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BIOAVAILABILITY OF BERYLLIUM OXIDE PARTICLES: AN IN VITRO STUDY IN THE MURINE J774A.1 MACROPHAGE CELL LINE MODEL

Gregory A. Day □ *Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, Division of Respiratory Disease Studies, Morgantown, West Virginia, USA; Los Alamos National Laboratory, Los Alamos, New Mexico, USA; and Department of Occupational and Environmental Health, College of Public Health, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, USA*

Mark D. Hoover □ *Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, Division of Respiratory Disease Studies, Morgantown, West Virginia, USA*

Aleksandr B. Stefaniak □ *Los Alamos National Laboratory, Los Alamos, New Mexico, USA; and Division of Environmental Health Engineering, Johns Hopkins University Bloomberg School of Public Health, Baltimore, Maryland, USA*

Robert M. Dickerson and Eric J. Peterson □ *Los Alamos National Laboratory, Los Alamos, New Mexico, USA*

Nurtan A. Esmen □ *Department of Occupational and Environmental Health, College of Public Health, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, USA*

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This work was performed at Los Alamos National Laboratory, Los Alamos, New Mexico, USA.

The former address of Mark D. Hoover is Lovelace Respiratory Research Institute, Albuquerque, New Mexico, USA.

The current address of Aleksandr B. Stefaniak is Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, Division of Respiratory Disease Studies, Morgantown, West Virginia, USA.

The current address of Nurtan A. Esmen is University of Illinois at Chicago, School of Public Health (Environmental and Occupational Health Sciences) and Institute of Environmental Science and Policy, Chicago, Illinois, USA.

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Address correspondence to Gregory A. Day, CDC/NIOSH, 1095 Willowdale Road, MS 2800, Morgantown, West Virginia 26505-2888, USA. E-mail: gdd2@cdc.gov

Ronald C. Scripsick □ *Los Alamos National Laboratory, Los Alamos, New Mexico, USA*

□ *Beryllium metal and its oxide and alloys are materials of industrial significance with recognized adverse effects on worker health. Currently, the degree of risk associated with exposure to these materials in the workplace is assessed through measurement of beryllium aerosol mass concentration. Compliance with the current mass-based occupational exposure limit has proven ineffective at eliminating the occurrence of chronic beryllium disease (CBD). The rationale for this research was to examine the mechanism of beryllium bioavailability, which may be pertinent to risk. The authors tested the hypothesis in vitro that dissolution of particles engulfed by macrophages is greater than dissolution in cellular medium alone. Physicochemical changes were evaluated in vitro for well-characterized high-purity beryllium oxide (BeO) particles in cell-free media alone and engulfed by and retained within murine J774A.1 monocyte-macrophage cells. The BeO particles were from a commercially available powder and consisted of diffuse clusters (aerodynamic diameter range 1.5 to 2.5 μm) of 200-nm diameter primary particles. Following incubation for 124 to 144 hours, particles were recovered and recharacterized. Recovered particles were similar in morphology, chemical composition, and size relative to the original material, confirming the relatively insoluble nature of the BeO particles. Measurable levels of dissolved beryllium, representing 0.3% to 4.8% of the estimated total beryllium mass added, were measured in the recovered intracellular fluid. Dissolved beryllium was not detected in the extracellular media. The BeO chemical dissolution rate constant in the J774A.1 cells was* $2.1 \pm 1.7 \times 10^{-8} g/(cm^2 \cdot day)$. *In contrast, the BeO chemical dissolution rate constant in cell-free media was* $< 8.1 \times 10^{-9} g/(cm^2 \cdot day)$. *In vivo, beryllium dissolved by macrophages may be released in the pulmonary alveolar environment, in the lymphatic system after transport of beryllium by macrophages, or in the alveolar interstitium after migration and dissolution of beryllium particles in tissue. These findings demonstrate a mechanism of bioavailability for beryllium, are consistent with previously observed results in canine alveolar macrophages, and provide insights into additional research needs to understand and prevent beryllium sensitization and CBD.*

Keywords beryllium oxide, beryllium sensitization, chronic beryllium disease, dissolution, particles, phagocytosis

Beryllium metal and its oxide and alloys are materials of industrial significance with recognized toxicological influences on worker health [1, 2]. Compliance with the current mass-based occupational exposure limit has proven ineffective at eliminating the occurrence of beryllium sensitization and chronic beryllium disease (CBD) [3, 4]. In addition, the degree of risk associated with exposure to beryllium materials in the workplace is inadequately assessed through measurement of beryllium aerosol mass concentration [5]. Sensitization occurs in some individuals exposed to beryllium compounds when their immune system is triggered to act against beryllium. This condition is thought to precede the development of CBD; however, not all individuals who become sensitized progress to disease [6]. CBD is

a slowly progressive respiratory disorder that is initiated by a cell-mediated beryllium-specific immune response and is characterized by the formation of inflammatory lesions known as granulomas [7, 8]. Evidence suggests that, in addition to inhalation of beryllium aerosols, skin contact with beryllium, including particles [9], may also play a role in exposures relevant to the development of sensitization.

Beryllium exposures in the workplace typically involve particles. However, at the cellular level, dissolved beryllium is the hypothesized input to the immune reaction associated with development of sensitization and CBD [10, 11]. In order to generate a beryllium-antigen complex, the body must dissolve the particles. Few researchers have studied the dissolution of insoluble beryllium compounds in either cell or cell-free models. The current work was part of a comprehensive evaluation of the bioavailability of beryllium oxide (BeO) particles [12]. We tested the hypothesis in vitro that dissolution of particles engulfed by macrophages is greater than dissolution in cell-free medium alone. The purposes of this research were to examine the dissolution of well-characterized BeO in an established murine macrophage cell model and to discuss the potential implications of the results for development of beryllium sensitization and CBD.

MATERIALS AND METHODS

Figure 1 presents our conceptual model of the macrophage as both a phagocytic and antigen-presenting cell. In this model, particles deposited in the alveolar region of the lung are phagocytized by macrophages and sequestered within phagolysosomes. Macrophages not cleared by mechanical processes will remain in the alveoli, enter the lymphatic system, or be sequestered in the alveolar interstitium. Dissolved beryllium in the macrophages may then be available to make antigen that can drive a cell-mediated immune response.

Our experimental design, summarized in Figure 2, evaluated the dissolution of well-characterized BeO particles in cell-free media and in an established and widely available murine macrophage cell model. Precharacterized BeO particles were placed into cell-free media (as a control) or were introduced to cells in culture. Particles were subsequently recovered and were recharacterized. Liquids were recovered from (1) media of the cell-free controls, (2) media of the BeO-exposed cells, and (3) lysate of the BeO-exposed cells. All liquids were analyzed for dissolved beryllium; chemical dissolution rate constants were calculated from the results. This design provides for the collection of baseline information about particle uptake and dissolution, which may offer insights to processes in human pulmonary alveolar macrophages (PAMs).

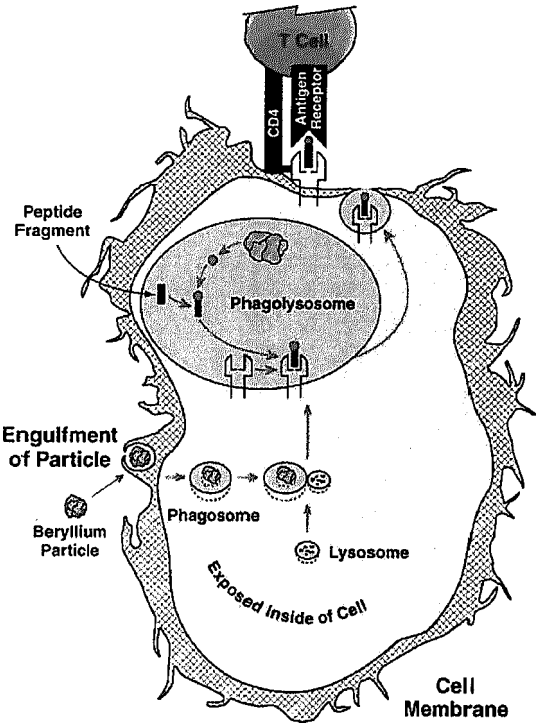

FIGURE 1 Hypothesized pathway for cellular processing of beryllium-containing particles from phagocytosis to antigen presentation.

Beryllium Oxide Particles

Commercially available bulk BeO powder (UOX-125; Brush Wellman, Elmore, OH) was aerosolized using a dry powder blower (Model 175; DeVilbiss, Somerset, PA) and aerodynamically separated into respirable size fractions using a 5-stage aerosol cyclone collector (Southern Research Institute, Birmingham, AL) as described by Hoover and colleagues [13]. Particles from stage 3 of the aerosol cyclone (aerodynamic diameter range 1.5 to 2.5 μm) were selected to provide a particle size known to be of concern for inhalation and deposition in alveolar airspaces where they may be engulfed by PAMs [14]. The specific surface area of the stage-3 BeO particles is 11.14 m^2/g [15]. Using detailed methods described by Stefaniak and colleagues [16], BeO suspensions for use in exposure and culturing were prepared from the stage-3 particles. Briefly, small masses (typically 5 mg) of BeO were placed into 10-mL phosphate buffered saline (PBS) (Gibco, Grand Island, NY) in 20-mL borosilicate glass screw-threaded vials (Kimble, Vineland, NJ).

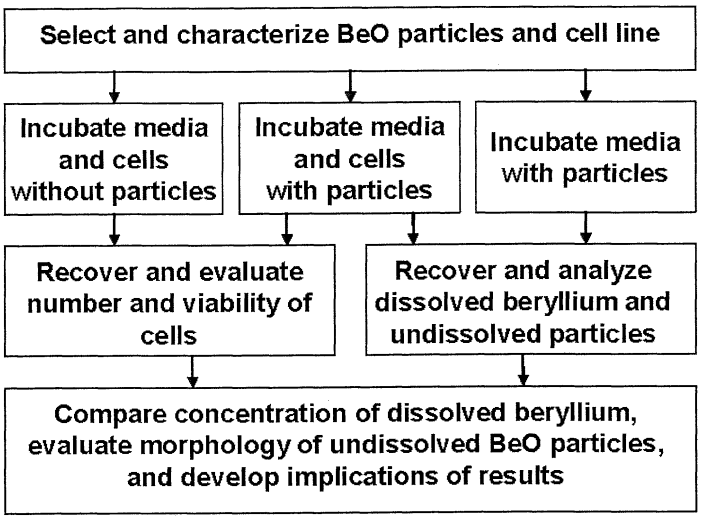

FIGURE 2 Experimental design for evaluation of the bioavailability of beryllium oxide particles in an in vitro study involving the murine J774A.1 monocyte-macrophage cell line model.

Analytical Techniques

Physicochemical properties of the BeO particles (stage 3) were characterized (before and after adding to cells in culture) using transmission electron microscopy (TEM). X-ray diffraction (XRD) was performed on BeO particles only before adding to cells in culture. Further details are described by Stefaniak and colleagues [17]. Levels of beryllium dissolved in culture were quantified using inductively coupled plasma–atomic emission spectroscopy (ICP-AES).

Transmission Electron Microscopy (TEM)

Particle size and morphology information were obtained using electron imaging on a Philips CM30 analytical TEM (Philips Electron Optics, Eindhoven, The Netherlands), operating at 300 keV. Samples were prepared on 300-mesh copper electron microscopy grids coated with a lacey carbon substrate (Ted Pella, Redding, CA).

Butnor and colleagues [18] used electron probe x-ray microanalysis to analyze the elemental composition of beryllium-containing particles in tissue samples. In our laboratory, the particular TEM-atmospheric thin window energy dispersive x-ray spectrometry system was capable of collecting and displaying chemical information for elements with an atomic number equal to or greater than 6; elemental beryllium (atomic number 4) could

not be directly detected. For this reason, electron diffraction analysis was used to determine the beryllium-containing phase of the particles.

Crystalline phase was assessed using a combination of TEM-selected area- and microelectron diffraction analyses. Selected area-electron diffraction evaluates multiple particles whereas microelectron diffraction evaluates individual particles. In electron diffraction, an electron beam probe is directed on a group of particles or on individual particles. As the beam interacts with a crystalline phase, the orientation of the atoms in the crystal planes of the specimen gives rise to coherent electron scattering, which is observed as intensity modulations forming a diffraction pattern specific to the lattice spacing of that crystal [19]. Electron diffraction patterns generated from BeO particle samples were compared to published BeO reference patterns [20].

X-Ray Diffraction

Crystalline structure and particle size information were also obtained by placing milligram quantities of BeO onto a single quartz crystal plate and analyzing with an XDS2000 Powder Diffractometer (Cu k-alpha, 45 kV, 40-mA x-ray source) (Scintag, Sunnyvale, CA). In XRD, a powder sample is bombarded with x-rays. Crystalline phases within the sample diffract the incident x-rays according to Bragg's equation, which relates crystal lattice spacing to the wavelength of the x-rays used as a probe [21]. Analysis of diffraction patterns to determine the phase composition of crystalline constituents was performed using the General Structure Analysis System computer software program [22, 23]. X-ray diffraction patterns generated from BeO particle samples were compared to published BeO reference patterns (ICDD, Newton Square, PA).

Inductively Coupled Plasma–Atomic Emission Spectrometry

Liquids recovered from culture were analyzed for beryllium using an ICP optical emission spectrometer (Optima Model 4300DV, Perkin Elmer Instruments, Überlingen, Germany). The recovered liquid samples (typically 20 to 50 mL) were acidified to pH $\leq$ 2, and aliquots were directly introduced to the instrument for determination of beryllium content. In ICP-AES, liquids are first nebulized into a fine aerosol that is injected into an argon-supplied inductively coupled plasma. The high temperature of the plasma atomizes the sample, thus promoting the transition of elemental constituents that are identified by characteristic wavelength emissions (beryllium = 313.107 nm) and intensities [24]. Emission intensities from the samples were compared to a calibration curve (prepared at beryllium concentrations of 0.005, 0.01, 0.5 and 1.0 μg/mL). Several quality

controls were analyzed, including sample blanks, verification standards (i.e., spiked samples), and interference correction standards.

Murine-macrophage Cell Line

The murine J774A.1 monocyte macrophage (American Type Culture Collection, TIB-67, Manassas, VA) was selected for use in this study because it is an established, highly phagocytic, adherent cell line that has been extensively used by researchers for more than 25 years [25–27]. The J774A.1 cell line was adapted to culture by Ralph and Nakoinz [28] and has an acidic phagolysosomal pH of 4.8 ± 0.1 [29]. This cell line has been used to demonstrate the phagocytosis of 1.4-μm diameter iron oxide particles [30] and to evaluate the morphology of phagocytized ultrafine metallic silver particle agglomerates [31].

Exposure and Culturing

Viable (>90% as assessed by 0.4% trypan blue dye exclusion using a hemacytometer) J774A.1 cells were incubated with and without BeO particles for periods of 5 to 6 days (n = 12 medium controls, n = 12 cell controls, and n = 24 replicates of cells with BeO particles). Sterile tissue culture dishes (150 × 25 mm; Falcon, San Jose, CA) were seeded with 1×10^6 cells in 50 mL (2×10^4cells/mL) of fresh minimum essential medium-alpha (Gibco) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 1% antibiotic (penicillin-streptomycin liquid combination; Gibco). Dishes were incubated at 37°C in a humidified 5% CO_2/95% air atmosphere for 2 hours to allow for cell adherence. During the 2-hour period, prepared suspension (BeO particles in PBS) was subjected to ultrasonic agitation for uniform mixing and deagglomeration, and then added to fresh medium. At the end of the 2-hour period, dishes were briefly taken from the incubator and media were removed and replaced by either 50 mL of fresh media supplemented with BeO particle suspension (exposed cells) or 50 mL of fresh media only (unexposed cells). BeO particle suspension was also added to cell-free media (exposed media).

Exposed cells received approximately 1.6 μg BeO/mL culture medium (6.4×10^{-5} M beryllium). The BeO concentration was selected based on published results of the in vitro cytotoxicity of BeO particles in canine PAMs [10, 32, 33] and met our analytical detection limit for dissolved beryllium (0.003 μg/mL) without reducing cell viability. This calculation was based on a desired nominal incubation period of 5 to 6 days, which allowed growth of the J774A.1 cells over the entire surface area of the culture dishes at a mean viability of greater than 90%. Cells in culture were periodically

observed by light microscopy during the incubation period to assess engulfment of particles.

Beryllium Recovery and Analysis

Exposed Media

After 124 to 144 hours, media were removed from dishes, transferred to separate bottles (Beckman Coulter, Fullerton, CA), and centrifuged (Model J2-21, Beckman Instruments, San Jose, CA) at $13775 \times g$ (high speed) for 1 hour to recover suspended particles. From the equation for terminal velocity due to centrifugal force [34], high-speed centrifugation for 1 hour was sufficient to sediment a 0.03-μm diameter BeO particle the full sedimentation distance of 4 cm in the centrifuge bottle. Supernatants were transferred to glass vials (Kimble, Vineland, NJ) for determination of dissolved beryllium. Concentrations of dissolved beryllium were determined by ICP-AES; limit of detection 0.003 μg beryllium/mL.

Adherent BeO particles were washed from dish surfaces with high-performance liquid chromatography–grade water from a syringe and 22-gauge needle. Resulting suspensions were centrifuged at high speed for 1 hour to recover particles. Recovered particles were rinsed and resuspended in water; sample grids were prepared from suspensions and allowed to dry prior to recharacterization by TEM.

Unexposed Cells

Media were removed from culture dishes after 124 to 144 hours, transferred to separate bottles, and centrifuged (Model GS-6R; Beckman Instruments, San Jose, CA) at $250 \times g$ (low speed) for 6 minutes at 4°C to recover suspended cells. Supernatants were transferred to glass vials for determination of dissolved beryllium by ICP-AES. Adherent cells were washed from dish surfaces with PBS from a syringe and combined with the other recovered cells, followed by determination of viability and number concentration.

Exposed Cells

Media were removed from culture dishes after 124 to 144 hours, transferred to separate bottles, and centrifuged at low speed for 6 minutes at 4°C to recover suspended cells. Supernatants were transferred to separate bottles and centrifuged at high speed for 1 hour to recover suspended particles. Final supernatants were transferred to glass vials for determination of dissolved beryllium by ICP-AES.

Adherent cells were washed from dish surfaces with PBS from a syringe and combined with the other recovered cells, followed by determination of viability and number concentration. Mean viability and number

concentration at harvesting were compared between unexposed and exposed cell cultures using the Wilcoxon 2-sample rank test.

Cells were then recovered by low-speed centrifugation and lysed in a 0.05% detergent solution (Triton X-100; Fisher Chemicals, Fairlawn, NJ). Lysate suspensions were agitated and then centrifuged at high speed for 1 hour to recover particles. Supernatants were transferred to individual glass vials for determination of dissolved beryllium by ICP-AES. Particles were rinsed and resuspended in water, collected on TEM grids, and allowed to dry prior to recharacterization.

Evaluation of Dissolution Data

As in a previous study [16], we used the traditional dissolution theory of Mercer [35] to evaluate dissolution results. For dissolution of particles with an initial specific surface area and log-normally distributed diameters with a geometric standard deviation of σ_g, Mercer describes an equation for the mass fraction remaining: $\frac{M}{M_0}$. Mercer's results for $\frac{M}{M_0} > 0.3$ and $\sigma_g = 1$ to 2.3 ($\sigma = \ln \sigma_g$ from 0 to 1) can be expressed as a single exponential:

$$\frac{M}{M_0} = e^{-\lambda k t SSA e^{-\frac{\sigma^2}{2}}} \tag{1}$$

where M = remaining mass of population of particles
M_0 = initial mass of population of particles
$\lambda = 1.18$
k = chemical dissolution rate constant $\left(\frac{\text{mass}}{\text{area}\cdot\text{time}}\right)$
t = time
SSA = specific surface area at time 0 $\left(\frac{\text{area}}{\text{mass}}\right)$
$\sigma = 0.5$

This equation can be expressed in terms of mass fraction dissolved, $\frac{M_D}{M_0}$:

$$\frac{M_D}{M_0} = 1 - e^{-1.04 k t SSA} \tag{2}$$

where M_D = mass dissolved from population of particles.

In turn, Equation 2 can be rearranged to solve for k:

$$k = \frac{\ln\left(1 - \frac{M_D}{M_0}\right)}{-1.04\, SSA t} \tag{3}$$

RESULTS

Beryllium Oxide Characteristics

TEM imaging of the original exposure material showed clusters of primary particles having average diameters ≅200 nm (Figure 3*a*). Selected area- and microelectron diffraction patterns (Figure 3*a* insets) showed that the specific orientation and dimensional arrangement of atoms in the sample were consistent with patterns for hexagonal BeO. This finding was also confirmed by XRD analysis. TEM imaging of particles recovered from exposed media (Figure 3*b*) were morphologically indistinguishable from the original exposure material; electron diffraction patterns (Figure 3*b* insets) were consistent with hexagonal BeO. Similarly, particles recovered from BeO-exposed cells (Figure 3*c*) were morphologically indistinguishable from the original exposure material and produced diffraction patterns consistent with hexagonal BeO (Figure 3*c* insets). Note, however, that a very thin coating of material was observed on surfaces of some recovered particles, most likely consisting of residual detergent and/or cellular protein not removed during rinsing.

Cell Viability and Number Concentration

At the time of recovery, mean cell concentrations were increased by factors of 36 (exposed) and 41 (unexposed) from the initial concentrations of 2×10^4 cells/mL culture medium. Using a significance criterion of $P \leq 0.05$, results of the Wilcoxon 2-sample rank test found no statistical differences between the mean viability and concentration of exposed cell groups ($V_{exposed} = 92.9\% \pm 5.7\%$; $C_{exposed} = 7.2 \pm 1.8 \times 10^5$ cells/mL) and unexposed cell groups ($V_{unexposed} = 94.8\% \pm 1.9\%$; $C_{unexposed} = 8.2 \pm 0.77 \times 10^5$ cells/mL).

Beryllium Dissolution

Exposed Media

BeO particles in all cell-free controls were observed without difficulty by visual inspection with a light microscope throughout each experimental period. Dissolved beryllium in the BeO-exposed media was below our limit of detection of 0.003 μg beryllium/mL. This result indicates that the BeO dissolution rate constant in cell-free media was $< 8.1 \times 10^{-9} g/(cm^2 \cdot day)$.

Exposed Cells

BeO particles were not observed in the culture media of exposed cells after the initial 48 hours; by this time all particles appeared to be cell associated. Dissolved beryllium was not detected in the culture media

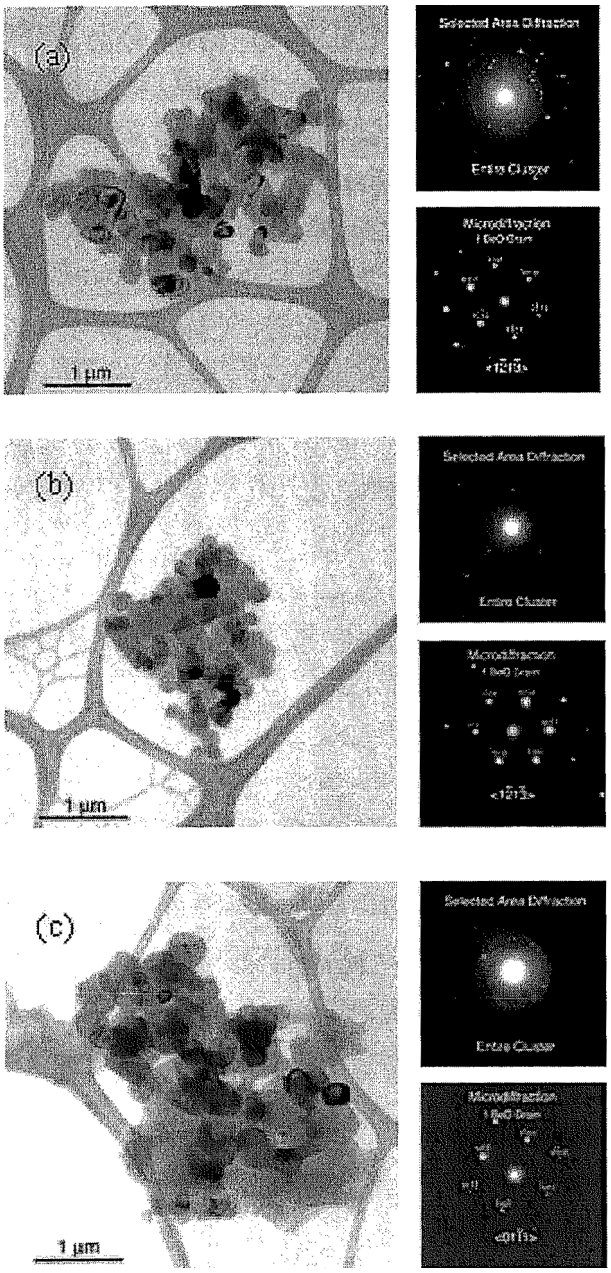

FIGURE 3 TEM images of BeO particles with corresponding selected area- and microelectron diffraction patterns for (*a*) the original exposure material, (*b*) particles recovered from exposed media, and (*c*) particles recovered from BeO-exposed cells. Figures illustrate that recovered particles were morphologically and chemically indistinguishable from the original exposure material, i.e., clusters of 200-nm diameter primary particles that produced diffraction patterns consistent with hexagonal BeO (*insets*).

associated with the BeO-exposed cells. Dissolved beryllium was detected in the supernatant of the lysed BeO-exposed cells in amounts equivalent to 0.3% to 4.8% (mean = 1.3% ± 1.1%) of the initial beryllium mass. Individual values of the dissolved beryllium recovered from cell lysate samples were 0.7%, 1.1%, 1.4%, 1.5%, 1.9%, 2.4%, 2.6%, and 2.8% for a group of 8 cell cultures incubated for 124 hours; 0.3%, 0.3%, 0.6%, 0.6%, 0.7%, 0.7%, 1.0%, and 1.3% for those incubated for 136 hours; and 0.3%, 0.3%, 0.5%, 0.6%, 1.4%, 1.7%, 2.5%, and 4.8% for those incubated for 144 hours. These results indicate that the mean BeO chemical dissolution rate constant in J774A.1 cells was $2.1 \pm 1.7 \times 10^{-8}$ g/(cm^2 · day).

DISCUSSION

Dissolution of BeO particles was greater in J774A.1 cells than in cell-free media. As discussed below, these results provide a perspective on the role of the macrophage in beryllium particle dissolution and clearance, including cellular aspects of beryllium immunotoxicity, the immunologic process of CBD, and the potential influence of beryllium bioavailability on beryllium sensitization and CBD.

Role of Macrophages in Particle Dissolution and Clearance

The immunopathologic process for CBD is thought to require the deposition of respirable particles in the alveolar region of the lung. Following pulmonary deposition, particles may be cleared by one or more routes that include (1) transport to the gastrointestinal tract via the pharynx, (2) absorption into the blood, and (3) transportation to lymph nodes via lymphatic channels [14]. The physicochemical form of the deposited particles plays an important role in the clearance process. Soluble particles may be cleared in a relatively short period of time, whereas insoluble particles clear very slowly, with retention times ranging from months to decades.

Particles deposited in pulmonary alveoli are rapidly engulfed by PAMs [36, 37]. As noted by Guilmette [38], because phagocytosis is a relatively rapid and efficient process for internalizing particles deposited on respiratory tract surfaces, the fate of all but the most soluble particles will depend on what happens to them after internalization in macrophages. The phagolysosomal medium that surrounds the ingested particle is an aqueous mixture of enzymes, reactive oxygen intermediates, and hydroxyl radicals having an acidic pH ranging from 4.5 to 5.0 [39–41]. This environment can be contrasted to an estimated neutral pH 7.4 in the extracellular environment of the lung [42].

Dissolution in PAMs is important to the clearance of particulate material from the lung [43] as well as to the presentation of the resulting

ions or antigens to surrounding tissues and the immune system. Lundborg and colleagues [44] observed that moderately insoluble particles of manganese dioxide dissolved 2 to 3 times more rapidly in rabbit PAMs than in culture medium. Lundborg and colleagues [45] also compared the in vitro dissolution of the same manganese dioxide particles in both human and rabbit PAMs, observing similar dissolution results in both species.

In studies with cobalt materials, Kreyling and colleagues [46] evaluated the in vitro dissolution of moderately soluble cobalt oxide particles in canine PAMs and in various cell-free control solvents. Results indicated the enhanced dissolution of particles in PAMs and in media buffered to pH 5.0, but only slight dissolution in media at pH 7.2. Kreyling and colleagues [47] later investigated the dissolution of cobalt oxide particles by human and canine PAMs, reporting that the rate of cobalt dissolution increased with time in cultured PAMs of both species, but remained constant with time in cell-free media.

In other work with metal oxide particles, Marafante and colleagues [48] evaluated the in vitro dissolution by rabbit PAMs of two poorly soluble arsenic compounds: lead arsenate and arsenic trisulfide. They observed that over a period of 3 days only 2% of the arsenate particles dissolved in a cell-free culture media, whereas 14% dissolved when incubated with PAMs. In contrast, the solubility of trisulfide particles was higher in the media alone than in PAMs. This work justifies material-specific studies of the influence of pH on particle dissolution.

Few researchers have investigated the in vitro or in vivo dissolution of beryllium particles. Using rat PAMs, André and colleagues [49] evaluated the in vitro dissolution of a commercially available beryllium metal powder and cuttings generated from the machining of hot-pressed beryllium parts. Dissolution was 10 to 20 times higher in the cell model than in the pH 6.8 to 7.0 cell-free media. They also conducted in vivo experiments with baboons and rats and concluded that bioassay studies of in vivo beryllium solubility should include cell models.

Eidson and colleagues [10] studied the in vitro dissolution of BeO particles that had been calcined (heat treated) at either 500°C or 1000°C. They observed that both forms of the BeO particles were phagocytized and partially dissolved within canine PAMs within a period of 48 hours. Over the same period, nearly 22% of the 500°C-calcined BeO was dissolved compared to only 9% of the 1000°C-calcined BeO. Using the same BeO materials in a canine in vivo model over a period of 180 days, Finch and colleagues [50] noted an initial rapid translocation of beryllium from the lung (attributed to a combination of mechanical clearance and dissolution), followed by a slower, long-term translocation (attributed to dissolution). Whereas the percentage of initial lung burden retained at 180 days post exposure was 90% for the 1000°C-calcined material, it was only 50%

for the 500°C-calcined material. The authors noted that the 1000°C-calcined material had a lower specific surface area and that the dissolution of the two materials was identical when normalized to surface area. Using a ^{7}Be radiolabel to provide a very low limit of detection, they were able to quantify dissolution rates of $6.9 \pm 1.1 \times 10^{-9}\,\mathrm{g/(cm^2 \cdot day)}$ for the 500°C material and $6.7 \pm 1.0 \times 10^{-9}\mathrm{g/(cm^2 \cdot day)}$ for the 1000°C material.

Finch and colleagues [51] also investigated the in vitro dissolution of insoluble beryllium particles in cell-free models involving neutral pH (serum ultrafiltrate [SUF], an extracellular lung fluid model) and acidic pH (0.1 N HCl). They observed that the BeO dissolution rate was much lower in SUF ($3.7 \pm 1.2 \times 10^{-9}\mathrm{g/(cm^2 \cdot day)}$) than in HCl ($6.1 \pm 2.2 \times 10^{-8}\,\mathrm{g/(cm^2 \cdot day)}$). The results of our study are consistent with those results. We estimated that the BeO chemical dissolution rate constant in J774A.1 cells (pH $= 4.8 \pm 0.1$) was $2.1 \pm 1.7 \times 10^{-8}\,\mathrm{g/(cm^2 \cdot day)}$. We also observed that the dissolution rate constant in culture media alone was $< 8.1 \times 10^{-9}\,\mathrm{g/(cm^2 \cdot day)}$.

Stefaniak [52] estimated that the chemical dissolution rate constant for BeO in a simulant of PAM phagolysosomal fluid having pH 4.5 ± 0.05 was $1.1 \pm 0.5 \times 10^{-8}\,\mathrm{g/(cm^2 \cdot day)}$. This observation indicates that the cell-free model provides a reasonable approximation of dissolution in the cellular environment, despite the absence of active biological factors such as digestive enzymes and reactive oxygen species.

Our findings suggest that beryllium bioavailability was substantially greater when BeO particles were combined with cells than when contained in cell-free medium alone. Although the study design did not include methods for determining the formation of beryllium-containing phagolysosomes, the data support our conceptual model of the macrophage as both a phagocytic and an antigen-presenting cell (Figure 1). Further research is needed to better understand beryllium particle uptake and phagolysosome formation.

Note that our experimental design was based on the observation of Eidson and colleagues [10] that dissolved beryllium accumulates within cells. These data suggest that dissolved beryllium is most appropriately measured in cell lysate. In contrast, Kreyling and colleagues [47] observed that dissolved material diffuses out of cells and into surrounding culture medium, suggesting that dissolved beryllium might also be measurable in culture medium. Data reported in our study are consistent with the observation reported by Eidson and colleagues [10]; however, because we did not concentrate liquid samples (e.g., by lyophilization) to achieve a more sensitive limit of detection, we cannot rule out the presence of dissolved beryllium in recovered media (cell-free controls and BeO-exposed). Thus, our calculated dissolution rate constant may underestimate the rate at which dissolved beryllium is made available for antigen formation.

Implications for Beryllium Sensitization and CBD

Sterner and Eisenbud [2] hypothesized that the development of CBD results from an immunologic reaction to beryllium ions in the lung. They believed that beryllium compounds were retained in the lung in immunologically significant quantities for very long periods of time, and that the immune response depended on not only the rate but also the quantity of beryllium ions released from the tissue deposit.

Nearly 20 years later, Hanifin and colleagues [53] experimentally demonstrated a cell-mediated immunologic reaction to BeO. Macrophage cells, exposed to insoluble BeO particles in vitro, were incubated together with lymphocyte cells obtained from either beryllium-sensitized or normal subjects. Results indicated that exposed macrophages (those cells containing engulfed BeO particles) induced transformation of the lymphocytes from sensitized but not from normal subjects. Unexposed macrophages did not induce transformation of lymphocytes from either group, thereby indicating a cell-mediated immunologic reaction to BeO.

Deodhar and colleagues [54] provided supporting evidence that the cell-mediated immunologic response to beryllium compounds results from the presence of beryllium ions. The authors observed the transformation of lymphocytes obtained from both healthy beryllium workers and CBD patients, noting a higher proportion and degree of response in the CBD group following exposure to beryllium sulfate. Because identical results were observed in a repeated experiment exposing cells to beryllium fluoride, the authors concluded that the cellular response resulted from the presence of beryllium ions rather than from sulfate or fluorine ions.

Jones and Amos [55] investigated the possibility that the beryllium ion combines with a protein to form an antigenic complex before reacting with the lymphocyte. The authors injected lymph node cells exposed in vitro to beryllium sulphosalicylate back into guinea pigs, thereby producing dermal sensitivity to beryllium fluoride in vivo. Direct injection of beryllium sulphosalicylate did not produce similar results, suggesting that an antigen may have been released from the beryllium-exposed cells in the form of a beryllium-protein conjugate.

Hart and Pittman [56] demonstrated the cellular uptake of beryllium compounds, and concluded that insoluble beryllium particles, but not soluble compounds, are phagocytized by PAMs. They also supported the hypothesis of Witschi and Aldridge [57] that particles, following phagocytosis, are sequestered within phagolysosomes. Haley [58] reported that PAMs are basic to granuloma formation, owing to their (1) engulfment of particles, (2) release of chemotactic mediators, and (3) presentation of antigen to T lymphocytes. Based on results of previous studies in laboratory animals [59, 60], they stated that the persistent antigenicity of

beryllium, perpetuated by the inability to eliminate the material from the lung, appeared key in the progressive development of CBD.

Collectively, in vitro and in vivo models may be useful for elucidating bioavailability of beryllium in human lung, including the rate at which dissolved beryllium is generated and particle dissolution lifetime. Our data indicate that relatively insoluble BeO particles release beryllium ions very slowly over time. Therefore, from a pathological standpoint, it is important to consider that BeO particles may persist in lung tissue and make dissolved beryllium bioavailable for decades after exposure ceases. Beryllium-containing particles have been identified in the granulomatous lung tissue of individuals with a history of beryllium exposure [e.g., 18, 61].

Evidence indicates that unusually large numbers of lymphocytes [62], specifically $CD4^+$ T lymphocytes [63], accumulate, persist, and respond to beryllium antigen in the lungs of CBD patients [64, 65]. Although the exact nature of the beryllium antigen is unknown [66], there is some evidence suggesting that beryllium ions may become complexed with peptides (fragments of proteins) that are ultimately recognized by $CD4^+$ T lymphocytes, thereby inducing beryllium sensitization [67–69]. Additionally, upon further contact with beryllium antigen, T cells maintain development of CBD through the release of proinflammatory cytokines such as interleukin-2, interferon-gamma, and tumor necrosis factor [70–73]. According to Fontenot and colleagues [74], although the exact mechanism of T-cell recognition of the beryllium antigen is unknown, "the ensuing immune response occurs in a self-propagating manner, which appears to be compartmentalized in the lung."

Critical Questions on Beryllium Bioavailability

The rationale for our research was to examine the mechanism of beryllium bioavailability in monocyte macrophages, which may be pertinent to the risk of beryllium sensitization and CBD. Insights were gained into differences between dissolution in cells and dissolution in extracellular media. In addition, the chemical dissolution rate constant for BeO in murine macrophages was shown to be consistent with previously observed results in canine PAMs. These results suggest several critical questions on beryllium bioavailability:

- How do the intensity and duration of bioavailable beryllium contribute to beryllium sensitization and CBD?
- Is the activation threshold for sensitization higher than the threshold for granuloma formation?
- What combinations of chemical dissolution rate constant, specific surface area, and deposited mass of respirable particles result in release of

beryllium ions at rates sufficient to activate T lymphocytes and *initiate* a granulomatous response?

- Similarly, what combinations of chemical dissolution rate constant, specific surface area, and deposited mass of respirable particles result in release of beryllium ions at rates sufficient to *maintain* a granulomatous response?
- How does intracellular particle dissolution affect the viability and function of antigen-presenting cells?
- To what extent do beryllium particles penetrate into the pulmonary alveolar interstitium? Do the particles remain free or are they sequestered in macrophages or some other cell type?
- In which biological compartments (e.g., macrophages, pulmonary alveolar interstitium, or other cell locations or types) does beryllium dissolution most contribute to the disease process?
- What is an appropriate exposure-response metric or dose-response metric for sensitization and CBD? Several metrics of lung deposition and bioavailability may need to be investigated, including peak, average, or cumulative beryllium ions/g lung/day.
- What is the role of skin exposure? How do beryllium ions or particles that penetrate the epidermis interact with immunologically active cells? Should there be similar concerns for the deposition and bioavailability of particles in the nasal and conducting airways?

The answers to these and other questions are relevant to (1) guiding the characterization and measurement of beryllium aerosols in the workplace; (2) applying this information in the design and interpretation of epidemiological studies of exposed workers; (3) designing and conducting targeted studies in laboratory animals to further understand the role of bioavailability in beryllium sensitization and CBD; and (4) using this understanding to help determine a safe level for beryllium exposure, if any. The answers may also point the way to interventions for exposed individuals. Success in these endeavors will require a continued collaborative research approach involving occupational health professionals and other scientists from many disciplines.

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