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DIAGNOSTIC RELEVANCE OF THE LYMPHOCYTE TRANSFORMATION TEST FOR SENSITIZATION TO BERYLLIUM AND OTHER METALS

(IUPAC Technical Report)

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Abstract: The lymphocyte transformation test (LTT) has been proven useful especially in the diagnosis of drug-induced allergic disorders. It is an in vitro test which is based on the fact that lymphocytes, which have been sensitized by a certain antigen, transform into blasts and proliferate when they are again exposed to this antigen. This proliferation is determined by measurement of the incorporation of [^{3}H]-thymidine or bromodeoxyuridine into replicating DNA. The test has the advantage over skin tests of avoiding re-exposure of individuals, and it was, therefore, hoped that it may also help to diagnose metal allergies and especially sensitization toward beryllium. However, the LTT measures only the sensitization of lymphocytes, but not the effector reaction, i.e., there may be positive results in exposed individuals even in the absence of clinical symptoms. There are several reports evaluating the LTT toward gold salts (Au), amalgam (Hg), nickel (Ni), beryllium (Be), and several other metals. With metals other than Be, the LTT appears to be of little use. In contrast, the LTT with Be may, indeed, define patients at risk of developing chronic beryllium disease (CBD), which affects mainly the respiratory tract and may even cause death. Beryllium sensitization progresses to CBD at a rate of 7–11 % per year. Since the Be-LTT can detect sensitization in workers who have not yet developed a disease it is an important diagnostic tool to detect individuals at risk. In conclusion, the LTT can detect a cell-mediated immunological response of an individual to metals. However, for most metals its usefulness is questionable, with the exception of Be; a positive Be-LTT can identify not only patients with CBD, but also persons at risk of developing CBD in later years.

1. INTRODUCTION

There is an important need to determine metal allergies in large numbers of individuals who may be exposed either occupationally or environmentally. An in vitro test has the significant advantage over skin tests of avoiding exposing people to the metal during testing, which may exacerbate, or even cause, sensitization. One of the most widely used test systems for this purpose is the lymphocyte transformation test (LTT). Its development was based on the observation in 1960 that incubation of lymphocytes with phytohemagglutinin, a mitogen, leads to cell activation and proliferation [1]. Therefore, it is sometimes also called the "lymphocyte proliferation test" (LPT). The test has been applied by different research groups for the evaluation of various cell-mediated immune reactions.

The principle of the LTT is based on the fact that lymphocytes, which have been sensitized by a certain antigen ("memory cells"), transform into blasts and proliferate when they are again exposed to this antigen. According to the different biological mechanisms occurring in a transforming cell, there are several chemical and physical methods to measure this transformation into blasts, as, for instance, determination of metabolic processes, biochemical alterations such as protein biosynthesis, or the synthesis of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA). The LTT, which measures the replication of DNA, is the most widely used and was proposed by the International Union of Immunological Societies (IUIS) [2].

This method is now used for the evaluation of lymphocyte function (lymphocytes are incubated with mitogens, thereby inducing a nonspecific transformation into blasts in vitro), as well as for the demonstration of specific sensitization of patients toward exogenous antigens (infectious agents, allergens) or autoantigens (autoimmune diseases). In the last 40 years, the test has been proven to be useful especially in the diagnosis of drug-induced allergic disorders and allergic disorders associated with exposures at the workplace [3–9]. In the field of environmental medicine, it was hoped that the LTT would give clues with respect to the etiopathogenesis of unknown disorders in which an allergic reaction toward "environmental substances" (metals, food, toxins, etc.) may play a role [10]. Several reports indicate, however, that the LTT may not be useful in the diagnosis of food-, pollen-, or mite dust-allergy or hypersensitivity reactions toward insect toxins [11–19].

At present, there is much debate about the usefulness of the LTT to detect sensitization of individuals exposed to metals. The aim of the present paper is to review critically the literature with respect to the relevance of the LTT for the diagnosis of metal hypersensitivity reactions, especially due to beryllium (Be). Guidelines are also cited for standardization and interpretation of the BeLTT. We prefer in the following text the term *LTT* instead of *LPT,* which is also quite often used in the literature in this context.

2. THE LTT METHOD IN GENERAL

Peripheral blood mononuclear cells (PBMCs) are isolated under strictly sterile conditions from heparinized blood by Ficoll-gradient centrifugation and cultured in a medium supplemented either with autologous serum, pooled homologous serum, or fetal calf serum.

For the determination of unspecific lymphocyte function, a mitogen is added to the cultures, for instance, phytohemagglutinin (PHA) or poke weed mitogen (PWM), which activates nonspecifically T- and B-lymphocytes (positive control). For the detection of a specific sensitization, the respective antigen or its chemically purified metabolite is added in increasing concentrations (typically three- or ten-fold stepwise increases). The different concentrations are necessary to obtain the optimal ratio between lymphocytes and antigen in the cultures, which induces the strongest proliferation. Finally, medium alone without the antigen is added as a control for "spontaneous proliferation". As a negative control, the test is also performed with lymphocytes from a healthy person who has had no known contact with the test substance.

The cells are incubated in suspension with the antigen for 4–7 days at 37 °C in an atmosphere of volume fraction 5–10 % carbon dioxide in air. Eighteen hours before the end of the culture period, radiolabeled [^{3}H] thymidine (typically, 1 μCi/ml = 3.7×10^4 Bq/ml) is added, which is incorporated into the DNA in proportion to the lymphocytes' proliferation. The cells are then collected onto glass fiber filters, washed, and the incorporation rate is measured in a beta scintillation counter. From the incorporation rate of thymidine (counts per minute, cpm) a stimulation index (SI) is calculated:

SI = (incorporated radioactivity in cultures with antigen)/(incorporated radioactivity in cultures without antigen)

An antigen-related SI-value has to be evaluated for each antigen separately, but in most instances SI-values between 2 and 3 are strongly suggestive of a positive reaction. Values greater than 3 may be taken as positive.

Proliferation of lymphocytes is determined by DNA replication, which is measured by incorporation of a tagged deoxynucleoside into the cellular macromolecular fraction. In principle, this could represent repair or turnover, but typically incorporation is allowed to occur for 18 h, more than enough time for all cells to pass through one cell cycle with DNA synthesis in S phase, and DNA replication is assumed to dominate the incorporation. Typically, the synthetic nucleoside bromodeoxyuridine (BrDU) [20,21] or radiolabeled [^{3}H] thymidine [22] are used. BrDU is detected with an *anti*-BrDU immunoglobulin, which has been coupled to peroxidase, and the complex is reacted with a chromophore such

as *o*-phenylenediamine for spectrophotometric detection. Alternatively, fluorescence-activated cell sorting (FACS) may be used. Thus, in a recent study beryllium sensitization has been determined by a FACS lymphocyte proliferation test, which gave results comparable to the conventional BeLTT [23]. Recently, a method based on mass spectrometric detection on [^{13}C,^{15}N]-labeled thymidine has also been reported for measuring the rate of DNA synthesis [24].

3. SPECIFICITY AND SELECTIVITY OF THE LTT

One has to be aware that the LTT only detects the sensitization of lymphocytes and not the effector reactions, i.e., it allows one to visualize a predisposition toward allergic reactions, which does not necessarily lead to clinical manifestations. Those false-positive reactions, which only indicate an exposure without development of any clinical signs or manifestations, are especially observed with those substances to which nearly everybody is exposed (e.g., food, pollen, metals, foreign proteins, etc.). Whether an effector reaction may appear depends upon other factors. Quite frequently, however, "false-negative" results are also obtained, where clinically there is a clear allergic reaction toward a certain substance but the LTT is negative (see below).

4. FACTORS INFLUENCING THE RESULTS OF THE LTT

The intensity of the lymphocyte proliferation response depends upon several factors such as the clinical manifestation (allergic reaction) itself, length of sensitization, and the time interval between appearance of clinical symptoms and performance of the test. It is general experience that the proliferation response usually decreases within 4–8 weeks after withdrawal of the inducing agent, probably because sensitized lymphocytes are homing to lymph nodes and can, therefore, no longer be detected in the peripheral blood [4,25]. On the other hand, in some instances the specific sensitization can be detected for several years [26,27].

However, the LTT can also be negative despite typical clinical manifestations of an allergy (fever, eosinophilia, rash, etc.). Probable causes are:

1. Lymphocytes are sensitized not to the parent compound, but toward haptens or metabolites, which are not available for the in vitro test [25,28–30].
2. The test was not performed within the optimal time interval of about 10 days to 6 weeks after manifestation of the allergic symptom, lymphocytes were not isolated within 24 h (at most, 48 h) after drawing the blood, and blood was not kept at room temperature [4].
3. The immune system of the subject is still activated at the time of drawing the blood, resulting in a strong spontaneous proliferation of the lymphocytes in the absence of any antigen, which may mask a specific reaction.
4. The observed clinical manifestation is not the result of an allergic but rather of a toxic or pseudo-allergic reaction, i.e., there is no sensitization of lymphocytes.

A disadvantage for the long-term stability of test results is certainly that no storage of any native specimens (as positive or negative controls for quality control purposes) is possible, i.e., the lymphocytes have to be prepared from fresh blood within 24–48 h under strictly sterile conditions.

From these considerations, it is clear that modifications of the LTT are necessary according to each antigen analyzed with respect to antigen concentration, length of time in culture, etc. Experience is required for interpretation of the results. Internal quality controls within the lab are especially important. External standards or controls are usually not available so that comparison of results between different laboratories is often difficult.

5. LTT FOR THE DIAGNOSIS OF METAL-INDUCED ALLERGIC DISORDERS

As mentioned above, the LTT has also been used for the detection of sensitization of lymphocytes toward metals, with the hope that it might help to identify patients with metal-induced disorders. Most studies in this respect are concerned with reactivity toward gold salts, amalgam or $HgCl_2$, Ni, and Be. With Au, Hg, and Ni, as well as several other metals that have been investigated, the reliability of the LTT must be carefully examined. An exception is the use of the LTT with beryllium, which may define patients at risk of developing chronic beryllium disease (CBD) [31–33].

In an early study with Ni, Svejgaard et al. [34] found that 7 of 8 patients with contact dermatitis and a positive patch test to Ni showed a significant response to $NiSO_4$ in the LTT. However, 3 of 15 patch test-negative controls showed a borderline response. The authors suggest that a weak nonspecific mitogenic effect of Ni might contribute to a false LTT response. The same group [35] found a significant increase in the strength of the Ni LTT in 8 allergic patients after oral challenge with Ni. In a series of 43 Ni allergic patients with a positive patch test, a linear correlation coefficient of only 0.42 was found between patch test and LTT [36]. Furthermore, some patients with strong skin reactivity had a weak LTT response, and vice versa. A poor correlation was further reported between patch test and LTT for Ni, with positive LTT in 63 % of individuals with, and 30 % of subjects without a history of metal allergy [37]. However, in the 60 % of individuals who had agreement between Ni LTT and a second in vitro test based on macrophage migration inhibition, correlation with the patch test was strong. For the other 40 %, the authors conclude skin testing remains indispensable for diagnosing Ni allergy. A Ni LTT was conducted with 15 orthopedic patients before and after implantation and after removal of stainless steel plates for mandibular fractures [38]. It was concluded that Ni LTT might be useful in this setting, although no adverse clinical effects attributable to sensitization were demonstrated. Using patch testing as a reference method to identify Ni-allergic patients, a specificity of 17 % was reported with a $NiCl_2$-based LTT [39]. In a more recent study, $NiSO_4$ caused a positive LTT in samples from most adults, regardless of the presence or absence of a positive patch test or clinical Ni allergy [40], casting further doubt on the usefulness of LTT for Ni. There is, however, evidence that identification of certain T-cell receptors for Ni may improve the correlation between in vitro analysis and clinical manifestations [41–43].

Several studies have evaluated the LTT for Hg [44–47]. The test was optimized to demonstrate a higher incidence of lymphocyte reactivity to Hg in patients with dental amalgams and oral lichen planus, compared to control subjects without amalgams, or to those with amalgams, but without oral changes [44]. That Hg was causative of the oral lesions was proven by removal of the amalgams. However, when the LTT was further evaluated in dental patients with amalgams, healthy blood donors with amalgams, volunteers without amalgams, and in patients with oral lichen planus adjacent to amalgams who had a positive patch test for HgO [45] results of the LTT with mass concentration of $HgCl_2$ ≤0.5 µg/ml did not correlate with the presence of amalgams. Sensitivity ranged from 33–67 % and specificity from 0–70 %, precluding a useful test. Patients claiming subjective symptoms from amalgams were divided into those with or without psychosomatic symptoms upon exposure to low-dose Hg patch testing [46]. These two groups together with a control group were investigated by LTT using various concentrations of Hg, Ni, and Pd chlorides and $Na_3Au(S_2O_3)_2$. Both LTT with Pd and Hg distinguished the controls from patient groups, but not the two patient groups themselves. Au was without effect on lymphocyte proliferation, and Ni showed a similar dose-dependent effect in all three groups.

The LTT appears to be of little use in demonstrating Au allergy, and the valence state of Au appears to be important in eliciting a positive LTT. Of 7 rheumatoid arthritis patients on long-term Au therapy, one gave a positive LTT with Au(III) (as gold trichloride), but not with sodium aurothiomalate. [48]. In one series of 52 patients, 73 % of those with a positive patch test had a positive LTT with $Na_3Au(S_2O_3)_2$ [49,50]. While some had shown symptoms from jewelry or dental work, most had subclinical allergy. Also using $Na_3Au(S_2O_3)_2$, as well as $PdCl_2$, Cederbrant et al. [39] reported a sen-

sitivity of 70–80 % and a specificity of about 55 % for both metals. They concluded that the LTT is not useful for testing allergy to either of these metals as a large number of false-positives can be expected. Vamnes et al. [51] evaluated the LTT with $Na_3Au(S_2O_3)_2$ and $AuCl_3$ in 8 patients with and 8 without positive patch test to Au salts. Although statistically significant differences were seen between the two groups, specificity and sensitivity were between 67 and 80 %. On the other hand, Raesaenen et al. [49] found a positive LTT in 12 of 13 patients with clinical Au allergy, and false-positives in only 2 of 15 nonallergic arthritis patients and 1 of 11 healthy controls. They concluded that the LTT is of use in this setting. Thus, while the view that LTT is of little use for Au allergy is not universally held, it must at present be interpreted with extreme caution.

Data with respect to the relevance of the LTT in chromium hypersensitivity are still conflicting [52,53]. A single case was reported [54] of a woman who developed lupus-like symptoms after implantation of metal plates, with a positive patch test to Mo and a positive LTT with Mo. Symptoms resolved after removal of the plates.

6. LTT FOR THE DIAGNOSIS OF BERYLLIUM-INDUCED DISEASES

6.1 Background on exposure and disease

Beryllium (atomic number 4) is a silver-gray metal existing naturally only as the isotope ^9Be, and is the lightest solid, stable chemical element. It forms divalent compounds and has a high melting point (1280 °C), excellent thermal and electrical conductivities, and a high strength-to-weight ratio. Its chemistry somewhat resembles that of aluminum, including a high affinity for oxygen, and a surface layer of BeO on its metallic form and alloys produces high corrosion resistance. It is permeable to X-rays. World production has been steady in recent years at 200–300 metric tons per year (metallic Be equivalent) (<http://minerals.usgs.gov/minerals/pubs/commodity/beryllium>). The United States is currently the major producer (bertrandite from Utah), followed by Russia and China (beryl, $Al_2O_3 \cdot 3BeO \cdot 6SiO_2$).

In the past, Brazil, Argentina, India, Portugal, and several central African countries have also been important, though smaller, producers of beryl.

In the earlier part of the 20th century, beryllium was used extensively in industry, mainly for the construction of atomic weapons and fluorescent lamps. In the last few decades, many additional uses have created further opportunities for human exposure. These include the electronics, aerospace, metal, and ceramics industries. In 2001, the major end uses were in computers and telecommunications equipment. Recently, beryllium exposure during refining and reclamation of metals from scrap has gained attention [55,56]. Gemstone cutting and grinding (e.g., beryls as aquamarines, emeralds) is another source of exposure for a relatively small group of highly specialized workers [57]. The National Institute for Occupational Safety and Health (NIOSH) has estimated that 14 000 workers are potentially exposed to beryllium in the United States [58].

Very high exposure to soluble compounds of beryllium (e.g., sulfate, fluoride) in the middle of the 20th century (solubilities up to mass concentrations of 100 or even 1000 $\mu g/m^3$) caused numerous cases of dermatitis as well as inflammation of the nasal mucous membranes, pharynx, trachea, and bronchi, with chemical pneumonitis developed in workers in the United States, the former Soviet Union, and Japan.

These cases of acute beryllium disease (acute berylliosis) could have a severe, sometimes rapidly fatal course, but recovery within several weeks was typical after cessation of exposure (DFG 1972–2001) [59]. The promulgation of an occupational exposure limit of mass concentration 2 $\mu g/m^3$ in many industrialized countries led to a distinct drop in the incidence of acute beryllium disease nearly to zero [60]. Today, the immunological (and potentially carcinogenic) properties of chronic beryllium exposure are of considerable relevance to the health of people exposed in the workplace.

The symptoms and clinical course of CBD were first described in the 1940s [61,62]. CBD has been observed after beryllium exposure in compliance with the air standard of 2 μg/m^3 [63–66]. Characteristic symptoms of CBD are dyspnoea on exertion, weight loss, nonproductive cough, fatigue, chest pain, anorexia, and weakness [67–69]. Neopterine [1-(2-amino-4-hydroxy-6-pteridinyl)-1,2,3-trihydroxy propane] levels can be elevated [70]. More severe CBD displays a combination of fibrosis and active pulmonary interstitial inflammation. Although skin, lymph nodes, liver, and spleen might be affected as well, the effect on the respiratory tract is decisive for the clinical symptoms [56,57,59,71–73]. Cor pulmonale may develop and may even cause death.

Characteristic tissue lesions in CBD are noncaseating granulomas, which are typically caused by irritants and persist due to immunological and biochemical mechanisms [74–76]. After inhalation and deposition of Be-containing particles in the lung, a proportion of Be is cleared rapidly, but some Be is cleared very slowly with a half-life of months or years. The amount that is deposited largely increases with decreasing particle size, and length of persistence in airways increases with decrease in solubility. Presumably, Be acts as a hapten, binding to a peptide. This Be-peptide-complex can be taken up by antigen presenting cells in the bronchopulmonary system, which then migrate to draining lymph nodes presenting here the antigen to lymphocytes via MHC class II-antigens. CBD is not directly related to the magnitude of exposure, indicating a lack of a direct dose-response relationship [77]. For example, persons living near a beryllium plant also contracted the disease to a certain extent from environmental pollution at exposure levels several orders of magnitude lower than experienced by the workers. Particle number rather than particle mass may be more reflective of target organ dose [78]. The amount of beryllium to which a person has been exposed can be determined by measuring the urinary beryllium concentration [55,79–84]. A genetic predisposition also appears to play an important role in the development of CBD [85–88].

CBD has a typical latency period of about 20 years (range from a few years to 30 or more years) [89,90], so former exposures still are cause for concern. In Germany, for example, only very few persons nowadays develop CBD that is approved and compensated as occupational disease. The disease remains a major concern in the United States, again mainly due to former exposures. At one time, about 14 000 workers were estimated to be exposed to beryllium in the United States. As a consequence, a lowering of the technical limit value (TLV) for the workplace to one-tenth the present value (to 0.2 μg/m^3) has been proposed [60].

CBD is clinically indistinguishable from pulmonary sarcoidosis [91]. Investigations by chest radiographs, histology, or pulmonary function tests do not differentiate CBD from sarcoidosis. Identification of an occupational beryllium exposure and determination of a beryllium sensitization are the decisive tools to recognize CBD.

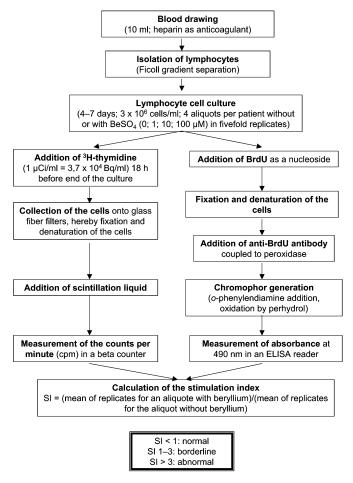## 6.2 Identification of beryllium sensitization by skin patch tests

Testing the skin with solutions of beryllium salts (patch or epicutane test) is a possibility to identify sensitized persons [92,93]. These tests are not without any hazard in contrast to the proliferation tests, which are performed in vitro. The skin test might introduce a sensitization in previously unsensitized persons or might provoke clinical symptoms in already sensitized persons. Therefore, the application of skin testing has been stopped in some countries, e.g., in the United States, since the LTT has emerged as an alternative.

6.3 Identification of beryllium sensitization by lymphocyte transformation test

The beryllium lymphocyte transformation test (BeLTT) investigates in vitro whether lymphocytes proliferate to a higher extent if challenged with soluble beryllium salts compared to the situation without beryllium [94]. If so, the BeLTT is considered to be positive, and this is interpreted as a beryllium sen-

sitization of the individual. The theory behind this test assumes that beryllium promotes the cell-mediated immune response in sensitized persons. Lymphocytes may be isolated from peripheral blood or broncho-alveolar lavage (BAL) [95]. The latter is derived by a process of instilling fluid into a portion of lung through a bronchoscope and of withdrawing the fluid. The beryllium reactivity of lymphocytes obtained by broncho-alveolar lavage is generally greater than the reactivity of lymphocytes obtained from the peripheral blood, but blood taking is less invasive. Therefore, the test is commonly performed with blood. The BeLTT can detect beryllium sensitivity in workers who have not yet developed a disease [96]. Beryllium sensitization progresses to CBD at a rate of 7–11 % per year. Therefore, BeLTT is an important diagnostic tool to detect workers at risk.

The principle of the analytical procedure is illustrated in Fig. 1. Isolated lymphocytes from blood or BAL are cultivated (positive control with a mitogen, e.g., phytohemagglutinine). Tritiated deoxythymidine or bromodeoxyuridine are added, which are taken up during the cell proliferation process. Aliquots of the cell cultures are run without and with several concentrations of a soluble beryllium salt (sulfate or fluoride). After termination of the culture, the amount of incorporated nucleosides for each of the cell cultures is measured either by a counter (in the case of tritiated thymidine) or pho-

Fig. 1 Scheme of an analytical run for BeLTT with peripheral blood lymphocytes (modified according to Wegner et al. [57]). The given SI used the calculation SI = (mean of replicates for an aliquot with beryllium)/(mean of replicates for the aliquot without beryllium) [4].

tometrically (bromodeoxiuridine; after addition of *anti*-BrdU, immunoglobulin-coupled peroxidase, and *o*-phenylendiamine as a chromophore).

The result of an LTT is expressed as a stimulation index (SI) [4]. The BeLTT is considered to be abnormal if the SI is in excess of 3, borderline if it is between 1 and 3, and normal if it is 1 or below. However, a specific problem of the LTT is the occasional occurrence of outliers. In order to reduce the influence of outliers, different statistical evaluations have been suggested. One is the calculation SI = $x_1/(x_2+3s)$ (x_1 = mean of replicates for an aliquot with beryllium, x_2 = mean for the aliquot without beryllium, s = standard deviation) [57].

Even more sophisticated is the least absolute value (LAV) method suggested by Frome et al. [97], which is described in the BeLTT document of the U.S. Department of Energy [98].

6.4 Analytical aspects of the BeLTT

The performance of the BeLTT requires laboratory personnel with great experience. The test consists of numerous manual steps, is time-consuming and prone to many sources of interferences during an analytical run. A standardization of the test is essential for interlaboratory comparison and a harmonization of test results and there are, indeed, already detailed protocols, as for instance by the U.S. Department of Energy or the ICPT [98,99]. The BeLTT is a screening test with a sensitivity and specificity that is not clearly defined.

6.5 Relation of BeLTT and CBD

It is generally accepted nowadays that sensitization to beryllium follows exposure and may develop into CBD [100] (Fig. 2). There seems to be an exposure level below which sensitization to beryllium seems not to occur [63,96,101–105]. The percentage of sensitized cases that evolve to CBD cases is not known, but some experts, admitting uncertainty, report as high as between 50–90 % [90,106]. 7–11 % of the sensitized individuals will develop CBD within the following year.
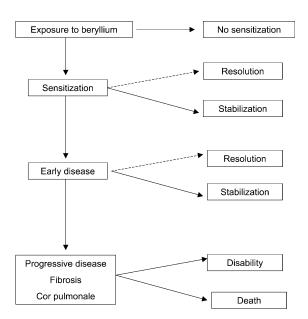

Fig. 2 Proposed scheme by Newmann et al. [32] for the events and outcomes for individuals exposed to beryllium (modification). Solid lines indicate known outcomes, broken lines indicate hypothetical outcomes.

In conclusion, the LTT for metals with peripheral blood lymphocytes is a noninvasive in vitro test for a cell-mediated immunological response of an individual to metals. It has a high predictive value in beryllium workers. A positive BeLTT can identify first of all patients with CBD, and secondly—as a biomarker of a biological effect—persons still free of CBD symptoms, but at risk of developing CBD later.

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