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Secondary Ion Mass Spectroscopy Demonstrates Retention of Beryllium in Chronic Beryllium Disease Granulomas

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Learning Objectives

- Explain possible mechanisms by which persistence of antigen in lung tissue could be important in the development of granulomatous inflammation.
- Compare the presence and amount of beryllium in lung tissue, within and outside of granulomas, in exposed ceramics workers with and those without chronic beryllium disease.
- Point out the conditions under which microanalysis of lung tissue for beryllium might be appropriate.

Abstract

Objective: *We hypothesized that beryllium (Be) might persist in lung granulomas in patients with chronic beryllium disease (CBD).* ***Methods:*** *A total of 33 Be-exposed ceramics workers underwent transbronchial biopsy. They were classified based on histopathology and Be-lymphocyte proliferation test as CBD or other categories. Lung tissue sections were analyzed using secondary ion mass spectroscopy.* ***Results:*** *Be was detected in the lungs of all Be-exposed groups. Be levels were increased within the granulomas of patients with CBD compared with the Be levels outside granulomas. Notably, Be was detectable in the lungs of CBD patients who had ceased exposure to Be an average of 9 years previously.* ***Conclusions:*** *Be was detected in the lungs of all Be-exposed subjects, with the highest levels of persistent Be inside CBD lung granulomas. Be antigen persistence may help explain the chronicity of this granulomatous disorder.* (J Occup Environ Med. *2005;47:1218–1226*)

Pulmonary interstitial mononuclear cell infiltrates occur in occupational diseases such as hypersensitivity pneumonitis and chronic beryllium disease (CBD), as well as other granulomatous disorders such as sarcoidosis. Over time, these mononuclear cell infiltrates can become organized into noncaseating granulomas. Antigen persistence is thought to play a key role in the pathogenesis of granulomatous inflammation, although there is little direct proof of this hypothesis. Antigen persistence could serve to drive both the proinflammatory cytokine production and the antigen-specific, adoptive immune response that ultimately gives rise to granulomas. CBD is a human granulomatous disease for which the causative antigen, beryllium (Be), is known.[1] Therefore, CBD provides a unique opportunity to examine the role of persistent metallic antigen in the pathogenesis of a human, antigen-driven lung disorder. Secondary ion mass spectroscopy (SIMS) is an extremely sensitive analytical technique that uses a beam of ions to generate a localized plasma on the tissue surface, from which ions are extracted and analyzed using a mass spectrometer. Under optimal conditions, single atomic or molecular ions, such as metallic Be, can be detected.

Be is a lightweight and durable metal used in a variety of industrial applications. Be-exposed workers may remain healthy with no evidence of immune reactivity to Be. Other Be-exposed workers develop a

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delayed-type hypersensitivity to Be and become sensitized. Be-sensitized (BeS) patients have a history of Be exposure and their Be-stimulated peripheral blood lymphocytes incorporate the DNA precursor tritiated thymidine in the Be lymphocyte proliferation test (BeLPT).[2] The lungs of BeS patients are normal, and their bronchoalveolar lavage (BAL) cells do not proliferate in response to Be stimulation in the BeLPT. After Be exposure, as many as 20% of workers develop BeS,[3] and although many of these have CBD at the time of their first evaluation, many others develop CBD progressing from BeS at a rate of 6–8% per year.[4] Individuals exposed to Be can develop disease even decades after their last occupational exposures have ceased.[5] CBD typically is characterized by a history of Be exposure, the proliferation of blood and/or BAL lymphocytes in the BeLPT, and by the presence of interstitial granulomas and/or mononuclear cell infiltrates upon lung biopsy. Taken alone, the presence of pulmonary granulomas by light microscopy is insufficient to distinguish CBD from sarcoidosis.[6,7]

The delayed onset of CBD and persistence of granulomas in CBD suggest the possibility that Be might persist within the lungs and within these immunologically active lesions. To test this hypothesis, we enrolled 33 Be-exposed subjects from a Be ceramics manufacturing company who met clinical and diagnostic criteria defining them as Be-exposed normal, CBD, negative BeLPT with granulomatous lung disease (NBeLPT-GLD), or Be-exposed with pulmonary fibrosis. These individuals were identified originally as part of a medical surveillance study.[8] In this workforce, we had identified not only cases of CBD but also an interesting cluster of biopsy-proven GLD cases that had both negative blood and BAL BeLPT. In 1993, by convention, they were not called CBD, but "sarcoidosis." In light of the possibility of false negative BeLPTs, in such instances, another goal of this study was to use secondary ion mass spectroscopy (SIMS) to determine if the granulomas in such NBeLPT-GLD cases might contain Be.

Materials and Methods

Study Population

We examined lung biopsies from 33 Be-exposed, Be ceramics manufacturing company employees/former employees. Exposures consisted mainly of beryllium oxide (beryllia) in the form of either fired or unfired dust.[8] Pulmonary pathologists' light microscopic interpretations were relied upon in classifying patients into one of four groups. In each case, we collected demographic information[9] and analyzed tissue samples by SIMS. All of the subjects who volunteered to participate in this study had a history of Be exposure and were separated based on previously published definitions.[2,6,7,10]

Be-Exposed Normal Subjects

Seven Be-exposed individuals from the Be ceramics company had normal lung biopsies showing no evidence of granulomas, fibrosis, or any other form of interstitial lung disease. Special stains and cultures for mycobacteria and fungi were all negative. All of the Be-exposed normal subjects had a normal blood and BAL BeLPT.

CBD

There were 15 individuals from the Be ceramics company who had a history of Be exposure, granulomatous inflammation, and/or mononuclear cell infiltrates on lung biopsy and abnormal blood and/or BAL BeLPT. Special stains and cultures for mycobacteria and fungi were all negative.

Be-Exposed Pulmonary Fibrosis

Four individuals from the Be ceramics company displayed varying degrees of lung fibrosis on their biopsies, all of unknown cause. Despite the fact that all of these subjects had a history of Be exposure, none had granulomatous inflammation, and all had a normal blood and BAL BeLPT. None of these subjects had evidence of asbestos-related fibrosis or a medical history suggestive of other known causes of lung fibrosis. Special stains and cultures for mycobacteria and fungi were all negative.

Negative BeLPT With Granulomatous Lung Disease (NBeLPT-GLD)

The last study group consisted of seven individuals from the Be ceramics company who had a history of Be exposure and who had noncaseating granulomas found on lung biopsy. All of these subjects had a negative blood and/or BAL BeLPT. These subjects meet all but one of the diagnostic criteria for sarcoidosis established by the American Thoracic Society.[11] However, an important criterion—the exclusion of other disease capable of producing a similar histologic or clinical picture—could not be fulfilled. Therefore, we designated this group as NBeLPT-GLD subjects. It should be noted that the NBeLPT-GLD subjects represents a cluster of seven patients among a total workforce of 505 Be ceramics workers, all of whom were Be-exposed.[8] In the original study by Kreiss et al.,[8] the nomenclature of sarcoidosis was used to describe this study group; however, in the present study we have elected to use this different nomenclature (NBeLPT-GLD) to be more descriptive. At the time of the study none of these subjects displayed immunologic evidence of Be sensitization. Special stains and cultures for mycobacteria and fungi were negative.

Demographics

Written, informed consent was obtained from each patient enrolled in this study, and the protocol was approved by the Human subject Institutional Review Board at National Jewish Medical and Research Center, Denver, Colorado. After informed consent, all participants completed a

modified version of the American Thoracic Society respiratory questionnaire.[9] This questionnaire included information on smoking status, self-reported race and ethnicity, and working history. A "never smoker" was defined as having smoked fewer than 20 packs of cigarettes or 12 oz of tobacco in a lifetime, or less than one cigarette per day for 1 year. A "current smoker" was defined as having smoked cigarettes within 1 month of study participation. A "former smoker" was defined as having quit smoking more than 1 month before study participation.[9] Corticosteroid use was defined as a subject who was using oral prednisone or its equivalent 1 month before bronchoscopy. Latency is defined as the time from first exposure to Be to the date of diagnosis (years). Time from last exposure to Be was calculated based on last year of employment or last year of self-reported Be work through date of diagnosis.

Preparation of BAL and Peripheral Blood Mononuclear Cells

At the time of bronchoscopy with BAL, all subjects underwent clinical evaluation consisting of a chest radiograph, pulmonary function testing, exercise testing, and venipuncture. BAL was performed by standard methods reported previously.[12] Cell viability, evaluated by trypan blue exclusion, ranged from 90% to 97%. Cell counts are reported as total white blood cells per milliliter of BAL fluid. Differential white blood cell counts were performed on cells prepared by cytocentrifugation (Shandon Southern, Sewickley, PA), stained with Diff-Quick (Fisher Scientific Co, Springfield, NJ) and included macrophages, lymphocytes, eosinophils, and neutrophils.

Be Lymphocyte Proliferation Test (BeLPT)

The blood peripheral blood mononuclear cells and BAL cell BeLPTs were performed according to the methods of Mroz et al.[2] Results are expressed as the Stimulation Index (SI) based on the ratio of the counts per minute (cpm) of the Be-stimulated cells to that of the untreated cells. At the time that testing was performed for this study, two Be-stimulated cell values greater than an SI of 2.0 was considered abnormal in our laboratory, based on the mean peak SI of 1.2 plus two standard deviations for nonexposed individuals' cells cultured under identical conditions.[8]

SIMS

We used SIMS to analyze lung tissue for the presence of Be as described previously.[13,14] SIMS is an extremely sensitive tool for detection of ions such as Be at $m/e^- = +9$, as the background noise in the mass spectrum is <0.1 cps.[13,14] Standard 5-μm thick, paraffin-embedded sections of formalin-fixed tissues were mounted on pure carbon discs and the paraffin removed with xylene. Correlation with light microscopy of adjacent/serial sections mounted on glass slides was used to facilitate identification of areas to be analyzed in the Atomika Ion Microprobe (FEI Deutschland GmbH, Munich, GR). In the Ion Microprobe sections were viewed by reflected light optics sufficient to identify different areas of the sections, for example, areas of normal tissue versus granulomatous lung parenchyma. The Ion Microprobe, which uses a focused beam of ions of one or more elements ($^{32}O_2^+$) to bombard the tissue specimen.[14] The ion beam current was 200nA, 10kV, with a spot size of approximately 30–40 micrometers and the scanning raster set at 200 × 200 micrometers. The ion beam creates a local plasma from which ions, neutral atoms, and molecules are ejected. These secondary ions are collected and amplified after passing through a mass spectrometer. The result is a mass spectrum with quantitative digital information related to the point or area being bombarded by the ion beam. Under these conditions, a standard of AuBe gave 1×10^6 counts per second (cps) at $m/e^- = +9$. Digital images and distribution maps of various ions can be constructed. These data thereby provide both quantitative and qualitative estimates for Be content within the lung biopsy specimens.

Measurements were obtained both inside lung granulomas and from areas of normal lung tissue outside areas of pathologic change. For each lung specimen analyzed by SIMS, we determined the average Be content, which is calculated as the average amount of Be (at $m/e^- = +9$) detected in cps for all areas of the lung biopsy examined. This method has been tested against tissue standards with known Be content and found to give reliable quantitation of Be in tissues where the average Be cps was shown to be directly related to the gravimetric Be concentration in the tissues.[14] We also determined the maximum Be content, which is calculated as the single greatest amount of Be detected in any of the areas of lung biopsy probed, expressed in cps. Thirty-three subjects provided lung tissue biopsy samples for this study and for particular SIMS analyses the number of individual samples used in each experiment is specified in the results section.

Statistical Analysis

We compared mean differences between groups using Student's *t* test, one-way analysis of variance (ANOVA), Wilcoxon rank sum test, or the Kruskal–Wallis test for nonnormally distributed data.[15,16] We compared group frequencies for presence or absence of Be using Fisher's exact and the chi square tests. All results are expressed as the mean and standard error unless otherwise indicated. A P value less than 0.05 was required for statistical significance. Small sample size in some comparison groups precluded formal statistical analysis.

Results

Demographics and Work History

Demographic profiles for the four groups are summarized in Table 1. The age and demographics of the subjects within the various groups reflect that of the Be workforce in the state in which the Be ceramics plant was located. The work history and job classifications for this group of subjects were described previously.[8] All of the subjects were Be-exposed, and the majority were Caucasian. For the CBD subjects, the last Be-exposure to time of diagnosis was 9 ± 2 years (13/15 CBD subjects, mean ± SEM), with a range of 1 year (3/13 subjects, minimum) to 26 years (1/13 subject, maximum). The latency from the initial Be exposure to time of diagnosis was 12 ± 2 years (mean ± SEM) with a range of 1 year (2/15 subjects, minimum) to 27.2 years (1/15 subjects, maximum) for the CBD subjects. Among the CBD subjects, 40% were never smokers, 33% former smokers, and 27% current smokers. We observed no statistically significant demographic or smoking differences among the groups. Two CBD subjects were being treated with methotrexate.

The immune response of the CBD subjects was confirmed by an elevated blood BeLPT peak SI = 29.5 ± 14.9 (mean ± SEM; interquartile range, IQR; 25% = 2.2 and 75% = 60.6), indicating the proliferation of blood lymphocytes in response to Be stimulation in vitro. The BAL BeLPT for the CBD group has been previously reported[8] and was significantly increased. The blood BeLPTs for the Be-exposed normal, NBeLPT-GLD, and Be-exposed pulmonary fibrosis groups also were previously reported in that study[8] and were not significantly increased. Additional cases in the present study came from the same place of employment but were not included in the original study by Kreiss et al[8] because of the timing of their evaluations.

Identification of Be Inside the CBD Granuloma Using SIMS

Standard light microscopic diagnostic criteria for histopathologic identification of granulomas and fibrosis were used by an experienced pulmonary pathologist (J.L.A.). Individual granulomas varied in cross sectional area from a few macrophages to more than 10 macrophages and giant cells. The areas of fibrosis also were variable, involving interstitial fibrosis and more dense consolidation in some cases. Because most of the biopsies were transbronchial, the actual tissue sampled in such cases was most limited to pieces less than 3 mm maximum dimension. The areas analyzed under the SIMS ion beam was a raster, which included portions of granulomas or portions of nongranulomatous tissues as previously described.[14] SIMS analyzed areas (rasters) were approximately 200–300 μm in diameter such that in some instances the ion beam could burn a tissue area encompassing roughly a single, well-formed, noncaseating granuloma, as shown in the example in Fig. 1A and B. We tested the hypothesis that SIMS would detect Be inside CBD granulomas. A representative tissue specimen obtained from the transbronchial biopsy of a CBD case demonstrates areas of normal alveolar histology in comparison with areas with mononuclear cell infiltration and well formed noncaseating granulomas, typically sampled dur-

TABLE 1
Demographics of the Patients Who Participated in this Study

	Be-Exposed Normal (*n* = 7)	CBD (*n* = 15)	NBeLPT- GLD (*n* = 7)	Be-Exposed Pulmonary Fibrosis (*n* = 4)
Age				
Mean years (SD)	51.2 (10.9)	53.1 (12.4)	51.2 (10.6)	64.1 (13.8)
Gender n, (%)				
Male	4 (57)	8 (53)	7 (100)	3 (75)
Female	3 (43)	7 (47)	0 (0)	1 (25)
Race n, (%)				
Caucasian	7 (100)	14 (93)	6 (86)	3 (75)
African American	0 (0)	1 (7)	1 (14)	1 (25)
Smoking status (%)				
Never smoker	0 (0)	6 (40)	2 (29)	0 (0)
Former smoker	3 (43)	5 (33)	2 (29)	3 (75)
Current smoker	4 (57)	4 (27)	3 (43)	1 (25)

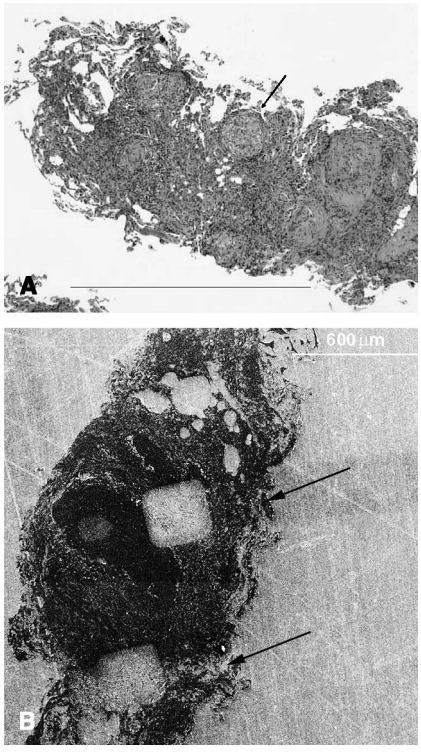

Fig. 1. (A) Light microscopic image of a transbronchial biopsy from one of the study participants showing noncaseating granulomas (arrow) and adjacent nongranulomatous lung parenchyma. Hematoxylin and eosin stain, scale marker = 1 mm. (B) Scanning electron micrographic image of a transbronchial biopsy section after SIMS analysis showing typical rasters where tissue was etched during SIMS analysis (arrows). Scale marker = 600 μm.

ing SIMS analysis (Fig. 1A). A scanning electron micrograph (Fig. 1B) shows typical rasters where a transbronchial specimen was etched during SIMS analyses.

For reference and analytical calibration, we used SIMS analysis of ^{9}Be in a pure Be standard containing 1×10^{6} ppm, and of tissue sections with known gravimetric Be concentrations. Analysis using two SIMS instruments shows a similar relationship between Be concentrations in the pure Be standard and known Be gravimetric tissue concentrations (Fig. 2). A SIMS mass spectrum in Fig. 3 shows positive Be peak and other ions collected from a single CBD lung tissue sample as measured inside a granuloma. The mass spectrum shows a peak at $m/e^{-} = +9$ identifying ^{9}Be.

We used SIMS to determine the Be levels, measured as counts per second (cps) inside the CBD granuloma versus outside the CBD granuloma, in normal appearing non-granulomatous CBD lung tissue (Fig. 4). For this analysis, granuloma-bearing lung tissue samples from 9 of the CBD participants were selected. We observed a significant increase in the Be cps inside CBD granulomas (Be cps median = 0.95, min. 0, max. 550 cps) versus the normal-appearing CBD lung tissue outside the CBD granuloma (Be cps median = 0, min. 0, max. 80 cps) ($P = 0.0035$ versus the Be cps inside the CBD granuloma, Student's t test). The maximum Be cps was determined from 6 individual SIMS analyses inside the CBD granuloma and from 7 individual SIMS analyses outside the CBD granuloma in normal lung tissue. There was a significant increase in the maximum Be cps inside the CBD granuloma (Be cps median = 61, min. 3, max. 550 cps) compared with the lung tissue outside the CBD granuloma (Be cps median = 10, min. 1.2, max. 80 cps; $P = 0.028$ versus the mean maximum Be cps inside the CBD granuloma, Student's t test).

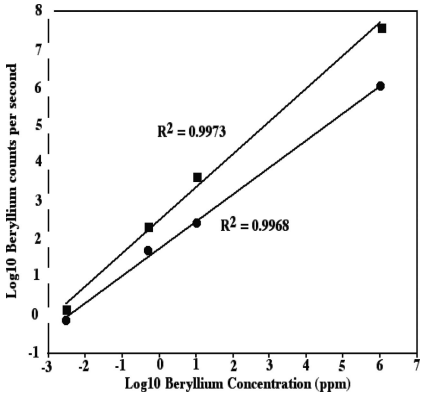

Fig. 2. SIMS analyses of Be in tissue sections with known gravimetric Be concentrations. Results of SIMS analysis for $^{9}Be^{+}$ using two SIMS instruments with different sets of tissue sections from the same cases, performed in 1976 (IMMA instrument, boxes) and in 1992 (ATOMIKA instrument, circles). Each show excellent correlation between the Log10 beryllium counts per second and known tissue concentrations (Log10 beryllium parts per million).

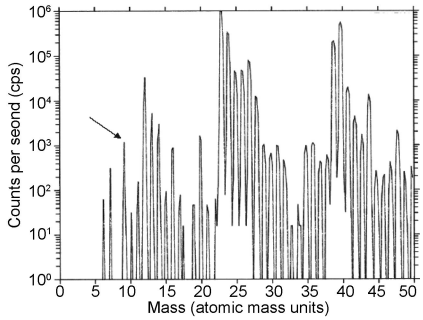

Fig. 3. A SIMS mass spectrum (atomic mass units, amu) of positive ions collected from inside a CBD lung tissue sample granuloma showing a Be concentration (count rate = 1/s) peak at $m/e^{-} = +9$, identifying $^{9}Be^{+}$ (arrow). Other peaks are observed caused by the presence of other elements and ions generated from within the same granuloma. Other typical peaks include ^{6}Li, ^{7}Li, $^{23}Na^{2+}$ (at $m/e^{-} = 11.5$), ^{12}C, ^{13}CH, ^{16}O, $^{40}Ca^{2+}$ (at $m/e^{-} = 20$), ^{23}Na, ^{27}Al, ^{28}Si, ^{39}K, ^{40}Ca, ^{44}SiO, and ^{44}Ca.

Prsence of Be in the Lungs of All Be Ceramics Workers

We tested the hypothesis that histologically normal lung tissue of all

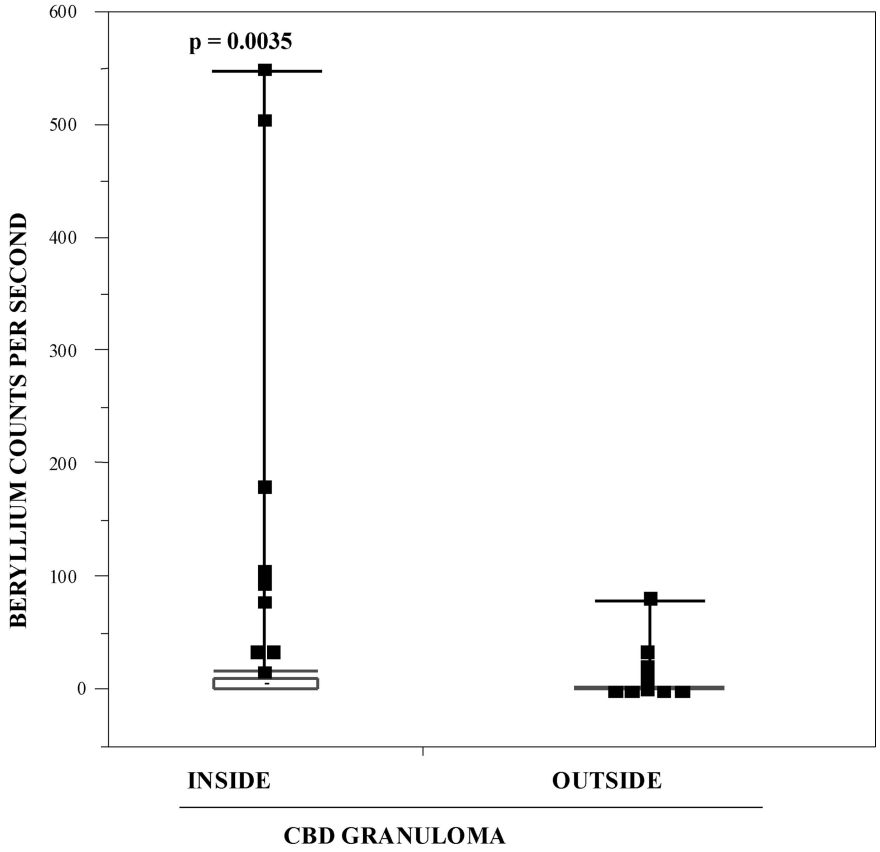

Fig. 4. The Be cps inside versus outside the CBD granuloma. The box and whisker plots show the median cps (middle box bar), the minimum and maximum cps (upper and lower bars) and the interquartile cps range at 25% and 75% (lower and upper box limits). $P = 0.0035$ for Be cps inside versus outside the CBD granuloma. The individual SIMS analyses (black boxes) are compressed because of the large number of SIMS analyses = 0 Be cps (see Results section).

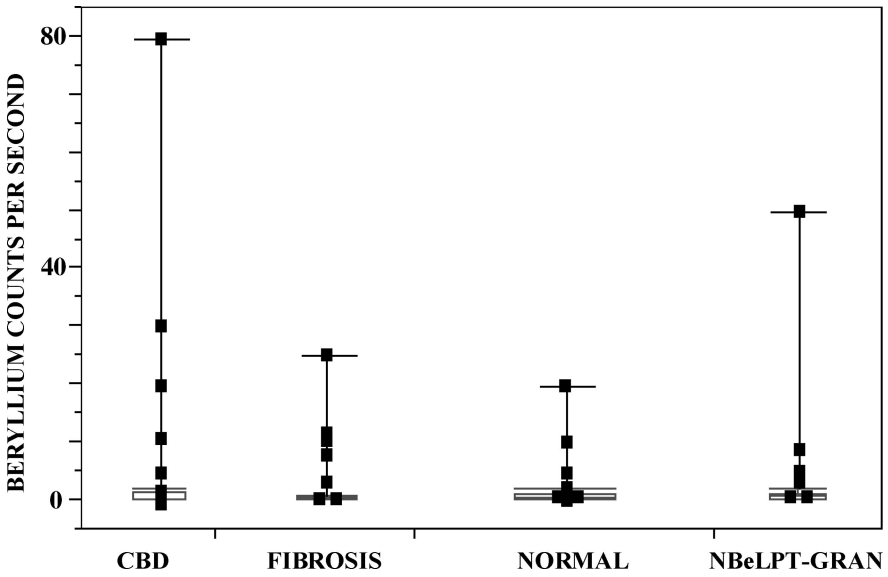

Fig. 5. The Be cps in nongranulomatous lung tissues among the various study groups. $P = 0.667$ for the CBD group as compared to all other groups. The box and whisker plots show the median cps (middle box bar), the minimum and maximum cps (upper and lower bars) and the interquartile cps range at 25% and 75% (lower and upper box limits). The individual SIMS analyses (black boxes) are compressed because of the large number of SIMS analyses = 0 Be cps (see Results section).

of the study groups would contain detectable Be. To do this, we used SIMS to determine the mean Be cps present only outside areas of pathologic change, in normal lung tissues, in subsets of the Be-exposed normal ($n = 7$), CBD ($n = 7$), NBeLPT-GLD ($n = 6$) and Be-exposed pulmonary fibrosis ($n = 4$) subjects (Fig. 5). Be was detected in the normal-appearing nongranulomatous lung tissue of all of the four study groups, regardless of disease status. We found no significant difference in the Be cps within normal (Be cps median = 0.3, min. 0, max. 20 cps, $n = 88$ samples), CBD (Be cps median = 0, min. 0, max. 80, $n = 46$ samples), fibrosis (Be cps median = 0, min. 0, max. 25, $n = 52$ samples), and NBeLPT-GLD (Be cps median = 0.65, min. 0, max. 290, $n = 50$ samples) nongranulomatous lung tissue samples among the four groups ($P = 0.667$ vs. the CBD group, ANOVA). In the normal lung tissues from the normal group, SIMS analysis showed that 41 of 88 samples (47%) had no detectable Be (ie, Be = 0 cps). The maximum Be cps in the normal subjects was based on a single sample = 20 Be cps. By comparison, in the CBD subjects' nongranulomatous lung tissues SIMS analysis showed that 24/46 samples (52%) had Be = 0 cps in which 2 of the 7 subjects studied had a maximum Be = 80 cps, and the maximum SIMS analysis for the other 5 subjects was Be = 20 cps. In the fibrosis subjects, SIMS analysis showed that 31 of 52 samples (59%) had a Be = 0 cps, maximum of one sample Be = 25 cps and in the NBeLPT-GLD group 15/50 samples (30%) had a Be = 0 cps. There was a single sample in the NBeLPT-GLD group, omitted in Fig. 5, in which Be = 290 cps and the next highest SIMS analysis showed a single sample in which Be = 50 cps.

Detection of Be Inside the Granuloma of Only the Be-Exposed Ceramics Workers With CBD

We tested the hypothesis that Be levels would be greater in the CBD granuloma compared with NBeLPT-GLD and pulmonary fibrosis groups despite the fact that all of the study participants were exposed to Be. To do this, we used SIMS to determine the Be cps inside CBD granulomas in comparison with the Be cps inside the NBeLPT-GLD granulomas and inside areas of fibrosis and/or mononuclear cell infiltrates in Be-exposed pulmonary fibrosis subjects. We compared the Be cps for the areas of lung tissues from these three groups that showed areas of pathologic change by light microscopy, to the Be cps inside the nongranulomatous tissues of the Be-exposed normal subjects (Be cps median = 0.3, min. 0, max. 20, $n = 88$ samples).

The Be levels inside the CBD subjects' granulomas (Be cps median = 0.95, min. 0, max. 550, $n = 32$ samples) were significantly increased in comparison with Be levels inside the nongranulomatous tissues of the Be-exposed normal subjects, and in comparison to the Be cps inside the granulomas of the NBeLPT-GLD subjects (Be cps median = 0.6, min. 0.6, max. 1.9, $n = 3$ samples) and the areas of Be-exposed pulmonary fibrosis and mononuclear cell infiltrates (Be cps median = 0, min. 0, max. 25, $n = 52$ samples) ($P = 0.05$ versus the Be cps inside the CBD subject's granulomas, multiple comparisons test using Tukey Kramer). A single outlier of Be cps = 290, for one of the NBeLPT-GLD study subjects, drove a seemingly high median Be cps value, and when this single outlier was dropped from the analysis, the median Be cps levels for this group was <0.5 cps ($P = 0.04$ vs. the Be cps inside the CBD subject's granulomas, multiple comparisons test using Tukey Kramer). Our data show that high Be levels were found principally inside the CBD granuloma.

Discussion

Be is detectable in the lung granulomas of CBD cases and persists even many years after occupational exposures have ceased. The majority of detectable Be is found inside the CBD granuloma, with less found in histologically normal lung tissue, as

shown by our ability to detect Be by SIMS in the lungs of all of the Be ceramics workers tested. There were no significant differences in the levels of Be associated with nongranulomatous lung tissue areas among the four study groups. Thus, little Be was found associated with lung tissues other than the CBD lung granuloma, despite the fact that all of the study subjects were Be-exposed Be ceramics plant workers. We conclude that in CBD, granulomatous inflammation is associated with elevated Be levels by SIMS.

Freiman and Hardy[17] used the chemical method of bulk tissue analysis (atomic absorption spectroscopy) to detect as little as 0.002 μg of Be per gram of dried control lung tissue. This method, however, does not distinguish between the active Be compounds and the inert beryllium silicate (beryl) that is present in certain fuels and dusts whereas the SIMS technique does allow distinguishing between Be silicates and Be oxides.[14] They found that individuals with acute Be disease, or with a history of Be exposure had a range of <5–20 μg and over, of Be per 100g of lung tissue, whereas 85% of individuals with no history of Be exposure had <5 μg of Be per 100g of lung tissue. Williams and Wallach[18] used a laser probe with mass spectroscopy to compare Be levels within lung biopsies of 33 CBD cases and 30 sarcoidosis cases. Be was identified in the lung granulomas of the CBD subjects but was not found in the adjacent normal appearing lung tissues. No Be was found in the 30 sarcoidosis cases that they studied. Unlike our study, their investigation did not examine the control group of non-affected, Be-exposed individuals. Also, the laser probe/mass spectrometry technique did not permit quantitative estimates of Be concentration. Recent development of polymeric atmospheric thin-window (ATW) energy-dispersive x-ray analysis (EDXA) overcomes the limitations of previous instrumentation making it possible to detect elements in tissue samples with an atomic number ≤ fluorine. Butnor et al[19] used ATW-EDXA to detect Be particles inside the cytoplasm of multinucleate giant cells in the lung granulomas of a single study subject, but not in the lung granulomas of a study subject with sarcoidosis and no history of Be-exposure. This technique (ATW-EDXA) is much less sensitive than the SIMS technique, that can detect Be in tissues in the sub-parts per million range. Taken together, these studies suggest that CBD in some circumstances may be distinguished from other granulomatous disorders that had been clinically classified as sarcoidosis by microprobe techniques. An earlier study using spectrographic analysis on bulk lung tissue samples had shown that Be levels are elevated in CBD lung and that Be was not associated with lung tissues from sarcoidosis subjects.[20] Animal studies using ion microprobe analysis have limited relevance to CBD, mainly because of the use of intraperitoneal injection of Be, lack of pulmonary granulomas, and their emphasis on cellular and subcellular localization.[21–25]

In our original epidemiological study of the Be ceramics plant workforce,[8] we identified a cluster of Be-exposed Be ceramics workers who had biopsy-proven granulomatous lung disease but who were consistently BeLPT negative. This group raised the question of whether there was another potential cause of GLD in this particular workplace or whether the BeLPT was producing false-negative results. The occurrence of seven NBeLPT-GLD cases among 505 Be-ceramics plant workers[8] is much greater than the expected sarcoidosis rate of approximately 10–30/100,000.[7,26] In the lungs of the NBeLPT-GLD group, we found that the detectable Be levels present in these granulomas equaled the Be levels present in the normal lung tissues of the Be-exposed normal subjects who were also BeLPT negative. On the basis of the combined blood and BAL BeLPT and SIMS data, we cannot fully exclude the possibility that some or all of these seven NBeLPT-GLD cases were, in fact, CBD cases. In follow up of this group, one subject later did develop a positive blood BeLPT and was designated as having CBD. Two others remained BeLPT negative on subsequent testing, and four were lost to follow up. The comparatively low levels of Be found in the granulomas of these cases, however, suggests that these patients either do not have CBD, or have such comparatively low levels of retained Be that they do not mount a detectable Be-specific immunologic response using the BeLPT. Although small quantities of Be may be found within granulomas of some Be-exposed NBeLPT-GLD patients by ultrasensitive techniques like SIMS, Be also can be found at similar low levels in the lungs of other Be-exposed ceramics workers. Although we measured Be levels in the lungs of a small group of Be-exposed ceramics workers, we did not examine Be content in normal, non-Be-exposed individuals' lungs in this study. However, the SIMS method is exquisitely sensitive for Be, and previous studies have shown that no Be is found in the lungs from non-Be-exposed individuals.[14]

Our data suggest that when levels of Be are elevated within granulomas relative to the surrounding lung tissue, the findings are indicative of CBD. Clinically if a patient with granulomatous lung disease and a history of Be-exposure has a negative blood and BAL BeLPT, microanalysis of tissue for Be could be considered. However, because the present study is small, this conclusion should be tempered until further studies confirm our observations. The limited number of subjects in this study emphasizes the need for future studies using SIMS analyses to determine if there is a defined cut off point for Be disease in comparison to the ratio of Be within the granuloma to outside the granuloma. In addition to these considerations,

follow-up immunologic testing should be performed to detect possible future conversion to an abnormal BeLPT.

In CBD, granuloma formation depends upon major histocompatibility class II-restricted presentation of Be-antigen to Be-specific $CD4^+$ T cells.[27–29] These Be-specific $CD4^+$ T cells undergo clonal expansion and display an effector memory T-cell phenotype.[28] Be stimulation also upregulates the production of proinflammatory cytokines believed to promote granulomatous inflammation.[28,30] The presence of Be in lung foci that exhibit granuloma formation suggests that retained Be may both promulgate and maintain the local adaptive immune response in CBD lung tissue. The data also suggest the possibility of a threshold of Be exposure, below which immunologic and immunopathologic abnormalities will not occur. In an intriguing study by Wang et al.[31] it was found that specific Glu^{69}-containing HLA-DPB1 gene alleles, and importantly their copy number, confer enhanced susceptibility to CBD in Be-exposed individuals. Restriction fragment length polymorphism analysis was recently used to confirm this observation in a population-based cohort of 884 Be workers.[32] Although it is not clear from either study how different genotypes might confer differential susceptibility to CBD, in light of our data showing an association between non-caseating CBD lung granulomas and high Be levels detected by SIMS, we hypothesize that an individual genotype may contribute to increased Be retention within the granuloma, even after low levels of Be exposure.

The physical properties of the Be in CBD granulomas have not been extensively characterized, but in at least one report actual respirable particles of beryllium oxide (BeO) have been demonstrated using SIMS plus electron microprobe analysis.[14] A recent study by Day et al[33] showed that high-purity BeO particles are engulfed and retained within murine macrophages. Be is detected in the intracellular fluid of these macrophages, resulting from a relatively slow BeO chemical dissolution rate constant. Thus, insoluble BeO, ingested by macrophages, may release low levels of bioavailable Be into local lung microenvironments, levels that drive chronic Be-specific T-cell–mediated responses and granuloma formation. Our data suggest that future studies should carefully address worker Be exposure levels, the relation of Be in exposed lungs as determined by SIMS, and Be exposure in relation to the worker's immunologic status. Our original study was unable to provide a defined characterization of workplace Be exposure levels.[8]

On average, our CBD group's last Be exposures occurred 9 years before the date of lung biopsy. Over time, this remaining Be could trigger both innate and adaptive immune responses, leading to granuloma formation.[34] The relationship between persistence of Be and the maintenance of Be-specific clonal $CD4^+$ T cells in the CBD granuloma[28] remains to be more fully explored. We do not know whether the findings from this study can be generalized to other forms of Be exposure encountered in the workplace. However, it is unlikely that Be ceramics is a special case, in light of the multiple studies demonstrating that similar rates of sensitization and CBD occur in workers exposed to Be metal, Be alloys, and Be-containing composite materials. It is interesting to note that in the two major studies of Be ceramics plants, a high proportion of the BeS workers developed CBD, often developing very severe forms of the illness.[8,35] Nonetheless, similarly severe cases of disease have been reported from all types of Be inhalation exposure, including low-percentage Be copper alloys.[36] Future studies may provide insight into the properties of the Be moiety and of the workplace environment that promote Be retention and sustain granulomatous pathology.

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