

## Skin as a Route of Exposure and Sensitization in Chronic Beryllium Disease

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Chronic beryllium disease is an occupational lung disease that begins as a cell-mediated immune response to beryllium. Although respiratory and engineering controls have significantly decreased occupational beryllium exposures over the last decade, the rate of beryllium sensitization has not declined. We hypothesized that skin exposure to beryllium particles would provide an alternative route for sensitization to this metal. We employed optical scanning laser confocal microscopy and size-selected fluorospheres to demonstrate that 0.5- and 1.0- $\mu\text{m}$  particles, in conjunction with motion, as at the wrist, penetrate the stratum corneum of human skin and reach the epidermis and, occasionally, the dermis. The cutaneous immune response to chemical sensitizers is initiated in the skin, matures in the local lymph node (LN), and releases hapten-specific T cells into the peripheral blood. Topical application of beryllium to C3H mice generated beryllium-specific sensitization that was documented by peripheral blood and LN beryllium lymphocyte proliferation tests (BeLPT) and by changes in LN T-cell activation markers, increased expression of CD44, and decreased CD62L. In a sensitization–challenge treatment paradigm, epicutaneous beryllium increased murine ear thickness following chemical challenge. These data are consistent with development of a hapten-specific, cell-mediated immune response following topical application of beryllium and suggest a mechanistic link between the persistent rate of beryllium worker sensitization and skin exposure to fine and ultrafine beryllium particles. *Key words:* beryllium, exposure, particle, sensitization, skin. *Environ Health Perspect* 111:1202–1208 (2003). doi:10.1289/ehp.5999 available via <http://dx.doi.org/> [Online 24 February 2003]

Beryllium is a strategic metal with unique physicochemical properties. It is light weight, nonsparking, noncorrosive, and stiffer than steel and has a high melting temperature. The dissolution half-life for beryllium particles ranges from tens to hundreds of days, depending on its chemical form (Hoover et al. 1988). The metal and its oxides and alloys are indispensable in the nuclear, aerospace, ceramics, and telecommunications industries. Its uses have expanded to include tools for emergency response teams, computers, dental implants, specialty golf clubs, and bicycle frames. Exposure to beryllium causes an incurable occupational lung disease, chronic beryllium disease (CBD), in approximately 3–5% of exposed workers (Henneberger et al. 2001; Kreiss et al. 1996, 1997; Newman et al. 2001; Schuler et al. 2002). Estimates of the current exposed worker population vary from 100,000 to 800,000 individuals (Cullen et al. 1986); however, decommissioning of beryllium-contaminated buildings at nuclear defense sites over the next 10 years may increase these numbers significantly.

CBD is a progressive granulomatous lung disease that is characterized by an MHC (major histocompatibility complex) class II–restricted, T-helper 1, CD4<sup>+</sup> lymphocytosis (Epstein et al. 1982; Saltini et al. 1989). Immunologic sensitization to beryllium can be detected in peripheral blood cells by the beryllium-stimulated lymphocyte proliferation test (BeLPT). A positive peripheral blood BeLPT frequently precedes a positive

bronchoalveolar lavage (BAL) cell BeLPT (Henneberger et al. 2001; Kreiss et al. 1993a, 1993b, 1996, 1997). A positive BAL cell BeLPT and histologic evidence of noncaseating granulomas on transbronchial lung biopsy support the diagnosis of disease (Newman et al. 1989).

Over the last decade, the prevalence of disease has not declined despite enactment of exposure limits by the Atomic Energy Commission and the implementation of improved control technology designed to eliminate respiratory exposure (Eisenbud 1982). Industrial hygiene studies demonstrated that disease prevalence correlates with beryllium ultrafine particle counts, not with beryllium mass measurements (Martyny et al. 2000; McCawley et al. 2001). Epidemiologic analyses showed that beryllium workers with the highest rates of disease are those involved in machining and grinding, tasks that generate significant amounts of dust (Kreiss et al. 1996, 1997). These workers had significant respiratory protection and engineering controls in their work environments, but no skin protection. Furthermore, cutaneous sensitization to beryllium is consistent with the clinical observation that beryllium-specific peripheral blood lymphocyte proliferation often precedes a positive pulmonary response, yet skin was not considered a route of exposure for worker sensitization. In this article we provide evidence that skin exposure may contribute to the persistence of beryllium sensitization in the workplace.

Due to the particulate nature of most exposures, the barrier properties of the skin, the low solubility of the metal and its alloys, and localization of disease in the lung, skin exposure to beryllium has not been considered hazardous. The external layer of the skin, the stratum corneum, is considered a mechanically strong and resilient structure that can withstand physical strain and stress. It is considered an effective barrier essential to the protection of the internal milieu from the external environment; however, there are several reports of particle penetration into the stratum corneum and hair follicles, and a report of increased titanium in the epidermis and dermis following the application of sunscreens containing titanium dioxide (Lademann et al. 1999; Tan et al. 1996). Microparticles have also been identified in the lymph nodes (LNs) and dermis of individuals in African rift valleys who walked barefoot and developed elephantiasis of the feet and legs (Blundell et al. 1989; Corachan et al. 1988). Using the guinea pig model, several researchers from 1950 through the 1980s used cutaneous application of beryllium salts and metal to confirm beryllium induction of a delayed-type hypersensitivity (DTH) response (Alekseeva 1965; Curtis 1951; Denham and Hall 1988; Jones and Amos 1974; Krivanek and Reeves 1972). Interestingly, only a subset of guinea pigs responded to beryllium in each study, and conclusions were sometimes based on data obtained from two or three animals (Alekseeva 1965; Krivanek and Reeves 1972). Attempts to develop a murine model of chronic disease were largely unsuccessful, although a single article reports the development of a beryllium DTH and granulomas in A/J mice (Huang et al. 1992). Although not widely considered to be a safe diagnostic procedure, several studies have evaluated elicitation of the cutaneous beryllium DTH response in beryllium-sensitized and CBD patients using the skin patch test (Bobka et al. 1997; Curtis 1951; Fontenot et al. 2002).

These multidisciplinary observations led us to propose skin exposure to beryllium particulates as a route of beryllium sensitization. Specifically, we hypothesized that fine particles

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are able to penetrate the stratum corneum and reach the immunologically active epidermis, and that flexing motion, as at the wrist, provides the kinetic energy necessary to move particles into the skin. This motion would be analogous to the pressure applied by walking barefoot or to the motion associated with machining and grinding. For these studies, we evaluated fluorescent dextran particle penetration of human skin *ex vivo* with laser scanning confocal microscopy, and cutaneous sensitization of mice using beryllium salts and beryllium oxide. We tested antigen specificity with a murine beryllium-specific lymphocyte proliferation assay, hapten-induced changes in LN cell activation markers with flow cytometry, and a well-established measurement of cutaneous sensitization, elicitation of the ear-swelling response.

## Materials and Methods

**Tissue handling parameters.** Human skin, 300–400  $\mu\text{m}$  thick, was obtained from the back or the thigh (Center for Organ Recovery and Education, Pittsburgh, PA) and stored on mesh in a 100-mm petri dish with the surface of the skin at the air–liquid interface. Tissue was stored in RPMI 1640 medium (Irvine Scientific, Santa Ana, CA) containing 25 mM HEPES, 2 mM L-glutamine, 10% (vol/vol) fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD), 100 U/mL penicillin, and 100  $\mu\text{g}/\text{mL}$  streptomycin at 4°C. Pilot studies verifying the health of the tissue samples revealed minimal release of the cytoplasmic enzyme lactate dehydrogenase into the medium for 5 days after arrival, and tissues were used within 24–48 hr (Wester et al. 1998).

Integrity of the stratum corneum was verified immediately before each experiment using the NOVA Dermaphase Meter, model 9008 (NOVA Technology Corp., Gloucester, MA), which measures the skin surface electrical impedance as the ratio of the current to the potential on an electrically charged isolated conductor. A cutoff of 300 units was determined by a pilot study and previous published literature (Newman and Seitz 1990; Okah et al. 1995). All skin tissues in the study had capacitance measurements of  $\leq 300$  units, signifying that the stratum corneum was intact. Additionally, systematic examination of each tissue sample by microscopy confirmed visually the integrity of the stratum corneum.

Humidity during experiments ranged between 56% and 70%, and room temperature was maintained between 21°C and 23°C.

**Flexing procedure.** Skin, 1.5  $\times$  0.5 inches, was fixed to the flexing device with double-sided tape and flexed at 45°, 20 flexes/minute, or left flat, for 15, 30, or 60 min. One hundred microliters of a 25% solid solution of 0.5-, 1-, 2-, or 4- $\mu\text{m}$ -diameter fluorescein isothiocyanate (FITC)-conjugated dextran

beads were applied to the surface of the tissue, directly over the flex area. Control tissues, obtained from matching skin samples, received the same application of beads under the same physical conditions as the flexed tissues, but were left flat and not flexed. After experimental manipulation, skin was rinsed in 1 $\times$  phosphate-buffered saline (PBS), fixed overnight in 10% buffered formalin, and flash frozen in liquid nitrogen. Twenty micrometer cross-sections were cut, stained with toluidine blue to visualize morphology, and coverslipped using a glycerine gel. Eight skin samples were tested at all time points under flexed and flat conditions. Additional samples were tested under flexed conditions only.

**Confocal microscopy.** We evaluated penetration of beads into the skin 20- $\mu\text{m}$ -thick cross sections at 1- $\mu\text{m}$  intervals by laser scanning confocal microscopy. Optical scans used in the data analysis were obtained from the center of the tissues, between 5 and 15  $\mu\text{m}$  only. Confocal images were recorded using a Sarastro 2000 laser scanning confocal microscope (Molecular Dynamics, Inc., Sunnyvale, CA) fitted with an argon-ion laser. Using a 40 $\times$  objective, images with a field size of 512  $\times$  512  $\mu\text{m}$  were generated by collecting 25 sections 1.0  $\mu\text{m}$  thick along the *z*-axis. Emission spectra  $> 535$  nm were diverted to one photodetector and used to image fluorescent beads. Transmitted light was simultaneously passed to a separate optical path and provided images of skin tissue. Images of skin tissue and fluorescent beads were combined to reveal the position of beads among skin structures. All scans were recorded at photomultiplier tube settings of 480–540, a pinhole aperture setting of 50  $\mu\text{m}$ , and a laser voltage setting of 20 mW.

**Scanning electron microscopy.** To prepare samples for scanning electron microscopy, flexed or flat tissue samples were rinsed in 1 $\times$  PBS, gently patted dry, and then subjected to repeat applications of D-Squame tape (Cuderm Corp., Dallas, TX). A fresh piece of D-Squame tape was used for each tape strip. Removal of 6–7  $\mu\text{m}$  of the stratum corneum by 30 tape strips using D-Squame tape and significant increases in transepidermal water loss after 30 tape strips have been documented (Dreher et al. 1998; van der Molen et al. 1997). Tissues were fixed in 10% neutral buffered formalin, dehydrated with ethanol, and dried with hexamethyldisilazane. The samples were mounted on aluminum stubs and coated with gold/palladium. We used a JEOL 6400 scanning electron microscope (JEOL, Tokyo, Japan) at 20 keV for imaging the samples.

**Murine care and treatment.** C3H/HeJ or C3H/HeOJ mice, 5–9 weeks of age, were housed (five per cage) according to American Association for Accreditation of Laboratory

Animal Care-approved guidelines (National Research Council 1996), and the protocols for these studies were approved by the National Institute for Occupational Safety and Health Animal Care and Use Committee. These mice have the putative beryllium presentation allele  $\text{Ia}^k$  and differ only at the CD14 locus (mutated in the HeJ mice), and pilot studies in our laboratory showed them to be equally responsive to beryllium. We first confirmed beryllium sensitization in HeJ mice because of their historical use, and then translated our studies to the OJ mice to avoid any potential complication due to the CD14 mutation.

**Lymphocyte proliferation studies.** Twenty-five microliters of a (4:1) mixture of acetone:olive oil (AOO) or a mixture of 0.5 M beryllium sulfate ( $\text{BeSO}_4$ ; 99.99% pure; Sigma, St. Louis, MO) dissolved in AOO was pipetted onto the dorsum of the ears of the mice for 3 consecutive days each week for 2 weeks. This application paradigm was determined by pilot studies in our laboratory that evaluated 0.01–0.5 M  $\text{BeSO}_4$ , the number of applications of chemical, and the optimal time point following application at which to obtain the cells. Forty-eight hours after the final application of chemical, mice (10 per treatment group) were euthanized by  $\text{CO}_2$  inhalation. Blood was collected immediately by cardiac puncture, and peripheral blood mononuclear cells (PBMCs) were isolated against a ficoll-hypaque gradient and resuspended in complete media containing 90% (vol/vol) RPMI 1640, 10% FBS, 100 U/mL penicillin, and 100  $\mu\text{g}/\text{mL}$  streptomycin (Sigma). Right and left auricular LNs were surgically removed. LN cells were dissociated sequentially through 400- $\mu\text{m}$  and 200- $\mu\text{m}$  nylon meshes, rinsed in RPMI 1640, and resuspended in complete media (Kimber and Dearman 1991). Viability ( $> 90\%$  for all studies) was determined by trypan blue exclusion. To obtain enough cells for a single proliferation experiment, we pooled cells from all mice in a treatment group.

Cells were cultured at  $1 \times 10^6$  cells/mL in triplicate or quadruplicate according to the established BeLPT protocol (Rossman et al. 1988). Culture conditions included stimulation with 10  $\mu\text{g}/\text{mL}$  phytohemagglutinin (PHA), 100  $\mu\text{M}$   $\text{BeSO}_4$ , or 50  $\mu\text{M}$   $\text{Al}_2\text{SO}_4$ , and cells were harvested at designated time points. Isotopic decay [disintegrations per minute (dpm)] for each experimental condition was averaged and is presented as mean  $\pm$  SEM. All proliferation experiments were repeated three to five times.

**Flow cytometric analysis.** We pipetted 25  $\mu\text{L}$  0.5 M  $\text{BeSO}_4$  in dibutylphthalate ( $\text{BeSO}_4/\text{DBPT}$ ; 1:1) or the positive control, 0.25% 2,4-dinitrofluorobenzene (DNFB; Sigma) in 4:1 AOO, onto the dorsum of the ears for 3 consecutive days. Auricular LN

single-cell suspensions were prepared. We performed flow cytometric analysis of surface phenotype by two- or three-color staining using FITC-, phycoerythrin-, and allophycocyanin-conjugated rat or hamster anti-mouse monoclonal antibodies. LN cells were stained with monoclonal antibodies against CD3, CD44, and CD62L (BD PharMingen, San Diego, CA). Nonspecific binding was assessed with isotype antibodies directed against immunoglobulins IgG1, IgG2a, and IgG2b. We determined the percentage of nonviable cells by propidium iodide uptake. Samples were analyzed in duplicate using a FACScalibur flow cytometer and CellQuest software (both from BD Biosciences, Franklin Lakes, NJ).

For beryllium oxide particle induction of sensitization, mice were shaved on the back and depilated with Nair (Carter Products, New York, NY), in a  $1.5 \times 3$  cm area, 24 hr before chemical treatment. BeO particles (99.98% pure; Sigma) were suspended in petrolatum (1 mg/g). This suspension was painted on the back, and the area was covered with Blendederm surgical tape (3M Health Care, St. Paul, MN). Tape was removed after 24–30 hr. The average amount of beryllium applied to each mouse was 70  $\mu\text{g}$ /Blendederm tape (range, 59–100  $\mu\text{g}$ /sample), and the average amount of beryllium in the flank skin 7 days after application of the BeO/petrolatum was 1.2  $\mu\text{g}$  beryllium/g skin tissue (range, 0.8–3.1  $\mu\text{g}$ /g skin tissue). Mice received 25  $\mu\text{L}$  0.5 M BeSO<sub>4</sub>/DBPT on the ear on day 7. Flow cytometric analysis was performed 48 hr after challenge.

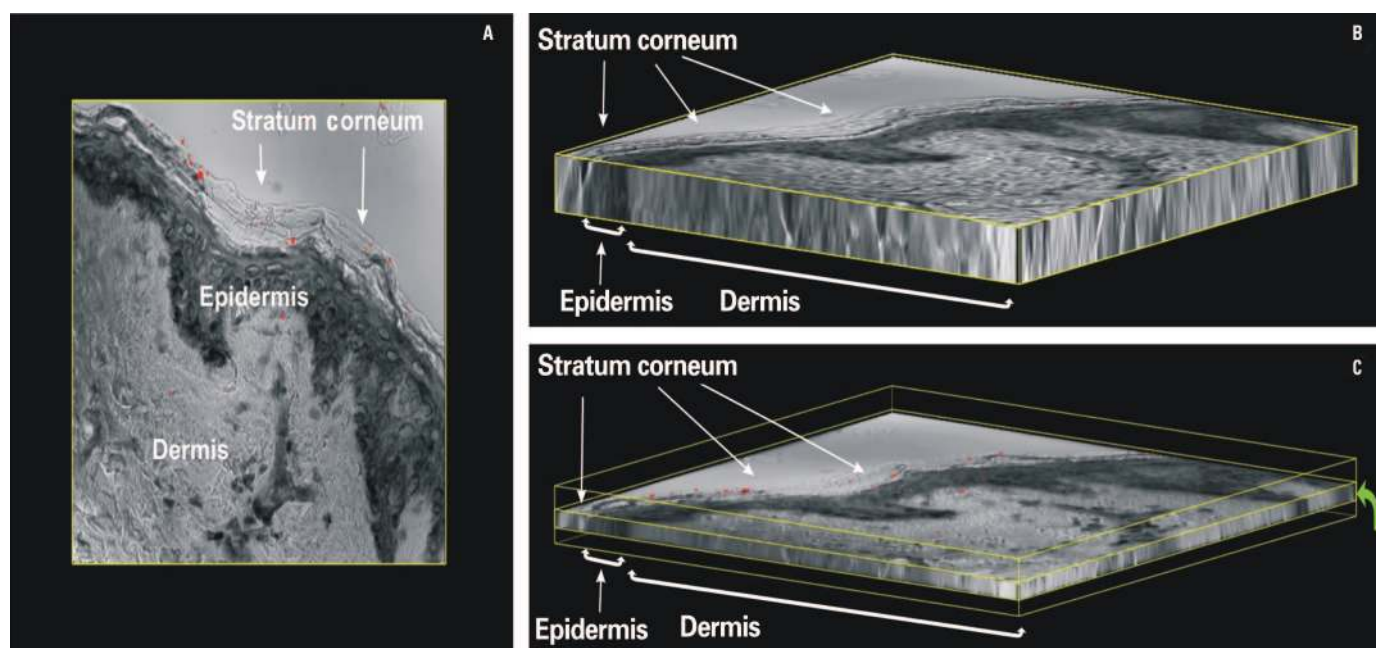
**Ear swelling analysis.** To determine the effects of beryllium on ear thickness, we pipetted 100  $\mu\text{L}$  0.5 M BeSO<sub>4</sub>/DBPT onto the backs of mice on days 1 and 2, or we painted BeO in petrolatum onto the backs and covered the area with dermal tape for 30 hr. On day 7, all mice were challenged on the ear with 25  $\mu\text{L}$  0.5 M BeSO<sub>4</sub>/DBPT. We measured ear thickness with a digital micrometer at 24, 48, and 72 hr postchallenge for BeSO<sub>4</sub> sensitization and at 24 and 48 hr postchallenge for BeO sensitization. Measurements for each treatment group are presented as mean  $\pm$  SEM.

**Statistical analysis.** Significant differences between treated and untreated groups were determined by analysis of variance for cell proliferation studies and by the *t*-test for flow cytometric analysis. Analyses of ear swelling data were conducted using PROC MIXED and LSMEANS software (SAS Institute, Cary, NC). Means and standard deviations were calculated and compared using PROC MIXED and adjusted for the covariate, initial ear thickness. The LSMEANS option was used for calculating means of treatment groups and for adjustment of unequal sample sizes among treatment groups. Statistical significance was defined as  $p < 0.05$ .

## Results

**Fluorescent bead penetration of intact skin samples.** The particulate BeO is the primary form of occupational exposure, and its half-life is tens to hundreds of days. For a material with a protracted dissolution half-life to provide a concentration of beryllium in the epidermis

sufficient to activate cutaneous immunity, BeO particles would have to colocalize with the Langerhans cell, the predominant antigen-presenting cell in the epidermis. Therefore, we hypothesized that beryllium particles would enter the epidermis to activate the cutaneous immune response and that flexing motion, as at the wrist, would provide the energy necessary for particle penetration of the stratum corneum. To test this hypothesis, we examined particle penetration in skin samples that were subjected to repeated 45° flexure, 20 flexures per minute, and control tissues taken from the same skin sample as the experimental tissues but not flexed. Penetration of fluorospheres into the skin was evaluated in 20- $\mu\text{m}$ -thick cross sections by laser scanning confocal microscopy. The principal anatomical features in a cross section, the stratum corneum, epidermis, and dermis, are shown in Figure 1A, and in side view in Figure 1B. Optical scans were acquired at 1- $\mu\text{m}$  intervals, and data were obtained from the center of the tissues, between 5 and 15  $\mu\text{m}$  only. The area for data collection is indicated by the yellow arrow in Figure 1C. One-micrometer red fluorospheres are clearly visible in the stratum corneum and epidermis after the 30-min application of beads and flexing motion (blue arrows, Figure 2A), and in the stratum corneum, epidermis, and dermis at 60 min (all arrows, Figure 2B). Occasional clusters of fluorospheres were observed in the epidermis and the dermis (yellow arrow, Figure 2B). To test the relationship of particle size to penetration, we also evaluated 0.5-, 2-, and 4- $\mu\text{m}$  spheres. We



**Figure 1.** Confocal microscopic imaging of human skin. (A) Following the flexing procedure (see “Materials and Methods” for details), optical scans were obtained at 1- $\mu\text{m}$  intervals through a 20- $\mu\text{m}$ -thick tissue section. (B) A three-dimensional image of the cross section of tissue shown in (A) reconstructed by Voxelfview software, version 2.5.4 (Vital Images Inc., Minneapolis, MN) from the optical scans obtained for each sample. (C) Data were obtained from the center of the tissue section as designated by the yellow arrow.

observed penetration of 0.5- $\mu\text{m}$  beads (Figure 2C) in addition to the 1- $\mu\text{m}$  beads, and exclusion of 2- and 4- $\mu\text{m}$  beads (Figure 2D).

A summary of all data in this study revealed that 0.5- and 1- $\mu\text{m}$  beads penetrated into the epidermis in 2 of 11 skin samples (18%) flexed for 15 min, in 5 of 12 samples (41%) flexed for 30 min, and in 9 of 16 samples (56%) flexed for 60 min. Penetration into the dermis occurred in two samples after flexing 60 min. The fluorospheres that penetrated through the stratum corneum represent only a small percentage of the applied beads, and the pattern of penetration was random. In contrast to skin samples with intact stratum corneum, discontinuous stratum corneum permitted entry of a bolus of beads directly under the tear (Figure 2E). No particle penetration was observed in nonflexed tissues at any time point.

We repeated this study by combining tape stripping of bead-exposed, flexed skin with

scanning electron microscopy (Figure 3). Although the technique does not permit the more precise tissue localization possible with the laser scanning confocal procedure, we observed beads in the skin after no tape stripping (Figure 3A) and tape stripping 10 times (Figure 3B) and 20 times (Figure 3C), to an approximate depth of 6–7  $\mu\text{m}$  into the stratum corneum.

#### Cutaneous sensitization by beryllium salts.

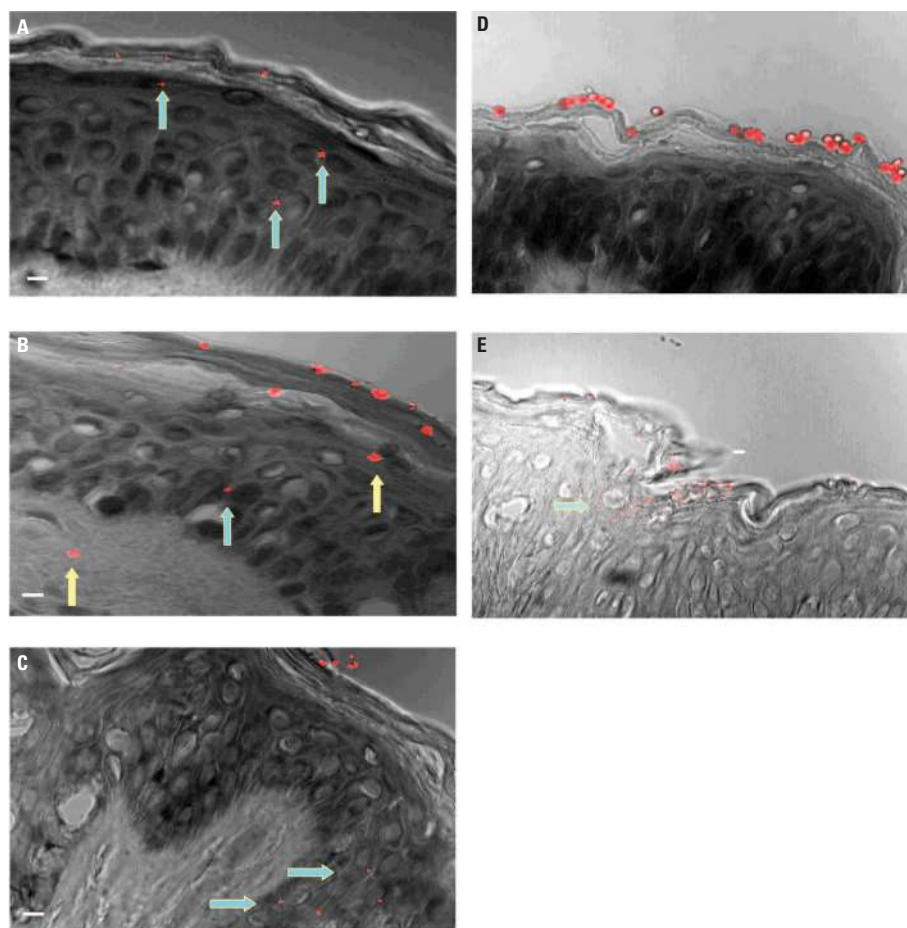
We next asked if cutaneous application of beryllium would initiate a beryllium-specific, cell-mediated immune response in mice. Most attempts to develop a CBD mouse model employed multiple subcutaneous or intradermal injections of  $\text{BeSO}_4$  for 2–6 weeks, followed by a single intratracheal instillation, or nose-only inhalation, of beryllium salts or respirable beryllium particulates (Finch et al. 1998; Huang et al. 1992). From these exposures, C3H/HeJ and A/J mice, but not BALB/c

or C57BL/6 mice, developed T-cell alveolitis, and a subset of these mice developed spontaneously resolving pulmonary microgranulomas. Beryllium-specific lymphocyte proliferation following the epicutaneous administration of beryllium salts was not evaluated. Therefore, we applied 50  $\mu\text{L}$  0.5 M  $\text{BeSO}_4$  dissolved in AOO vehicle, or vehicle only, to the dorsal side of the ear for 3 consecutive days per week for 2 weeks. We obtained auricular LN cells and PBMC for each exposure group 48 hr after the final application of chemical, and the lymphoproliferative response was measured by tritiated thymidine incorporation at designated time points. We confirmed the proliferative ability of the cell preparations with the mitogen PHA and beryllium specificity with beryllium and aluminum salts.

Evaluating the beryllium responsive C3H/HeJ mice, we measured a 34.8-fold beryllium-stimulated increase in auricular LN cell proliferation at 48 hr ( $\text{BeSO}_4$ -stimulated cells vs. unstimulated cells:  $29,691 \pm 6,916$  dpm vs.  $517 \pm 62$  dpm, mean  $\pm$  SEM;  $p < 0.01$ ; Figure 4) and a 30.7-fold increase in PBMC proliferation at 120 hr ( $\text{BeSO}_4$ -stimulated cells vs. unstimulated cells:  $51,342 \pm 16,135$  dpm vs.  $1,673 \pm 385$  dpm;  $p < 0.03$ ; Figure 5). Beryllium salts did not stimulate LN cell or PBMC proliferation in cells harvested from mice treated with vehicle only. In both treatment groups, the PHA response peaked at 24 hr in LN cells preparations and at 48 hr in PBMC. BAL cells taken from beryllium skin-sensitized mice produced no beryllium-specific proliferation (data not shown).

**Lymph node T-lymphocyte activation in beryllium-sensitized mice.** Expression of CD44, a membrane glycoprotein involved in cell adhesion and extravasation, is up-regulated on activated and memory T cells. CD62L, or L-selectin, is expressed on the majority of B and T lymphocytes, and its expression is rapidly lost upon T-cell activation. We next asked if cutaneous beryllium, the salt solution and the oxide particles, could induce LN T-lymphocyte activation, as measured by changes in CD44 and CD62L expression. The well-studied skin sensitizer DNFB served as the positive control. In the first series of experiments,  $\text{BeSO}_4$  or the control chemicals were applied to the dorsum of the ears daily for 3 days, and auricular LNs were harvested 48 hr later. In beryllium-treated mice, we measured a 13% increase in CD44<sup>+</sup> LN cells ( $p < 0.008$ ), an increase that closely matched CD44 expression on LN cells derived from DNFB-treated mice ( $p < 0.008$ ; Figure 6). In contrast, the percentage of CD3<sup>+</sup> LN cells expressing CD62L decreased approximately 12% for  $\text{BeSO}_4$ - ( $p < 0.008$ ) and DNFB-treated mice ( $p < 0.01$ ).

Because of the difficulty applying  $\text{BeO}$ /petrolatum under an occlusive patch to



**Figure 2.** Confocal microscopic images of skin containing FITC-conjugated dextran beads (red). Images presented were obtained from the center of the tissue cross section at a depth of 10  $\mu\text{m}$ . (A) One-micrometer beads were observed at the stratum corneum–epidermal interface and in the epidermis following 30 min of flexure (blue arrows). (B) One-micrometer beads were observed at the stratum corneum–epidermal interface and in the epidermis after 30 min of flexure, and in the epidermis and dermis after 60 min (all arrows); beads were found randomly distributed in the tissue, and occasional clusters of beads were identified (yellow arrows). (C) Beads (0.5- $\mu\text{m}$ ; blue arrows) were present in the epidermis after 30 min. (D) Four-micrometer beads remained on the surface of the skin (tissue depth 13  $\mu\text{m}$ ). (E) Discontinuous stratum corneum permitted entry of a bolus of 0.5- $\mu\text{m}$  beads into the skin directly under the tear (blue arrow). Bar = 10  $\mu\text{m}$  for A–E.

the dorsum of the ears, we tested BeO in a sensitization-challenge paradigm. To induce sensitization, BeO/petrolatum was applied to the back for 24–30 hr, and 6 days later, mice received one treatment of beryllium salts on the ear. A single application of BeSO<sub>4</sub> to the ear did not change the percentage of LN cells expressing CD44/CD62L as demonstrated by the DBPT/BeSO<sub>4</sub>-treated mice ( $p > 0.05$ ; Figure 6B). Mice treated with BeO on the back before application of BeSO<sub>4</sub> on the ear displayed a 10% increase in CD44<sup>+</sup> LN cells ( $p < 0.006$ ) and a 20% decrease in CD62L<sup>+</sup>/CD3<sup>+</sup> cells ( $p < 0.001$ ).

**Parameters of cutaneous sensitization.** To evaluate elicitation of cutaneous sensitization, we tested the skin antigen recall response, as measured by increased ear thickness. We tested the irritant/inflammatory properties of beryllium salts with a single application of this solution on the ear (DBPT/BeSO<sub>4</sub>-treated

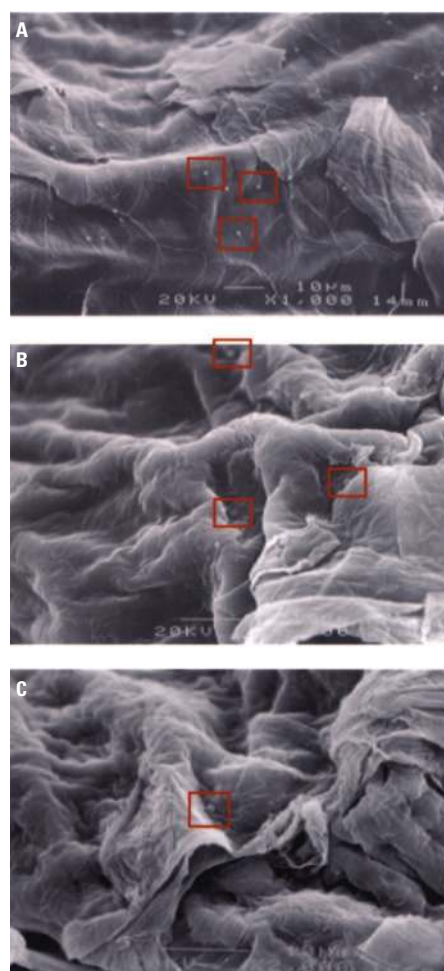
mice) and, compared to mice receiving vehicle on the ear (BeSO<sub>4</sub>/DBPT-treated mice), we measured no significant increase in ear thickness at any time point (Figure 7). In contrast, sensitization and challenge with beryllium salts (BeSO<sub>4</sub>/BeSO<sub>4</sub>-treated mice) increased ear thickness 25–30% at 24 and 48 hr ( $p < 0.04$ ). Mice receiving epicutaneous BeO and challenged once on the ear with the salts (BeO/BeSO<sub>4</sub>-treated mice) displayed a 30% increase at 24 hr that remained elevated significantly at 48 hr ( $p < 0.04$ ). Because a single application of beryllium to the ear does not increase ear thickness unless preceded by beryllium sensitization on the back, these data illustrate that BeO particles induced sensitization in the BeO/BeSO<sub>4</sub>-treated mice, analogous to the increase measured for BeSO<sub>4</sub>/BeSO<sub>4</sub>-treated mice.

## Discussion

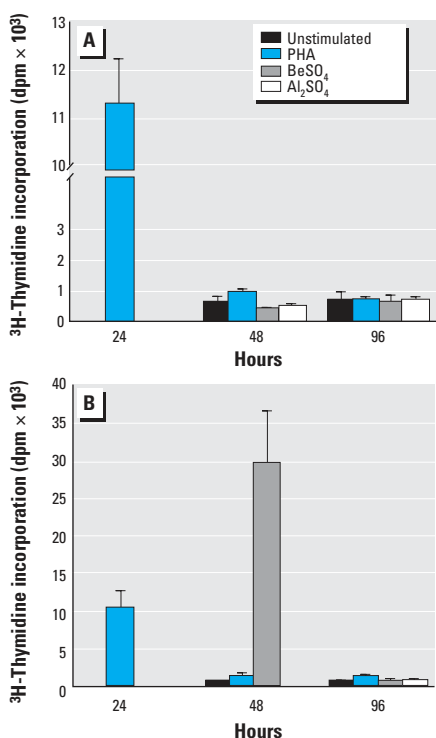
The lung is the primary location for development of noncaseating granulomas in beryllium disease, and respiratory protection and engineering controls, not skin protection, were considered good and sufficient industrial

hygiene practice to mitigate development of sensitization and disease. However, over the last decade, the rates of sensitization and disease did not decline. Because the most common worker exposure is to the particulate BeO, we investigated cutaneous exposure to particulates as a potential route for sensitization to beryllium. The data presented in this study demonstrate that, in conjunction with motion, fine particles penetrate the stratum corneum and lodge in the epidermis, the anatomical location of the cutaneous antigen-presenting cell, the Langerhans cell. Further, we provided evidence for a murine beryllium-specific, cell-mediated immune response following cutaneous application of BeO and BeSO<sub>4</sub>.

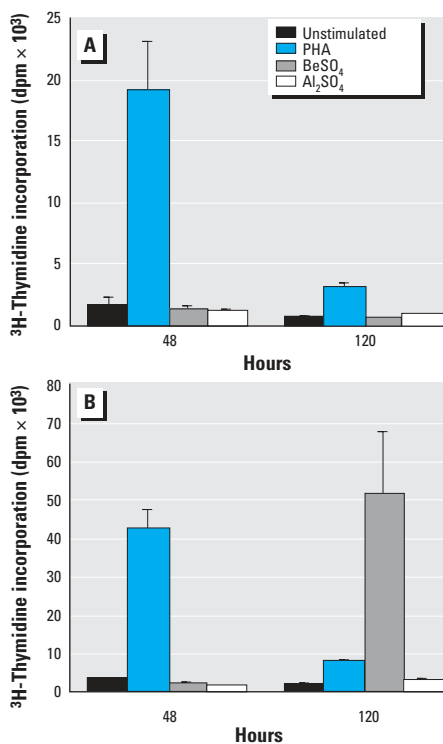
Activation of cutaneous immunity by percutaneous absorption of chemical through the stratum corneum is well established; however, given the long dissolution half-life for beryllium metal and its alloys, we hypothesized that beryllium particles would have to colocalize with the cutaneous antigen-presenting cell in the epidermis in order to activate a cell-mediated immune response. To reach the epidermis, particles would have to traverse the



**Figure 3.** Scanning electron microscopic images of the surface of skin samples tape stripped 0 (A), 10 (B), and 20 (C) times. Skin treated with 1- $\mu$ m beads was flexed for 60 min, rinsed, and subjected to repeated applications of D-Squame tape as described in “Materials and Methods.” The red boxes indicate location of beads.



**Figure 4.** Lymphocyte proliferation measured by <sup>3</sup>H-thymidine incorporation in auricular LN cell preparations derived from (A) AOO-treated mice and (B) BeSO<sub>4</sub>-treated mice. Single-cell suspensions of auricular LNs were prepared and stimulated as described in “Materials and Methods” and harvested at the designated time points. No BeSO<sub>4</sub>-stimulated cell proliferation was measured in LN cells obtained from AOO-treated mice. Data are presented as the mean  $\pm$  SEM and are representative of three experiments;  $n = 10$  mice per group.



**Figure 5.** Lymphocyte proliferation measured by <sup>3</sup>H-thymidine incorporation in PBMC preparations derived from (A) AOO-treated mice and (B) BeSO<sub>4</sub>-treated mice. PBMCs were isolated and stimulated as described in “Materials and Methods” and harvested at the designated time points. No BeSO<sub>4</sub>-stimulated cell proliferation was measured in PBMCs obtained from AOO-treated mice. Data are presented as the mean  $\pm$  SEM and are representative of five experiments;  $n = 10$  mice per group.

stratum corneum. The stratum corneum is composed of several layers of randomly stacked corneocytes surrounded by lipids. The lipids are arranged in overlapping lamellar sheets and form pathways for molecular transport into and through the stratum corneum. These channels follow a winding but continuous path from the surface of the skin into the epidermis (Menon and Elias 1997). Our data demonstrate the passage of fluorospheres,  $\leq 1 \mu\text{m}$ , through stratum corneum, into the epidermis. Although we are unable to demonstrate definitively that the particles are within these intercellular pathways, it is reasonable to consider that these lipids would be sufficiently malleable that fine particles, in conjunction with motion, could enter. Lacunae, or bulges, identified in these lipid pathways may explain the occasional clustering of fluorescent beads that we observed. Once in these lipid channels, over time particles could continue to move into the skin and be phagocytosed by the Langerhans cells. Murine Langerhans cell phagocytosis of 0.5–3.5- $\mu\text{m}$  microspheres has been demonstrated *in vitro* by Reis e Sousa et al. (1993). These data provide the link between our proof of concept experiments for particle entry into the stratum corneum and BeO-stimulated skin sensitization.

The application of external force is a consistent component of particle penetration studies. Lademann et al. (1999) and Tan et al. (1996) documented titanium dioxide penetration of the stratum corneum following repeated rubbing of sunscreen onto the volar forearm. The podoconiosis literature ascribes development of disease to soil microparticles driven through the soles of the feet. Individuals

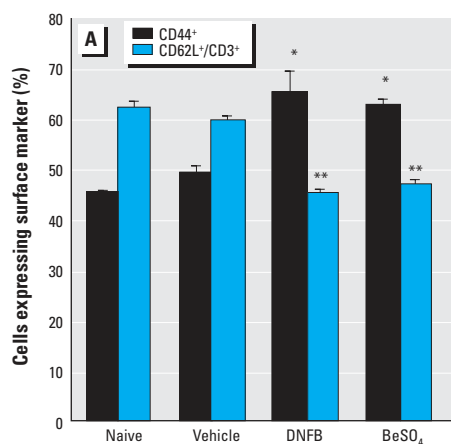
walked barefoot, and over time, microparticles, frequently 0.4–0.5  $\mu\text{m}$  but as large as 25  $\mu\text{m}$ , were observed in the femoral and inguinal LNs by scanning electron microscopy (Blundell et al. 1989; Corachan et al. 1988). In our experimental system, we employed flexing motion, as at the wrist, as the external force, and observed no particle penetration in its absence. The differences in particle size in these studies may reflect the difference in the magnitude of the force applied and perhaps the lipid composition of the skin area.

We also documented a particle size cutoff in our *ex vivo* system, at a diameter of 1  $\mu\text{m}$ . Other researchers tested particle penetration of the skin using particles with diameters of  $\geq 3\text{--}8 \mu\text{m}$  (Andersson et al. 2002; Lademann et al. 1999, 2001). These studies identified clusters of particles on the surface of the skin, at the hair follicle orifice, or within the follicle, but no documentation of particles in the epidermis. These data are consistent with our 1- $\mu\text{m}$  size cutoff and may explain others' inability to make observations similar to our findings.

We have shown that cutaneous application of beryllium induces a beryllium-specific murine DTH. Historical studies from the 1950s–1980s used intradermal injection or epicutaneous beryllium solutions to evaluate a diagnostic skin patch test and to demonstrate the immunologic underpinnings of the beryllium cellular response. Beryllium salts, especially beryllium fluoride, cause a dose-related erythema and edema, and although there are several clinical reports of beryllium-induced contact dermatitis (Curtis 1951), a strong association between skin rash and the development of sensitization and disease was

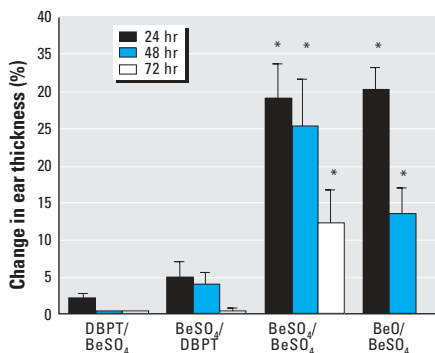
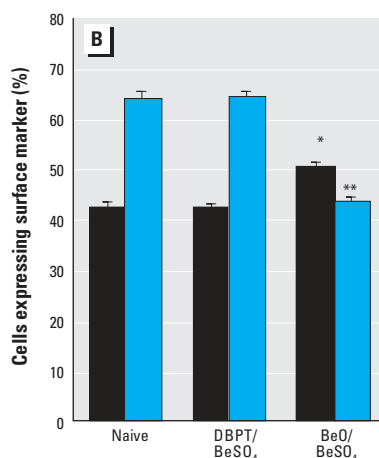
not observed. Belman (1969) documented preferential sequestration of topically applied beryllium fluoride in guinea pig epidermis and increased beryllium binding affinity for denatured proteins. Reeves and Krivanek (1974) concluded that biweekly intradermal injection of beryllium salts into guinea pig abdominal skin for 6 weeks conferred resistance to pulmonary sensitization. This repeated exposure treatment paradigm is consistent with studies on cutaneous induction of tolerance, not sensitization; however, extrapolation of the findings to human exposure was not explored.

Several studies demonstrated that the murine beryllium-MHC class II restriction is Ia<sup>k</sup> (Huang et al. 1992), the isoform present in C3H mice, and conceptually analogous to the human beryllium HLA-DPB restriction identified by Richeldi et al. (1993) and Wang et al. (1999, 2001). Using flow cytometry and the BeLPT, we demonstrated that the murine response to topical beryllium occurs in the LN draining the site of chemical application, the auricular LN in our study, and that T cells are activated and released into the peripheral blood. Using a classical measure of skin sensitization, we have shown elicitation of the ear-swelling response in BeO-sensitized mice. In a pilot study, we removed excess BeO from the surface of the flank skin immediately following the 24-hr exposure by gentle washing and tape stripping three times. On day 7 or 14 following the exposure, the amount of beryllium in the flank skin of BeO-treated mice was, on average, 1.2  $\mu\text{g/g}$  tissue, thus confirming that BeO is present in the skin (data not shown). Interestingly, application of BeO produced no erythema or edema in the sensitized mice. Shaved flank skin maintained a normal color and appearance following topical application



**Figure 6.** T-Lymphocyte activation induced by topically applied BeSO<sub>4</sub> (A) and BeO (B). BeSO<sub>4</sub> ( $n = 5$  mice), DNFB (positive control;  $n = 2$  mice), DBPT (vehicle;  $n = 2$  mice), or BeO ( $n = 5$  mice) was applied to the dorsum of the ear; single-cell suspensions of auricular LNs were prepared; and the percentage of LN cells expressing CD44 and CD62L was analyzed by flow cytometry 48 hr later (see “Materials and Methods” for details). In (B), BeO induction of T-cell activation was evaluated in a sensitization–challenge paradigm as described in “Materials and Methods.” The increase in CD44 and the decrease in CD62L was measured only on cells obtained from BeO-sensitized mice. Data are presented as the mean  $\pm$  SEM and are representative of three experiments.

\*Significant increase with respect to vehicle-treated mice. \*\*Significant decrease with respect to vehicle-treated mice.



**Figure 7.** Elicitation of an ear-swelling response following sensitization by topical application of BeSO<sub>4</sub> or BeO. Mice were sensitized with BeSO<sub>4</sub> or BeO/petrolatum and challenged on the ears 1 week later as described in “Materials and Methods.” Mice sensitized with BeSO<sub>4</sub> or BeO displayed a significant increase in ear thickness following challenge on the ear with BeSO<sub>4</sub>. Data are presented as the mean  $\pm$  SEM and are representative of three experiments.

\*Significant increase with respect to control mice.

of BeO or following intradermal injection, and within days, hair growth was evident.

The formation of noncaseating granulomas in beryllium disease occurs primarily in the lung, and skin exposure to beryllium particulates had not been considered hazardous to the workers. We have demonstrated that BeO initiates cutaneous sensitization in mice and have provided proof of concept experiments for fine particle penetration of skin as an initiating event in this process. Because the concentration of antigen required for elicitation of a cell-mediated immune response is significantly smaller than the concentration required for sensitization, our data may provide insight into the immunopathology underlying the failure of respiratory exposure limits to lower the rate of disease over the last decade. Our findings suggest that, for workers with significant beryllium skin exposure, the pulmonary exposure required to elicit a subsequent immune response and granuloma formation would be significantly smaller. We are currently characterizing the murine peripheral immune response to beryllium and investigating the relationship between skin sensitization and pulmonary disease. Although these studies were performed in the context of beryllium disease, evaluation of cutaneous particle exposures in other occupational settings may be warranted.

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