Beryllium Research Symposium: Basic Mechanisms and Human Health
June 25- 26, 2002
National Library of Medicine, Bethesda, MD

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Office of Biological and Environmental Research,
The Department of Energy in cooperation with

The National Institute for Occupational Safety and Health, and

The National Jewish Medical and Research Center

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- **Chemicals and Immunologic Lung Disease:Gene and Environment**
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Date Published: June 2002

Prepared for the
U.S. Department of Energy Office of Science
Biological and Environmental Research Program
in cooperation with
National Institute for Occupational Safety and Health
and
National Jewish Medical and Research Center

Prepared by
Human Genome Management Information System
Oak Ridge National Laboratory
Oak Ridge, TN 37830

Managed by
UT Battelle, LLC
for the
U.S. DEPARTMENT OF ENERGY
UNDER CONTRACT DE-AC05-00OR22725

Introduction

Welcome to the Beryllium Research Symposium: Basic Mechanism and Human Health, sponsored by the Department of Energy (DOE) Office of Biological and Environmental Sciences, in conjunction with the National Institute for Occupational Safety and Health (NIOSH) and National Jewish Medical and Research Center. This is the second symposium DOE has supported to stimulate a dialogue among scientists, physicians, and government officials about current and productive new research into Chronic Beryllium Disease (CBD). CBD is a very serious progressive occupational lung disease, and its occurrence in current and former beryllium workers is of continuing interest and concern to DOE and other federal agencies. To put the issue in perspective, about 1% to 6% of exposed beryllium workers develop CBD or beryllium hypersensitivity, which can progress to CBD.

This workshop provides a unique opportunity for beryllium health-effect investigators to discuss and share the successes, problems, and challenges of their research as well as new resources and capabilities. The meeting also provides scientists and administrative staff with a chance to assess the impact of new technologies and, perhaps most important, to initiate new collaborations.

Many challenges lie ahead, particularly in understanding basic disease mechanisms. We are optimistic, nevertheless, that if we can bring to bear the increasingly sophisticated arsenal of techniques, technology, and new approaches in areas such as genomics and immunology, we can answer seminal question about the nature of CBD. Yet we cannot afford to be complacent, and workshop presenters on ethical, legal, and social implications (ELSI) will remind and challenge all of us that science has societal impacts that we must confront. These are real-life issues that need to be considered in the context of research, testing, and treatment with the active participation of all involved physicians and scientists.

We look forward to a very interesting and productive meeting and offer our sincere thanks to all the organizers and to you whose vision and hard work will bring to fruition our efforts to reach our ultimate goal, protecting and effectively treating beryllium workers.

Dr. Marvin E. Frazier, Director
Life Sciences Division
Biological and Environmental Research Program
Office of Science
U.S. Department of Energy

Welcome from the Planning Committee

June 25, 2002

Welcome!

On behalf of the planning committee, welcome to the Beryllium Research Symposium: Basic Mechanisms and Human Health. This meeting represents an exciting opportunity to bring new and established researchers in beryllium disease together with those from related areas of science.

Chronic Beryllium Disease presents a unique research opportunity—an occupational lung disease for which we have exposure data, clinical data, and a known hapten. While the immunological underpinnings of the disease have been identified, the molecular and cellular events underlying progression from sensitization to granulomatous disease have yet to be elucidated. Determination of these events is critical to reduction of disease risk and development of therapeutic interventions. This is the challenge we bring to you today.

Thank you for joining us in the presentation of your research, the exchange of ideas, and the development beryllium-related collaborations.

Sincerely,

Sally Tinkle, National Institute for Occupational Safety and Health

Lee Newman, National Jewish Medical and Research Center

Lisa Maier, National Jewish Medical and Research Center

Beryllium Research Symposium Planning Committee

Session 1. Immunopathology of Beryllium-Induced Granulomatous Disease

Characterization of Beryllium-Specific Memory CD4+ T Cells in Patients with Chronic Beryllium Disease

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Evidence suggest that CD4+ T cells play a critical role in the development of chronic beryllium disease (CBD). Using intracellular cytokine staining, we found that the frequency of beryllium-specific CD4+ T cells in the lungs of 10 CBD patients ranged from 1.4-29% (mean 18.8%), and these T cells expressed a Th1-type phenotype in response to beryllium sulfate ($BeSO_4$). Few, if any, beryllium-specific CD8+ T cells were identified. In contrast, the frequency of beryllium-responsive CD4+ T cells in the blood of these subjects ranged from undetectable to 1 in 500. No correlation was observed between the frequency of beryllium-responsive BAL CD4+ T cells detected with intracellular cytokine staining and lymphocyte proliferation in culture after $BeSO_4$ exposure. The number of $BeSO_4$-responsive CD4+ T cells appeared to reflect the industry of beryllium exposure. Staining for surface marker expression showed that nearly all BAL T cells exhibit an effector memory cell phenotype. These results demonstrate a dramatically high frequency and compartmentalization of antigen-specific effector memory CD4+ cells in lungs of CBD patients. These studies provide insight into the phenotypic and functional characteristics of antigen-specific T cells invading other inaccessible target organs in human disease.

HLA-DP-Unrestricted TNF-a Release in Be-Stimulated PBMC from Berylliosis Patients

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Berylliosis is a granulomatous disorder of the lung caused by inhalation of beryllium (Be) and dominated by

the accumulation of CD4+ Th1 memory T-cells proliferating in response to Be in the lower respiratory tract. Two gene markers have been associated with susceptibility to berylliosis: the HLA-DP gene whose allelic variants carrying glutamate in position 69 of the b chain (HLA-DPGlu69), which play a central role as they can bind Be directly and present it to IFN-g releasing Th1 T-cell clones from patients with berylliosis, and the cytokine gene TNF-a which has been shown to increase berylliosis risk independent of HLA-DPGlu69. In order to determine whether TNF-a release was triggered by TH1 T-cell activation by Be stimulation in the context of HLA-DPGlu69 molecules, we quantified the proliferation and IFN-g, TNF-a, Rantes, GM-CSF, IL-4, IL-6, IL-8, IL-10 and IL-12 release by of $BeSO_4$-stimulated blood mononuclear cells in 11 individuals with berylliosis using an anti-HLA-DP antibody as a probe for HLA-DP restricted T-cell activation.

While proliferation and IFN-g was completely abrogated by HLA-DP inhibition (inhibition with anti-HLA-DP MoAb: 88+16% and 77+16% respectively; anti-HLA-DR, 29+38% and 14+10% respectively, $p<0.05$) the release of TNF-a was not (inhibition with anti-HLA-DP MoAb: 8.9+7.8); no other cytokine was detected at significant levels. Moreover, Be was able to induce TNF-a production in healthy no Be-exposed control in the absence of T-cell proliferation and IFN-g production. Altogether this data suggests that, consistently with the finding that the TNFA2 and the HLA-DPGlu69 genetic markers are independently interacting in increasing berylliosis risk, the TNF-a response of mononuclear cells is independent of the activation of Be-specific HLA-DP restricted T-cells.

Design, Engineering and Production of Human Recombinant T Cell Receptor Ligands Derived from HLA-DP

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Chronic beryllium disease (CBD) is a lung disease similar clinically to other granulomatous diseases, such as sarcoidosis, schistosomiasis and tuberculosis. Approximately 800,000 individuals are currently at risk for developing the disease, which is caused by metal and relatively insoluble compounds of beryllium. The disease process begins as a sensitizing cell-mediated immune response to beryllium antigen which develops into a non-caseating granuloma. Evidence strongly suggests that CD4+ T cells and the MHC class II allele HLA-DPB1*0201 are important in the immunopathogenesis of CBD. How the T cell receptor (TCR) on the T cells interacts with beryllium and the MHC, and the mechanism that gives rise to the pathogenesis of CBD, remains unknown. We have developed a family of novel recombinant HLA-DP-derived T cell receptor ligands (RTLs) to test critically the hypothesis that a specific MHC class II allele interacts with beryllium and induces T cell responses that contribute directly to the pathogenesis of CBD. Development and characterization of these novel constructs will provide the opportunity to identify unique points of intervention for controlling T cells and in turn the T cell immune response and repertoire. These molecules may provide a template for engineering a novel treatment of CBD.

A Flow Cytometric Assay for Beryllium Sensitization: Screening And Mechanistic Applications

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Beryllium inhalation in humans can result in chronic beryllium disease (CBD). Medical monitoring relies on the in vitro assessment of beryllium sensitization using the lymphocyte proliferation test (LPT) that measures tritiated thymidine incorporation into the DNA of proliferating cells. In this study, we demonstrate the utility of a flow cytometry-based method to test for beryllium sensitization and provide information on the mechanism of beryllium disease at the cellular and molecular levels through multiparametric analysis of cellular DNA and proteins. The four-color flow cytometry assay allows the simultaneous measurement of cumulative cell proliferation and surface marker expression for the purpose of identifying the types of cells responding to beryllium exposure. Peripheral blood mononuclear cells (PBMC) from known CBD patients and normal controls were isolated and tested in culture for their response to beryllium. Increased cell proliferation to beryllium was only seen with blood cells from sensitized individuals but not normal controls. Beryllium elicited a positive proliferative response in cultured PBMC from known CBD patients that ranged from 2-7% at day 4 and 3-10% at day 6 of culture. The lymphocyte composition of resting (G0/G1) and proliferating subpopulations was determined by immunolabeling specific markers of T (CD4, CD3) and B (CD19) cells. The resting subpopulation included 40-60% $CD4^+$ T cells, 60-80% $CD3^+$ T cells, and 5-10% B lymphocytes. Beryllium caused a selective stimulation of $CD4^+$ T cells since the majority of proliferating cells were $CD4^+$ T cells. The versatility of this assay may improve our ability to detect beryllium health effects and advance our understanding on the causes of CBD.

(Supported by Department of Energy Cooperative Agreement #DE-FCO1-95EW55084).

Session 2. Pulmonary Signaling Pathways Activated by Beryllium

Beryllium Stimulates a Local Angiotensin System in Chronic Beryllium Disease

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Presentation PDF

Background: Increased serum angiotensin converting enzyme (ACE) is associated with granulomatous lung diseases such as sarcoidosis and chronic beryllium disease (CBD). CBD is marked by a beryllium specific cellular immune response, which results in granuloma formation and may progress to fibrosis. High serum ACE has been associated with disease severity in CBD, although the source and function of a local angiotensin system is not known. Mast cells found in fibrosis surrounding granulomas are a source of basic fibroblast growth factor (bFGF). Angiotensin II (ATII), an enzymatic product of ACE, stimulates bFGF production.

Objective: This study tested the hypothesis that ACE and its enzymatic product angiotensin II (ATII) are stimulated by beryllium during granuloma formation in individuals with CBD.

Methods: Following informed consent, 25 subjects with CBD and 5 with sarcoidosis were enrolled in this study. Of the 25 with CBD, 5 underwent beryllium (Be) skin patch testing, using aluminum as a negative control. Skin patch biopsies were collected at various intervals between 0 and 35 days. Immunohistochemistry was used to evaluate CD3, CD4, CD8, CD68, ACE and ATII in skin patch biopsies and morphometric analysis was used for quantification. The remaining 20 subjects with CBD underwent bronchoscopy with bronchoalveolar lavage (BAL). Five subjects with sarcoidosis were used as BAL controls. BAL cells were stimulated with and without beryllium sulfate at 10-4M and 10-5M for 0, 24, 72, and 120 hours. Aluminum sulfate at 10-4M was used as a negative control. ACE and ATII were determined in cell supernatants by ELISA and RIA respectively. Immunohistochemistry was performed on unstimulated cells to localize CD3, CD4, CD68, ACE and ATII in BAL cells.

Results: CD3, CD4, CD68, ACE and ATII were present by 48 hours in Be skin patch biopsies. ATII continued to increase over time, while ACE staining peaked at 96 hours. Double immunohistochemistry revealed predominant colocalization of CD4 with ACE and ATII with CD68. CBD BAL cells produced constitutive levels of ACE (median 12 ng/ml) and ATII (median 42 pg/ml). Sarcoidosis BAL cells also produced constitutive levels of ACE (median 15 ng/ml) and ATII (median 36 pg/ml). Be stimulated CBD BAL cells produced significantly more ACE compared to unstimulated cells (median 20 ng/ml vs. 12 ng/ml). ACE production did not increased in response to Be in sarcoidosis BAL cells (median 13 ng/ml vs. 15 ng/ml). Be did not stimulate ATII in CBD BAL cells (median 34 pg/ml vs. 42 pg/ml). In sarcoidosis BAL cells, ATII was not stimulated by Be (median 33 pg/ml vs. 36 pg/ml). Immunohisto-chemistry of BAL cells revealed colocalization of ACE with CD4 and ATII with CD68.

Conclusion: Be stimulates ACE production by T cells and ATII by monocytes in the skin during granuloma formation. Significant ACE is stimulated in BAL cells in response to Be, with T cells producing ACE and monocytes producing ATII. This suggests that Be stimulates a local angiotensin system in CBD It is possible

that the angiotensin system is important in the immune response to beryllium and/or progression to fibrosis, which will be the topic of future studies.

K08 HL03887, R01 ES06358-06, M01 RR00051

High Levels of Nitric Oxide (NO) in Exhaled Breath in Patients with Chronic Beryllium Disease

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Chronic Beryllium Disease (CBD) is an occupationally-acquired lung disease that begins as a sensitizing T cell-mediated immune response to beryllium antigen which develops into non-caseating granulomatous lung inflammation. Nitric oxide (NO) measurement in exhaled gases has been proposed as a noninvasive marker of lung inflammation. In this context, we hypothesized that NO may be high in patients with CBD due to active granulomatous lung inflammation. To evaluate this hypothesis, exhaled NO was measured in beryllium-exposed workers who were referred for evaluation of CBD after a positive blood lymphocyte proliferation test to beryllium. Following a 15 sec breath hold at total lung capacity, individuals exhaled against 10 cm of water pressure into a Mylar balloon while wearing a nose clip. Measures of NO in the exhalate were determined in duplicate using a chemiluminescent analyzer (NOA 280). All individuals had lung biopsies to determine granulomatous lung disease. Granuloma-positive individuals had higher exhaled NO levels than granuloma-negative individuals [CBD 12±2 ppb (n=8) vs granuloma-negative 7±1 ppb (n=8); p=0.04]. These results provide further support for an association between active lung inflammation and increased NO in exhaled breath.

Nitric Oxide (NO) Attenuation of Interferon Gamma (IFN-Gamma) Responses in Chronic Beryllium Disease (CBD): Evidence for Mechanisms Independent of Interleukin (IL)-18

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Mechanisms regulating bronchoalveolar lavage cell (BAL) IFN-gamma responses to beryllium salts (BE) were investigated in CBD, and BE-hypersensitive subjects without CBD. BE exposure (24h) elicited IFN-gamma (746 +/- 245 SEM pg/ml) in CBD BAL but none in untreated cells (<25.6 pg/ml, $p<0.001$,n=14) or in BE-treated BAL from BE-hypersensitive Individuals without lung disease ($p<0.001$, n=15). Because NO is elevated in CBD airways (Dweik et al AJRCCM, 161,A731, 2000), we examined effects of DETA NONOate (DNO), an NO generator, on BAL IFN-gamma. DNO reduced BE-stimulated IFN-gamma levels by 74% ($p<0.01$; n=3) vs. untreated. Analysis of IL-18 and IL-12 (cytokines known to augment IFN-gamma) indicated that BE elevated BAL IL-18 (229 +/- 62 vs. control 76 +/- 31 pg/ml, $p<0.05$, n=7) but IL-12 was

undetectable (<15.4 pg/ml, n=6). Since DNO also reduced BE-stimulated IL-18 (55.7%, $p<0.01$, n=4), the role of IL-18 in BAL IFN-gamma was examined. Antibody to IL-18 drastically reduced IL-18 levels (95%, $p<0.01$, n=2) but IFN-gamma was only marginally affected (19%). Results suggest that NO represents a potent blocker of BE-stimulated IFN-gamma but mechanisms may involve sites other than IL-18 or IL-12.

Session 3. Physico-Chemical Properties of Beryllium Metal and Alloys

Dosimetry of Beryllium in an Animal Model by Accelerator Mass Spectrometry

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Presentation PDF

A method using accelerator mass spectrometry (AMS) was developed to measure low levels (femtomoles) of beryllium in biological samples. This method provides the sensitivity to investigate macromolecular complexes formed with beryllium at low exposure doses and will provide further understanding about the molecular targets involved in beryllium disease. Berylliosis is a debilitating, progressive and potentially fatal lung disease that develops in individuals exposed to beryllium. Proof of the method was tested by administering 0.05, 0.5 and 5.0 µg ^{9}Be and ^{10}Be by IP injection to 30 g male ICR mice. The mice were euthanized after 24 h and blood, femurs, feces, urine, kidneys, spleen, liver, thymus and lung were prepared for AMS analysis by acid digestion. Highest levels of Be were found in the liver and the spleen (6.0 and 2.0% of whole mouse dose, respectively) while the lowest levels were found in blood, lung and thymus. Beryllium levels were dose-dependent in the spleen and liver. The detection limit of Be in tissue by this method is approximately 2 amol and the analysis was linear over 2 orders of magnitude. Possible sample size effects for measuring Be by AMS showed that similar results were obtained when using samples that were between 3.0 and 150 mg of dosed liver tissue. Precision of 8 replicates of pooled liver tissue was 5% while the variability between 8 dosed livers was 10%. These results show that routine quantification of atto- to femtomole levels of Be in tissues is possible. This method should enable future studies to understand the molecular dosimetry and mechanisms of Be toxicity in biological studies.

This work performed under the auspices of the USDOE by LLNL (W- 7405-ENG-48) with support from the USDOE/OBER.

Identification of Non-Toxic Beryllium-Chelating Porphyrins and Method of Delivery to Mice in an Attempt to Reduce the Lung Beryllium (Be) Burden in Chronic Beryllium Disease (CBD)

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We have proposed that Be entering the lungs is chelated by hemoglobin (Hb), some of which is fixed in place and constitutes a Be lung burden, and that such in-situ Be enhances the genetic and biological responses manifest as CBD. We have conducted in-vitro competitive binding experiments to evaluate (1) the interaction of Be with Hb to establish whether Be can replace the heme iron and thereby be chelated by the Hb; and, if

this occurred, (2) the interaction of each of five different candidate porphyrin compounds with the Be-Hb complex to establish whether the Be could be chelated by and removed from the Be-Hb complex by one or all of these porphyrins. We observed that chelation of Be by Hb and subsequently from Hb by the porphyrin compounds does indeed occur. We have also examined parameters of toxicity of these porphyrins and intend to deliver via aerosols those identified as non-toxic into the lungs of Be-lung-burdened mice to determine whether Be can be chelated by these porphyrins and removed via urinary excretion. In our attempt to establish the parameters of toxicity associated with aerosol administration of these porphyrins, we (1) aerosolized aqueous solutions of the porphyrins using a constant-flow Pulmo-Mist pump and nebulizer system and (2) delivered these solutions into cages each housing five female 20+week-old C3H/HEJ mice over a period of twenty days. The group bodyweights of these mice were measured each morning prior to aerosol treatment. Regimens included multiple, constant dosing, and dose escalation. Mice in cages receiving multiple doses of 9mg of one of the select porphyrins began to lose weight (up to 14%) by the third day of treatment. When treatment was suspended after Day 5 to allow bodyweight normalization, and then resumed on Day 14, the weight loss was delayed compared to the first sequence, suggestive of some tolerance induction. Tolerance was also observed with the dose-escalation study, in that treatment with 18 mg on Day 6 resulted in a ~21% weight loss on Day 15; whereas, following weight normalization, treatment with 27 mg on Day 20 only resulted in ~12% weight loss. Treatments with two of the porphyrins generally reflected lower and delayed evidence of toxicity compared to that observed with one of the select porphyrins. Younger mice (~6 weeks old) were generally more tolerant than older mice, but the rank order of porphyrin toxicity was the same. Our results suggest that three of the select five beryllium-binding porphyrins may be safely delivered as aerosols to C3H/HEJ mice, to allow evaluation of reduction of a beryllium load in the lungs of mice as an intervention in development of CBD.

Surface Area of Respirable Beryllium Metal, Oxide, and Copper Alloy Aerosols and Implications for Assessment of Exposure Risk of Chronic Beryllium Disease

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The continued prevalence of chronic beryllium disease (CBD) suggests the current occupational exposure limit of 2 µg beryllium per cubic meter of air does not adequately protect workers from CBD. An understanding of the role of particle surface area, including its influence on beryllium particle dissolution kinetics, could allow for development of an exposure metric based on bioavailability of dissolved beryllium. Therefore, we examined the morphology and measured the particle surface area of aerodynamically size-separated powders and process-sampled particles of beryllium metal, beryllium oxide, and copper-beryllium alloy. The beryllium metal powder consisted of compact particles, while the beryllium oxide powder and particles were clusters of smaller primary particles. Large relative differences in SSA were observed as a function of particle size for the beryllium metal powder, from 4.0± 0.01 m2/g (for the particle size fraction >6 µm) to 20.8± 0.44 m2/g (for the particle size fraction <=0.4 µm). In contrast, little relative difference in SSA (<25%) was observed as a function of particle size for the beryllium oxide powder and particles collected from the screening operation. The SSA of beryllium metal powder decreases with increasing particle size, as expected for compact particles, and the SSA of the beryllium oxide powders and particles remains constant as a function of particle size, which might be expected for clustered particles. These associations illustrate how process-related factors can influence the morphology and SSA of beryllium materials. To avoid errors in predicting bioavailability of beryllium and the associated risks for CBD, the mechanisms of particle formation should be understood and the SSA of beryllium particles should be measured directly.

Examining Beryllium Chemistry with Modern Analytical Techniques

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There are increasing numbers of cases of occupational Chronic Beryllium Disease (CBD) being reported and there is currently no cure for CBD. Be body burden has been identified in many CBD patients and may play a role in CBD pathogenesis. Chelation therapy has been suggested to provide a potential therapeutic treatment for the reduction of a beryllium body burden.

Currently available thermodynamic data has been used to assess the solubility, speciation and chelation of beryllium particles in a number of biological matrices. In cases where thermodynamic data is not available, laboratory experiments such as potentiometric titrations have been used to measure formation constants for potential chelators to add to the model database. In addition, multi-scale quantum molecular dynamics has been used to investigate the interaction of chelators with beryllium on the quantum level, and in the process, predicting free energy data that can be compared with previously reported constants and those determined in the laboratory. Modern imaging techniques to identify small quantities of Be in tissues will be discussed.

A large number of possible chelators have been assessed and a ranking system developed based upon the effectiveness of the chelator in dissolving beryllium particles and competition for metal chelation with other chemical systems in the body. This has led to the selection and development of three candidates for selective and effective chelators for further study — sulfo-naphthoic acids, diphosphonic acids and disulfonic acids.

The research currently being undertaken at LLNL brings together a number of scientific capabilities to study the physical-, analytical- and biological- chemistry of beryllium particles in both the body and the workplace environment.

Session 4. Gene-Exposure Interactions

Functional TNF-a Promoter Polymorphisms Associated With Chronic Beryllium Disease and Beryllium-Induced TNF-a

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Background: Beryllium (Be) stimulates high levels of TNF-a from chronic beryllium disease (CBD) bronchoalveolar lavage (BAL) cells. Previously we have shown that Be-stimulated TNF-a production is associated with a G to A transition in the TNF-a promoter located at position -308 and with markers of disease severity. Although not statistically significant, lower Be-stimulated TNF-a was produced from subjects with a C to T transition at -856 in the TNF-a promoter. We questioned whether the functional -308 polymorphism was a risk factor for CBD and whether other TNF-a promoter variants were associated with high Be-stimulated TNF-a production.

Objective: This study tested the hypothesis that the -308 A TNF-a promoter polymorphism was present at a higher frequency in CBD compared to beryllium-exposed non-diseased subjects (Be-non-diseased).

Methods: Following informed consent, demographic information was obtained from CBD (n=85) and Be-non-diseased subjects (n=67) subjects. Genomic DNA was extracted from peripheral blood cells. A Taqman allelic discrimination assay specific for the -308 variants was used to determine the subjects' genotypes. Direct sequencing of the TNF-a promoter from +64 to -1045 was used to confirm the Taqman results and detect other known TNF-a promoter variants, including those at -238, -376, -572, -856, -862 and -1031, in CBD subjects (n=43). HLA-DPB1 and -DRB1 genotyping was determined by sequence specific PCR in a subset of the cases (n=31). Bronchoalveolar lavage cells were obtained from CBD subjects (n=31) and cultured in the presence and absence of beryllium sulfate. TNF-a supernatant concentrations were measured from the BAL cells by ELISA.

Results: The -308A variant was found at higher frequency in CBD subjects (34%) compared to the Be-non-diseased subjects (19%, p=.04), with an odds ratio of 2.2 (95% confidence interval 1.01, 4.57). High TNF-a production was associated with the -308 A variant (median 6,000 pg/ml vs. 1,142 pg/ml from -308 GG variants, p=0.01) and the -856 CC variant (median 2,636 pg/ml vs. 528 pg/ml from -856 T variants, p=0.04), but not with the -238, -376, -572, -862 or -1031 TNF-a promoter variants. Individuals homozygous for HLA-DPB1 with a glutamic acid at position 69 (Glu69) produced higher beryllium stimulated TNF-a (median 6113 pg/ml vs. 1320 pg/ml from nonGlu69 heterozygotes or homozygotes, p=0.04). HLA-DRB1 DR2, DR3 and DR4 genotypes were not associated with Be-stimulated TNF-a production in CBD.

Conclusion: The -308 A variant (AA or AG) is a risk factor for disease susceptibility in CBD. It is likely that this association is due in part to the functional role the -308 polymorphisms plays in TNF-a production. Whether the association between the -308A variant and CBD is related to an extended TNF-a haplotype, including the -856 CC variant, and/or association with the Glu69 genotype, will require further investigation.

K08 HL03887, R01 ES06358-06, M01 RR00051

Association of HLA Class II Markers and Beryllium Hypersensitivity and Disease: Reevaluation of Previously Published Data after 4-8 Years of Follow-up

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After the first report of the association of the genetic marker HLA-DPB1 Glu-69 with susceptibility to chronic beryllium disease (CBD), a number of observations have associated beryllium hypersensitivity with a number of HLA locus genetic markers. In particular, HLA-DPB1*0201, *0601, *0901, *1001, 1601, 1701, 1901 and HLA-DQB1 Gly86 have been associated with CBD, HLA-DRB3 Arg74 and HLA-DPB1 Glu-69 with susceptibility to beryllium sensitization, and TNF-a -308A with beryllium hypersensitivity in the presence or the absence of granulomatous disease. However, the studies published so far are limited by: 1) the small size of the population groups, sometimes too small to assess associations with less frequent HLA-DRB1, B3, B4, B5, HLA-DP rare alleles; 2) a follow-up period insufficiently long to assess progression from sensitization to disease with a condition known for the long latency from exposure to clinical presentation; 3) non uniform phenotypic description i.e., lack of uniformity in the use of descriptors such as “lymphocytic alveolitis,” “positive BAL Be-LPT,” or lacking pulmonary function data as diagnostic criteria. These limitations hamper the ability of investigators to combine data from different studies to make a comprehensive assessment of the strength of the association of genetic markers with sensitization and/or disease. The aim of the present study was to assess the association between HLA class II (HLA-DP, DQ, and DR) genetic markers and beryllium hypersensitivity. To determine if the association is between these markers and beryllium hypersensitivity, beryllium hypersensitivity without disease or with clinical chronic beryllium disease. Subjects with beryllium hypersensitivity and exposed controls that have been previously published will be reevaluated. Follow-up studies will be performed to determine whether these subjects progressed to CBD or had beryllium hypersensitivity without clinical disease. The resulting population that was comprised of 62 hypersensitive subjects, 53 diseased subjects and 175 control subjects. Both populations, which had been HLA class II typed with standard molecular methods, were re-characterized using a common phenotypic criteria. Follow up will be from 4-8 years.

Electrostatic Potential on HLA-DP*1701 and HLA-DP*0401: Implications for Putative Mechanism of Chronic Beryllium Disease

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Presentation PDF

The pathobiology of chronic beryllium disease (CDB) involves MHC class II human leukocyte antigen (HLA). Molecular epidemiological studies indicate interaction of specific HLA-DPB1 alleles as a factor in

disease susceptibility. We have studied three-dimensional structural models of HLA-DP proteins encoded by these genes. The extracellular domains of HLA-DPA1*01031/B1*1701 and HLA-DPA1*01031/B1*0401 were modeled from the X-ray coordinates of HLA-DR as a template. Using these models the electrostatic potential at the molecular surface of HLA-DP was calculated and compared for both isoforms. These data show the distinguishing characteristics of the isoforms in the vicinity of the antigen-binding groove. The presence of a positively charged lysine in position 69 in HLA-DPB1*0401 was compared with the negatively charged glutamate in the same position in the 3D-structure of HLA-DPB1*1701. We calculated the pKa values for the titratable sites in these proteins. Using these predicted values we estimated the mean charge on relevant titratable residues at physiological pH, and assigned an approximate total charge to the protein. The total charges based on the standard pKas for amino acid residues are -17/-9 and -17/-3 for HLA-DPA1*01031/B1*1701 and HLA-DPA1*01031/B1*0401, respectively (and compared to -14/-6 on the HLA-DR template). Hence, the combination of CBD-susceptible alleles HLA-DPA1*01031/B1*1701 may encode proteins that carry the largest negative charge. The protein charges based on the calculated pKa values are lower, but the highest negative charge still remains on HLA-DPA1*01031/B1*1701.

The calculation of pKa values also permits examination of the interaction energies between the titratable sites. Specific pairs of residues in proximity to the binding pocket are shown to have large interaction energies, indicating electrostatic coupling between these residues. The majority of these residues are on the B chain encoded by the HLA-DPB1*1701 and HLA-DPB1*0401 alleles, and 6/7th of the difference in the total negative charge results from substitutions in positions B55, B56, B69, B84 and B85. Interestingly, the results of epidemiological studies have implicated these positions as being potentially important for conferring susceptibility to either CBD [1-3] or to other hard metal lung disease [4]. These results are useful for interpreting the molecular recognition in MHC class II proteins, and for investigating its interactions with Be2+ in chronic beryllium disease.

1. Wang Z., et al. (1999) J. Immunol. 163, 1647-53; 2. Wang Z., et al. (2001) Toxicology 165, 27-38; 3. Rossman M.D., et al. (2002) Am. J. Respir. Crit. Care Med. 165, 788-94; 4. Potolicchio I., et al. (1999) Eur. J. Immunol. 29, 2140-7.

Session 5. New Directions in Pulmonary Research

Cutaneous Application of Beryllium Salts and Oxide Particles Produces Beryllium-Specific Peripheral Sensitization in the C3H/HeOuJ Mice

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Chronic Beryllium Disease is an occupational lung disease that begins as a cell-mediated immune response to beryllium. Because major improvements in respiratory protection did not decrease the rate of sensitization or disease over the last decade, and skin exposure to environmental chemicals is a well established route for immunologic sensitization, we hypothesized that skin exposure to beryllium could provide an alternative route for sensitization to this metal. Furthermore, recent evidence suggests that ultrafine beryllium particle concentration, not mass measurements, is the appropriate metric for relating exposure to risk of disease, and BeO particles are a common form of industrial exposure. We have tested fine particle penetration of human skin, and beryllium salt and beryllium oxide particle induction of peripheral sensitization in C3H/HeOuJ mice. We have demonstrated, by confocal microscopy, that 0.5 and 1 micron fluorescently-labelled dextran beads, in conjunction with flexing motion, as at the wrist, can penetrate stratum corneum and reach the epidermis. These data provided proof of concept for particle penetration into intact skin. To determine if epicutaneous beryllium causes peripheral sensitization, we applied $BeSO_4$ solution or BeO particle suspension to the shaved backs of mice, and a single $BeSO_4$ challenge on the ear. Forty-eight hours post-challenge, we measured by flow cytometric analysis of auricular lymph node cells, a significant increase in the B220 ratio and in the T cell activation marker, CD44. No change in surface marker expression was observed in mice sensitized with vehicle and challenged with $BeSO_4$. Furthermore, we measured a concomitant decrease in CD62L on both CD4 and CD8 T lymphocytes, a cellular event also associated with T lymphocyte activation. To evaluate the antigen specificity of this response, we applied beryllium salts to the ears of mice and evaluated beryllium-induced lymphocyte proliferation (murine BeLPT) *in vitro*. We observed significant beryllium-induced auricular lymph node lymphocyte proliferation and peripheral blood mononuclear cell proliferation, with stimulation indices of ten and fifteen, respectively. In combination, these data provide evidence for beryllium salt and BeO particle induction of peripheral sensitization in mice and are consistent with development of an antigen-specific, cell-mediated immune response.

In Vitro Transformation of Phagocytized Beryllium Oxide Particles in the Murine J774A.1 Cell

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Beryllium (Be) metal, its oxide (BeO), 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 Be aerosol mass concentration, the control of which has proven ineffective at eliminating the occurrence of chronic beryllium disease (CBD). This might be due to intracellular change in BeO particles from crystalline to amorphous form as a potential mechanism by which dissolution occurs, thereby producing dissolved Be associated with the onset and progression of CBD. We examined changes in pre-characterized BeO particles engulfed by and retained within murine J774A.1 cells *in vitro*.

The physicochemical properties of commercially available BeO powder were analyzed by transmission electron microscopy (TEM) and x-ray powder diffraction (XRD). J774A.1 cells were dosed with BeO particles *in vitro* and incubated from 124 to 144 hours, followed by recovery of engulfed particles for post-characterization. Dissolved Be in recovered intracellular fluid was analyzed and quantified using inductively-coupled plasma atomic emission spectrometry (ICP-AES).

Pre-analytical TEM and XRD results showed a highly pure BeO powder consisting of diffuse clusters of primary particles measuring approximately 200 nm in diameter. Post-analytical TEM results did not show an observable change in morphology, chemical composition, or size. ICP-AES of the cell lysate indicated measurable levels of Be ranging from 3.2 to 27.4 parts per billion. These findings demonstrate the intracellular dissolution of BeO particles thereby showing that dissolved Be, concentrated within the cell, may serve as input to immunopathologic response in the host.

The Mouse Deep Lung is Exposed to Respirable Particles Administered by Pharyngeal Aspiration

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A pharyngeal aspiration technique has recently been described as a method for exposing the mouse lung to soluble antigens. This new technique overcomes the technical difficulty and potential trauma associated with intratracheal instillation methods in the mouse. In this study, we investigated the potential for the pharyngeal aspiration technique to expose the mouse deep lung to respirable particulates. Ten, 6-8 week old C3H/HeOujMMTV-, male mice were anesthetized with isoflurane using a bell jar. The mice were removed from the bell jar when anesthetized, placed on a dosing board with the mouth fully opened and the tongue gently held in full extension. A suspension of 1 micron blue fluorescent polystyrene amine-modified latex beads in a 50 microliter volume was pipetted onto the base of the tongue in the pharyngeal region. The tongue was maintained in fixed full extension until the mouse completed a minimum of two full breaths. Evaluations were made at 5 bead concentrations, representing a 100-fold range, with 2 animals per group. Anesthetic recovery occurred within one minute. Mice were necropsied four hours after exposure and no gross lesions were noted. Fluorescent beads were morphometrically quantified in frozen sections of lung. For each dose group, the percent of each microscopic field occupied by fluorescent beads directly and highly significantly correlated with number of beads administered ($r^2 = 0.99$). Both the left and right lungs were

exposed in all individuals, although the concentration of beads in the two sides varied in some mice. Beads were distributed in alveoli and alveolar ducts as free or phagocytized particles. We conclude that pharyngeal aspiration of particles is a simple, humane and effective technique that exposes the mouse deep lung to respirable particles over a wide range of exposure concentrations.

Beryllium-Induced Macrophage Apoptosis in Chronic Beryllium Disease

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Presentation PDF

The link between metal-induced apoptosis and granulomatous inflammation in human disease pathogenesis is not established. The presence of TUNEL positive nuclei in chronic beryllium disease (CBD) pulmonary granulomas suggested the possibility that beryllium (Be) could induce apoptosis in CBD bronchoalveolar (BAL) cells. Apoptosis was measured in unstimulated and Be-stimulated BAL macrophages from CBD (n = 21) and Be-sensitized (BeS, n = 16) subjects. Be-stimulated CBD and BeS macrophages underwent cytoplasmic membrane blebbing, surface CD14 loss, and nuclear fragmentation. Nuclear fragmentation was blocked by the general caspase inhibitor BD-fmk. Apoptosis, CD14 loss and TNF-a production were not observed in unstimulated BAL cells. Be induced apoptosis of two mouse macrophage cell lines, only one of which produced Be-stimulated TNF-a. Be induced apoptosis of bone marrow derived macrophages from TNF-a (-/-) knockout mice. Thus, Be-induced macrophage apoptosis was caspase-mediated, TNF-a independent, and it occurred whether cells were derived from patients with granulomatous inflammation or not. Our data suggest that Be-induced macrophage apoptosis could contribute to the host's continued re-exposure to Be. Beryllium-antigen induced macrophage apoptosis may represent a fundamental mechanism that separates antigen elimination and the resolution of inflammation, from antigen persistence and progression to chronic granulomatous inflammation.

(Supported by NIH grants ES06538 and GM60449)

Agenda

8 - 8:30	**Registration**
8:30 - 8:40	**Welcome from DOE** Susan Rose
8:40 - 8:50	**Welcome on Behalf of the Planning Committee** Sally Tinkle
8:50- 9:35	**Multi-Disciplinary Research-the Key to Solving the Beryllium Disease Puzzle** Kathleen Kreiss
Session 1. Immunopathology of Beryllium-Induced Granulomatous Disease **Session chair: Brian Kotzin**	
9:35 - 10:15	**Immunopathogenesis of Sarcoidosis** Gianpietro Semanzato
10:15 - 10:30	**Break**
10:35 -10:55	**Characterization of Beryllium-Specific Memory CD4+ T Cells in Patients with Chronic Beryllium Disease** Andrew P. Fontenot, Scott J. Canavera, Laia Gharavi, Lee S. Newman, and Brian L. Kotzin
10:55 - 11:15	**HLA-DP-Unrestricted TNF-a Release in Be-Stimulated PBMC from Berylliosis Patients** Massimo Amicosante, Floriana Berretta, Alberto Franchi, Paola Rogliani, Chiara Dotti, Monica Losi, Raed Dweik, and Cesare Saltini
11:15 - 11:35	**Design, Engineering and Production of Human Recombinant T Cell Receptor Ligands Derived from HLA-DP** Gregory G. Burrows, Roberto Meza-Romero, Jeff Mooney, Justin W. Chang, Hans Peter Bachinger, and Jianya Huan
11:35 - 11:55	**A Flow Cytometric Assay for Beryllium Sensitization: Screening and Mechanistic Applications** A.J. Jabbour, R.A. Ponce, M.T. Rosato, T.J. Kavanagh, L.S. Newman, T.K. Takaro, and E.M. Faustman
12:00 - 1:00	**Lunch**

Session 2. Pulmonary Signaling Pathways Activated by Beryllium **Session chair: Lisa Maier**	
1:00 - 1:45	**Second Messenger Pathways Activated by Endotoxin and Asbestosis in Alveolar Macrophages** Gary Hunninghake
1:45- 2:05	**Beryllium Stimulates a Local Angiotensin System in Chronic Beryllium Disease** T. Hendry-Hofer, A.F. Fontenot, E.A. Barker, M. Boguniewicz, L.S. Newman, and L.A. Maier
2:05 - 2:25	**High Levels of Nitric Oxide (NO) in Exhaled Breath in Patients with Chronic Beryllium Disease** R.A. Dweik, D. Laskowski, and S.C. Erzurum
2:25 - 2:45	**Nitric Oxide (NO) Attenuation of Interferon Gamma (IFN-Gamma) Responses in Chronic Beryllium Disease (CBD): Evidence for Mechanisms Independent of Interleukin (IL)-18** M.J. Thomassen, C. Farver, R.A. Dweik, D. Culver, B. Yen-Lieberman, M. Kavuru, and B.P. Barna
2:45 - 3:00	**Break**
Session 3. Physico-Chemical Properties of Beryllium Metal and Alloys **Session chair: Babetta Marrone**	
3:00 - 3:45	**Physicochemical Determinants of Beryllium Toxicity Using In Vitro and In Vivo Models** Greg Finch
3:45 - 4:05	**Dosimetry of Beryllium in an Animal Model by Accelerator Mass Spectrometry** M.L. Chiarappa-Zucca, R.C. Finkel, J.E. McAninch, R.E. Martinelli, and K.W.Turteltaub
4:05 - 4:25	**Identification of Non-Toxic Beryllium-Chelating Porphyrins and Method of Delivery to Mice in an Attempt to Reduce the Lung Beryllium (Be) Burden in Chronic Beryllium Disease (CBD)** Jim Klostergaard and George P. Sakalosky
4:25 - 4:45	**Surface Area of Respirable Beryllium Metal, Oxide, and Copper Alloy Aerosols and Implications for Assessment of Exposure Risk of Chronic Beryllium Disease**

	A.B. Stefaniak, M.D. Hoover, R.M. Dickerson, E.J. Peterson, G.A. Day, P.N. Breysse, M.S. Kent, and R.C. Scripsick
4:45 - 5:05	**Examining Beryllium Chemistry with Modern Analytical Techniques** M. Sutton, S.R. Burastero, C. Mundy, and J. Quong
5:05	**Social gathering-location to be announced**
Wednesday, June 26, 2002	
Session 4. Gene - Exposure Interactions **Session chair: Cesare Saltini**	
9 - 9:45	**Chemicals + Immunologic Lung Disease: Gene + Environment** Anthony Newman-Taylor
9:45 - 10:05	**Functional TNF-** a **Promoter Polymorphisms Associated with Chronic Beryllium Disease and Beryllium-Induced TNF-** a L.A. Maier, R.T. Sawyer, T. Hendry-Hofer, C. Parsons, R. Bauer, D. McGrath, P. Lympany, R. duBois, and L.S. Newman
10:05 - 10:25	**Association of HLA Class II Markers and Beryllium Hypersensitivity and Disease: Reevaluation of Previously Published Data After 4-8 Years of Follow-up** C. Saltini and M. Rossman
10:25 - 10:45	**Electrostatic Potential on HLA-DP*1701 and HLA-DP*0401: Implications for Putative Mechanism of Chronic Beryllium Disease** James A. Snyder, Ainsley Weston, Sally T. Tinkle, and Eugene Demchuk
10:45 - 11:00	**Break**
Session 5. New Directions in Pulmonary Research **Session chair: Milton Rossman**	
11:00 - 11:45	**Effects and Fate of Ultrafine Particles** Günter Oberdörster
11:45 - 1:00	**Lunch**
1:00 - 1:20	**Cutaneous Application of Beryllium Salts and Oxide Particles Produces Peripheral Sensitization in the C3H/HeOuJ Mice** Sally S. Tinkle, James M. Antonini, Jenny R. Roberts, Rebecca Salmen, Karyn Depree, and Melanie Flint

1:20 - 1:40	**In Vitro Transformation of Phagocytized Beryllium Oxide Particles in the Murine J774A.1 Cell** G.A. Day, A.B. Stefaniak, M.D. Hoover, R.M. Dickerson, E.J. Peterson, N.A. Esmen, and R.C. Scripsick
1:40 - 2:00	**The Mouse Deep Lung is Exposed to Respirable Particles Administered by Pharyngeal Aspiration** A.F. Hubbs, G.V.S. Rao, M. Kashon, R. Salmen, L.A. Battelli, P. Willard, J. Antonini, D.N. Weissman, and S. Tinkle
2:00 - 2:20	**Beryllium-Induced Macrophage Apoptosis in Chronic Beryllium Disease** R.T. Sawyer, V.A. Fadok, L.A. Maier, L.A. Kittle, J.M. Routes, and L.S. Newman
2:20 - 2:35	**Break**
2:35 - 3:35	**Ethical, Legal and Social Implications of Beryllium Exposure and Research: Case Presentation** Panel: Reed Durham, beryllium chemist; Myron Harrison, occupational medicine; Jon Moreno, bioethicist; Susan Rose, bioethicist
3:35 - 3:55	**Meeting Overview** - Lee Newman
3:55 - 4:00	**Closing Remarks** - Sally Tinkle

Invited Speakers

"Chemicals + immunologic lung disease: gene + environment"
Anthony Newman-Taylor
Imperial College School of Medicine
National Heart & Lung Institute

"Immunopathogenesis of sarcoidosis"
Gianpietro Semenzato, MD
Padua University School of Medicine

"Multidisciplinary research- the key to solving the beryllium disease puzzle."
Kay Kreiss, MD
Division of Respiratory Disease Studies
National Institute for Occupational Safety and Health

"Physicochemical determinants of beryllium toxicity using in vitro and in vivo models"
Gregory L. Finch, PhD
Pfizer Global Research & Development

"Effects and fate of ultrafine particles"
Günter Oberdörster, PhD
Environmental Medicine
University of Rochester

Gary Hunninghake
University of Iowa
"Second messenger pathways activated by endotoxin and other environmental exposures."

Panel discussion on the Ethical Legal and Social Implications of Beryllium Disease:

- Myron C. Harrison, MD
  Exxon Mobil Corp.

- Reed Durham
  Y12 Plant

- Susan L. Rose, Ph.D.
  Office of Biological and Environmental Research, Office of Science
  U.S. Department of Energy

- Jonathan D. Moreno, Ph.D.
  Center for Biomedical Ethics
  University of Virginia

Minisymposia Topics

- Physico-chemical properties of beryllium
- Gene-exposure interactions
- Immunopathology of sensitization and disease
- The biology of ultrafine particles
- Development of animal models of granulomatous disease
- Novel therapeutics
- Round Table Discussion: Ethical, legal and social implications of beryllium research

Attendees

(*Presenters)

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The U.S. Department of Energy invites scientists from all disciplines to an open workshop that will explore Chronic Beryllium Disease (CBD), a very serious occupational lung disease. CBD in current and former beryllium workers is of continuing interest and concern to federal agencies such as the U.S. Department of Energy. It is of potential broader interest as an immunological based disease that may have significant genetic associations and has similarities to other granulomatous diseases such as sarcoidosis and tuberculosis.

The current revolution in molecular biology and genetics offers new tools to reveal CBD's basic biology and genetics, leading to better prevention, diagnosis, and treatment options. The workshop will gather a diverse community from molecular biology, immunology, genetics, analytical science, and ethics to formulate a multifaceted approach.

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- Physico-chemical properties of beryllium
- Gene-exposure interactions
- Immunopathology of sensitization and disease
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- Round Table Discussion: Ethical, legal, and social implications of beryllium research

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Dr. Gary Hunninghake, *University of Michigan*
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