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# Immune System and Environmental Xenobiotics - The Effect of Selected Mineral Fibers and Particles on the Immune Response

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## 1. Introduction

Mineral fibers and particles are finding growing applications in industry and thus entering into the human environment. The utility of using such products for various purposes is promising but detailed information related to immune safety is needed. Immunotoxic effects may be displayed as immunosuppression, immunostimulation, hypersensitivity and autoimmunity. Humans may be exposed to fibers and particles from a variety of sources, including occupational settings, ambient air, consumer products, drinking water and food. This chapter is dedicated to the effect of inhalation exposure to asbestos, rock wool, glass wool, ceramic fibers and nickel oxide particles on the immune system.

Findings of *in vitro* studies, *in vivo* animal experiments and molecular epidemiological studies conducted during the period of several years are summarized. *In vitro* studies comprised studies on alveolar macrophages and alveolar epithelial type II cells. Refractory ceramic fibers, asbestos and stone wool fibers were tested *in vitro*. *In vivo* testing involved both inhalation and intratracheal instillation studies using amosite, wollastonite, rock wool and glass fibers. Moreover, three population based studies in workers occupationally exposed to asbestos, rock wool and glass fibers were performed.

Finally, options and pitfalls to the use of immune assays as sensitive biomarkers of possible immunotoxic effects are discussed. Since, in human studies, specimens from living people used to examine the effects of particles and fibers on the immune response are typically limited to minimally invasive (whole blood, plasma or serum by venipuncture, sputum) or moderately invasive techniques (bronchoalveolar lavage or nasal lavage), human blood

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leukocytes are the most appropriate specimens for *in vitro* cellular assays. Macrophages and lymphocytes are appropriate models for examining the effects of xenobiotics on cell functions. Serum cytokines, chemokines or soluble adhesion molecules have potential to contribute to the panel of biomarkers used to assess immunotoxicity.

### 1.1 Immunotoxicology

Immune dysregulation resulting from inhalation, skin exposure or ingestion of chemicals in the workplace and general environment is an important health problem in industrialized an industrializing societies (National Research Council, 1992). Immunotoxicity is an important aspect of the safety evaluation of drugs and chemicals (Descotes, 2005). It has been generally accepted that all new chemicals require safety evaluation before marketing and sale. This is a difficult task due to the large number of chemicals directly consumed by man, such as drugs and food additives, and those that are widely used such as pesticides, household chemicals, and industrial products (De Rosa et al., 2002; IPCS/WHO, 1999).

Immunotoxicity refers to any adverse effect on the structure or function of innate and adaptive immunity. It can be divided into immunosuppression, immunostimulation, hypersensitivity and autoimmunity (Duramad & Holland, 2011; Descotes, 2005; Fig. 1). The outcome of immunotoxicity is influenced by the dose of the immunotoxicant as well as mechanism of action of exposure to other agents, such as bacteria, viruses, parasites, or chemicals normally harmless. Direct immunotoxic effects of xenobiotics including particles and fibers can lead to the suppression or stimulation of immune response. Immunosuppression can result in increased occurrence of infectious diseases or and neoplasias, in particular lymphomas, as shown in both transplant and cancer patients treated with potent immunosuppressive drugs (Descotes, 2000; Vial & Descotes, 1996). Immunosuppression caused by chemicals, may make the course of infections more severe, atypical and or likely to relapse. The target organ systems affected could be the respiratory, gastrointestinal tracts, CNS or the skin (Descotes, 2005). Flu-like reactions, autoimmune diseases and hypersensitivity reactions to unrelated allergens are among the adverse effects related to immunostimulation (Descotes, 2005). Hypersensitivity reactions are the most frequently detected immunotoxic effects of chemicals. They include immune-mediated ('allergic') and non immune-mediated ('pseudoallergic') reactions. Particles and mineral fibers are recognized causes of hypersensitivy reactions provoked mostly within respiratory tract and skin (D'Amato et al., 2005; Di Giampaolo et al., 2011). A large number of drugs and an increasing number of environmental agents can result in the appearance of a number of autoantibodies or even autoimmune diseases. Systemic lupus erythematosus, scleroderma or dermal vasculitis have been associated with exposure to a variety of chemical agents (Hess, 2002; Van Loveren et al., 2001).

### 1.2 Models and methods in immunotoxicology

The immune system is a complex network comprised of several cell types (i.e., lymphocytes, macrophages, granulocytes, and natural killer cells) whose diversity of functions includes maintaining homeostasis and health (Luster et al., 1989). Scientists use immunocompetent cells as models for studying the toxic mechanisms of xenobiotics at the cellular and

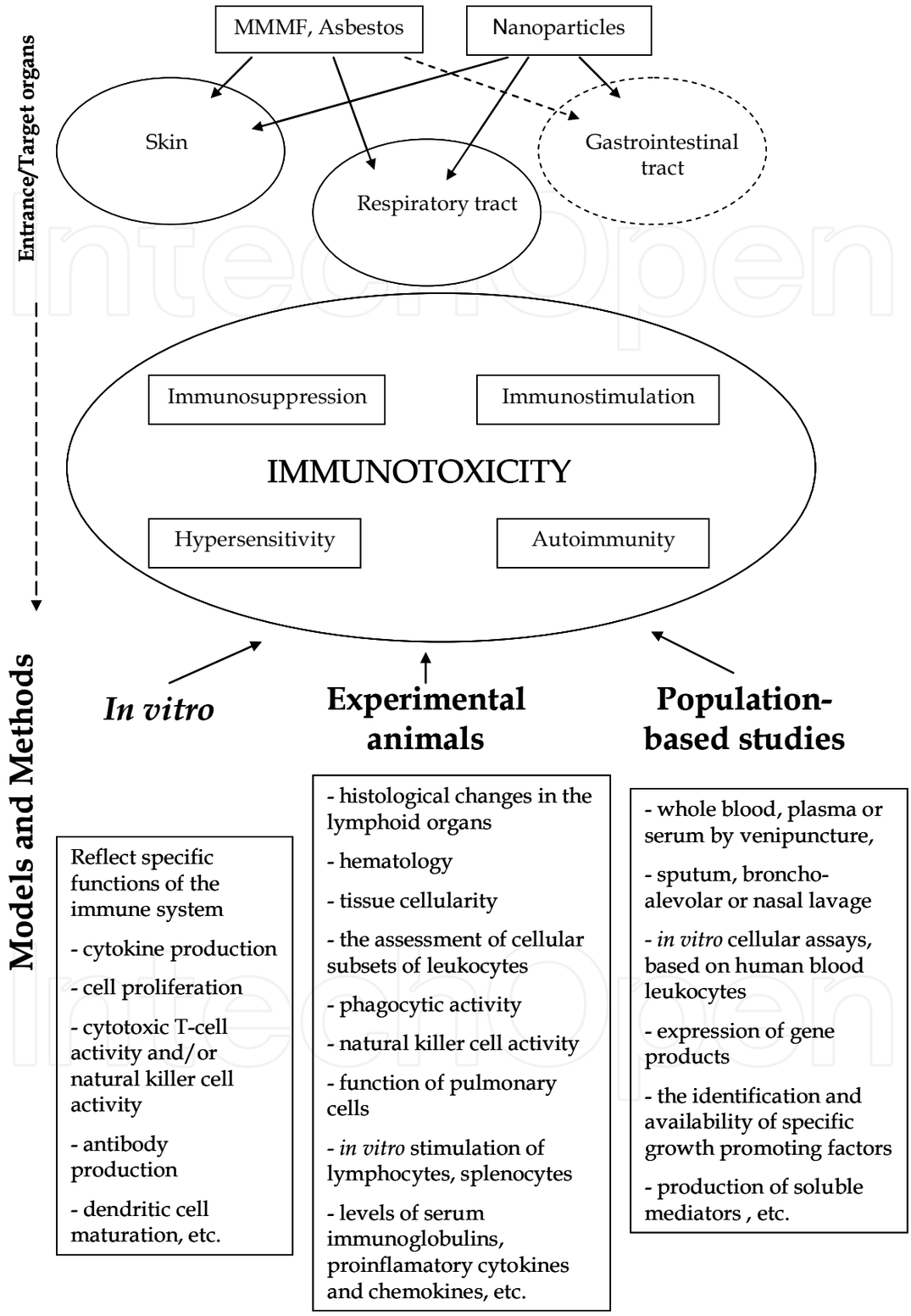



Fig. 1. Strategy for immunotoxicity testing in particle and fiber toxicology. Options for *in vitro, in vivo* and population studies.

molecular levels. In general, biological effects of xenobiotics (including particles and fibers) depend on several factors, e.g., chemical composition and physico-chemical properties such as solubility, chemical reactivity, size, length:width ratio, persistence in the organism, surface properties, dose, duration of exposure, the ability of a material to interact with body proteins etc. Host factors such as underlying health status, individual susceptibility to xenobiotics, metabolism, age, nutrition status, life style (smoking, etc.), presence of immune disease (asthma, allergic rhinitis, immunosuppression etc.) and other factors are also important determinants of immunotoxicity.

Increasing evidence that the immune system is a frequent target of xenobiotics following chronic, subchronic, or acute exposure underlines the need for development of models and immune assays suitable for use in screening potential immunotoxic compounds. For hazard assessment of xenobiotics, *in vitro* studies, experimental studies in laboratory animals, as well as epidemiological studies may provide necessary information (Fig. 1). Recently, several review papers have been published on design and methods used in immunotoxicological studies (Dietert & Holsapple, 2007; Lankveld et al., 2010; Oostingh et al., 2011). Haley published best practice guidelines for routine pathology evaluation of the immune system (Haley et al., 2005). Study methods of immunotoxicology have been reviewed and guidance documents developed by United States and European regulatory agencies (Committee for Proprietary Medicinal Products, 2000; Food and Drug Administration, 2004; Gopinath, 1996; ICICIS 1998; Kuper et al., 2002; Schuurman et al., 1994). In addition, harmonization of immunotoxicity guidelines in the ICH (International Conference on Harmonisation) process has been discussed by Ruehl-Fehlert (2005). One of the first steps in planning and conducting immunotoxicity studies is the identification and characterization of fibers/particles of interest. Secondly, attention must be paid to choosing proper *in vitro* immune cell models, sensitive animal models or occupationally or environmentally exposed human populations to assess the effect of xenobiotics on the immune system. Furthermore the selection of suitable immune assays as sensitive biomarkers of immunotoxic effect is also important.

### *In vitro* assessment of immunotoxicity

*In vitro* testing has several advantages over *in vivo* animal testing. Among others, 3R requirements - reduction, refinement, and replacement of animal experiments are fulfilled, detailed mechanistic understanding of target immune cell/molecule is a clear benefit to consider and costs are lower. Most assays that are currently used to analyze immunotoxicity were originally designed for diagnostic purposes to examine hereditary or acquired immune disease in humans. Subsequently, these methods have been adapted for analysis of immunotoxicity of xenobiotics. *In vitro* assays may reflect specific functions of the immune system (cytokine production, cell proliferation, cytotoxic T-cell activity, natural killer cell activity, antibody production, and dendritic cell maturation). To avoid inter-species extrapolation, assays should preferably use human primary cells. They reproduce the response of normal cells of normal individuals. However, the use of primary cells is not always feasible (e.g., in the case of primary lung epithelial cells). As an alternative, the use of animal primary cells or human cell lines (transformed or tumor cells with unrestrained proliferative capacity) is applicable to first line screening of immunomodulatory effects. Furthermore, whole blood has the advantage of comprising multiple cell types in their natural proportion and environment (Lankveld et al., 2010).

***In vivo* models in experimental animals**

In investigating potential effects of compounds on the immune system in experimental animals, a tiered approach is recommended. Studies aimed on the identification of histologic changes in the lymphoid organs and functional immune alterations in laboratory animals are useful for detecting probable immunotoxicants and may play an important role as a first indicator of direct immunotoxicity, i.e. immunosuppression (De Jong & Van Loveren, 2007). First tier, general toxicity studies may include parameters for detection of relatively gross toxic effects on the immune system. Hematology, tissue cellularity and the assessment of cellular subsets of T- and B-leukocytes by flow cytometry as non-functional assay are common initial tests. Some authors consider such bioeffects as insensitive indicators of immunotoxicity.

Second tier consists of studies of immune function. Phagocytic activity and determination of Natural Killer (NK) cell activity may be used in evaluation of direct immunotoxicity. In animal models, there is no limitation to obtain cell suspensions from lung tissue or bronchoalveolar lavage to look at function of pulmonary cells affected by particles and fibers. Several other possibilities are presented by thymus, bone marrow or spleen tissues for *in vitro* stimulation of lymphocytes by potential mitogens. These methods may indicate effects of xenobiotics on the functionality of splenic cell populations. Concanavalin A (Con A) and phytohemagglutinin (PHA) activate T-cells, while lipopolysaccharide (LPS) activates primarily B-cell populations. In addition, serum can be obtained for determination of serum immunoglobulins or proinflamatory cytokines and chemokines. Comparison of treated and control groups may give a first indication of possible direct immunotoxic effects (De Jong & Van Loveren, 2007).

**Population-based studies**

Biological endpoints used in molecular epidemiology are called biomarkers. Several definitions of biomarkers as tools used in human or animal studies to assess exposure and disease risks have been published (Benford et al., 2000). Bottrill defined biomarkers as "parameters which can be evaluated quantitatively, semi-quantitatively or qualitatively and which provide information on exposure to a xenobiotic or on the actual or potential effects of that exposure in an individual or in a group" (Bottrill, 1998). There is a high degree of complexity of the immune system and an enormous variety of responses and mechanisms involved in immunotoxic injury. Therefore it is a challenge to identify a key parameter to develop as a biomarker. The inclusion of several immune endpoints applicable to man is thus essential. Specimens from living people to examine the effects of particles and fibers on the immune response are typically limited to minimally invasive (whole blood, plasma or serum by venipuncture, sputum) or moderately invasive techniques (bronchoalveolar or nasal lavage). *In vitro* cellular assays are typically based on human blood leukocytes. In particular, macrophages and lymphocytes are appropriate models for examining the effects of various agents on cell maturation and function. The expression of gene products can be used as markers of differentiation, the identification and availability of specific growth promoting factors (e.g., interleukins), and their potential to undergo terminal differentiation resulting in production of soluble mediators (e.g., monokines, lymphokines, or antibodies) or indicating effector function (e.g., tumor target cell killing) (Luster et al., 1989).

### 1.3 Immunotoxicity studies of mineral fibers

The adverse effects that arise from exposure to asbestos have stimulated the development of substitute materials, man-made mineral fibers. However, little is known about the health effects of these fibers. The potentially harmful effects of all types of respirable fibers are at present one of the most important fields of interest in industrial toxicology. The production, sale and use of asbestos are no longer permitted in Europe. Some of the properties of asbestos (e.g. as an insulation material) can be substituted by alternative man-made fibers. In view of the importance of the possible biological effects of fibers we have conducted *in vitro*, animal and a molecular epidemiology studies to examine the relationship between relevant biomarkers and exposure to asbestos, mineral wool and glass fibers. We have measured a range of biomarkers of exposure, effects and individual susceptibility. In this chapter, biomarkers of immunotoxicity will be presented.

#### 1.3.1 Asbestos

Asbestos has long been recognized as a cause of both benign and malignant lung disease (interstitial and pleural fibrosis, lung cancer and mesothelioma). Asbestos refers to a group of naturally occurring mineral fibers with a ≥ 3:1 length to diameter ratio. These fibers once inhaled and displaced by various means to lung tissues, can cause a spectrum of diseases including cancer and disorders related to inflammation and fibrosis (American Thoracic Society, 2004; Mossman et al., 1996). Mechanisms of asbestos-induced carcinogenesis are thought to be multiple, including generation of reactive oxygen (ROS) and nitrogen species (RNS), alteration of mitochondrial function, physical disturbance of cell cycle progression, and activation of several signal transduction pathways (Jaurand, 1997; Nymark et al., 2008). Asbestos fibers having iron (or even chrysotile) and producing ROS/RNS can cause DNA damage to nearby cells, and fibers are sometimes directly inserted into the cells and injure chromosomes, while retained fibers may adsorb other carcinogens on their surface (known asbestos bodies) (Toyokuni, 2009a, 2009b).

The extrapulmonary consequences of asbestos exposure were discussed in Bunderson-Schelvan et al., (2011). Authors used several hundred epidemiological, *in vivo* and *in vitro* studies and finally they supported a strong association between asbestos exposure and peritoneal neoplasm. On the other hand, the correlations between asbestos exposure and immune-related disease were less conclusive and effects of asbestos exposure to the GIT (gastrointestinal tract) appeared to be minimal. Immunomodulatory effects of asbestos have been well established in patients with asbestosis and mesothelioma (American Thoracic Society, 2004; Corsini et al., 1994; Mascagni et al., 2003; Rosenthal et al., 1999) however there is limited information on effects in individuals with minimal evidence for asbestos related lung disease or exposure only. Our study offered an opportunity to assess biomarkers which may represent individual susceptibility to and/or early evidence for asbestos related health effects.

#### 1.3.2 Man made fibers

**Rock wool, glass fibers and ceramic fibers**

The evidence for adverse health effects following exposure to asbestos has prompted a drastic reduction in the use of asbestos, resulting in the increased use of substitutes

composed of both naturally occurring and synthetic materials which are thought to have lower toxicity. Man-made mineral fibers include glass fibers (used in glass wool and continuous glass filament), rock (stone)/slag wool and refractory ceramic fibers. Rock (stone) wool, slag wool and glass wool are used extensively in thermal and acoustic insulation, typically in buildings, vehicles and appliances. Refractory ceramic fibers are designed for high-temperature applications, mainly in industrial settings. Continuous glass filament is used primarily in reinforced composite materials for the insulation, electronics and construction industries (IARC, 2002; National Toxicology Program, 2009). Man-made vitreous fibers have some physical similarities to asbestos, in particular, their fibrous character which gives them the same aerodynamic properties and leads to their deposition throughout the respiratory tract. Unlike amphibole asbestos, however, they are synthetic and amorphous, and generally have a lower biopersistence in lung tissues. Also, unlike serpentine asbestos, they tend to break transversely rather than cleaving along the fiber axis (IARC, 2002).

Data on respiratory cancer of man-made mineral (MMMFs) and vitreous fibers (MMVFs) are not consistent. Statistically significant increases in respiratory cancer mortality were observed among glass wool-exposed workers in unadjusted analyses in the United States (Marsh et al., 2001), European (Boffetta et al., 1997), and Canadian cohorts (Shannon et al., 2005). Excesses of lung cancer incidence were observed among the European workers (Boffetta et al., 1997) and Canadian workers (Shannon et al., 2005), but not among French workers (Moulin et al., 1986). Marsh et al., (2001) concluded that the US cohort study of man-made vitreous fiber workers has not provided consistent evidence of a relationship between man-made vitreous fiber exposure and mortality from malignant or non-malignant respiratory disease. Gillissen et al. (2006) stated that MMMFs or MMVFs including glass wool, rock wool, slag wool, glass filaments, microfibers, refractory ceramic fibers are bioactive under certain experimental conditions. Although it has been shown that MMMFs may cause malignancies when injected intraperitoneally in high quantities in rodents, inhalation trials and human studies have not shown such effects. The amorphous structure of synthetic vitreous fibers facilitates designing fibers with low biopersistence. In 2001, IARC reclassified these fibers from Category 2b to Category 3 (with RCF and special purpose fibers remaining in 2b) based on epidemiological data and the animal studies database indicating that there is little if any health risk associated with the use of SVFs of low biopersistence (Bernstein, 2007).

Occupational or environmental exposures to many inhaled particles and fibers have been linked with immunotoxicity. First of all, silica and silicates have been associated with the development of lung inflammation, interstitial fibrosis, bronchitis, small airway disease, emphysema, and vascular diseases as well as immunologic reactions (Song & Tang, 2011). Recent studies showed that Th1 and Th2 cytokines may be involved in silicosis and regulatory T-cells (Treg cells) have crucial role in modulation of immune homeostasis by regulating Th1/Th2 polarization. Studies in animals provided knowledge that depletion of Tregs may attenuate the progress of silica-induced lung fibrosis and enhance Th1 response and decelerate Th1/Th2 balance toward a Th2 phenotype in silica-induced lung fibrosis (Liu et al., 2010). Exposure to diesel exhaust particles (Inoue & Takano, 2011), coal dust (Ates et al., 2011), soil dust (Schenker et al., 2009), beryllium (Martin et al., 2011; Mikulski et al., 2011; Sood, 2009), heavy metal fumes (Montero et al., 2010) can evoke new or facilitate existing immune-mediated pulmonary inflammation.

### 1.3.3 NiO nanoparticles

Nickel and nickel compounds are widely used in industry. In occupational settings, exposure to nickel and nickel compounds occurs primarily during nickel refining, electroplating, and welding. In addition to nickel, workers in metal mining and processing are exposed to diesel emissions, oil mists, blasting agents and also to various other substances prevalent in the mine or industry (Lightfoot et al., 2010). Some of them, such as silica (Costantini et al., 2011; Huaux, 2007), radon (Chauhan et al., 2011) or arsenic (Burchiel at al. 2009) are known to be potent immunotoxic agents thus implicating possible synergistic effects on the immune response. The most common airborne exposures to nickel in the workplace are to insoluble nickel species, such as metallic nickel, nickel sulfide, and nickel oxides from dusts and fumes. The chemical and physical properties of nickel and nickel compounds strongly influence their bioavailability and toxicity. The lung and the skin are the principal routes of entry and target organs of occupational exposure. The most serious adverse health effects due to occupational exposure to nickel and its compounds are lung fibrosis and lung cancer, nickel is also hematotoxic, hepatotoxic and nephrotoxic. Allergic skin reactions are relatively common in individuals who are exposed to nickel (Brüske-Hohlfeld, 2009; Das & Buchner, 2007; Panizza, 2011; Zhao, 2009).

Recently, nickel nanoparticles are increasingly used in modern industries such as catalysts, sensors and electronic applications (Ahamed, 2011). Due to known toxic effects of "bulk" nickel products, caution in industrial applications of new nickel nanoproducts is important. Several *in vivo* studies in rats demonstrated that nickel oxide nanoparticles (NiO NP) have inflammatory effects in lungs by transient increase in cytokine expression (IL-1alpha, IL-1beta in lung and monocyte chemotactic protein-1 in bronchoalveolar lavage fluid) and persistent increase in CC chemokine (macrophage inflammatory protein-1alpha in lung and bronchoalveolar lavage fluid - BALF) (Morimoto et al., 2010). Cytokine-induced neutrophil chemoattractant-1 (CINC-1), CINC-2 alpha, beta, and CINC-3 were involved in the persistent pulmonary inflammation by NiO NP (Nishi et al., 2009) but NiO NP did not induce the gene expression of MMP-2 and TIMP-2 mRNA in rat lungs (Morimoto et al., 2011). *In vitro* assessment of the toxic effect of nickel nanoparticles in human lung epithelial A549 cells showed reduced mitochondrial function, induction of the leakage of lactate dehydrogenase (LDH) and induction of oxidative stress in dose and time-dependent manner (Ahamed, 2011).

Other airborne and engineered nanoparticles in addition to nickel, such as carbon nanotubes (He et al., 2011), titanium dioxide (Morimoto et al., 2011), cobalt - $Co_3O_4$ (Cho et al., 2011a) or quantum dots (Jacobsen et al., 2009) has been reported to induce lung inflammation. Anoher example is ZnO nanoparticles (ZnO NP) discovered to induce eosinophilia, proliferation of airway epithelial cells, goblet cell hyperplasia, and pulmonary fibrosis. Fibrosis was associated with increased myofibroblast accumulation and transforming growth factor-beta positivity. Serum IgE levels were up-regulated by ZnO NP along with the eosinophilia whilst serum IgA levels were down-regulated by ZnO NP (Cho et al., 2011b).

## 1.4 Hazard assessment of mineral fibers and particles

Humans may be exposed to fibrous particles from a variety of sources, including occupational settings, ambient air, consumer products, drinking water and food (De Vuyst et al., 1995). Potential effects of airborne fibers in humans can only occur after a complex

process of inhalation, deposition, elimination, retention and translocation. The biological effects of inhaled fibers are highly dependent on dose (fiber exposure concentration - numbers of long fibers), fiber size (diameter/length), (Donaldson & Tran, 2004; Kohyama et al., 1997; Yamato et al., 1998), durability of material in the organism (biopersistence) (Mossman et al., (2011), duration of exposure, chemical composition and properties, solubility, chemical reactivity, surface properties of the material, ability of a material to interact with body proteins etc. Host factors such as efficiency of defense mechanisms of the respiratory tract between the initial deposition and the ultimate contact of the fibers with the target cell, individual susceptibility to xenobiotics, metabolism, age, nutrition status, life style (smoking), presence of immune disease (asthma, allergic rhinitis, immunosuppression etc.) and other factors influence the development of immunotoxicity.

## 2. *In vitro* studies on lung cells

The lung consists of more than 40 different cell types; each type has its special function, localization and morphology. From the toxicological point of view, the most important cell types being alveolar macrophages (AM - free living cells, whose function is to phagocyte the inhaled particles and maintain the alveoli clean and sterile) and alveolar epithelial type II cells (TII - localized on the inner surface of lung alveoli and which play an important role in tissue renewal). For this reason we focused on these cell types, isolated them from rats and maintained them in cell culture (Hoet et al., 1994; Richards et al., 1987). After 20 h cultivation the cells were exposed to different dose (1, 5, 10 $\mu g.ml^{-1}$) of mineral fibers and the cultivation was prolonged for another 20 h period when the experiment was terminated and the analyses were done.

### 2.1 Lectin histochemistry

*Bandeiraea simplicifolia* agglutinin (BSA) and *Maclura pomifera* agglutinin (MPA) are able to bind to the terminal N-acetyl-α-galactosaminyl or α-D-galactose/galactosamine residues in the membranes of AM and TII cells which makes them suitable for detection of cell membrane injuries (Tatrai et al., 1994). Control cells showed regular, linear staining with BSA or MPA. Stone wool at the concentration 5 $\mu g.ml^{-1}$ caused moderate injury to membranes of AM and incomplete phagocytosis in a small fraction of AM. Alveolar epithelial type II cells did not develop detectable membrane damage at any tested fiber dose. Refractory ceramic fibers (RCF) evoked changes in both cell types only at the highest dose: the membranes were not continuous and reduplicated. Wollastonite caused a decreased reaction in the membranes only at the highest dose. After exposure to the lowest dose of crocidolite the membranes of both cell types were fragmented irregularly and frustrated phagocytosis could be found in AM (Tátrai et al., 2004; 2006a; 2006b).

### 2.2 Effect of mineral fibers on cells using TEM

The control cells and those exposed to fibers at a dose of 1 $\mu g.ml^{-1}$ were examined. TII cells did not show any alterations after RCF or stone wool exposure. AM cells phagocyted RCF fibers without injuries of cell organelles, intact organelles remained also after exposure to stone wool. In both cell types crocidolite evoked severe damage in the organelles and necrobiosis of whole cells (Tátrai et al., 2004; 2006a; 2006b).

### 2.3 Immunological studies

Production of proinflammatory peptides MCP-1 and MIP-1α was assayed in growth media after termination of cell cultivation. Exposure to wollastonite did not change production of MCP-1 and MIP-1α in TII cells, but in AM the production was significantly enhanced: MCP-1 at the concentration 10 $\mu g.ml^{-1}$, MIP-1α at the concentration 5 $\mu g.ml^{-1}$. The results after exposure to stone wool were different: in TII cells the production of MCP-1 was enhanced at all concentrations, MIP-1α at doses of 5 and 10 $\mu g.ml^{-1}$; in AM the production of both cytokines was statistically significantly enhanced after doses of 5 and 10 $\mu g.ml^{-1}$. Crocidolite evoked statistically significant dose dependent enhancement of the production of MCP-1 in AM, for MIP-1α and both cytokines in TII at doses of 5 and 10 $\mu g.ml^{-1}$ in all cases (Tátrai et al., 2004; 2006a; 2006b). Comparing the results from different fibers on 2 various primary cell types the following differences are clearly seen: crocidolite (asbestos) evoked the greatest changes, both morphologically and functionally. Effects of wollastonite were seen more significant comparing to stone wool. AM cells are more sensitive to the fibers exposure than TII cells.

## 3. Animal model

### 3.1 Intratracheal instillation studies in rat model

Four types of fibers: asbestos and three types of ASMF fibers (asbestos substitute mineral fibers): wollastonite, rock wool and glass fibers were intratracheally instilled at 2 doses (2 mg or 8 mg of fibers) to Fisher 344 rats. Dose of 2 mg was suspended in 0.2 ml of saline solution per animal or control group with 0.2 ml saline only. A dose of 8 mg was divided and instilled 4 times (weekly 2 mg/0.2 ml saline solution). The assays were performed 4 or 16 weeks after last instillation of the fibers. After sacrifice, markers of immune response and hematology were analyzed. Immunotoxic effects were examined using a panel of immune and hematological assays. Phenotypic analysis of leukocytes (T-lymphocytes, activated T-cells, B-lymphocytes, NK-cells, T-helpers, T-cytotoxic cells) and expression of adhesion molecules (CD11b, CD54) were performed by flow cytometry. Immune functions were evaluated by proliferative activity of T and B-lymphocytes *in vitro* stimulated with mitogens and antigen and phagocytic activity of leukocytes.

Our findings demonstrate the immunomodulatory effect of mineral fibers in the rat animal model 4 and 16 weeks after intratracheal exposure to amosite, wollastonite, rock wool and glass fibers. Significant changes were observed in total white blood cell count and percentages neutrophils in all fiber-treated, especially high-dosed, animals after 4 weeks of exposure. The percentage of lymphocytes was altered in rock wool fiber-treated especially in high-dosed animals after 4 weeks of exposure (Table 3.1-1).

Analysis of lymphocyte subsets showed significantly increased percentage of T-lymphocytes, mainly cytotoxic cells and decreased percentage of B-lymphocytes in peripheral blood of animals exposed to amosite. Rats exposed to wollastonite had increased percentage of T-helper cells. Exposure to mineral fibers decreased expression of adhesion molecule CD54 (ICAM-1) on granulocytes (amosite, glass fibers) and monocytes (rockwool). Suppressed expression of adhesion molecules CD11b was found on granulocytes (wollastonite, glass fibers) and monocytes (glass fibers) (data not shown).

| | | Amosite | Wolastonite | Rock wool | Glass Fibers |
|---|---|---|---|---|---|
| White blood cells ($10^9$/l) | 4 weeks, 2mg | | | | |
| | 4 weeks, 8mg | ↓ * | ↓ * | | ↑ * |
| | 16 weeks, 2mg | | | ↓ * | |
| | 16 weeks, 8mg | ↓ * | | | |
| Neutrophils (%) | 4 weeks, 2mg | | | | |
| | 4 weeks, 8mg | ↑ * | ↑ * | ↑ * | ↑ * |
| | 16 weeks, 2mg | | ↑ * | | |
| | 16 weeks, 8mg | | | | |
| Lymphocytes (%) | 4 weeks, 2mg | | | | |
| | 4 weeks, 8mg | | | ↓ * | |
| | 16 weeks, 2mg | | | | |
| | 16 weeks, 8mg | | | | |

* $p<0.05$; ** $p<0.01$; *** $p<0.001$; ↓ - decrease ↑ - increase in comparison with relevant control;

Table 3.1-1. White blood cell (WBC) count and differential WBC in Fisher 344 rats administered with 2 mg or 8 mg of amosite, wollastonite, rock wool or glass fibers.

Although amosite seems to be most potent suppressor of T- and B-lymphocyte proliferation, especially in high-dosed animals, wollastonite and rock wool also interfered with lymphocyte proliferation and suppressed the response of T-lymphocytes. The opposite stimulative effect on proliferative capacity of B-cells was found in animals exposed to glass fibers (Table 3.1-2). Phagocytic activity was dramatically affected by exposure to rock wool and glass fibers. A highly significant dose-dependent suppression was found in neutrophils and monocytes. Rats exposed to wollastonite fibers also had decreased phagocytic activity of peripheral blood phagocytes 4 weeks after instillation of either dose. Surprisingly, the phagocytic activity of animals exposed to amosite was affected only in high dosed rats (Table 3.1-2).

In conclusion, animal exposure to mineral fibers leads to alterations in systemic immune response. Immune dysregulation consisted of changes of the main lymphocyte subsets. Moreover, the function of immunocompetent cells that are responsible for the specific immune response (T- and B-lymphocytes) and phagocytic cells was impaired. Our results correspond with the hypothesis of Hurbánková (1994), who observed that the phagocytic activity of granulocytes and monocytes is altered in asbestos-treated rats up to one year following treatment, displaying a two-phase progress: an initial increase (phase I) followed by a decrease below the average values of the control animals (phase II).

### 3.2 Inhalation studies in rat model

The effects of industrial fibrous dusts on the respiratory system represent a potential environmental and occupational health hazard for humans. Chronic asbestos exposure can cause pleural plaques, asbestosis and cancer diseases. These effects stimulated research activities aiming at the study of the health effects of fibrous substitutes as well as combined effects with other noxious materials respectively (Boor et al., 2009; Donald & Gardner, 2006; IARC, 2002; 2004). This study gives information about the dose-response relationships after

| Function of lymphocytes | | Amosite | Wolastonite | Rock wool | Glass Fibers |
|---|---|---|---|---|---|
| Proliferative activity of T-lymphocytes | 4 weeks, 2mg | ↑ Con A * | | ↓ PHA ** | |
| | 4 weeks, 8mg | ↓ Con A *** ↓ PHA ** | ↓ Con A *** ↓ PHA *** | | |
| | 16 weeks, 2mg | | | ↓ CD3 * | |
| | 16 weeks, 8mg | ↓ Con A ** | | | |
| Proliferative activity of B-lymphocytes | 4 weeks, 2mg | | | | ↑ PWM *** |
| | 4 weeks, 8mg | ↓ PWM * | | | ↑ STM * |
| | 16 weeks, 2mg | | | | |
| | 16 weeks, 8mg | ↓ PWM *** | | | |
| Function of phagocytes | | | | | |
| Phagocytic activity of neutrophils | 4 weeks, 2mg | | ↓ * | ↓ *** | ↓ *** |
| | 4 weeks, 8mg | ↓ *** | ↓ ** | ↓ ** | ↓ *** |
| | 16 weeks, 2mg | | | ↓ * | ↓ * |
| | 16 weeks, 8mg | | | ↓ ** | ↓ * |
| Phagocytic activity of monocytes | 4 weeks, 2mg | | ↓ * | ↓ *** | ↓ *** |
| | 4 weeks, 8mg | ↓ *** | ↓ * | ↓ *** | ↓ *** |
| | 16 weeks, 2mg | | | ↓ * | |
| | 16 weeks, 8mg | | | ↓ ** | ↓ * |

* $p<0.05$; ** $p<0.01$; *** $p<0.001$; ↓ - decrease in comparison with relevant control; ↑ - increase in comparison with relevant control

Table 3.1-2. Activity of immune cells measured via lymphocyte proliferation test and phagocytic test in Fisher 344 rats administered with 2 mg or 8 mg of amosite, wollastonite, rock wool or glass fibers.

inhalation of two concentration levels of amosite asbestos and wollastonite alone or combined with daily exposure to cigarette smoke together with the basic lung inflammation and cytotoxic parameters. Male Fisher 344 rats were exposed for 6 months. Animals inhaled amosite asbestos or wollastonite fibers in a nose-only inhalation device (In-Tox, USA). Amphibole asbestos - amosite and wollastonite fibers belong to naturally occurring silicate inorganic fibers. Wollastonite is used as a substitute of asbestos. Dust aerosol was produced at two dosages: 30 mg/m$^3$ air and 60 mg/m$^3$ air for one hour per exposure. Exposure of animal groups to dusts proceeded every second day, 5 days per week. Six groups, each of 11 animals were exposed to:

- 60 mg/m$^3$ amosite fibers for one hour every two days; combined with exposure to mainstream smoke from three cigarettes daily;

- 60 mg/m$^3$ amosite fibers for one hour every two days;
- 30 mg/m$^3$ amosite fibers for one hour every two days, combined with exposure to mainstream smoke from three cigarettes daily;
- 30 mg/m$^3$ amosite fibers for one hour every two days;
- exposure to mainstream smoke from three cigarettes daily plus immobilization stress as for animals exposed to dust;
- immobilization stress as for animals exposed to dust.

Cigarette smoke exposure: Standard research cigarettes of the 1R1 type (Tobacco and Health Research Institute - THRI, Lexington, KY, USA) were used in all experiments. A whole-body actively ventilated exposure chamber was used, with a cigarette smoke generator and pumps (THRI, Lexington, KY, USA) allowing all smoker animal groups to breathe at the same time diluted main-stream tobacco smoke at the target concentration 30 mg of total particulate matter (TPM)/m$^3$ air for one hour daily (an exposure requiring to burn three cigarettes).

| Length [μm] | % | Diameter [μm] |
|---|---|---|
| < 20 | 5 | |
| 20 – 30 | 75 | 0.71 |
| >30 | 20 | |

| Diameter [μm] | % |
|---|---|
| = 1 | 47 |
| <1 | 22 |
| <3 | 21 |
| =3 | 6 |
| >3 | 4 |
| Length [μm] | % |
| 1 – 10 | 48 |
| 11 – 30 | 40 |
| >30 | 12 |

Tables 3.2-1. and 3.2-2. Length, diameter and percentage of wollastonite fibers (top) and amosite fibers (bottom).

The aim of our study was to find and compare the combined effect of amosite or wollastonite (asbestos substitute) with cigarette smoke on the selected immune, inflammatory and cytotoxic parameters. The rats inhaled two doses: 30 and 60 mg/m$^3$ of amosite (asbestos) and wollastonite fibers (mineral asbestos substitute) for 1 hour every 2 days and cigarette smoke from 3 cigarettes/day. They were sacrificed after 6 months exposure.

### 3.2.1 Combined effect of mineral fibers and tobacco smoke on respiratory tract

Six months after the beginning of the inhalation exposures, the animals were anesthetized and BAL was performed.

The following BAL parameters were examined:

Inflammatory response biomarkers

- Total cell count/ml BAL (bronchoalveolar lavage) fluid
- AM count/ml BAL fluid
- Differential cell count (alveolar macrophages - AM, lymphocytes - Ly, granulocytes - Gr)

Cytotoxic parameters

- Phagocytic activity of AM
- Viability of AM
- Lactate dehydrogenase activity (in the cell - free lavage fluid)
- Acid phosphatase activity (in the cell- free lavage fluid and in the BAL cell suspension)
- The cathepsin D activity (in the cell - free lavage fluid and in the BAL suspension)

Methods are described in papers of Hurbánková & Kaiglová (1999) and Černá et al. (2004). The results were statistically evaluated using Mann-Whitney test.

| | Fibers alone | | | Fibers/Tobacco smoke | | |
|---|---|---|---|---|---|---|
| | Control | 30 mg/m$^3$ | 60 mg/m$^3$ | Tobacco smoke alone | Tobacco smoke + fibers 30 mg/m$^3$ | Tobacco smoke + fibers 60 mg/m$^3$ |
| N | 7 | 7 | 7 | 7 | 7 | 6 |
| Total cell count/ml BALF ($10^3 \cdot ml^{-1}$) | | | | | | Am ↑ ** |
| AM count/ml BALF ($10^3 \cdot ml^{-1}$) | | | | | | Am ↓ ** |
| Ly % | | Am ↑ * | Am ↑** | | | Am ↑ *** |
| AM % | | Am ↓ * | Am ↓ * | Am ↓ * | Am ↓ ** | Am ↓ *** |
| PMN % | | | Am ↑ * | | Am ↑ * | Am ↑ ** |
| Immature forms of AM (%) | | Am ↑ * | | Am ↑ ** | Am ↑ * | Am ↑ ** |
| Multinucleated cells (%) | | | Am ↑** | | | |

Comparison of exposed group with the control group; *p<0.05, **p<0.01, ***p<0.001; ↑ - increase against compared group; ↓ - decrease against compared group; abbreviations: Le - leukocytes; AM - alveolar macrophages; PMN – polymophonuclear leukocytes, BAL - bronchoalveolar lavage

Table 3.2-3. Amosite – inhalation exposure - with/without tobacco smoke; inflammatory response parameters in BAL.

Increased numbers of bronchoalveolar lavage fluid (BALF) cells after asbestos or other fiber-exposure as a result of inflammatory response have been described by numerous authors (Hurbankova & Kaiglova, 1999; Greim et al., 2001; Morimoto & Tanaka, 2001; Osinubi et al., 2000). In our study, a significantly increased number of BALF cells after exposure to amosite in comparison with the control group was observed in the smoker plus 60 mg/m$^3$ fiber group (by 11.4 %) as well as in the corresponding-dose, non-smoker group (by about 16%). This increase could be ascribed to the increase of lymphocyte population proportions. These changes were accompanied by an inverse change in the AM count in BALF, which significantly decreased in the same group exposed to combined higher dust plus cigarette smoke. A very similar but shorter exposure only to cigarette smoke has been reported to lead to a higher (35%) difference of BALF cell counts in comparison with the control values (Hurbánková et al., 2010; Ishihara et al., 1997; Nelson & Kelsey, 2002). The higher

| | Fibers alone | | | Fibers/tobacco smoke | | |
|---|---|---|---|---|---|---|
| | Control | 30 mg/m$^3$ | 60 mg/m$^3$ | Tobacco smoke alone | Tobacco smoke + fibers 30 mg/m$^3$ | Tobacco smoke + fibers 60 mg/m$^3$ |
| N | 7 | 7 | 7 | 7 | 7 | 6 |
| Phagocytic activity of AM (%) | | | | Am ↓** Woll ↓** | Am ↓** | Am ↓* |
| Viability of living AM (%) | | | Am ↓* | | | Am ↓* |
| LDH μkat.g prot.$^{-1}$ | | | | | | |
| ACP nkat.g prot.$^{-1}$ | | | | | Woll ↑* | |
| ACP nkat.10$^{-6}$ cells | | | | | | |
| Cathepsin D $U_{tyr}$.mg prot.$^{-1}$ | | | | | Am ↑* Woll ↑* | Woll ↑* |
| Cathepsin D $U_{tyr}$.10$^{-6}$ cells | | Woll ↑* | Am ↑* Woll ↑* | Am ↑ ** | Am ↑ ** | Am ↑ ** |

Comparison of exposed groups with the control group: *p<0.05, **p<0.01, ***p<0.001; ↑ - increase against compared group, ↓ - decrease against compared group; (1) enzyme activity expressed as μmol of p-nitrophenol.hour$^{-1}$ mg protein$^{-1}$; abbreviations: LDH: lactate dehydrogenase; ACP: acid phosphatase; $U_{tyr}$ : μg of thyrosine released in an hour time

Table 3.2-4. Amosite and wollastonite - inhalation exposure - with/without tobacco smoke; cytotoxic parameters in BAL.

proportions of PMN and lymphocytes in the BALF than control values indicate the presence of inflammation in the lungs at sacrifice. The magnitude of the increase of these parameters was dose-dependent. AM are the predominant cells present in BALF and changes in their number or function are important factors determining the lung inflammatory response and characterizing the pathogenesis of such response. A decrease in macrophage number or phagocytic capacity may result in the reduction of the clearance of inhaled materials and thus can lead to an increase of the effective dose of the potentially injurious agent (Aoshiba et al., 2001; Dziedzic et al., 1993). A significant reduction in the number of AM after intratracheal instillation of amosite has been observed also in our previous experiments (Hurbankova & Kaiglova, 1999). Associated with inflammatory changes, a dose-dependent increase in multinuclear cells (MNC) proportions was found in the BALF as well as in the lung tissue suspensions. MNC were increased after exposure (separate or in combination) to tobacco smoke as well as both fiber concentrations but significantly only after higher dose without smoking. Similarly, immature forms of AM in all exposed groups were increased in comparison with control (Beňo et al., 2005). Strongly dose dependent decrease of AM viability (higher dose with and without smoking) as well as phagocytic activity of AM (all group with smoking) was found in this experiment. That is in accordance with previously described effect of asbestos (Hurbánková & Kaiglová, 1999).

Increased LDH and ACP activity in extracellular fluids are generally accepted as good markers of cell or tissue injury and used for evaluation of the cytotoxic effect. We did not

find significant changes in activities of measured enzymes in our experiment. Cathepsin D activity was significantly changed after amosite inhalation. These results are in good accordance with study of Sjörstrand et al. (1989). Wollastonite inhalation confirmed the lower cytotoxicity in comparison with asbestos. Significant changes were found only by measurement of cathepsin D activity in BAL cells (increased levels), decreased percentage of phagocytic activity of AM in "tobacco smoke alone" group and increased levels of ACP in "tobacco smoke + wollastonite fibers 30mg/m$^3$"group.

**Amosite**

- Inflammatory parameters were mostly changed after 60mg/m$^3$ in combined group (amosite exposure and tobacco smoke).
- Tobacco smoke alone induced changes in inflammatory parameters. It confirms that smoking alone might play an important role in inflammatory processes.
- Smoking alone caused some changes of cytotoxic parameters and intensified the harmful effect of amosite exposure.
- Mild dose dependence between 30mg/m$^3$ and 60mg/m$^3$ in groups without tobacco smoke was seen.

**Wollastonite**

- No dose dependence of inflammatory parameter changes in this study was recorded in groups without smoking and very weak in combined exposure groups.
- Mild dose dependence of cytotoxic parameters changes in groups without or with tobacco smoke was observed.
- Influence of tobacco smoke on cytotoxic parameters was not explicit.

### 3.2.2 Combined effect of mineral fibers and tobacco smoke on immune parameters

Cellular immunity was examined by phenotypic analysis of leukocytes ($CD3^+$, MHC II, $CD4^+$, $CD8^+$, $CD161^+$, B-lymphocytes) and by expression of adhesion molecules (CD11b, CD54) on leukocytes (Table 3.2-5). Inhalation of high dose of amosite fibrous dust resulted in a significantly increased percentage of B-lymphocytes and elevated expression of adhesion molecule CD11b on lymphocytes of peripheral blood in non-smoking rats. Similarly, inhalation of high dose of wollastonite increased the percentage of B-lymphocytes, and this elevation was reinforced with combined exposure to lower dose of wollastonite and tobacco smoke. Moreover, the combined exposure to wollastonite and smoking caused a significant, dose-dependent increase of the percentage of cytotoxic cells and enhanced expression of adhesion molecule CD11b on granulocytes in peripheral blood. On the other hand, cigarette smoke and higher dose of wollastonite resulted in decrease of T-cells ($CD3^+$). The stimulative effect of exclusive exposure to smoking on the immune system was shown as significantly elevated percentage of some lymphocyte subsets (T-cytotoxic, T-helper lymphocytes, B-lymphocytes) and elevated expression of adhesion molecule CD11b in comparison with non-smoking animals.

Immune function assays included proliferative response of T- and B-lymphocytes and phagocytic activity of blood leukocytes (Table 3.2-6). The proliferative activity of T-lymphocytes stimulated with CD3 antigen and T-dependent B-cell response in rats exposed to amosite was significantly decreased. The immunosuppressive effect was more pronounced

| Proportion of lymphocyte subsets in peripheral blood | 30 mg/m$^3$ | 60 mg/m$^3$ | Tobacco smoke alone | Tobacco smoke + fibers 30 mg/m$^3$ | Tobacco smoke + fibers 60 mg/m$^3$ |
|---|---|---|---|---|---|
| $CD3^+$ – T-lymphocytes (%) | | | | | Woll ↓ $^a$ |
| $CD3^+$/MHC II – activated T-lymphocytes (%) | | | | | |
| $CD4^+$ – T-helper lymphocytes (%) | | | ↑ ** | | |
| $CD8^+$ – T-cytotoxic lymphocytes (%) | | | ↑ * | Woll ↑ * | Woll ↑ * |
| $CD161^+$- Natural killer cells (%) | | | | | |
| B-Lymphocytes (%) | | Am ↑ * Woll ↑ * | ↑ * | Woll ↑ * | |
| Adhesion molecules on leukocytes | | | | | |
| Expression of CD11b on lymphocytes (%) | | Am ↑ * Woll ↑ ** | ↑ * | | |
| Expression of CD11b on monocytes (%) | | | | | |
| Expression of CD11b on granulocytes (%) | | Am ↑ * | | | Woll ↑ * |
| Expression of CD54 on lymphocytes (%) | | | | | |
| Expression of CD54 on monocytes (%) | | | | | |
| Expression of CD54 on granulocytes (%) | | | | | |

Ly – lymphocytes; Mono – monocytes; Gr – granulocytes; $*/^a$ $p<0.05$; $**/^{aa}p<0.01$; $***/^{aaa}p<0.001$; *– significant level calculated in exposed rats in comparison with control rats without tobacco smoke exposure; $^a$ – significant level calculated in exposed rats in comparison with control rats with tobacco smoke exposure; ↓ - decrease in comparison with relevant control; ↑ - increase in comparison with relevant control; Am – amosite; Woll – wollastonite

Table 3.2-5. Cellular immunity of rats treated via inhalation exposure with two doses of amosite/wollastonite fibers and with/without tobacco smoke.

in low-dosed rats. No effect of exposure to amosite fibers alone on proliferative activity of B-cells stimulated with STM (lipopolysacharide from *Salmonella typhimurium*) was seen in non-smoking rats, while a moderate enhancement was recorded in animals exposed to amosite and tobacco smoke. A marked suppressive effect of amosite on phagocytic activity of leukocytes was also found. Stimulation of the immune system was observed as increased phagocytic activity of leukocytes in animals exposed to cigarette smoke. Animals exposed to wollastonite or cigarette smoke alone caused enhancement of proliferative activity of T-lymphocytes stimulated with concanavalin A. All animals exposed to wollastonite fibers had suppressed phagocytic activity of monocytes and granulocytes. Moreover, decrease of phagocytosis was recorded also in combined exposure to wollastonite and cigarette smoke. In conclusion, inhalation of amosite and wollastonite mineral fibers resulted in marked changes in specific and non-specific immunity. Moreover, findings indicate mutual

| Function of lymphocytes | 30 mg/m$^3$ | 60 mg/m$^3$ | Tobacco smoke alone | Tobacco Smoke + fibers 30 mg/m$^3$ | Tobacco smoke + fibers 60 mg/m$^3$ |
|---|---|---|---|---|---|
| Proliferative activity of T-lymphocytes stimulated with Con A (cpm) | | Woll ↑ * | ↑ * | | |
| Proliferative activity of T-lymphocytes stimulated with PHA (cpm) | | | | | Am ↓ $^a$ |
| Proliferative activity of T-dependent B-lymphocytes stimulated with PWM (cpm) | Am ↓ * | | | | |
| Proliferative activity of B-lymphocytes stimulated with STM (cpm) | | | | | Am ↑ * |
| Proliferative activity of T-lymphocytes stimulated with CD3 (cpm) | Am ↓ * | Am ↓ * | | | |
| Function of phagocytes | | | | | |
| Phagocytic activity of neutrophils (%) | Woll ↓ * | Am ↓ ** Woll ↓ * | ↑ * | Am ↓ $^a$ | Am ↓ $^{aaa}$ |
| Phagocytic activity of monocytes (%) | Am ↓ ** Woll ↓ * | Am ↓ *** Woll ↓ ** | | Am ↓ $^{aaa}$ Woll ↓ * | Am ↓ $^{aaa}$ Woll ↓ * |

*/$^a$ p<0.05; **/$^{aa}$p<0.01; ***/$^{aaa}$p<0.001; *– significant level calculated in exposed rats in comparison with control rats without tobacco smoke exposure; $^a$– significant level calculated in exposed rats in comparison with control rats with tobacco smoke exposure; ↓ - decrease in comparison with relevant control; ↑ - increase in comparison with relevant control; Woll – wollastonite; Am – amosite; Con A-concanavalin A; PHA-phytohemmagglutinin; PWM-pokeweed mitogen; STM-lipopolysaccharide from *Salmonella typhimurium*; CD3-alloantigen

Table 3.2-6. Proliferative activity of lymphocytes and phagocytic activity in rats treated via inhalation exposure with two doses of amosite/wollastonite fibers and with/without tobacco smoke.

interference of mineral fibers and smoking in the modulation of the systemic immune response during combined exposure.

### 3.3 Assessment of Immunotoxicity of ceramic fibers and NiO nanoparticles

The aim of the study was the assessment of immune effects of exposure to ceramic fibers and/or NiO nanoparticles in experimental animals – male Sprague-Dawley rats. Rats were treated by intratracheal instillation with 1 mg of refractory ceramic fibers and/or 1mg NiO nanoparticles. Controls were treated with 1 ml physiological saline (1ml per animal). One and six months after instillation, the animals were killed. The blood samples were taken and the spleen was aseptically removed and placed into RPMI medium. Panel of immune assays was performed. The phagocytic activity of blood monocytes and granulocytes was assessed by ability to ingest bacteria *Staphylococcus aureus* (Tulinska et. al., 2005). One month after exposure of animals to ceramic fibers and/or NiO nanoparticles no alterations in phagocytic activity and respiratory burst was shown. However 6 months after exposure, situation was

different. Exposure to NiO nanoparticles and combined exposure to ceramic fibers and NiO nanoparticles caused significantly increased phagocytic activity of granulocytes, as well as percentage of cells with respiratory burst (Table 3.3-1). NiO nanoparticles and combined exposure of ceramic fibers and NiO nanoparticles stimulated this important function of nonspecific immune response.

| Function of phagocytes | Ceramic fibers 1 month | NiO nanoparticles 1 month | Ceramic fibers and NiO nanoparticles 1 month |
|---|---|---|---|
| Phagocytic activity of monocyes (%) | | | |
| Phagocytic activity of granulocytes (%) | | | |
| % of phagocytic cells with respiratory burst | | | |
| | Ceramic fibers 6 months | NiO nanoparticles 6 months | Ceramic fibers and NiO nanoparticles 6 months |
| Phagocytic activity of monocyes (%) | | | |
| Phagocytic activity of granulocytes (%) | | ↑ * | ↑ *** |
| % of phagocytic cells with respiratory burst | | ↑ * | ↑ ** |

* $p<0.05$, ***$p<0.001$; ↑ - increase in comparison with relevant control

Table 3.3-1. Phagocytic activity and respiratory burst of peripheral blood cells in male Sprague-Dawley rats administered with 1 mg refractory ceramic fibers, NiO nanoparticles and combined exposure to both elements.

Function of T- and B-lymphocytes was studied using lymphoproliferation assay in spleen cells derived from rats exposed to ceramic fibers and/or NiO nanoparticles. Cells were *in vitro* stimulated with mitogens - concanavalin A (Con A), phytohemagglutin (PHA) and pokeweed (PWM) mitogen. One month after exposure, significant decrease of proliferative activity of lymphocytes stimulated with all three mitogens was found in animals exposed to ceramic fibers. To the contrary, 6 months after exposure, significant increase of lymphocyte proliferation stimulated with phytohemagglutin and pokeweed mitogen was recorded. The effect of combined exposure to ceramic fibers and NiO nanoparticles on spleen cells was manifested as significant increase of proliferative activity of T-lymphocytes after stimulation with Con A. Moreover, significant increase of basal proliferative response of spleen cells derived from rats 1 month after exposure to NiO nanoparticles alone and combined exposure to fibers and nanoparticles was seen. (Table 3.3-2).

Immunophenotypic analysis of leukocytes was examined using panel of surface markers: CD3, CD4, CD8, CD161 and MHC II. Six months after exposure, immunophenotypic analysis of leukocytes performed by flow cytometry revealed statistically significant decrease of expression of marker for T-lymphocyte subpopulations (CD4, CD8) in rats administered with ceramic fibers. On the other hand, increase of expression of CD4 marker after combined exposure was observed. 6 months after exposure to NiO nanoparticles, significant increase of expression of molecule MHC II on lymphocytes, monocytes and granulocytes was shown. Similar effect of combined exposure on expression MHC II on monocytes and granulocytes was found (Table 3.3-3).

| Function of lymphocytes | Ceramic fibers 1 month | NiO nanoparticles 1 month | Ceramic fibers and NiO nanoparticles 1 month |
|---|---|---|---|
| Proliferative activity of T-lymphocytes stimulated with Conncanavalin A – Con A (cpm) | ↓ * | | ↑ * |
| Proliferative activity of T- lymphocytes stimulated with Phytohemmagglutinin - PHA (cpm) | ↓ *** | | |
| Proliferative activity of T-dependent B-lymphocytes stimulated with Pokeweed mitogen - PWM (cpm) | ↓ * | | |
| Basal proliferative activity (cpm) | | ↑ *** | ↑ *** |
| | Ceramic fibers 6 months | NiO nanoparticles 6 months | Ceramic fibers and NiO nanoparticles 6 months |
| Proliferative activity of T- lymphocytes stimulated with Con A (cpm) | | | |
| Proliferative activity of T- lymphocytes stimulated with PHA (cpm) | ↑ * | | |
| Proliferative activity of T- dependent B-lymphocytes stimulated with PWM (cpm) | ↑ * | | |
| Basal proliferative activity of lymphocytes (cpm) | | | |

* $p<0.05$, ***$p<0.001$; cpm-counts per minutes after $^{3}H$ incorporation into lymphocytes, ↓ - decrease in comparison with relevant control; ↑ - increase in comparison with relevant control

Table 3.3-2. Proliferative response of lymphocytes in male Sprague-Dawley rats administered with 1 mg refractory ceramic fibers, NiO nanoparticles and combined exposure to both elements.

| Proportion of leukocyte subsets in peripheral blood | Ceramic fibers 6 months | NiO nanoparticles 6 months | Ceramic fibers and NiO nanoparticles 6 months |
|---|---|---|---|
| $CD3^+$ – T-lymphocytes (%) | | | |
| $CD4^+$ – T-helper lymphocytes (%) | ↓ * | | ↑ ** |
| $CD8^+$ – T-cytotoxic lymphocytes (%) | ↓ * | | |
| $CD4^+/CD8^+$ lymphocytes (%) | ↓ * | | |
| Expression of MHCII marker on lymphocytes (%) | | ↑ * | |
| Expression of MHCII marker on monocytes (%) | | ↑ ** | ↑ * |
| Expression of MHCII marker on granulocytes (%) | | ↑ ** | ↑ *** |

* $p<0.05$; **$p<0.01$; ***$p<0.001$; ↓ - decrease in comparison with relevant control; ↑ - increase in comparison with relevant control

Table 3.3-3. Proportion of leukocyte subsets in peripheral blood in male Sprague-Dawley rats administered with 1 mg refractory ceramic fibers, NiO nanoparticles and combined exposure to both elements.

Phagocytosis is a major host defense mechanism of the innate immune system. The specific molecular pathways that direct the process of ingestion depend on the size of the particle (Hazenbos & Brown, 2006). Several other mechanisms, such as release of inflammatory mediators, antigen presentation (Garcia-Garcia & Rosales, 2005; Rabinovitch, 1995) and expression of different membrane receptors (Garcia-Garcia, 2005; Johansson et. al., 1997) are also involved. It is known that different pulmonary macrophages (airway, alveolar, interstitial, pleural, intravascular) are an important part of the lung's defenses against particles deposited by inhalation (Oberdorster, 1994). After phagocytic stimulation, macrophages release various chemotactic factors for neutrophils and other inflammatory cells including TNF, neutrophil chemotactic factor and many proinflammatory mediators such as prostaglandins, leukotrienes, thromboxane. Apart from that, macrophages produce free radical oxygen and release lysosome enzymes which may cause lung tissue injury. Published literature, studying influence ceramic and metal nanoparticles *in vitro,* showed that ceramic nanoparticles had effect on production of cytokines in monocytes. This effect resulted to the shift of cytokine balance towards inflammation. Moreover, obtained results showed that nanoparticles have significant effects on the expression of some TLR molecules, suggesting that they could affect cell reactivity to infections by altering the expression of innate receptors. Particularly interesting is the finding that ceramic nanoparticles can enhance expression of TLR chains important for viral-dependent stimulation (Lucarelli et al., 2004). Another study (Yagil-Kelmer et al., 2004) compared influence of ceramic particles on monocytes of peripheral human blood and human monocytes cell line U937. They found out the higher variability of expression of cytokines of primary human blood monocytes from donors in compared with cell line. Importantly, studies consistently demonstrated that smaller, sub-micrometer ceramic particles provoke relative1y larger amounts of the cytokines IL-l alpha, IL-I beta, IL-8, TNF-alpha and IL-10 when compared to 1.5 um particles. The variation in the reactivity of different human individuals to particle stimulation may have highlighted another major contributory factor - genetic capacity of an individual to express related cytokines with their susceptibility to, and subsequently, the severity of, a particular disease (Matthews et al., 2000). Nkamgueu et al., (2000) recorded suppression of phagocytic activity and respiratory burst after *in vitro* exposure of cells to ceramic particles. The results of another study indicated that refractory ceramic fibers 6 months after intratracheal instillation significantly changed the majority of examined BAL parameters. The presence of inflammatory and cytotoxic response in lung may signalize beginning or developing disease process (Hurbankova et al., 2005).

Observations that nickel oxide-induced changes may contribute to significant immunodysfunction are known from immunotoxicity studies examining “bulk” nickel oxide aerosols. 65-day inhalation study in mice showed, that exposure to nickel oxide resulted in increased numbers of lung-associated lymph nodes (LALN), enhanced numbers of nucleated cells in lavage samples, increased antibody-forming cells (AFC) in LALN, but decreased AFC/$10^6$ spleen cells and suppressed alveolar macrophage phagocytic activity (Haley et al., 1990). Significant alterations of humoral immune system and alveolar macrophages were found also in rats after 4 weeks or 4 months of exposure to nickel oxide aerosols, respectively (Spiegelberg et al., 1984). Nanoform might have substantial impact on toxic effects including immunotoxicity. *In vitro* studies demonstrated that ultrafine NiO particles showed higher cytotoxicities toward human keratinocyte HaCaT cells and human lung carcinoma A549 cells *in vitro* than fine NiO particles (Horie et al., 2009). Transmission

electron microscope observations revealed uptake of both ultrafine and fine NiO particles into HaCaT cells. Cellular uptake of NiO nanoparticles (NiO NP) was found to be associated with the release of $Ni^{2+}$ ions after 24-48 h (Pietruska et al., 2011). The intracellular $Ni^{2+}$ release could be an important factor that determines the cytotoxicity of NiO. Pathological features of different sizes of nickel oxide following intratracheal instillation in rats were studied by Ogami et al. (2009). Submicrometer nano-nickel oxide was associated with greater toxicity, as for crystalline silica, than micrometer-sized nickel oxide. Biological effects of factors of particle size reduction, when dealing with finer particles such as nanoparticles, were reconfirmed to be important in the evaluation of respirable particle toxicity.

*In vivo* studies in experimental animals showed persistent high level of inflammation in lungs even at low doses of NiO NP. Cho et al. (2010) described chronic neutrophilic/lymphocytic cytotoxic inflammation in rats 4 weeks after instillation NiO NP accompanied by increased MIP-2, IFN-γ, and LDH in BALF. The alveolar lipoproteinosis evident in NiO NP-exposed lungs was reflected in very high protein and LDH levels in the BALF. Increased levels of neutrophils and macrophages have been observed from 3 days to 3 months after instillation of agglomerated NiO NP suspended in distilled water in Wistar rats (Nishi et al., 2009; Ogami et al., 2009). Gene expression profiling of the rat lung after whole-body inhalation exposure to ultrafine NiO particles induced high expression of genes associated with chemokines, oxidative stress, and matrix metalloproteinase 12 (Mmp12), suggesting that Uf-NiO particles lead to acute inflammation (Fujita et al., 2009). *In vitro* studies conducted to test the possible toxic effects (Ada et al., 2010) bring evidence that one of the contributing underlying mechanisms is oxidative stress. The levels of intracellular reactive oxygen species and lipid peroxidation in A549 cells enhanced with increasing exposure to NiO nanoparticles and growth in gene expressions of HO-1 and SP-D were observed in A549 cells (Horie et al., 2011).

Our data of suppressed proliferative activity of T-lymphocytes and decreased T-dependent B-cell response indicate fiber-induced changes in systemic immune response. The hypothesis that inhaled particles or fibers can exert adverse effects outside of the lung is supported by several studies. Although, most of findings refer to systemic effect of particles, similar influence of fibers can be assumed. For example, ultrafine particles were found to decrease the number of blood PMNs and increase the intracellular oxidation of a fluorescent dye (DCFD) in blood PMNs (Elder et al., 2004). Diesel exhaust particles and carbon black particles had significant adjuvant effect on the local immune-mediated inflammatory response in the draining popliteal lymph node and on the systemic specific IgE response to model allergen ovalbumin in BALB/c mice (Lovik et al., 2003). The data of van Eeden (van Eeden et al., 2002) showed the effects of particulate air pollution on bone-marrow stimulation in animals. Acute exposure to ambient particles accelerates the transit of polymorphonuclear leukocytes (PMN) through the marrow whereas chronic exposure expands the size of the bone marrow pool of PMN. A communication between the fiber-induced processes in the pulmonary compartment and peripheral tissues can be mediated by: 1) leakage of reactive oxygen species and stress-induced cytokines directly into the peripheral blood, 2) (pre)activation of peripheral blood leukocytes that can result in aberrant homing and activation of inflammatory cells in distant tissues, and 3) the liberation of proinflammatory mediators by leukocytes and/or stromal cells present in the pulmonary tissues (Oudijk et al., 2003).

## 4. Molecular epidemiological studies in human population

The possible immunomodulatory effects of mineral fibers, in workers occupationally exposed to asbestos, rock wool and glass fibers, were examined in the context of a large-scale molecular epidemiology study (Ilavska et al., 2005; Tulinska et al., 2004). In addition to biomarkers of immunotoxicity, biomarkers of genotoxicity (Beňo et al., 2005; Dusinska et al., 2004; Horská et al., 2006; Topinka et al., 2004; 2006), oxidative damage and antioxidant defense (Staruchova et al., 2008) were also examined in the same cohorts. The studies involved workers with at least 5 years' exposure to asbestos, rock wool and glass fibers, respectively, at 3 industrial plants in Slovakia. A control group of clerical workers, matched for sex, age, smoking habits and alcohol use were also studied. All workers underwent clinical examination, including functional spirometry testing, and radiological examination.

**Exposure:** Fiber samples were used for asbestos fiber and ASMF identification, fiber morphology and quantification, using a microscope with phase contrast (Nikon, Japan) according to the Reference Method for the Determination of Airborne Asbestos Fiber Concentration at Workplaces by Light Microscopy (Membrane Filter Method), AIA 1979, London, UK. Exposure assessment had been based on personal and environmental monitoring.

**Subjects and health status**: In each plant, 61, 98 and 80 exposed workers and 21, 43 or 36 control clerical subjects, respectively, were recruited. In the case of the asbestos-exposed subjects, an additional town-control group of 49 people was included. Evidence of pulmonary fibrosis was found in 42% of the asbestos-exposed workers, while evidence of pleural fibrosis was found in 24%. The asbestos-exposed cohort had significantly decreased forced vital capacity of lungs as well as forced expiratory volume per first second.

**Immune parameters:** Markers of lymphocyte function were found to differ significantly between fiber-exposed cohorts and corresponding controls. Workers from the former asbestos cement plant had significantly decreased proliferative capacity of lymphocytes stimulated by T-cell mitogen PHA. In contrast, the proliferative activity of T-lymphocytes in subjects from the rock wool and glass fiber factories was stimulated (Table 4). A significant *in vitro* stimulatory effect was observed in cultured B-lymphocytes stimulated with PWM from peripheral blood obtained from the glass fiber workers, while no such effect was found in workers from the asbestos and rock wool plants in comparison with the corresponding controls (Table 4). Although no other published data on functional changes of lymphocytes has been published in rock wool and glass fiber workers, depression of cell mediated immune response with a clear relationship between defective T-cell function and pulmonary fibrosis was seen in asbestos-exposed individuals. *In vitro* studies have clarified that asbestos fibers inhibit proliferation at an early stage ($G_0$ phase) of the cell cycle of PHA-stimulated cells. Besides the evidence for an important role of specific immunity in chemically induced pulmonary disease, including asbestosis, and published results on the protective role of T-lymphocytes especially in asbestos-induced pulmonary inflammation, our data also suggest immunomodulatory effects for two man-made fibers. We propose that the different patterns of T-cell proliferative activity found in workers exposed to asbestos versus rock wool and glass fibers may be due to differences in the duration of exposure to the different fibers as well as differences in the underlying health status of the populations studied. In contrast to the relatively good clinical status, shorter duration and low level of

fiber exposure in rock wool and glass fiber workers, the former asbestos cement workers had historically high levels of exposure to fibrogenic dust and showed clinical evidence of asbestosis and a high prevalence of low forced vital capacity. It is notable that a biphasic immune response has been reported with silica exposure. In a rat model two distinct phases were noted in development of silicosis: in early stages, silica activates both humoral and cellular immunity; however, in late phases no activated adaptive immune system effects were observed.

The phagocytic activity of polymorphonuclear leukocytes and monocytes as well as respiratory bursts of cells did not differ significantly between the exposed and control groups. Similarly, the results of the natural killer cell assays indicated no significant differences in cytotoxic activity of NK-cells between exposed and controls in the cohort exposed to asbestos and rock wool (cytotoxicity assays were not done in the glass fiber workers). Phenotypic analysis of peripheral blood leukocytes was performed to assess the proportions of the main lymphocyte subsets. Flow cytometry analysis revealed significantly decreased expression of markers $CD16^{+}56^{+}$ (natural killer cells) in exposed workers from the glass fiber plant in comparison with the corresponding controls (Table 4). No significant alterations between workers exposed to asbestos, rock wool and glass fibers exposed and controls have been found in proportion of $CD3^{+}$, $CD4^{+}$, $CD8^{+}$, $CD19^{+}$ cells in peripheral blood.

| Table 4 | **Parameters** | **Asbestos** | **Rock wool** | **Glass Fibers** |
|---|---|---|---|---|
| **Hematology** | White blood cell count ($x10^9/l$) | | | ↓ * |
| | Lymphocytes (%) | | | |
| | Basophils and eosinophils (%) | | | |
| | Neutrophils (%) | | | |
| | Lymphocyte count ($x10^9/l$) | | | ↑ ** |
| | Basophil and eosinophil count ($x10^9/l$) | | | ↑ * |
| | Neutrophil count ($x10^9/l$) | | | |
| | Erythrocyte count ($x10^{12}/l$) | | | |
| | Hemoglobin (g/l), hematocrit (%) | | | |
| | Mean cell volume (fl) | | | ↓ * |
| | Platelets ($x10^9/l$) | | | |
| **Function of lymphocytes** | Proliferative activity of T-lymphocytes stimulated with Concanavalin A – ConA (cpm) | | ↑ * | ↑ ** |
| | Index Con A | | ↑ ** | ↑ ** |
| | Proliferative activity of T-lymphocytes stimulated with Phytohemagglutinin - PHA (cpm) | | | ↑ * |
| | Index PHA | ↓ * | ↑ ** | ↑ ** |
| | Proliferative activity of T-dependent B-lymphocytes stimulated with Pokewed mitogen - PWM (cpm) | | | ↑ * |
| | Index PWM | | | |

| Table 4 | **Parameters** | **Asbestos** | **Rock wool** | **Glass Fibers** |
|---|---|---|---|---|
| | Proliferative activity of T-lymphocytes stimulated with CD3 antigen (cpm), Index CD3 | | | |
| | Proliferative activity of lymphocytes stimulated with Tetanus antigen - TET (cpm), Index TET | | | |
| | Basal proliferative activity of lymphocytes (cpm) | | | ↑ ** |
| **Function of phagocytes** | Phagocytic activity of monocytes and granulocytes (%) | | | |
| | Respiratory burst of granulocytes (%) | | | |
| **Function of NK cells** | Natural killer cell activity (%) | | | not done |
| **Proportion of lymphocyte subsets in peripheral blood** | $CD3^+$ – T-lymphocytes (%)<br>$CD3^+$/HLA DR – activated T-cells (%) | | | |
| | $CD4^+$ - T-helper lymphocytes (%) | | | |
| | $CD8^+$ - T-cytotoxic lymphocytes (%) | | | |
| | $CD16^+56^+$ - Natural killer cells (%) | | | ↓ * |
| | CD $19^+$ - B-lymphocytes (%) | | | |
| | CD25, CD81 – activated T-lymphocytes (%) | | | |
| **Adhesion molecules on leukocytes** | Expression of CD62L on lymphocytes (%) | ↑ ** | | |
| | Expression of CD62L on granulocytes (%) | ↑ *** | | |
| | Expression of CD62L on monocytes (%) | ↑ *** | | |
| | Expression of adhesion molecules CD11b, CD11c, CD18, CD49d and CD54 – ICAM on lymphocytes, granulocytes and monocytes (%) | | | |
| **Activation markers on eosinophils** | Expression of CD66b on eosinophils (%) | ↑ *** | | ↑ * |
| | Expression of CD69 on eosinophils (%) | ↑ *** | | |
| **Soluble adhesion molecules** | E-Selectin (ng/ml) | | | ↑ *** |
| | Intercellular Adhesion Molecule - ICAM (ng/ml) | ↑ * | ↑ *** | |
| **Immunoglobulins** | Immunoglobulin A – IgA (mg/dl) | ↑ ** | | |
| | Immunoglobulin E – IgE (U/ml) | ↑ ** | ↑ * | |
| | Immunoglobulin G – IgG (mg/dl) | | | |
| | Immunoglobulin M - IgM (mg/dl) | | ↓ ** | |
| **Complement** | C3 and C4 Components of Complement (mg/dl) | | | |
| **Proinflammatory cytokines** | Interleukin 1 beta – IL1β (pg/ml) | | | |
| | Interleukin 6 – IL6 (pg/ml) | ↑ ** | | |
| | Interleukin 8 - IL-8 (pg/ml) | ↑ *** | ↑ *** | ↑ *** |

* $p<0.05$; ** $p<0.01$; *** $p<0.001$; ↓ - decrease ↑ - increase in comparison with relevant control;

Table 4. Immune parameters measured in population study- humans occupationally exposed to asbestos and two man-made mineral fibers (rock wool and glass fibers).

The expression of adhesion molecules on blood leukocytes was analyzed using flow cytometry. In workers from the former asbestos cement plant, expression of adhesion molecule CD62L (L-selectin) on monocytes and granulocytes was significantly increased (Table 4). Increased levels of soluble adhesion molecules ICAM-1 were found in sera from the cohorts who worked with asbestos and rock wool (Table 4). The chi square test confirmed a significantly increased proportion of people with high levels of soluble ICAM-1 (>306 ng/ml) not only among the asbestos cohort but also in glass fiber workers (asbestos $p<0.02$, glass fibers $p<0.03$) compared with the controls. Exposure to glass fibers enhanced the level of soluble E-selectin in workers' sera (Table 4). Pathologically relevant increases in the expression and function of adhesion molecules have been observed in humans with such pulmonary disease/conditions as bronchial hyperreactivity, allergic rhinitis, idiopathic pulmonary fibrosis or neoplasia.

Analysis of serum levels of proinflammatory cytokines revealed increased serum concentrations of interleukin 6 (IL-6) in former asbestos workers. Significantly elevated serum concentrations of IL-8 were found in workers exposed to all three types of fibers, while no changes in IL-1β were recorded in exposed populations. Inflammatory cytokines are rapidly induced and expressed early in a disease or injury process. They mediate and modulate the healing processes but, if overexpressed, may exacerbate the severity of a disease condition as well as give rise to oxidative stress. Up-regulation of IL-8 secretion has been found in patients with fibrosing lung disease and, because IL-8 is the main chemotactic and activation factor for neutrophils, secretion of IL-8 was associated with neutrophil accumulation in the lower respiratory tract. Since the presence of neutrophils in BAL fluid is frequently reported in humans with asbestosis changes in levels of inflammatory cytokines were examined in the context of the present study.

Exposure to asbestos and rock wool was associated with significantly increased levels of immunoglobulin E. The results of the analysis of expression of markers CD66b and CD69 on eosinophils are summarized in Table 4, where it can be seen that workers from the former asbestos cement plant and glass fiber factory had significantly elevated expression of marker CD66b, while significantly increased expression of CD69 on eosinophils was found only in asbestos workers. Immunoglobulin E is well known as being involved in the mechanisms of development of allergic diseases. The observation of significantly increased levels of total immunoglobulin E in asbestos workers is in agreement with published results of Rosenthal et al., (1999) who concluded that asbestos appears to produce a hyperresponsive state, with chronically exposed individuals manifesting an elevation in circulating immunoglobulins (IgG, IgM, IgE). No data are available on populations occupationally exposed to rock wool for comparison.

## 5. Biomarkers

### 5.1 Proliferative activity of lymphocytes (lymphocyte transformation test)

Lymphocytes are important cells of the adaptive immune response. T-cells are involved in cell-mediated immunity whereas B-cells are primarily responsible for humoral immunity (relating to antibodies). The function of T-cells and B-cells is to recognize specific "non-self" antigens, during a process known as antigen presentation. Our study revealed high sensitivity of T-lymphocyte response to exposure to mineral fibers. Meanwhile in workers

exposed to asbestos, significant suppression of proliferative response of T-cells *in vitro* stimulated with phytohemagglutinin was found, stimulative effect of rock wool and glass fibers on activity of T-lymphocytes in peripheral blood of exposed population were recorded. Our findings indicate that one of the immune targets of mineral fiber exposure seems to be specific cellular immunity. Proliferative activity of lymphocytes might be a sensitive indicator of immunomodulatory effects of mineral fibers; however the limitations of use it as a biomarker of individual susceptibility are interindividual differences.

### 5.2 Phagocytic activity of leukocytes

Pulmonary macrophages are crucial cells in contact with mineral fibers and nanoparticles representing the first line of defense in the lung alveoli. Expansion of macrophages in the lung is a typical characteristic of that type of exposure in both humans and experimental animals. Total macrophage numbers in the lung may increase by migration of blood monocytes, local proliferation of the alveolar macrophages or induced generation of chemotaxins (Rosenthal et al., 1998). Phagocytosis of asbestos fibers has been shown to be accompanied by the activation of macrophages, which results in the generation of ROS as well as a variety of chemical mediators and cytokines. These mediators amplify the local inflammatory reaction. Persistence of asbestos fibers in the lung interstitium or in the sub-pleural connective tissue may lead to a sustained chronic inflammatory reaction accompanied by fibrosis and proliferation of epithelial and mesenchymal cells (Branche, 2009). Surprisingly, in contrast to a marked suppressive effect of mineral fibers on the activity of phagocytes observed in our animal studies, no dramatic influence was found in worker populations. A statistically significant deterioration of phagocytic activity of monocytes was observed only among smoking workers exposed to asbestos, in comparison with exposed non-smokers (Tulinska et al., 2004).

### 5.3 Percentage of $CD16^{+}56^{+}$ cells (natural killer cells – NK cells) and cytotoxic activity of NK cells

Natural killer cells (NK cells) are crucial members of innate immunity responsible for killing of virus infected cells, overseeing of mutated or other way transformed cells and as a first defense line toward cancer cells. They kill cells by releasing small cytoplasmic granules of proteins called perforin and granzyme that cause the target cell to die by apoptosis (programmed cell death). Several authors have reported increased numbers of circulating NK cells and their reduced activity in asbestos exposed humans (Froom et al., 2000; Rosenthal et al., 1999). The results from our study do not confirm these findings. Neither asbestos cement nor rock wool workers were noted to have significant changes in NK-cell activity or the percentage of cells with NK phenotype. However, significantly decreased expression of marker $CD16^{+}56$ was found in glass fiber-exposed workers in comparison with controls. Although the effect was not dramatic, this observation suggests that exposed workers need to be screened preventively for this marker. This finding is surprising because glass fiber exposure has not as yet been connected with malignant tumors as has asbestos. Synthetic vitreous fibers (that include insulation glass wool and continuous glass filament) were reclassified by International Agency for Research on Cancer (IARC) commission from category 2b (*possibly carcinogenic to humans*) to category 3 (*not classifiable as to their carcinogenicity to humans*) in 2001 (Bernstein, 2007; IARC, 2002). Regardless, that in our study population no significant differences in cytotoxic activity of natural killer cells were found

between workers exposed to asbestos and rock wool and corresponding controls (assay in glass fiber workers not done), the assay is considered an important member of a panel to assess antitumor immunity in workers exposed to probable, possible or susceptible carcinogens. We assume that both numbers and activity of NK cells are important in individual health surveys of workers exposed to mineral fibers.

### 5.4 The phenotypical analysis of peripheral blood leukocytes

**T-lymphocytes, CD3, CD4, CD8, HLA DR markers**

Published data suggest that asbestos may affect immunocompetent cells such as $CD4^+$ responder T-cells, $CD4^+$ regulatory T-cells, Th17 T-cells, $CD8^+$ cytotoxic T-cells (CTL) or dendritic cells (DC). Continuous exposure to chrysotile produces a stronger Treg function, at least with the capacity to produce soluble functional factors (i.e., IL-10 and TGF-β) (Kumagai-Takei et al., 2011). Recent research indicates that asbestos is able to act as a superantigen (Otsuki et al., 2007). The increased expression of T-cell receptor Vβ without a clonal expansion of T-lymphocytes has been demonstrated after asbestos exposure. This is in line with our results. We did not detect changes either in absolute or relative number of T-lymphocytes and activated T-lymphocytes in the asbestos exposed workers in comparison to controls. Previous papers referred changes in Th/Ts ratio as well as decreased relative and absolute number of circulating T-lymphocytes (Kagan et al., 1977; Tsang et al., 1988). These parameters were without change also in case of rock wool or glass fiber exposure.

**Expression of CD81 and CD25 on activated T-lymphocytes**

In spite of inhibition of T-cell proliferation observed in the case of asbestos or stimulation in case of glass fibers and rock wool we did not find changes in expression of early activation markers CD81 and CD25 on $CD4^+$ and $CD8^+$ T-cells after PHA stimulation (data not published). Their spontaneous stimulation was not damaged either. Chronic exposure to all of three fibers had no effect on these tested parameters. Similar results were recorded by Wu et al. (2000). The marker CD81 was not expressed on peripheral T-lymphocytes after *in vitro* cultivation with chrysotile asbestos. Dysregulation and long-term T-cell activation can lead to survival of self-recognizing cells, and consecutively to initiation of autoimmune responses. We assume that synthetic mineral fibers do not impact the human organism in the same manner as in the case of asbestos. The expression of T-cells activation markers was not changed after glass fibers and rock wool exposure.

**B- lymphocytes, CD19 marker, and immunoglobulins IgG, IgA, IgM, IgE**

An increased number of B-cells have been reported in patients with asbestosis, fibrosis or malignant diseases after asbestos exposure (Gaumer et al., 1981; Ozesmi et al., 1988). We did not detect this change after asbestos, glass fiber or rock wool exposure. However, among those with asbestos exposure we confirmed a hyperresponsive B-lymphocytes as seen by Rosenthal et al., (1999) with increased levels of immunogloulins IgE and IgA. Exposure to rock wool was also associated with increased IgE levels and in contrast with decreased levels of IgM. Exposure to glass fibers did not affect these parameters. An elevation in serum immunoglobulins (IgA, IgG, IgM, IgE) and mucosal (salivary) IgA and the presence of autoantibodies, antinuclear antibody and rheumatoid factor is one of the most consistent findings in individuals chronically exposed to asbestos (Doll, 1983). IgE is well known for being a central regulator in the allergic reactions. The increased level of IgE and a higher

production of proinflammatory interleukins, IL-6 and IL-8, suggest inflammation with a shift from Th1 to Th2 immune response. Our findings correspond to other studies which confirm a shift towards a Th2 mediated immune response in BAL fluid after asbestos exposure (elevated levels of cytokines IL-1b, Il-4, Il-5, IL-6, Il-13) (Sabo-Attwood et al., 2005; Shukla et al., 2007).

**Activation markers on eosinophils**

Eosinophils are known for their participation on allergic reactions. Pulmonary diseases as asthma or allergic rhinitis are associated with elevated number of circulating eosinophils (Venarske & deShazo, 2003). The expression of activation markers on eosinophils can indicate a growing allergic status. Workers from the World Trade Center crash (with high exposure to asbestos and synthetic mineral fibers) had enormously increased numbers of eosinophils in BAL fluid but circulating eosinophils were not changed (Rom et al., 2002). Also in our study, we did not detect increased number of peripheral blood eosinophils after mineral fibers exposure, but we observed evidence of their activation. The expression of CD69 and CD66b markers was associated primarily with asbestos exposure, and glass fibers enhanced only CD66b. Rock wool did not have impact on these parameters.

**Expression of adhesion molecules CD11b, CD11c, CD18, CD54, CD62L, and CD49d on lymphocytes, monocytes and granulocytes**

Transendothelial migration of leukocytes into tissues is a multistep process. Leukocytes express adhesion molecules as mediators. We evaluated the expression of adhesion molecules CD11b, CD11c, CD18, CD54, CD62L and CD49d on leucocytes and detected the increased expression of L-selectin (CD62-L) on monocytes and granulocytes in workers exposed to asbestos. Selectins mediate the rolling of leukocytes on the stimulated endothelium. Increased numbers of alveolar macrophages in the lower human airways is a typical finding after asbestos exposure (Rosenthal et al., 1999). Circulating monocytes transmigrate to tissue in response to chemotactic factors and become tissue macrophages. The over-expression of CD62L as well as IL-8, a chemotactic factor for neutrophils, may be an important part of this process.

### 5.5 The assessment of soluble markers

**Complement components C3 and C4**

Sabo-Attwood et al., (2005) showed changes in the gene expression of C1 complement component in a mouse model. The incubation of human plasma with asbestos fibers induced production of C5a fragment of C5 component of complement (Governa et al., 2000). We did not detect any changes of C3 and C4 complement components in human serum of workers exposed to mineral fibers (data not published).

**Interleukins IL-1β, IL-6, and IL-8**

Asbestosis is accompanied by persistent inflammation and by production of mediators of inflammation. Certain asbestos substitute fibers, e.g. wollastonite fibers are potential angiogenic agents that can induce regenerative cytokine (IL-6, IL-8) and angiogenic factor production (VEGF-A) resulting in the formation of new blood vessels (Carbonari et al., 2011). Many *in vitro* studies showed that measurement of interleukin levels is equally sensitive for testing of cell activation after air-transmitted particles exposure *in vitro*

(Mitschik et al., 2008). In connection with asbestosis, there are cytokines, mainly IL-1β, IL-6, IL-8, which appear to have a role in pathology of this disease (Mossman & Churg, 1998; Tsuda et al., 1997). In spite of the fact that IL-1β is a proinflammatory cytokine required for the synthesis of others cytokines (e.g. IL-8), we did not detect differences in exposed groups in comparison to controls. Our findings were in accordance to observations of Simeonova and Luster (Simeonova & Luster et al., 1996) who noted an enhancement of IL-8 without IL-1β stimuli. IL-6 was previously known as a factor for B-cell differentiation and immunoglobulin production. The increased level of IL-6 may be associated with the increased IgE and IgA levels seen in asbestos exposed individuals. Monitoring of IL-8 in peripheral blood could serve as an early and sensitive marker of developing pulmonary inflammation in consequence of asbestos, glass fibers and rock wool exposure. Across all three exposed groups we observed an increase of cytokine IL-8. Despite the highly significant ($p<0.001$) differences in IL-8 between exposed workers and human control subjects, these interleukin levels were still in normal reference range.

**Soluble adhesion molecules sICAM-1, sVCAM-1 and sE-selectin**

The soluble adhesion molecules are products of activated endothelial cells. They are known for their involvement in processes of inflammation. Ciebiada et al., (2011) declared that concentrations of sICAM-1 are significantly higher in patients with asthma, and are dependent on a seriousness of disease. Our observations of increased adhesion molecules are in agreement with findings of Kristovich who stated that in the context of the pulmonary microenvironment, TNF-α elaborated by particulate-laden alveolar monocytes could act upon proximal septal capillary endothelial cells, inducing their expression of endothelial leukocyte adhesion molecules ICAM-1, vascular cell adhesion molecule (VCAM -1 and E-selectin (Kristovich et al., 2004). Based on this fact we can speculate that levels of sICAM-1 corresponded with inflammation of the airways. Levels of sICAM-1 were increased in the asbestos exposed group. This was not surprising because asbestos fibers are persistent and insoluble in the lungs and are known as causative factor of inflammation. Although in the case of rock wool was a rather disturbing finding for a reason of better elimination of these inhaled synthetic mineral fibers from organism. Usually they have a high solubility and short-term durability in the airways. Among others, there was a shorter duration and lower concentration of fiber in the case of rock wool than asbestos exposure. We noted a statistically significantly higher elevation of sICAM-1 levels in individuals exposed to rock wool compared to the group exposed to asbestos. Glass fibers were not associated with differences in sICAM-1 levels. Adhesion molecules seem to be a sensitive indicator of activation of the immune system and inflammatory response in humans exposed to mineral fibers. Oxidative stress and production of ROS is an important component of the multiple effects of asbestos on human airways (Manning et al., 2002; van Helden et al., 2009). ROS modulate receptor signals and immune responses under physiological conditions, but their overproduction mediates endothelial damage through growth and migration of inflammatory cells, over-expression of inflammatory cytokines and adhesion molecules such as ICAM-1, VCAM-1, and E-selectin (Urso et al., 2011). The elevated production of IL-8, sICAM-1 (rock wool exposure) and sE-selectin (glass fibers exposure) signify immunotoxic effects of synthetic fibers from the airways and increased production of ROS.

## 6. Summary

This chapter addresses the effects of asbestos, man made mineral fibers (rock wool, glass wool, ceramic fibers) and nickel oxide nanoparticles on the immune system using *in vitro* model, animal model and molecular-epidemiological studies. Data from *in vitro* studies contained results of experiments on alveolar macrophages (AM) and alveolar epithelial type II cells (TII). Stone wool, refractory ceramic fibers (RCF), asbestos (crocidolite) and wollastonite have been tested by lectin histochemistry. Stone wool caused moderate membrane injury of AM and incomplete phagocytosis in a small fraction of AM. RCF caused gaps and reduplicated changes in membranes of both cell types (high dose). Wollastonite caused a decreased reaction in the membranes (high dose). After exposure to the lowest dose of asbestos (crocidolite), the membranes of both cell types were fragmented irregularly and frustrated phagocytosis could be found in AM. Analysis using transmission electron microscopy found severe damage in the organelles and cell death of both cell types exposed to crocidolite. No alterations were found after RCF or stone wool exposure. Analysis of proinflammatory peptides showed that exposure to wollastonite did not change production MCP-1 and MIP-1α in TII cells but in AM the production was significantly enhanced. Different doses of stone wool enhanced production of both peptides in TII cells and AM cells. Crocidolite evoked statistically significant dose dependent enhancement of the production of MCP-1 in AM, for MIP-1α; and both cytokines in TII cells. Comparing the results from different fibers on 2 various primary cell types the following differences are clearly seen: crocidolite (asbestos) evoked the greatest changes, both morphologically and functionally. Increased effects in wollastonite were seen when compared to stone wool. AM cells are more sensitive to the fiber exposure than TII cells.

**Intratracheal instillation studies in rat model**

Four types of mineral fibers were administered intratracheally to rats. Four (4w) and 16 weeks (16w) later, immune parameters were examined. Amosite, wollastonite (4w) and rock wool (16w) significantly decreased number of white blood cells; while opposite effect of glass fibers was seen (4w). A consistent increase in percentage of neutrophils was found in animals exposed to all fibers (4w) while decreased percentage of lymphocytes was observed only in rock wool fiber-treated rats (4w). Analysis of lymphocyte subsets in amosite exposed rats showed significantly increased percentage of T-lymphocytes (4w, 16w), mainly cytotoxic cells (4w) and decreased percentage of B-lymphocytes (4w). An increased percentage of T-helper cells was seen in wollastonite group (4w). Exposure to mineral fibers decreased expression of adhesion molecule CD54 (ICAM-1) on peripheral blood leukocytes (amosite, glass fibers and rockwool; all 4w) and CD11b (glass fibers, wollastonite; 4w). Although amosite (4w, 16w) seems to be most potent suppressor of T- and B-lymphocyte proliferation, especially in high-dosed animals, wollastonite (4w) and rock wool (4w, 16w) also interfered with lymphocyte proliferation and suppressed the response of T-lymphocytes. The opposite, stimulative, effect on proliferative capacity of B-cells was found in animals exposed to glass fibers (4w). A highly significant dose-dependent suppression of phagocytic activity of neutrophils and monocytes was found mainly in rock wool and glass fiber exposed animals (4w, 16w), but present also in wollastonite and amosite group (4w).

**Inhalation studies in rat model - combined effect of mineral fibers and tobacco smoke on inflammatory response and cytotoxicity**

In rats administered with amosite, weak dose-dependence was seen in simple exposure to fibers without smoking but inflammatory parameters were mostly changed in animals with combined exposure to high dose of fibers and tobacco smoke. In case of wollastonite exposure, no clear dose-dependence in changes of inflammatory parameters was recorded in those administered with fibers alone and very weak in combined exposure groups (fibers and tobacco smoke). Additionally, mild dose dependence of cytotoxic parameters changes in groups without or with tobacco smoke was observed. Tobacco smoke alone induced changes predominantly in inflammatory parameters; alterations in cytotoxic parameters were not explicit.

**Combined effect of mineral fibers and tobacco smoke on immune parameters**

Inhalation of high dose of both fibers (amosite and wollastonite) resulted in a significantly increased percentage of B-lymphocytes in peripheral blood of exposed rats. Except the percentage the B-cells, the combined exposure to wollastonite and smoking caused a significant, dose-dependent increase of cytotoxic cells, but total T-lympocytes were decreased. Exposure to amosite and wollastonite increased expression of adhesion molecule CD11b on peripheral blood leukocytes. The proliferative activity of T-lymphocytes and T-dependent B-cell response in animals exposed to amosite in simple or combined exposure with smoking was mostly suppressed. The only exception was combined exposure to amosite fibers and smoking resulting in significant increase of proliferative activity of B-cells. Enhanced proliferative reponse of T-cells was found in animals given high dose of wollastonite. A marked suppressive effect of amosite and wollastonite on phagocytic activity of leukocytes was observed. Moreover, decrease of phagocytosis was recorded in combined exposure to wollastonite and cigarette smoke.

**Assessment of immunotoxicity of ceramic fibers and NiO nanoparticles**

Immunophenotypic analysis of leukocytes was examined only 6 months after exposure to fibers and nanoparticles. Analysis revealed statistically significant decreased expression of marker for T-lymphocyte subpopulations ($CD4^+$, $CD8^+$) in rats administered with ceramic fibers. On the other hand, increased expression of $CD4^+$ marker after combined exposure was observed. Exposure to NiO nanoparticles significantly increased expression of MHC II on leukocytes. A similar effect was found on expression of MHC II with combined exposure. A significant decrease of proliferative activity of lymphocytes stimulated with all three mitogens was found in animals exposed to ceramic fibers one month after exposure. To the contrary, 6 months after exposure, opposite effect was seen. Moreover, significant increase of basal proliferative response of spleen cells derived from rats was seen 1 month after exposure to NiO nanoparticles alone and combined exposure to fibers and nanoparticles. Combined exposure to nickel oxide nanoparticles manifested a significant increase of proliferative activity of T-lymphocytes after stimulation with Con A. No alterations in phagocytic activity and respiratory burst were shown one month after exposure of animals to ceramic fibers and/or NiO nanoparticles. However 6 months after exposure, situation was different. Exposure to NiO nanoparticles and combined exposure to ceramic fibers and NiO nanoparticles caused significantly increased phagocytic activity of granulocytes, as well as percentage of cells with respiratory burst.

**Molecular epidemiological studies in human population**

In the context of a large-scale molecular epidemiology study, the possible immunomodulatory effects of mineral fibers, in workers occupationally exposed to asbestos, rockwool and glass fibers, were examined. Results of hematological evaluation shown decreased white blood cell count and increased number of lymphocytes and (common) eosinophil and basophil count in glass fiber exposed population. Our findings indicate that exposure to all three types of fibers examined the modulation of immune response to a different degrees. Suppression of T-cell immunity was found in the workers from a former asbestos cement plant, while stimulation of T-cell response was observed in rockwool workers. In addition to an elevated T- lymphocyte response, stimulated T-dependent B-cell response and basal proliferative activity of lymphocytes was seen in workers from glass fiber factory. Changes in lymphocyte subpopulation of CD $16^{+}56$ (natural killer cells) in peripheral blood may indicate negative effects of glass fibers on natural cellular immunity. No significant alterations between workers exposed to asbestos, rock wool and glass fibers and controls were found in proportion of $CD3^{+}$, $CD4^{+}$, $CD8^{+}$, $CD19^{+}$ cells in peripheral blood. Significantly increased serum levels of immunoglobulins IgA (asbestos), IgE (asbestos, rockwool) and decreased levels of IgM (rockwool) were recorded in people exposed to fibers. Increased levels of proinflammatory cytokines (IL-6 asbestos; IL-8 all three fibers), expression of adhesion molecule L-selectin on granulocytes and monocytes (asbestos), levels of soluble adhesion molecules in sera (ICAM-1 asbestos, rockwool; E-selectin glass fibers), increased levels of immunoglobulin E (asbestos and rockwool) and elevated expression of activation markers on eosinophils (CD66b asbestos, glass fibers; CD69 asbestos) may indicate hypersensitivity and an elevated inflammatory status in workers exposed to mineral fibers.

## 7. Conclusions

With the increasing commercial needs for substitutes of asbestos fibers, a number of man-made and other naturally occurring mineral fibers will appear as a part of living and occupational environments. Fibers discussed in this chapter can enter the human body mainly via the lungs, significance of exposure through digestive tract is less clear. Asbestos has long been recognized as a cause of both benign and malignant lung disease. Man made mineral fibers, once inhaled and displaced to lung tissues, can cause respiratory diseases related to inflammation and fibrosis. Skin diseases have been also reported. In reference to nanoparticles besides the lung and digestive tract, penetration via the skin also occurs (Fig. 1). Knowledge from air pollution showing increased risk of cardiopulmonary, respiratory, hypersensitivity disease and cancer requires specific assessments to be performed for newly produced nanoparticles. The assays currently used to test the safety of materials might be applicable to identify hazards of nanoparticles. Special attention is needed for nanoparticles designed for drug delivery or food components.

To optimize risk assessment for immune system toxicity, it is still necessary to increase our understanding of the underlying immunomodulatory mechanisms which cause negative effects and the quantitative relationships between the immunological tests conducted in the laboratory and manifestation of disease in human populations. There is no universal "consequence of exposure", each type of immunotoxicant should be treated individually when health risks are expected. As mentioned throughout this chapter, the immune system

has been identified as a potential target organ for chemicals including particles and fibers. The immune system plays a critical role in host defense from disease as well as in normal homeostasis; thus identification of immunotoxic risk is important in the protection of human, animal and wildlife health. Clear understanding of normal development of cellular components of the immune system, the means by which they interact, and the known parameters by which their structure and function can be modified is necessary for designing investigations into how environmental agents may affect health through the immune system.

A growth of knowledge in immunology and cell biology connected with an explosion in methodologic and technologic capabilities is very promising for the science of immunotoxicology. There are several challenges yet to be solved within the discipline of immunotoxicology: (1) to improve traditional tests and establish a new tests, which reflect the variety of potential impacts of immunotoxicity; (2) to identify valid, sensitive human biomarkers of immunotoxicity; (3) to interpret minor, moderate, or significant immunotoxic effects in animal models in relation to human risk assessment; (4) better integration of methods of exposure assessment and immunotoxicological risk assessment, especially for simultaneous exposure to multiple agents ; (5) to design better human studies to assess the impact on the immune system in the species of the greatest interest in the context of risk assessment and (6) the better understanding of the role of genetic predisposition and susceptibility in identifying sensitive subpopulations to immune-altering agents (Kaminiski et al., 2008). These challenges are not unique to immunotoxicology, but they are critical, and need to be addressed through intensive and systematic efforts to improve human immune testing strategies.

## 8. Acknowledgment

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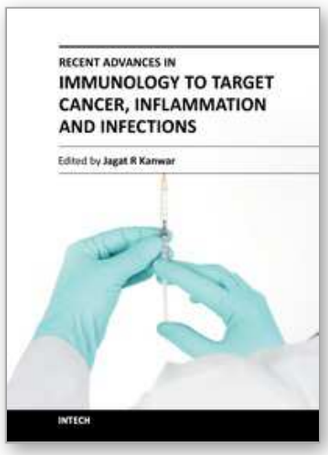
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**Recent Advances in Immunology to Target Cancer, Inflammation and Infections**

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Immunology is the branch of biomedical sciences to study of the immune system physiology both in healthy and diseased states. Some aspects of autoimmunity draws our attention to the fact that it is not always associated with pathology. For instance, autoimmune reactions are highly useful in clearing off the excess, unwanted or aged tissues from the body. Also, generation of autoimmunity occurs after the exposure to the non-self antigen that is structurally similar to the self, aided by the stimulatory molecules like the cytokines. Thus, a narrow margin differentiates immunity from auto-immunity as already discussed. Hence, finding answers for how the physiologic immunity turns to pathologic autoimmunity always remains a question of intense interest. However, this margin could be cut down only if the physiology of the immune system is better understood. The individual chapters included in this book will cover all the possible aspects of immunology and pathologies associated with it. The authors have taken strenuous effort in elaborating the concepts that are lucid and will be of reader's interest.

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