC, Larios JM, Fanburg BL. Ras-dependent and -independent regulation of reactive oxygen species by mitogenic growth factors and TGF- β_1 . *FASEB J* 2000;14: 1741–1748.
 17. Mahadev K, Zilbering A, Zhu L, Goldstein BJ. Insulin-stimulated hydrogen peroxide reversibly inhibits protein-tyrosine phosphatase 1b *in vivo* and enhances the early insulin action cascade. *J Biol Chem* 2001;276:21938–21942.
 18. Touyz RM. Reactive oxygen species and angiotensin II signaling in vascular cells: implications in cardiovascular disease. *Braz J Med Biol Res* 2004;37:1263–1273.
 19. Williams MS, Kwon J. T cell receptor stimulation, reactive oxygen species, and cell signaling. *Free Radic Biol Med* 2004;37:1144–1151.
 20. Williams MS, Henkart PA. Role of reactive oxygen intermediates in TCR-induced death of T cell blasts and hybridomas. *J Immunol* 1996; 157:2395–2402.
 21. Tatla S, Woodhead V, Foreman JC, Chain BM. The role of reactive oxygen species in triggering proliferation and IL-2 secretion in T cells. *Free Radic Biol Med* 1999;26:14–24.
 22. Kwon J, Devadas S, Williams MS. T cell receptor-stimulated generation of hydrogen peroxide inhibits MEK-ERK activation and Lck serine phosphorylation. *Free Radic Biol Med* 2003;35:406–417.
 23. Jackson SH, Devadas S, Kwon J, Pinto LA, Williams MS. T cells express a phagocyte-type NADPH oxidase that is activated after T cell receptor stimulation. *Nat Immunol* 2004;5:818–827.
 24. Novogrodsky A, Ravid A, Rubin AL, Stenzel KH. Hydroxyl radical scavengers inhibit lymphocyte mitogenesis. *Proc Natl Acad Sci USA* 1982;79:1171–1174.
 25. Fidelus RK, Ginouves P, Lawrence D, Tsan MF. Modulation of intracellular glutathione concentrations alters lymphocyte activation and proliferation. *Exp Cell Res* 1987;170:269–275.
 26. Fidelus RK, Tsan MF. Enhancement of intracellular glutathione promotes lymphocyte activation by mitogen. *Cell Immunol* 1986;97:155–163.
 27. Chaudhri G, Hunt NH, Clark IA, Ceredig R. Antioxidants inhibit proliferation and cell surface expression of receptors for interleukin-2 and transferrin in T lymphocytes stimulated with phorbol myristate acetate and ionomycin. *Cell Immunol* 1988;115:204–213.
 28. Dornand J, Gerber M. Inhibition of murine T-cell responses by antioxidants: the targets of lipo-oxygenase pathway inhibitors. *Immunology* 1989;68:384–391.
 29. Forman HJ, Torres M, Fukuto J. Redox signaling. *Mol Cell Biochem* 2002;249:234–235.
 30. Cavellier G. Theory of malignant cell transformation by superoxide fate coupled with cytoskeletal electron-transport and electron-transfer. *Med Hypotheses* 2000;54:95–98.
 31. Boonstra J, Post JA. Molecular events associated with reactive oxygen species and cell cycle progression in mammalian cells. *Gene* 2004;337:1–13.
 32. Sen CK, Packer L. Antioxidant and redox regulation of gene transcription. *FASEB J* 1996;10:709–720.
 33. Sawyer RT, Fontenot AP, Barnes TA, Parsons CE, Maier LA, Gillespie MM, Gottschall EB, Hagman JA, Newman LS. Beryllium-induced TNF- α production is transcription-dependent in chronic beryllium disease. *Am J Respir Cell Mol Biol* 2007;36:191–200.
 34. He HT, Lellouch A, Marguet D. Lipid rafts and the initiation of T cell receptor signaling. *Semin Immunol* 2005;17:23–33.
 35. Peterson JD, Herzenberg LA, Vasquez K, Waltenbaugh C. Glutathione levels in antigen-presenting cells modulate Th1 versus Th2 response patterns. *Proc Natl Acad Sci USA* 1998;95:3071–3076.
 36. Staal FT, Anderson MT, Staal GE, Herzenberg LA, Gitler C. Redox regulation of signal transduction: tyrosine phosphorylation and calcium influx. *Proc Natl Acad Sci USA* 1994;91:3619–3622.
 37. Staal FT, Roederer M, Herzenberg LA. Intracellular thiols regulate activation of nuclear factor- κ B and transcription of human immunodeficiency virus. *Proc Natl Acad Sci USA* 1990;87:9943–9947.
 38. Mihm S, Ennen J, Pessara U, Kurth R, Droge W. Inhibition of HIV-1 replication and NF- κ B activity by cysteine and cysteine derivatives. *AIDS* 1991;5:497–503.
 39. Fernandez A, Kiefer J, Fosdick L, McConkey DJ. Oxygen radical production and thiol depletion are required for Ca²⁺ mediated endogenous endonuclease activation in apoptotic thymocytes. *J Immunol* 1995;155: 5133–5139.
 40. Treitinger A. Effect of N-acetyl-L-cysteine on lymphocyte apoptosis, lymphocyte viability, TNF- α and IL-8 in HIV-infected patients undergoing anti-retroviral treatment. *Braz J Infect Dis* 2004;8:363–371.
 41. Reimund JM. Antioxidants inhibit the *in vitro* production of inflammatory cytokines in Crohn's disease and ulcerative colitis. *Eur J Clin Invest* 1998;28:145–150.
 42. Malorni W, Rivaben R, Santini MT, Donelli G. N-Acetylcysteine inhibits apoptosis and decreases viral particles in HIV-chronically infected U937 cells. *FEBS Lett* 1993;327:75–78.
 43. Rhee SG. H₂O₂, a necessary evil for cell signaling. *Science* 2006;312: 1882–1883.
 44. Sawyer RT, Parsons CE, Fontenot AP, Maier LA, Gillespie MM, Gottschall EB, Silveira L, Newman LS. Beryllium-induced tumor necrosis factor- α production by CD4⁺ T cells is mediated by HAL-DP. *Am J Respir Cell Mol Biol* 2004;31:122–130.