White Paper – Developing Alternative Medical Testing Procedures for CBD Detection

Research Needs Subcommittee

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PURPOSE

The purpose of this paper is to present the current state of clinical testing for chronic beryllium disease (CBD) and to provide guidelines and references to be considered for funding or for developing, enhancing, validating, and evaluating clinical test procedures with the ultimate goal of having a diagnostic test that can:

1. Relate to disease burden and extent
2. Change in accordance with the clinical evolution, reflecting the current status of disease
3. Add information about the risk or prognosis
4. Anticipate clinical changes, i.e. indicating the presence of relapse before it becomes obvious at a clinical level
5. Increase pathologically in the presence of the disease (high sensitivity)
6. Not increase in the absence of the disease (high specificity)
7. Be reproducible (as determined by the low coefficient of variation)
8. Be of easy and cheap determination

Currently, absent a less invasive and more sensitive test, the beryllium blood lymphocyte proliferation test (BeLPT) has become a surveillance tool and the accepted method of testing for beryllium sensitization even though the sensitivity of the test is not clear.[35] Test results are subject to substantial intra and inter-laboratory variability.[19] There continues to be no uniform protocol that commercial laboratories use to perform the test nor is there a quality control program that insures the various testing laboratories generate consistent findings for the same sample. Studies of beryllium-exposed workers demonstrate that the positive predictive value of a positive blood lymphocyte proliferation test to detect sub clinical CBD (sensitization and granuloma in lung parenchyma) ranges from 11 to 100%.[14, 18, 19, 20]

A new clinical test that can be used as a screening tool or a surveillance tool and having any of the characteristics detailed in the above elements (1-5) is clearly needed. Additionally, a clinical surveillance test that offers improved sensitivity, specificity, and reproducibility and is cost competitive when compared to an existing method is also desirable. It is clear that criteria are needed to define guidelines for clinical test methods with the ultimate endpoint being the establishment of a gold standard that can clearly quantify the relationship between a test result and an individual's likelihood of having clinical CBD. Establishing these guidelines will also allow for sensitivity, specificity, positive predictive value, and cost comparisons as clinical test methods are developed.

1. Background

Beryllium has unique characteristics that make it a superior material for many specialized applications. Compared to other metals, beryllium is very light, has a high melting point, low electrical conductivity, superior strength and stiffness, high thermal conductivity, and most alloys are highly resistant to corrosion. In addition, it is transparent to X-rays, absorbs neutrons, and is non-magnetic. Beryllium is used in several forms: as a pure metal, as beryllium oxide, and as an alloy with copper, aluminum, or nickel.

The original demand for beryllium came from the Department of Defense and the Atomic Energy Commission, where beryllium metal, compounds and alloys were important in the development of nuclear weapons and in naval applications. Our nation's defense and energy programs continue to rely on beryllium.[1, 2] Beryllium and beryllium alloys are used by the aerospace industry in the manufacture of high performance military aircraft as well as commercial aircraft components. Beryllium alloys are used by manufacturers of electrical components to make springs, switches, and electrical connectors that are used in automobiles, computers, cell phones, telecommunications equipment and other electronics.

Processes that can create employee exposure involve operations that generate airborne particulates, e.g., machining, sanding, grinding, crushing, lapping, welding and hot working.

1.1 Description of Beryllium Disease

Beryllium oxide, beryllium metal and beryllium alloys are the forms of beryllium present in the work place today with inhalation being the primary route of exposure concern.[1,3] Skin and lung reactions have occurred with exposures to the soluble beryllium salts such as beryllium sulfate and beryllium fluoride. Soluble beryllium compounds are not used in consumer products, are rarely found in industry, have very limited use as analytical standards for chemical analysis in the laboratory, and are occasionally used in research.

Acute Beryllium Disease (ABD) is a pneumonitis resulting from high exposure to soluble beryllium compounds or low-fired beryllium oxide that has not been seen for decades and low-fired beryllium oxide has not been commercially available since 1950.[4] The onset of symptoms was usually immediate, but could be delayed from several hours up to 3 days. Symptoms included dypsnea, fatigue, fever, night sweats and cough. Pulmonary function tests revealed obstructive lung disease with impaired gas exchange. Most of the cases of ABD usually resolved completely, however, there were incidents of fatal outcomes or subsequent development of chronic beryllium disease.[5,6] Airborne exposures to beryllium metal, beryllium oxide or beryllium alloy fumes or dust are not associated with acute or short-term respiratory reactions.

*Chronic beryllium disease (C*BD) *Clinical cCBD* was diagnosed, before the late 1980's, when clinical symptoms were observed along with changes in chest x-rays or lung function tests. In 1951, it was suggested that CBD was an immune-mediated disease and subsequently the term beryllium sensitization was initially defined by the beryllium skin patch test (BePT).[7] Patch tests results were 100% positive in beryllium workers who had previously been diagnosed with clinical cCBD. [8] The use of the BePT was curtailed because simultaneous experimental application of multiple tests sensitized members (positive patch test) of control populations and because it was suggested that the test might exacerbate existing cCBD.[9]

The clinical course of cCBD is considered highly variable since the symptomatic disease may not develop or it may develop slowly over time. The earliest manifestations of clinical chronic beryllium disease (cCBD) are the symptoms of shortness of breath, dry cough, or wheeze, and in some, night sweats or fatigue. Chest radiographs can be normal, but often range from small nodular opacities, with an upper level predominance, to formation of conglomerate masses. [10] Also, a chest x ray may demonstrate abnormalities with the person being asymptomatic relative to pulmonary functions. Progression may lead to weight loss, acrocyanosis (blueness or pallor of the extremities usually associated with pain and numbness), heart failure, and possible death. In addition to cCBD, these symptoms may be found in persons with other lung diseases and in persons with no diagnosable disease. [11]

Pulmonary responses to cCBD also vary. Some have normal lung volume, but abnormal gas exchange in either diffusing capacity for carbon monoxide or by arterial blood gas analysis. Some have air flow limitations and later followed by restrictions in lung volumes. In the early stages of the disease, alterations in lung function include airflow obstruction, later developing a mixed pattern of obstruction and restriction. Pure restriction develops toward the end stage of the disease. The most common abnormalities seen early in the course of the disease are a post exercise increase in the alveolar-arterial oxygen gradient and diffusing capacity and a reduction in lung capacity.

Clinical evaluations generally include a pulmonary function test (PFT) with forced vital capacity (FVC) and forced expiratory volume at one second (FEV1) performed according to American Thoracic Society (ATS) standards and a chest x-ray. An abnormal FVC or FEV1 result, or a reduction greater than 15% in either compared to prior tests is considered to be significant. X-rays in early cCBD are often normal, but may show marginally enlarged lymph nodes in the chest, sub-pleural tenting lesions, or ground glass changes (diffuse hazy appearance). In the more advanced stages, fine opacities or frank scarring similar to other interstitial lung diseases such as sarcoidosis or idiopathic pulmonary fibrosis may be observed. In very late disease, there are signs of pulmonary hypertension or right heart failure.

Subclinical CBD was a term that originated in the late 1980s and the criterion for diagnosis of CBD was changed.[12] The diagnosis of Subclinical CBD is based on abnormal lymphocyte proliferation tests for beryllium sensitization and the presence, upon lung biopsy, of non-caseating granulomas. Granuloma formation can exist with no symptomology or physical impairment of health or proliferation of granulomas may lead to symptoms of cCBD. The link between the presence of granulomas and the development of clinical disease symptoms can be elusive because the latency from time of first beryllium exposure to the development of clinical disease ranges from a few years to several decades.[12, 13] With Subclinical CBD, there are no clinical symptoms and there is no measurable impairment.

Sensitization is a term used in occupational medicine that has two distinct meanings. A sensitizer is defined by OSHA as: "a chemical that causes a substantial proportion of exposed people or animals to develop an allergic reaction in normal tissue after repeated exposure to the chemical." A chemical allergy is an adverse reaction to a chemical resulting from previous sensitization to that chemical or to one that is structurally similar. A chemical allergy is initiated by the immune system and expressed as hypersensitivity; after an initial allergic reaction to a chemical, very small subsequent exposures can evoke a severe response. The range of *chemical sensitization* response is broad and can manifest itself in forms such as a skin rash, eye irritation, allergic asthma, or even anaphylactic shock. *Chemical sensitization* has not been demonstrated in persons exposed to insoluble forms of beryllium either in massive or particulate forms.

The term *beryllium sensitization*, as it is used today, refers to the accepted theoretical recognition of beryllium by the immune system which may be detected only via an in-vivo patch test, an in-vitro blood test, or in-vitro bronchial lavage testing using soluble salts of beryllium such as beryllium sulfate or beryllium fluoride.[7,8,12,14,16,17] Beryllium sensitization is only definable as a test result. Beryllium sensitization is not a health effect, illness or disability nor does it predict clinical chronic beryllium disease. Only workers who have immune sensitization to beryllium are believed to develop CBD (clinical and subclinical), however, persons have been diagnosed with cCBD who have not been found positive using the blood lymphocyte proliferation test (BeLPT) or bronchial lavage lymphocyte proliferation test (BALLPT) for sensitization. Studies of beryllium-exposed workers demonstrate that the positive predictive value of a positive blood lymphocyte proliferation test to detect sub clinical CBD (sensitization and granuloma in lung parenchyma) ranges from 11 to 100%.[14, 18, 19, 20] However, absent a gold standard and the knowledge of prevalence, the PPV of the BeLPT will continue to be a range of estimates.

It is not yet known if all beryllium sensitized individuals will eventually develop CBD (cCBD or subclinical CBD). Medical screenings of beryllium-exposed workers consistently demonstrate that a larger percentage of individuals will have

a positive blood lymphocyte proliferation test to beryllium (become sensitized) than will be diagnosed with sub clinical CBD (sensitization and granuloma in lung parenchyma).[21, 22, 23, 24] Studies have indicated that there are factors that may influence whether a worker may develop CBD. Genetic susceptibility, particle size, particle numbers, particle surface area, chemical form, peak exposure profiles have been suggested as possible contributors to the risk of clinical disease.[25,26,27,28,29]

While many aspects of the etiology of CBD are still unclear, researchers have identified a genetic marker that appears to significantly increase the probability that a worker will develop CBD. CBD has been associated with the allelic substitution of glutamic acid for lysine at position 69 in the HLA-DPB1 protein. [29, 40] The results of this study indicated that CBD cases were more likely to have *HLA-DPB1* alleles coding for aspartic acid (D) and glutamic acid (E) in positions 55 and 56 compared to the controls who where more likely to code for an alanine (A) in those positions 79% vs. 41%. Alleles, characterized by a codon for glutamic acid residue at position 69 (E69) in the amino acid sequence, also occurred more often in individuals with CBD than in those without (97% vs. 27%. Allele specific analysis implicated an association between CBD and the inheritance of the common *HLA-DPB1*0201*, glutamic acid 69 containing allele.

Wang investigated the presence and absence of both *HLA-DPB1* and *HLA-DPA1* alleles in beryllium exposed individuals with and without CBD.[41] This study verified the association between *HLA-DPB1*^{E69} and CBD; and it evaluated allele specific relations including the effect of homozygosity versus heterozygosity and disease status. The study found that 95% of participants having CBD were found to carry at least one *HLA-DPB1*^{E69} variant (or putative disease allele) compared to (45%) without disease. The data suggested that individuals homozygous for *HLA-DPB1*^{E69} were at an increased risk of disease compared to heterozygous individuals.

Because the role of the Glu69 gene in the development of CBD is not well understood and a significant portion of the general population express the genetic marker, future research is needed on these and other issues before it can be determined if definitive genetic tests will be predictive of CBD.

1.2 Medical Surveillance, Screening, and Medical Testing – Epidemiological Considerations

Medical surveillance can be defined as the systematic collection, analysis and dissemination of disease data on groups of workers. It can also be defined as the ongoing systematic evaluation of employee health to monitor for the early occurrence of disease. Simply stated, a medical surveillance program is designed to detect early signs of work-related illness and disease. A well designed and well-documented program may aid in early recognition of a

relationship between exposure to a hazardous substance and disease, in the assurance of the safety of new substances, and as an indicator of the effectiveness of existing control measures.[43]

The first purpose of medical surveillance is to prevent adverse health effects, otherwise known as primary prevention. Methods of analysis and interpreting results should be focused on this purpose. Medical surveillance can also be useful for detecting occupational health problems at an early stage (secondary prevention), and for clinical management of occupational illness and rehabilitation (tertiary prevention).[43]

Medical surveillance testing oftentimes is designed to be of benefit to the individual worker tested. In some situations, medical surveillance testing is performed for the benefit of a group of workers.

Medical surveillance program optimally should be linked with and informed by environmental and workplace surveillance and testing.

The utility of a medical surveillance program is determined by the adequacy of interpretation of the results, and the actions based upon them to prevent work-related disease and illness.[43] Consequently, the establishment of a careful plan for interpretation is as important as actual data collection.

When the goal of medical surveillance is to identify work-related disease at an early stage, it is considered to be a type of screening. Screening is the search for a previously unrecognized disease or abnormal physiologic or pathologic condition at a stage at which intervention can slow, halt, or reverse the progression of the disease. In short, an effective screening test should identify disease at a stage at which intervention truly matters.

The risks of medical tests and screening directly point to the ‘appropriateness” of the test itself. What is meant by appropriate? One must go back and ask what is the context in which it, the test, is being conducted? What is the purpose to begin with? For example,

- Is it to be done as a diagnostic test? i.e., the evaluation of patient or worker with signs and symptoms associated with the disease (e.g., CBD).
- Is it being done for surveillance? i.e., the follow-up screening in patient who has already been diagnosed with and treated for the disease (e.g., CBD).
- Or is it being used as a screening test? i.e., the early detection of preclinical disease in persons without signs or symptoms suggestive of the target condition (e.g., CBD).

Screening is considered a secondary preventive measure in the control of occupational illness. Screening is based on a number of principles, including the following:[43]

1. The screening test must be selective and geared to the population at risk.
2. The disease should be identified in its latent stage, not when symptoms appear.
3. Adequate follow-up study is necessary.
4. The screening test is both valid (accurate) and reliable (precision, variability, reproducibility).
5. Benefits outweigh the costs, and where feasible, tests are noninvasive.
6. Treatment should be both available and effective at a stage when the disease is detectable.

These principles coincide with the World Health Organization's evidence-based criteria for appropriateness of screening. Screening must be shown to be effective before screening guidelines for widespread implementation are issued. A screening test must be affirmatively demonstrated to be effective based on these criteria before it is considered appropriate. Consider these principles to evaluate the appropriateness of screening and medical testing:

1. **Burden of suffering**. The health problem to be averted by screening and testing must be sufficiently common in the screened population and pose a substantial risk to health to justify routine screening. For example, before medical testing, ask, "What is being identified? CBD? Or a precondition? Sensitization?"

2. **Accuracy and reliability of test.** A screening test must be available that has the following:
   a. Sufficient **sensitivity** (the proportion of cases with disease that correctly test positive, i.e., ability of the test to correctly identify the sick as sick) to find the condition earlier in its clinical course than if screening was not performed;

      $$\text{Sensitivity} = \frac{\text{true positives}}{\text{All those with the disease}} = \frac{\text{true positives}}{\text{true positives + false negatives}}$$

   b. Sufficient specificity (the proportion of cases without disease that correctly test negative, i.e., ability of the test to correctly identify the well as well) to avoid producing a large proportion of false-positive results;

      $$\text{Sensitivity} = \frac{\text{true negatives}}{\text{All those without the disease}} = \frac{\text{true negatives}}{\text{true negatives + false positives}}$$

c. Sufficient reliability (the ability to produce a similar result with consistency, i.e., reproducibility or precision). This includes standardization of the test, quality control and assurance measures, inter- and intra- laboratory variability, as well as standardized criteria for what constitutes a positive result.
d. Sufficient positive predictive value, or PPV (the proportion of abnormal results that represent true disease). The numerator is the number of persons who actually have disease, and the denominator is the number of persons with an abnormal test result.

$$PPV = \frac{\text{true positives}}{\text{All those with a positive test result}} = \frac{\text{true positives}}{\text{true positives + false positives}}$$

A low PPV means that many persons in the screened population will receive false-positive results for every individual with true disease. Because the PPV is directly proportional to the prevalence of the disease (or the proportion of people who have the disease in the population at risk at a given point of time), the lower the prevalence, the lower the PPV, and the greater chances of false-positive results. This is important because even tests with high sensitivity and specificity can have low PPV when the prevalence of the disease is low.

3. **Effectiveness of early detection.** Finding the condition earlier in its clinical course compared to if screening was not performed (or early removal from exposure) must be known to improve health outcomes. Implicit in this requirement is evidence that an effective treatment is available for persons found to have the condition.

4. **Acceptable harms.** The harms of screening, including those affecting the large proportion of the screened population that is ultimately determined not to have disease (anxiety, labeling effects, side effects and complications from screening procedures and follow-up tests and treatments) and those affecting the subset found to actually have disease (harms of diagnostic work-up and treatments) must be acceptable.

Harms of screening process may include the following:
- Harm of test itself
- Psychological and labeling effects of positive results
- Morbidity of follow-up tests and treatments triggered by the initial screening

These must be counterbalanced vs. benefits to determine if tradeoff is worthwhile.

The potential psychological consequences are real, and include the anxiety over the possibility of having or getting the disease in the future. In addition, even though a positive test result may prove to be a false-positive, a subset of workers will continue to believe that something is wrong with their health. For

example, higher work absenteeism and other labeling effects such as decreased insurance eligibility have been shown in workplace Be Screening programs.[46]

Other potential harms of treatment may include:

- Effects of being removed from exposure
- Effects of medical treatment

The effects of being removed from exposure include the very real possibility of job transfer, which may or may not be desirable or acceptable to the worker considering and comparing the pay and seniority and status of the jobs.

5. **Tradeoff between benefits and harms.** The tradeoffs between benefits and harms must be worthwhile. In most cases, this means that the harms incurred by the large proportion of the screened population without disease are small enough to be outweighed by the benefits obtained by the few persons who are found to have the disease.

   Other variables to consider that may affect perceptions of medical testing include:

   - Current health status (healthy, have a diagnosed disease, have unexplained symptoms, psychological status)
   - Anxiety about health status
   - Knowledge about diseases and diagnostic tests
   - Clinical counseling methods
   - Timing of counseling

2. Beryllium Lymphocyte Proliferation Test (BeLPT)

Absent a less invasive and more sensitive test, the BeLPT has become the accepted method of testing for beryllium sensitization even though the sensitivity of the test is not clear.[35] Test results are subject to substantial intra and inter-laboratory variability.[19] There continues to be no uniform protocol that commercial laboratories use to perform the test nor is there a quality control program that insures the various testing laboratories generate consistent findings for the same sample. Similarly, evaluations of the BeLPT conducted by various branches of the Department of Defense (Air Force, Army, Navy) have also concluded the BeLPT suffers from highly variable estimates of sensitivity, specificity and positive predictive value.[37,38,39] The Department of Energy has guidelines (DOE-SPEC-1142-2001) for the conduct of the BeLPT.

Borak applied the approach used by the U.S. Preventive Services Task Force (USPSTF) in its expansion and refinement of the guidelines for screening first adopted by the World Health Organization to objectively evaluate the effectiveness of the BeLPT test as a screening tool. He concluded, “The accuracy and reliability of BeLPT are uncertain, and the test itself has demonstrated marked intra- and inter-laboratory variability. The prognosis of

BeS and subclinical CBD is essentially unknown and the clinical benefits of early intervention have not been studied. It is therefore difficult to judge whether potential benefits outweigh the harms of testing and treatment. Accordingly, there is currently insufficient scientific evidence to support recommendations that BeLPT be adopted as a clinical screening tool in asymptomatic individuals. Beryllium exposed workers may be eligible for worksite screening in the context of occupational health surveillance, although the clinical benefit of such testing has not been determined." [36]

Morinello also evaluated the performance of the beryllium blood lymphocyte proliferation test from general workforce survey data and a five-year survey of new employee data. More than 10,000 results, from nearly 2,400 participants over a 12 year period, were analyzed using consistent criteria to describe the performance characteristics of the BeBLPT. There was no correlation between time of employment and an increasing prevalence of confirmed BeBLPT positive results in individual surveys. The study concluded: "The detection of confirmed BLPT results in non-occupationally exposed persons, the apparent reversions of confirmed BeBLPT results, the identification of a positive BeBLPT peak prevalence period and variation in intra- and inter-laboratory test methods and test interpretation should be considered with caution when interpreting results from studies utilising the BeBLPT, especially when considering policy interventions such as worker removal. Additional research to refine BeBLPT performance or develop a new test is needed to reliably identify the relationship of sensitized workers to subclinical or clinical indicators of chronic beryllium disease."[45]

The BeLPT is typically performed using mononuclear cells collected from venous blood by gradient centrifugation. A fixed number of cells are suspended in tissue culture medium and incubated in microtiter plates with 1, 10, or 100 μM beryllium sulfate. The cells are harvested at two time points and [3H]-thymidine is added during the day prior to collection. A stimulation index (SI), believed to correlate with beryllium sensitization, is calculated by comparing the extent of thymidine incorporation in DNA from beryllium-treated lymphocytes to that of lymphocytes cultured in the absence of beryllium. Positive control samples are incubated with non-specific mitogens, such as hytohemagglutinin A or concanavalin A, or antigen-specific mitogens, such as tetanus toxoid or *Candida albicans*. A test is determined to be positive (abnormal), negative (normal), or borderline based on the total number of wells with SIs above a threshold value. Workers who test positive in successive BeLPT tests may be referred for bronchoalveolar lavage to assess the immune status of lymphocytes (BAL-LPT) in the lung and bronchial biopsy to determine if granuloma formation has occurred in the lungs.

3. Review of Current Possible Alternatives to BeLPT

The use of biomarkers in medicine lies in their ability to detect disease and support diagnostic and therapeutic decisions. The initial evaluation of a serum

biomarker concerns its expression in patients with the disease and in normal individuals in order to define sensitivity and specificity. Consequently the serum level of an ideal marker should: 1) increase pathologically in the presence of the disease (high sensitivity), 2) not increase in the absence of the disease (high specificity), 3) add information about the risk or prognosis 4) change in accordance with the clinical evolution, reflecting the current status of disease, or better 5) anticipate clinical changes, i.e. indicating the presence of relapse before it becomes obvious at a clinical level and finally 6) relate to disease burden and extent 7) be reproducible (as determined by the low coefficient of variation), 8) be of easy and cheap determination. [42]

To understand how both the BeLPT and other potential clinical tests might detect biomarkers of disease it is necessary to understand the role of T cells in disease and how already developed tests might use that understanding of T cells to detect beryllium disease. T cells belong to group of white blood cells known as lymphocytes and play a central role in cell-mediated immunity. They can be distinguished from other lymphocyte types, by the presence of a special receptor on their cell surface that is called the *T cell receptor* (TCR). The abbreviation "T", in T cell, stands for thymus since it is the principal organ for their development.

Several different subsets of T cells have been described, each with a distinct function.

- **Cytotoxic T cells** (T_c cells) destroy virally infected cells and tumor cells, and are also implicated in transplant rejection. These cells are also known as $CD8^+$ T cells, since they express the CD8 glycoprotein at their surface.

- **Helper T cells**, (T_h cells) are the "middlemen" of the adaptive immune system. Once activated, they divide rapidly and secrete small proteins called cytokines that regulate or "help" the immune response. These cells (also called $CD4^+$ T cells) are a target of HIV infection; the virus infects the cell by using the CD4 protein to gain entry. The loss of T_h cells as a result of HIV infection leads to the symptoms of AIDS.

A cytotoxic T cell (also known as T_C, CTL or killer T cell) belongs to a sub-group of T lymphocytes (a type of white blood cell) which are capable of inducing the death of infected somatic or tumor cells; they kill cells that are infected with viruses (or other pathogens), or are otherwise damaged or dysfunctional. CD8+ T cells are recognized as T_C cells once they become activated and are generally classified as having a pre-defined cytotoxic role within the immune system.

Helper T cells (also known as effector T cells or Th cells) are a sub-group of lymphocytes (a type of white blood cell or leukocyte) that play an important role in establishing and maximizing the capabilities of the immune system. These cells are unusual in that they have no cytotoxic or phagocytic activity; they cannot kill infected host (also known as somatic) cells or pathogens, and without other

immune cells they would usually be considered useless against an infection. The cells are involved in activating and directing other immune cells, and are particularly important in the immune system. They are essential in determining B cell antibody class switching, in the activation and growth of cytotoxic T cells, and in maximizing bactericidal activity of phagocytes such as macrophages. It is this diversity in function and their role in influencing other cells that give helper T cells their namesake.

Mature Th cells are believed to always express the surface protein CD4. T cells expressing CD4 are also known as called CD4+ T cells. CD4+ T cells are generally treated as having a pre-defined role as helper T cells within the immune system, although there are known rare exceptions. For example, there are sub-groups of suppressor T cells, natural killer T cells, and cytotoxic T cells that are known to express CD4 (although cytotoxic examples have been observed in extremely low numbers in specific disease states, they are usually considered non-existent). All of the latter CD4+ T cell groups are not considered T helper cells.

Considering the diverse and important role helper T cells play in the immune system, it is not surprising that these cells often influence the immune response against disease. They also appear to make occasional mistakes, or generate responses that would be politely considered non-beneficial. In the worst case scenario, the helper T cell response could lead to a disaster and the fatality of the host.

The immune system must achieve a balance of sensitivity in order to respond to foreign antigens without responding to the antigens of the host itself. When the immune system responds to very low levels of antigen or to metals which should not be recognized as an antigen, a hypersensitivity response occurs. Hypersensitivity is believed to be the cause of allergy and some auto-immune disease such as chronic beryllium disease.

Hypersensitivity reactions can be divided into four types:

Type 1 hypersensitivity includes common immune disorders such as asthma, allergic rhinitis (hay fever), eczema (hives), and anaphylaxis. These reactions all involve IgE antibodies, which require a Th2 response during helper T cell development. Preventative treatments, such as corticosteroids and montelukast, focus on suppressing mast cells or other allergic cells. T cells do not play a primary role during the actual inflammatory response. It's important to note that the numeral allocation of hypersensitivity "types" does not correlate (and is completely unrelated) to the "response" in the Th model.

Type 2 and Type 3 hypersensitivity both involve complications from auto-immune or low affinity antibodies. In both of these reactions, T cells may play an accomplice role in generating these auto-specific antibodies, although some of

these reactions under Type 2 hypersensitivity would be considered normal in a healthy immune system (for example, Rhesus factor reactions during child-birth is a normal immune response against child antigens). The understanding of the role of helper T cells in these responses is limited but it is generally thought that Th2 cytokines would promote such disorders. For example, studies have suggested that lupus (SLE) and other auto-immune diseases of similar nature can be linked to the production of Th2 cytokines.

Type 4 hypersensitivity, also known as delayed type hypersensitivity, is caused via the over-stimulation of immune cells, commonly lymphocytes and macrophages, resulting in chronic inflammation and cytokine release. Antibodies do not play a direct role in this allergy type. T cells play an important role in this hypersensitivity, as they activate against the stimulus itself and promote the activation of other cells; particularly macrophages via Th1 cytokines.

Other cellular hypersensitivities include cytotoxic T cell mediated auto-immune disease, and a similar phenomenon; transplant rejection. Helper T cells are required to fuel the development of these diseases. In order to create sufficient auto-reactive killer T cells, interleukin-2 must be produced, and this is supplied by CD4+ T cells. CD4+ T cells can also stimulate cells such as natural killer cells and macrophages via cytokines such as interferon-gamma, encouraging these cytotoxic cells to kill host cells in certain circumstances.

The mechanism that killer T cells use during auto-immunity is almost identical to their response against viruses, and some viruses have been accused of causing auto-immune diseases such as Type 1 Diabetes mellitus. Cellular auto-immune disease occurs because the host antigen recognition systems fail, and the immune system believes, by mistake, that a host antigen is foreign. As a result, the CD8+ T cells treat the host cell presenting that antigen as infected, and go on to destroy all host cells (or in the case of transplant rejection, transplant organ) that express that antigen.

3.1 ELISPOT

Beryllium sensitization is characterized by a CD4+ T-cell alveolitis and granulomatous inflammation. Pott et al. (2005) found that patients with CBD have a significantly elevated number of IFN-gamma-producing and IL-2-producing beryllium-specific CD4+ T cells in blood compared with both BeS and normal control subjects. In contrast, no difference in beryllium-induced proliferation of blood T cells was seen between Beryllium sensitized (BeS) patients and patients with CBD. Compared with the blood beryllium lymphocyte proliferation test, which detected beryllium-induced proliferation in 65% of BeS patients and patients with CBD, ELISPOT analysis detected IFN-gamma secretion in 80% of these subjects. Higher numbers of beryllium-specific cells in blood were also associated with the extent of alveolar inflammation, as measured by both bronchoalveolar lavage white blood cell and lymphocyte counts.

ELISPOT is an immunological assay based on ELISA (Enzyme-Linked Immunosorbent Assay). Basically, the difference between the two is that in ELISA, the substance containing the "unknown" is stuck at the bottom of the well, whereas in ELISPOT the substance with the "unknown" is placed in the well after the bottom of the well has been coated with cytokine-specific antibody. In both cases, the wells are typically contained within a generic microtiter plate. The ELISPOT method is most often used to determine the amount (i.e. the concentration) of activated antigen-specific cytotoxic T-cells in a given sample of splenocytes harvested from immunized animals, usually mice.

ELISPOTs rely on the principle that T cells secrete cytokines following activation. In this assay, a given number (around 10^6) splenocytes or peripheral blood lymphocytes are plated in a 96-well nitrocellulose plate with antigen. The T cells settle to the bottom of the plate and, if they are specific for the given antigen, they will become activated. Because the plates are pre-coated with antibodies to the cytokine of interest, cytokines secreted by activated T cells will be “captured” locally. Typically, CD4 responses are measured by Interleukin-4 capture, while CD8 responses are measured by Ifn-γ (Interferon-gamma) capture.

Following incubation, the T cells can be washed away and a secondary antibody to the same cytokine will be added. This secondary antibody is usually biotinylated and can be visualized by adding streptavidin-alkaline phosphatase reagent. This reagent catalyses the conversion of a substrate to a deep purple stain, causing purple spots to appear wherever an activated T cell was. By counting these spots, the fraction of T cells that can be activated by a given antigen can be ascertained.

ELISA tests are generally highly sensitive and specific and compare favorably with other methods used to detect substances in the body, such as radioimmune assay (RIA) tests. They have the added advantages of not needing radioisotopes (radioactive substances) or a costly radiation counter.

3.2. Serum Neopterin determined by ELISA

Harris et al. evaluated serum neopterin as a biomarker of chronic beryllium disease (CBD), for use in conjunction with the beryllium lymphocyte proliferation test (BeLPT) in workplace screening. Neopterin is a catabolic product of GTP(guanosine triphosphate). It is synthesised by macrophages upon stimulation with interferon-gamma. It belongs to the chemical group known as pterins. Serum neopterin levels were determined by radioimmunoassay, and levels in three groups were compared: CBD (n = 86), beryllium sensitized (BeS) (n = 22), and normal (Nor) (n = 20). Those in the diseased group underwent pulmonary function tests, bronchoalveolar lavage (BAL), and maximal exercise testing. To evaluate the optimum sensitivity, specificity, and neopterin cut-off value, receiver operator characteristic (ROC) curves were generated. The

median serum neopterin level in CBD was significantly higher than in BeS or in Nor [median 1.45, 25th, 75th percentiles (1.00, 2.7) ng/ml, 0.82 (0.67, 1.16) ng/ml, and 0.92 (0.86, 1.16) ng/ml, respectively] ($P < 0.05$). I n CBD, there were statistically significant associations between neopterin and measures of gas exchange and BAL cellularity. Using a neopterin value of 1.27 ng/ml, test specificity is 88%. In those workers with an abnormal BeLPT, serum neopterin has high positive predictive value (92%), and can identify disease, helping to distinguish it from BeS without the risks of biopsy.

Maier et al. (2005) took peripheral blood mononuclear cells (PBMN) that were cultured in the presence and absence of beryllium sulfate. Neopterin levels were determined from cell supernatants by enzyme linked immunosorbent assay (ELISA). CBD patients produced higher levels of neopterin in both unstimulated and Be-stimulated conditions compared to all other subjects ($P < 0.0001$). Unstimulated neopterin mononuclear cell levels overlapped among groups, however, Be-stimulated neopterin levels in CBD showed little overlap. Using a neopterin concentration of 2.5 ng/ml as a cutoff, Be-stimulated neopterin had a sensitivity of 80% and specificity of 100% for CBD and was able to differentiate CBD from BeS. Be-stimulated neopterin was inversely related to measures of pulmonary function, exercise capacity, and gas exchange.

3.3. Flow Cytometry (ImmunoLPT)

Flow cytometry is a technique for counting, examining and sorting microscopic particles suspended in a stream of fluid. It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of single cells flowing through an optical and/or electronic detection apparatus. A beam of light (usually laser light) of a single frequency (color) is directed onto a hydro-dynamically focused stream of fluid. A number of detectors are aimed at the point where the stream passes through the light beam; one in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter (SSC) and one or more fluorescent detectors). Each suspended particle passing through the beam scatters the light in some way, and fluorescent chemicals in the particle may be excited into emitting light at a lower frequency than the light source. This combination of scattered and fluorescent light is picked up by the detectors, and by analyzing fluctuations in brightness at each detector (one for each fluorescent emission peak) it is then possible to extrapolate various types of information about the physical and chemical structure of each individual particle. FSC correlates with the cell volume and SSC depends on the inner complexity of the particle (i.e. shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness). Some flow cytometers on the market have eliminated the need for fluorescence and use only light scatter for measurement.

Milovanoa et al.(2004) applied a 5,6-carboxyfluorescein diacetate succinimidyl ester flow cytometric technique for measurement of mitogen- and antigen-

induced T-lymphocyte proliferation to a group of beryllium-exposed sensitized individuals and beryllium-unexposed controls. They detected mitogen and antigen proliferative responses in CD3+, CD4+, and CD8+ subpopulations. Phytohemagglutinin and Candida stimulated CD4+ and CD8+ T-cell responses, but beryllium appeared to stimulate only CD3+/CD4+ responses.

Farris et al. (2000) developed a reliable and simple flow cytometric assay for lymphocyte proliferation testing (Immuno-Be-LPT) by combining immunophenotyping with bromodeoxyuridine (BrdU) incorporation or DNA content using propidium iodide (PI) or 4'6'-diimidazolin-2-phenylindole (DAPI). Evaluation of beryllium-induced lymphocyte proliferation in blood cells from seven patients with chronic beryllium disease (CBD) and 120 beryllium workers by both the Bc-LPT and the Immuno-Be-LPT showed agreement between the tests. The Immuno-Bc-LPT provided additional information about the specific type of lymphocytes responding. CD4+ lymphocytes proliferated in response to beryllium in blood samples from all seven CBD individuals and CD8+ lymphocytes proliferated in six of the seven. Four beryllium workers without CBD had positive responses to beryllium primarily in the CD8+ cells. The use of the individual's own plasma supported a greater beryllium or tetanus-induced proliferation of CD4+ lymphocytes when compared to commercial human serum.

3.4. MELISA (MEmory Lymphocyte Immuno Stimulation Assay)

MELISA® is a blood test measuring hypersensitivity to metals. It works by placing a range of metals into contact with white blood cells and monitoring the reaction.

Patients with a metal allergy will have, in any blood sample, memory cells formed by original exposure to the offending metal. These memory cells will also grow outside the body in a tissue culture plate. An immunologic reaction is demonstrated when the lymphocytes start to grow into lymphoblasts.

In this technique, venous blood is collected in citrate tubes. White blood cells (lymphocytes) are isolated and tested against allergens chosen accordingly to the patient’s anamnesis.

The lymphocyte reaction is measured by two separate technologies: one based on the uptake of radioisotope by dividing lymphocytes (Stimulation Index); the other by classical evaluation by microscopy (presence of enlarged lymphocytes called Lymphoblasts). Cells from the 5-day cultures are analyzed morphologically after staining cytospin preparations with Rapid Differential Hematology Staining solutions (Dade Behring AG, Marburg). Only tests in which the radioactively positive results shows the presence of lymphoblasts and the radioactively negative results shows only viable, small lymphocytes (non-cytotoxicity and non-stimulation) are accepted as valid.

MELISA has not been tested with beryllium. Early work with MELISA® indicates a subset of the general human population, at least 14% form a T-cell mediated immune response specific to common dental metals, and/or pharmaceutical preservatives and/or colorants, at the quantities these metals are entering the human blood stream.

Valentin-Thon and Schiwara (2003) tested 250 patients. Their reactivity to 0, 1, 2, 3, 4, or ≥ 5 metals was 26%, 36%, 15%, 12%, 6%, and 5%, respectively. Reactivity was most frequent to nickel (73%), followed by titanium (42%), cadmium (18%) gold (17%), palladium (13%), lead (11%), beryllium (9%), inorganic mercury (8%), tin (8%), and phenylmercury (6%). All patients (n=15) with confirmed or suspected nickel allergy were positive in MELISA®, while patients with no suspicion of nickel allergy were either negative (n=6) or very low positive (n=4) in MELISA® . MELISA® reactivity is directly dependent on lymphocyte concentration: the higher the lymphocyte concentration per test, the stronger the reactivity. Concentrations of inorganic mercury > 0.5 µg/ml cause non antigen-specific (mitogenic) reactions in a majority of patients. The reproducibility rate was 94% using a cut-off of Stimulation Index ≥ 3 or 99% using a cut-off of ≥ 5. While the absolute intra- and interassay Stimulation Index values may vary, the qualitative results are highly reproducible.

4. Principles of Good Clinical Trials

4.1 Clinical trials should be conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki, and that are consistent with GCP and the applicable regulatory requirement(s).[44]

4.2 Before a trial is initiated, foreseeable risks and inconveniences should be weighed against the anticipated benefit for the individual trial subject and society. A trial should be initiated and continued only if the anticipated benefits justify the risks.

4.3 The rights, safety, and well-being of the trial subjects are the most important considerations and should prevail over interests of science and society.

4.4 The available non-clinical and clinical information on an investigational product should be adequate to support the proposed clinical trial.

4.5 Clinical trials should be scientifically sound, and described in a clear, detailed protocol.

4.6 A trial should be conducted in compliance with the protocol that has received prior institutional review board (IRB)/independent ethics committee (IEC) approval/favorable opinion.

4.7 The medical care given to, and medical decisions made on behalf of, subjects should always be the responsibility of a qualified physician.

4.8 Each individual involved in conducting a trial should be qualified by education, training, and experience to perform his or her respective task(s).

4.9 Freely given informed consent should be obtained from every subject prior to clinical trial participation.

4.10 All clinical trial information should be recorded, handled, and stored in a way that allows its accurate reporting, interpretation, and verification.

4.11 The confidentiality of records that could identify subjects should be protected, respecting the privacy and confidentiality rules in accordance with the applicable regulatory requirement(s).

4.12 Investigational products should be manufactured, handled, and stored in accordance with applicable good manufacturing practice (GMP). They should be used in accordance with the approved protocol.

4.13. Systems with procedures that assure the quality of every aspect of the trial should be implemented.

5. Epidemiology Guidelines

5.1. The population studied should be pertinent to the risk assessment at hand, and it should be representative of a well-defined underlying cohort or population at risk.

5.2. Study procedures should be described in sufficient detail, or available from the study's written protocol, to determine whether appropriate methods were used in the design and conduct of the investigation.

5.3. The measures of exposure(s) or exposure surrogates should be: (a) conceptually relevant to the risk assessment being conducted; (b) based on principles that are biologically sound in light of present knowledge; and (c) properly quantified to assess dose-response relationships.

5.4. Study outcomes (endpoints) should be clearly defined, properly measured, and ascertained in an unbiased manner.

5.5. The analysis of the study's data should provide both point and interval estimates of the exposure's effect, including adjustment for confounding, assessment of interaction (e.g, effect of multiple exposures or differential susceptibility), and an evaluation of the possible influence of study bias.

5.6. The reporting of the study should clearly identify both its strengths and limitations, and the interpretation of its findings should reflect not only an honest consideration of those factors, but also its relationship to the current state of knowledge in the area. The overall study quality should be

sufficiently high that it would be judged publishable in a peer-reviewed scientific journal.

6. Resources for Method Development

6.1. NIH, the Office of Extramural Programs can be accessed at the following web sites:

http://grants1.nih.gov/grants/oer.htm

http://grants1.nih.gov/grants/new_investigators/pathway_independence.htm

6.2. There is a category for multiple principle investigators should PI's want to combine their research interests and conduct a validation on multiple tests at one time:

http://grants1.nih.gov/grants/multi_pi/index.htm

6.3. NIEHS may be another potential funding source, it being with the NIH.

http://www.niehs.nih.gov/

6.4. With regards to DOE, the office most likely to fund this type of research is Office of Biological and Environmental Research or OBER.

http://www.er.doe.gov/ober/