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Takemi Otsuki
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# Allergy and Immunotoxicology in Occupational Health - The Next Step


Springer

# Current Topics in Environmental Health and Preventive Medicine

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# Preface

We published the second volume of this EHPM eBook series in 2017: *Allergy and Immunotoxicology in Occupational Health*. And this is a new eBook that, 3 years later, describes the next step.

As I wrote last time, the three editors will be working together in the Allergy and Immunotoxicology Scientific Committee (AISC) of the International Congress of Occupational Health (ICOH). The ICOH plenary meeting is held once every 3 years and will be held in Melbourne (Australia) in March 2021. Before that it was held in Dublin (Ireland) in 2018. In 2015 it was held in Seoul (Korea), and the previous eBook was published as a mid-term AISC activity between 2015 and 2018.

And this time, as a mid-term activity from 2018 to 2021, we decided to write this book again.

As in the previous case, we will also introduce new discoveries, following the previous case, regarding basic experimental systems related to allergy and immunotoxicity and immune abnormalities in respiratory disorders (pneumoconiosis) such as silica and asbestos. In addition, we talk about pesticide immunotoxicity—an old and new problem.

In addition, this time, a chapter was also set up on regulations on occupational medicine in relation to skin diseases and respiratory diseases. It does not specialize in occupational medicine at present, but also mentions immune reconstitution inflammatory syndrome.

In addition, taking into account clinical control and basic experiments, we devoted a number of chapters to nanomaterials to raise awareness of nanomaterials, which is a topic of recent times, as an AISC.

Readers of this book who are interested in ICOH, or who are already members of ICOH but did not know about AISC, should definitely stop by the AISC session at the 2021 meeting in Melbourne. I want you. During Dublin in 2018, I had many poster presentations with two symposiums and two oral presentation sessions. Participants from Europe also actively asked questions about basic aspects and controls in the field of occupational medicine. In addition, presentations from Africa were presented at the symposium.

In particular, from the viewpoint of allergy and immunotoxicity, there is a complex background to pay attention to in the basic occupational health activities and in the health care of workers. Therefore, it is necessary to study carefully on the issues of normal occupational health and health and also to understand the basics of allergology, immunology, and toxicology before responding. And it is also important to take precautionary measures, so further consideration of all AICS members will be important.

Among these perspectives, the eBook published in 2017 and the book *Allergy and Immunotoxicology in Occupational health: The Next Step* are intended to improve the health of readers, all researchers related to occupational health, and even workers. We hope to contribute to disease prevention.

Kurashiki, Japan — Takemi Otsuki
Chieti, Italy — Mario Di Gioacchino
Chieti, Italy — Claudia Petrarca

# Introduction

This eBook highlights the importance of allergy and immunotoxicity, especially in occupational medicine. Examples of the substance include nanomaterials, pesticides, fibers, and particulate matter (silicic acid and asbestos). An important viewpoint is respiratory and skin diseases and their clinical regulation or prevention of health disorders due to allergies and immunotoxicity. This year, the second tier of the eBook, published in 2017 as *Allergy and Immunotoxicology in Occupational Health*, added the phrase "The Next Step" to the title. Also, the three editors are the same as the previous one, but all are active members of the Allergy and Immunotoxicology Scientific Committee (AISC) of the International Congress in Occupational Health (ICOH), and the previous book was ICOH 2015 and 2018 The Next Step will be published as an activity of the AISC during this year's main competition and this year's The Next Step. Many thanks to readers who are interested in occupational health and allergies and immunotoxicity.

# Contents

# Chapter 1
# Mechanisms of Action of Inhaled Particulates on Allergic Lung Inflammation

**Etsushi Kuroda**

**Abstract** The incidence of allergic diseases is on the rise, especially in developed countries, and airborne particulate pollution from fine particulate matter and sand dust has been suggested as a factor in the exacerbation of allergic responses. These particulates function as adjuvants to induce allergic responses such as immunoglobulin E induction and eosinophil activation. This chapter summarizes data on the mechanisms by which particulates stimulate immune responses in the lung, including alveolar macrophage function and interleukin 1α release.

**Keywords** Particulate · Inflammasome · Immunoglobulin · Lymphoid tissue · Interleukin · Adjuvant · Alum · Apoptosis

## Abbreviations

| | |
|---|---|
| DAMP | damage-associated molecular pattern |
| iBALT | inducible bronchus-associated lymphoid tissue |
| IgE | immunoglobulin E |
| IL | interleukin |
| MyD88 | myeloid differentiation primary response 88 |
| NLRP | nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing |
| OVA | ovalbumin |

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PAMP pathogen-associated molecular pattern
$PM_{2.5}$ fine particulate matter with a diameter of 2.5 microns
PRR pattern-recognition receptor
Tfh T follicular helper
Th2 type 2 T helper

## 1.1 Introduction

The rising incidence of allergic diseases has become a severe public health problem, especially in developed countries, and epidemiological studies have reported that airborne particulate matter is associated with increased risk of hospitalization for asthma [1–3]. Typical allergic reactions are mediated by the induction of immunoglobulin E (IgE) responses and the activation of eosinophils; these are allergen-specific immune responses that involve type 2 T helper (Th2) cells [4]. Since these Th2 cells are maintained as memory cells in allergic subjects, allergen-specific immune responses are evoked by reexposure to the identical allergen even if individuals remain in an allergen-free environment for a long period of time [5]. In general, the induction of memory cells, termed acquired immunity, is known to require the activation of innate immune responses [6, 7]. However, the mechanisms by which allergens stimulate innate immune cells and induce Th2 responses are unclear. Multiple studies have suggested that the induction of allergen-specific Th2 cells is promoted by innate immune cells activated by an adjuvant-like substance in the environment, such as fine particulate matter with a diameter of 2.5 microns ($PM_{2.5}$) or sand dust [8–14]. Some particulates are known to exert strong adjuvant activity and induce Th2 responses [15]. This chapter introduces the putative immunological mechanisms of action of particulates and their role in lung immune responses.

## 1.2 Adjuvants and Innate Immune Responses

Adjuvants are substances that induce or enhance antigen-specific immune responses. Some vaccines contain adjuvants that function as effective inducers of antigen-specific acquired immune responses, such as antigen-specific antibodies and cytotoxic T cells [16–18]. Activation of innate immune responses is necessary for the induction of acquired responses, and many adjuvants are known to activate innate immune cells. In general, innate immune cells are activated through the stimulation of pattern recognition receptors (PRRs) by pathogen-associated molecular patterns (PAMPs) such as pathogen-derived lipopolysaccharide or nucleic acid [19–22]. In addition, some factors from dying or stressed cells, termed damage-associated

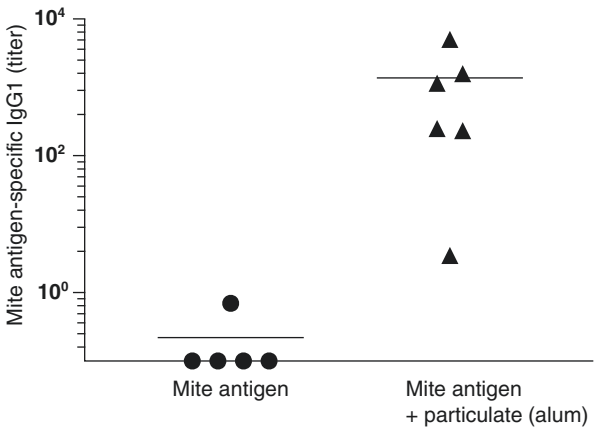


**Fig. 1.1** Adjuvant activity of particulates enhances allergen-specific antibody responses. Example from a study in mice sensitized by mite antigen in the presence or absence of particulate alum. Serum levels of immunoglobulin (Ig) G1 were quantified

molecular patterns (DAMPs), can also activate innate immune cells [23–25]. Thus, PAMPs and DAMPs are thought to function as adjuvants and induce acquired immune responses.

Particulate matter such as $PM_{2.5}$ and sand dust has been reported to function as an adjuvant [15], and to preferentially activate Th2 responses characterized by the induction of IgE and activation of eosinophils. Thus, some airborne particulates may be involved in the recent increase in the incidence of allergic diseases. In mice, low-dose exposures to allergens do not induce allergen-specific IgE, but administration of an allergen concomitantly with particulate aluminum salt (alum) as an adjuvant induces allergen-specific antibody (IgG1 and IgE) responses, indicating that particulates trigger and exacerbate allergic inflammation (Fig. 1.1). Unlike PAMPs, these particulates are not thought to stimulate PRRs on innate immune cells directly. Therefore, understanding their mode of action is key to identifying the immunological mechanisms underlying particulate-induced allergic inflammation.

## 1.3 Particulate and Adjuvant Effect

Alum has been used as an adjuvant in human vaccines and its immunological action is well-characterized and known as the "depot effect." Glenny et al. first noted that antigens adsorbed to alum exhibited persistence and prolonged release at the injection site [26], suggesting that alum is a long-term and effective stimulator of immune cell action [27]. However, alum adjuvanticity was shown to be normal even when alum nodules were removed several weeks after immunization [28], and ablation of the injection site after immunization with antigen/alum has been reported to not alter the magnitude of the immune responses [29], implying that the antigen depot is not necessary for the adjuvanticity of alum and other particulate adjuvants.

Particulate adjuvants such as alum and silica were shown to stimulate innate cells (macrophages and dendritic cells) to activate the inflammasome, an intracellular complex of PRRs [30–32]. Particulate adjuvants mainly activate the nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing (NLRP) 3 inflammasome, and then release interleukin (IL)-1β and IL-18 from innate cells via the action of caspase-1. Activation of the NLRP3 inflammasome was at first thought to be involved in the adjuvant activity of particulates [33], but the role of the NLRP3 inflammasome and caspase-1-dependent cytokines was questioned in several studies using NLRP3-, caspase recruitment domain-, and caspase-1-deficient mice [34, 35], and the inflammasome's involvement in the adjuvanticity of particulates is still unclear.

Since particulates such as alum and silica have been reported to induce cell death in phagocytes following phagocytosis [36–38], cell death has also been hypothesized to affect the adjuvant activity of particulates. DNA and uric acid are released from dying cells and may induce immune responses. Uric acid is a purine catabolite, and forms crystals at saturated concentrations. Both uric acid and its crystal are well-known DAMPs and exert strong adjuvant activity [39–44]. However, the mechanisms by which uric acid and its crystal stimulate innate immune cells and induce acquired immunity are unknown. Host cell-derived DNA is also a DAMP and functions as an adjuvant [16, 45–48]. Marichal et al. demonstrated that host DNA was released at the site of alum injection and induced IgG1 and IgE responses, while coadministration of DNase and alum significantly reduced adjuvant activity [45]. A recent study also indicated that DNA release from neutrophils recruited to the site of alum injection was involved in alum adjuvanticity [48]. DNA released by activated neutrophils forms fibers termed neutrophil extracellular traps, which can contribute to the adjuvant activity of particulates, although the detailed mechanisms of innate cell activation by DNA are unclear.

In addition to DAMPs, recognition of dead cells by a molecule expressed on dendritic cells has been reported to be involved in alum adjuvanticity [49]. Administration of alum induced apoptosis in cells that display phosphatidylserine on their surfaces, and CD300a expressed on innate cells bound to phosphatidylserine to clear apoptotic cells. CD300a-deficient mice exhibited reduced levels of antigen-specific IgE and lower Th2 responses after immunization with alum and the antigen. CD300a is mainly expressed on inflammatory dendritic cells, and these cells are recruited at the site of alum injection. Inflammatory dendritic cells induced Th2 cells, suggesting that recognition of dead cells by specialized dendritic cells is involved in induction of acquired immune responses by alum [49].

Prostaglandin, a lipid mediator released from macrophages, also participates in alum-triggered IgE responses [37]. Thus, cell death and released DAMPs play a pivotal role in activating innate cells and inducing adequate acquired immunity. Table 1.1 summarizes the modes of action of particulate adjuvants reported so far.

**Table 1.1** Modes of action of particulate adjuvants

| Adjuvanticity factor | Mechanisms of action | References |
|---|---|---|
| Depot effect | Antigen absorbed to alum persists to stimulate immune cells over time. The depot effect has been questioned | [28, 29] (not required) |
| NLRP3 inflammasome | Some particulates stimulate innate cells to activate the NLRP3 inflammasome, releasing interleukin 1β and interleukin 18. The importance of the inflammasome in the adjuvanticity of particulates is unclear | [33–35] |
| Uric acid and its crystal | Known factors in damage-associated pattern recognition and show strong adjuvant activity. Detailed immunological mechanisms are unknown | [39–44] |
| DNA | Released from dying cells and functions as an adjuvant. DNA released from activated neutrophils also exhibits strong adjuvant activity | [45–48] |
| Apoptotic cells | Inflammatory dendritic cells are activated via the interaction of CD300a and apoptotic cells, preferentially inducing type 2 T helper cell responses | [49] |
| Lipid mediators | The lipid mediator prostaglandin is released from particulate-activated innate immune cells and induces the production of immunoglobulin E | [37] |
| MyD88 signaling | Signaling depends on the administration route. MyD88 signaling is required for immunoglobulin E responses when particulates are administered directly to the airway | [50, 51] (not required) [38, 52] (required) |

## 1.4 Particulates and Lung Diseases

Asbestosis and silicosis are representative lung diseases caused by inhalation of chemical particulates. The effect of silica in particular on the immune system has been demonstrated in animal models [53–56]. Inhaled particulates that are deposited in the lungs are generally engulfed by phagocytes such as alveolar macrophages and then excreted [57, 58]. Engulfing particulates has been reported to activate the inflammasome and subsequently IL-1β -via the action of caspase-1 [30–32, 59, 60]. In the lung model of inflammation caused by silica, inflammasome deficiencies have been shown to attenuate the magnitude of inflammation [55]. Exposure to tobacco smoke is a rodent model for chronic obstructive pulmonary disease, as tobacco smoke contains fine particulate matter, and long-term exposure has been shown to cause lung inflammation and the formation of ectopic lung lymphoid tissue. These responses are mediated by IL-1 and myeloid differentiation primary response 88 (MyD88) signaling [61–64]. Environmental particulates such as sand dust, diesel exhaust particles, and $PM_{2.5}$ and industrial particulates such as carbon nanotubes have been assessed for their toxicity and allergenicity [8–14, 65–75]. Although multiple studies reported that these particulates also function as adjuvants to promote Th2-type immune responses similar to those induced by alum and silica,

the underlying mechanisms are unclear [15]. Inhalation of particulates causes chronic lung inflammation and allergic asthma, so understanding the immunological mechanisms of particulate-induced inflammation would be required for prevention and effective treatment of chronic lung diseases.

## 1.5 Particulates and Alveolar Macrophages

Alveolar macrophages act as sentinels against inhaled foreign substances [53, 76]. Some particulates, such as $PM_{2.5}$, are deposited deep in the lungs. These particulates are thought to be engulfed by alveolar macrophages and cleared from the respiratory system [57, 58]. An in vitro study in the author's laboratory used murine alveolar macrophages exposed to either inflammatory (alum, silica, and nickel oxide nanoparticles) or noninflammatory (aluminum oxide and hydroxyapatite) particulates. Inflammatory particulates stimulated alveolar macrophages to release IL-1α, which was closely linked to cell death caused by phagocytosis of the inflammatory particulates [38]. IL-1α has been reported to be constitutively stored in alveolar macrophages and rapidly released as a DAMP following cell death [53]; alveolar macrophage death therefore appears to trigger lung inflammation by releasing IL-1α [77], and was detected in vivo in bronchoalveolar lavage fluid. Noninflammatory particulates did not induce macrophage death or IL-1α release in vitro or in vivo [38].

Multiple studies have reported that particulates activate the NLRP3 inflammasome after engulfment by dendritic cells and macrophages and then induce the release of IL-1β via caspase-1. Stimulation by low concentrations of lipopolysaccharide is known to be required for IL-1β release following activation of the NLRP3 inflammasome [30–32, 36, 59, 60], but this priming step is not necessary for IL-1α release from alveolar macrophages. IL-1β release was not detected in non-primed alveolar macrophages stimulated with alum. Unlike IL-1α, pro-IL-1β has no biological activity, and is therefore not considered a DAMP cytokine [77–80].

## 1.6 IL-1α Release and IgE Responses in the Lungs

IL-1 is known to function as an adjuvant and induce antigen-specific IgE [38, 81, 82]. The author's laboratory therefore studied whether IL-1α release from dying alveolar macrophages in response to particulate exposure was related to IgE. Particulates were administered to mice by intratracheal instillation, followed by exposure to the allergen ovalbumin (OVA). Levels of OVA-specific IgE increased but only following administration of inflammatory particulates such as alum and silica. Mice deficient in the IL-1 receptor or IL-1α exhibited reduced levels of OVA-specific IgE,

indicating that inflammatory particulates induce the release of intracellular IL-1α, which functions as an adjuvant to induce antigen-specific IgE responses [38].

IL-1-dependent IgE responses were observed in mice following inhalation of peanut flour, and serum IgE levels decreased significantly in IL-1 receptor-deficient mice, in accordance with the decreased numbers of T follicular helper (Tfh) cells [83]. Tfh-derived but not Th2-derived IL-4 has been shown to be necessary for class switching to IgE in B cells [84], suggesting that IL-1α participates in IgE responses via the differentiation of IL-4-producing Tfh cells. Activation of inflammasomes and IL-1β production do not appear to be involved in IgE responses caused by inhaled particulates [38].

## 1.7 Particulates and Unique Immune Responses in the Lungs

Several studies have reported that MyD88 signaling is not required for the adjuvanticity of alum when it is administered intraperitoneally or subcutaneously [50, 51]. However, IL-1 receptor signaling is necessary for IgE responses when alum is administered by intratracheal instillation, suggesting that MyD88 signaling is involved in the adjuvanticity of alum in the lungs. Matsushita and Yoshimoto also reported that MyD88 signaling was necessary for IgE responses by intranasal administration of alum [52]. These data suggest that direct administration of inflammatory particulates to the airway induces IgE responses via mechanisms specific to the administration route. As mentioned above, alveolar macrophages constitutively express intracellular IL-1α, and release it as a dead cell factor induced by phagocytosis of inflammatory particulates. This is unique to alveolar macrophages and not observed in macrophages from other tissues stimulated with inflammatory particulates [38, 53].

Lung tissues from mice exposed to particulate alum and OVA exhibited infiltrations of inflammatory cells and lymphoid cluster formations. These lymphoid clusters were mainly composed of B cells and hypothesized to be inducible bronchus-associated lymphoid tissue (iBALT) [38]. iBALT is known to be induced in chronic inflammation evoked by viral infection, administration of lipopolysaccharide, or autoimmune diseases such as rheumatoid arthritis [85–90]. iBALT formation was also observed in the lungs of mice with allergic inflammation [91, 92], and pathogenic Th2 cells have been associated with iBALT formation [5, 93], suggesting that lung ectopic lymphoid tissues are closely linked to allergic inflammation. The lymphoid clusters induced by alum and antigen exposure contain a germinal center of B cell maturation and antibody class-switching, areas of T cells, and clusters of plasmablasts. These iBALT formations are regulated by IL-1 and are correlated with IgE levels [38].

iBALT structures are involved in local lung immune responses and dendritic cells recruited to the lungs induce and maintain the iBALT structures [87]. Mice deficient in the IL-1 receptor have decreased numbers of iBALT formations and

recruited dendritic cells in their lungs [38, 94]. This suggests that dead cell-derived IL-1α is involved in dendritic cell infiltration and subsequent formation of iBALT structures, inducing IgE responses. Both iBALT structure formation and IgE induction have been shown to be reduced following depletion of lung dendritic cells [38]. Furthermore, Tfh cells have also been reported to participate in the formation of iBALT structures [89], and particulate-induced iBALT formations were reduced in Tfh cell-deficient mice, mirroring antibody responses [38]. Dead cell-derived IL-1α may therefore induce the activation of both Tfh cells and dendritic cells, resulting in iBALT formation and IgE responses as immune responses unique to the lung. Figure 1.2 summarizes the model of particulate (alum)-induced allergic lung inflammation.

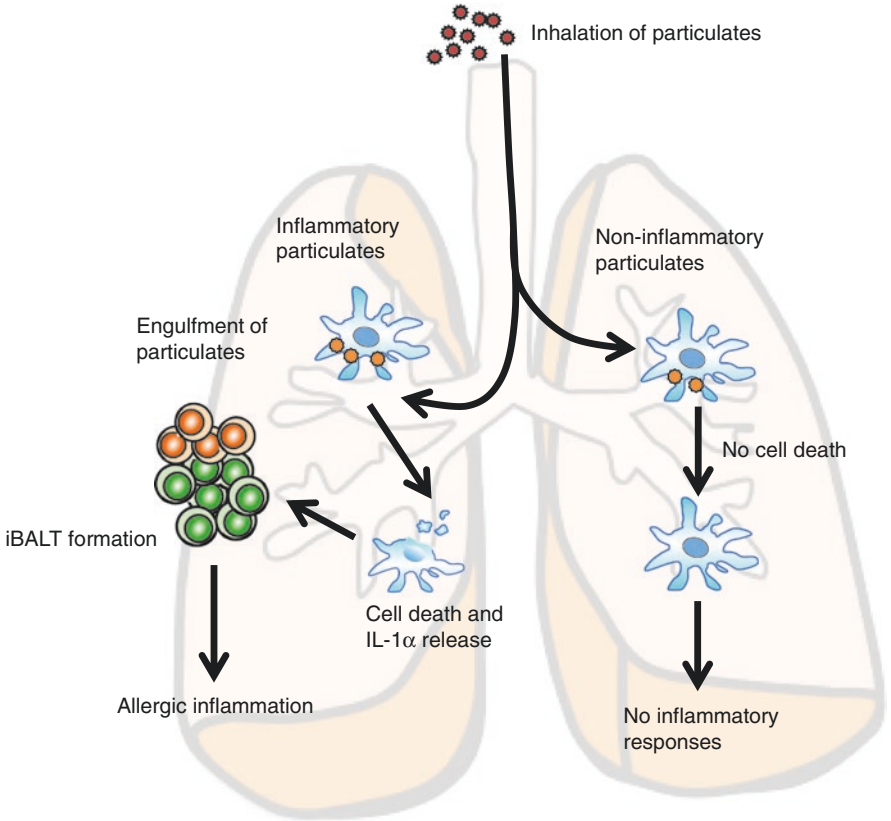


**Fig. 1.2** Model of particulate-induced lung allergic inflammation. Inhaled inflammatory particulates kill alveolar macrophages and induce the release of interleukin (IL)-1α, which functions as an adjuvant to promote the formation of inducible bronchus-associated lymphoid tissue (iBALT) and production of immunoglobulin E. Modified figure from Kuroda et al. [38]

## 1.8 Therapeutic Strategies for Particulate-Induced Lung Inflammation

iBALT structures in the lungs have been observed in infants but not in healthy adults [95, 96], indicating that infants are sensitive to particulate-induced allergic asthma. Apart from environmental particulates, tobacco smoke constituents can also induce iBALT structures in the lungs, suggesting a similar inflammatory mechanism [63, 64]. Targeting IL-1 signaling may therefore be a valid therapeutic strategy for particulate-induced lung inflammation. Corticosteroids are generally used to treat allergic asthma, and have been demonstrated to hamper the maturation of iBALTs induced after particulate and antigen exposure [97]. Administration of corticosteroids may control allergic asthma in part by inhibiting iBALT formation.

## 1.9 Conclusion

This chapter summarized the mechanisms of allergic lung inflammation caused by inhalation of particulates. Allergic lung inflammation diseases are frequently observed in urban areas of developed and fast-growing countries, and particulate pollution is hypothesized to be their major trigger. However, not all particulates function as adjuvants to induce inflammation, and some do not activate immune responses in the lungs following direct administration to the airway. These particulates have no effect on macrophage function and its subsequent IL-1α release. Effective treatment and prevention of inflammatory diseases caused by inhaling particulates require the elucidation of the immunological mechanisms involved.

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# Chapter 2
# Metal Nanoparticle Health Risk Assessment

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**Abstract** The widespread application of nanomaterials confers enormous potential for human exposure and environmental release particularly for workers producing nanoparticles or making nano-based objects. The various routes by which nanoparticles could be taken up by the body (respiratory, skin, and digestive) complicate the definition of NPs to be used in risk assessment.

The present review describes the difficulties in making a sufficiently correct risk assessment and management for nanomaterials, addressing the various problems that render difficult the risk management of nanomaterials in the occupational setting, in particular the exposure scenario, the exposure appraisal, and the hazard identification and characterization.

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**Keywords** Nanoparticles · Environmental monitoring · Exposure appraisal · Risk assessment · Risk management · Control banding

## 2.1 Introduction

Nanoparticles are defined as "a material with one, two, or three external dimensions at the nanoscale (from ~ 1 to 100 nm) metrics." In this form, they achieve unique mechanical, optical, electrical, and magnetic properties. Recent rapid advancements in nanotechnology have led to wide applications of nanomaterials in a number of areas, including material science, energy, and medicine. Therefore, the widespread application of nanomaterials confers enormous potential for human exposure and environmental release particularly for workers producing nanoparticles or making nano-based objects. The wide variety of routes by which NPs could be taken up by the body (respiratory, skin, and digestive) complicates the definition of NPs to be used in risk assessment. It is probably necessary to consider multicomponent and multiphase particles of any size and composition that can be absorbed by the body.

Although the potential for these nanomaterials to affect the ecosystem function, to exert cytotoxic and pro-inflammatory effects in vitro as well as to induce early alterations in different target organs in vivo models has been reported, further investigations appear absolutely necessary to confirm such preliminary findings [1].

Depending on the conditions of manufacture, formulation, use, and final disposal, a risk assessment of NPs may need addressing: (1) Worker safety: typically workers are exposed to higher levels of chemicals and for more prolonged periods of time compared with the general population; (2) Safety of consumers using products that contain NPs; (3) Safety of local human populations due to chronic or acute release of NPs from industry; (4) The potential for human reexposure through the environment. Focusing on products that are deliberately used in nanoparticle form in the environment, such as biocides, or environment-improving agents; (5) The environmental and human health risks involved in the disposal or recycling of nanoparticle-based products. In principle, the traditional risk assessment procedure is an appropriate tool for assessing the risks from exposure to NPs under specified exposure conditions. The traditional risk assessment methodology comprises the following stages: (a) exposure appraisal; (b) hazard identification and characterization; and (c) risk characterization and management. This framework has not yet been applied to NPs, in terms of either their potential human or environmental impact. There is an unclear situation with regard to regulatory requirements for risk assessment. As a consequence, there are no official guidelines on what constitutes an appropriate testing regimen. This review will focus on metal nanoparticles due to the large and already increasing use of these nanoparticles in industries.

## 2.2 Exposure Scenarios

Essentially workers are exposed to NP by inhalation, ingestion, and contact. In a room, the air can contain 10,000–20,000 NPs/cm$^3$, in a street the quantity on NPs are extremely higher reaching 100,000 NPs/cm$^3$. In a working environment, their

concentration is extremely variable, according to the type of working activity and the hygienic measures applied [2]. Therefore, workers can be in contact with millions on NPs per hour. It is estimated that half of them can reach the alveoli or are in contact with the skin. A part of them are ingested and adsorbed through the gastrointestinal system. NPs are adsorbed and can be found in tissues, penetrate cells (through diffusion or endocytosis), and exert their toxic potential. It has been observed that NPs may trigger oxidative stress, inflammation, and indirect DNA damage in living systems, and that the size and shape of NPs could have an important role in determining the cellular damage. In many cases the toxicity of NPs is exerted by released ions [3]. In some cases, the released ions are neutralized by forcing or interacting with the cell component leading to depletion of dissolved oxygen and generating reactive oxygen species (ROS) together with reactive nitrogen species (RNS) leading to molecular and biochemical alterations [4].

### *2.2.1 Lung Exposure*

The respiratory tract is the most important route of exposure to NPs. Their deposition in the alveolar region gives the possibility of their absorption in the lung. Although NPs tend to agglomerate, potentially making their aerodynamic characteristics similar to those of larger particles [5], size remains a characteristic, which correlates with toxic responses. However, even though surface area has been completely studied for inflammatory responses, it has not been similarly validated for cytotoxicity or oxidative stress effects [6]. NPs, deposited in the respiratory tract, easily translocate from the alveolar region to epithelial and interstitial sites [7]. Muhlfeld [8] demonstrated that when NPs reach the alveoli, they have a high probability of encountering the alveolar epithelium, because the uptake of NPs by alveolar macrophages seems to play a minor role than for larger sized particles [9, 10]. Essentially monocytes/macrophages transport NP across a confluent endothelial cell layer [11]. NPs enter easily interstitial spaces after alveolar deposition, compared with bulk particles [12].

Inhaled NPs can spread more like gas molecules and, thanks to their size, NPs pass through the alveoli into the bloodstream, reaching sensitive sites such as bone marrow, liver, kidneys, spleen, and heart [8] and specifically accumulate at sites of vascular disease [13]. Videira et al. [14] evidenced inhaled 200 nm solid lipid NPs (SLNPs) rediolabeled with 95Tc into the lymphatics, and a high rate of distribution in periaortic, axillar, and inguinal lymph nodes. It has been showed that, in the blood of rats exposed to 15 nm Ag NPs by inhalation, or to agglomerated Ag NPs or Ag+ ions intratracheally, the significant amounts of silver detected initially decrease rapidly, and this shows that systemic distribution occurred [15]. Hint of silver were found in the liver, kidney, spleen, brain, and heart, while the nasal cavities, such as the posterior portion, and lung-associated lymph nodes showed relatively high concentrations of silver. Exposure via inhalation by rats of 40 and 51 nm CdO resulted in efficient deposition in the lung [16], with a fraction from the lung translocated to

the blood, liver, and kidneys. Kwon et al. [17] studied the body distribution of inhaled 50 nm fluorescent magnetic NPs in mice. Magnetic resonance imaging and confocal laser scanning microscope analysis demonstrated that NPs were distributed in various organs, for example, the liver, testis, spleen, lung, and brain, indicating that these NPs could penetrate the blood–brain barrier. Dumková [18] showed that sub-chronic inhalation of lead oxide nanoparticles favors their broad distribution and tissue-specific subcellular localization in target organs.

Thanks to a single 4-h nose-only exposure to freshly emitted or aged CeO2 in different gaps of time, ICP-MS detection of Ce in the lungs, gastrointestinal tract, spleen, kidneys, heart, brain, liver, blood, olfactory bulb, urine, and feces were studied to have a complete vision of the distribution. Their research found Cerium mostly in the lungs and feces, with extrapulmonary organs' contributing less than 4% to the recovery rate at 24 h post exposure. No significant differences were found in biodistribution patterns between fresh and aged CeO2 nanoparticles [19].

Oberdorster et al. [20] showed that after inhalation of (20–29 nm) 13C NPs, a transportation from the olfactory mucosa of the rat to the olfactory bulb occurred, supplying a portal of entry into the central nervous system (CNS) for solid NPs. The same group of the former study [21] performed experiments in rats inhaled with 36 nm 13C NPs. During inhalation about 20% of the particles deposited into the nasopharyngeal region reached the olfactory mucosa and further translocated to the olfactory bulb where they persisted. The NPs were also able to cross the blood–brain barrier in some regions, targeting the CNS. The translocation of NPs to the brain was also found in mice exposed by inhalation to 20–200 nm $TiO_2$ NPs [22], gold NPs [23], and Ag NPs [24].

We can assert that it is mostly accepted that the translocation of NPs from lung to other tissues can occur. Nevertheless, only a small part of inhaled NPs translocates across the air–blood barrier, entering the circulation and reaching other organs. To understand the significance of this translocation, research priorities should be: (1) to study the metabolic fate of the translocated NPs; (2) to assess their cardiovascular toxicity: cardiovascular system is currently considered an important potential target for NPs; (3) to study the toxic effects of the translocated NPs with the help of in vitro systems such as perfused organs and cell cultures; effects investigated would involve systems which are particularly sensitive to long-term low-dose exposure, such as the CNS and reproductive system; and (4) to study mechanisms of NP penetration into cells and their intracellular distribution in relation to the immunotoxic responses and other effects, particularly the production of reactive oxygen species (ROS) and inflammation proteins.

### *2.2.2 Dermal Exposure*

Many studies have suggested that metallic NPs can trigger sensitization reactions. However, there is an urgent need to clarify the immunologic effects of skin exposure to metallic NPs, especially in how they aggravate allergies. Although many studies

have been conducted to explore toxicity following skin exposure to metallic NPs, there is a lack of knowledge of immunologic mechanisms. In general, haptens are involved in immunoreactivity, but are not immunogenic. They have the potential to modify self-protein binding to obtain immunogenicity. In contrast to classic haptens, transition metals produce coordination complexes instead of stable covalent modifications with binding proteins. These coordination complexes are reversible and exchange allergenic metallic ions among different sites [25].

When foreign substances enter the body, immune cells, such as antigen-presenting cells and leukocytes, recognize them and activate immunodefenses. Smulders et al [26]. showed an increased Ti concentration in draining lymph-node cells after topical application of $TiO_2$ NPs, indicating that $TiO_2$ NPs penetrated the skin and were transferred to the lymph nodes. Human macrophages were found partially to dissolve ZnO NPs based on evaluations of ZnO NP counts with X-ray fluorescence and SEM [27]. Therefore, one of the potential immunologic mechanisms after metallic NPs are topically applied might be that the NPs move to the lymph nodes, where they are engulfed by macrophages. On the other hand, metallic NPs activate adaptive immune responses. Metallic ions, such as Ni ions, were capable of activating metallic ion-specific $CD4^+$ T cells in the lymph nodes, and IL17 was produced in response to metallic ions. On the basis of these studies, metallic NPs have been speculated to act as carriers to transport metallic ions into the lymph nodes for $CD4^+$ T cell- and IL17-mediated immune responses. In the case of inflammatory skin diseases, such as atopic dermatitis and psoriasis, the exact immune responses and whether these responses can affect skin inflammation are not yet fully understood.

Increasing skin exposure of metallic NPs from a variety of nanotechnology applications has raised concerns regarding potential adverse effects on human health. Of all possible entry routes, skin absorption may serve as the first portal for metallic NP exposure. As mentioned, transdermally applied metallic NPs can penetrate damaged or even intact skin. The high activity of metallic NPs has raised debate on their interactions with skin cells. Although some studies have been conducted to assess these interactions, their results were inconclusive. The proposed mechanism is that metallic NPs generate oxidative stress, mitochondrial damage, and DNA damage, and accelerate the apoptosis of skin cells [28] and have synergistic biological effects [29].

The effects of $Fe_3O_4$ and ZnO NPs on mouse dermal fibroblast cells have been evaluated in vitro. After exposure, a number of endocytic vesicles were detected on the cell membrane, and metallic NPs were visualized in either the cytoplasm or cytoplasmic vesicles, indicating that metallic NPs seemed to be phagocytosed by cells. In addition, NP-treated cells formed irregular shapes due to cytoplasmic shrinkage or were even completely necrotic at high NP concentrations [30]. Furthermore, NP attachment caused membrane rupture, increased cell-membrane permeability and influenced cell-membrane fluidity [31]. Moreover, the combination of electrostatic attraction and hydrogen bonding between NPs and membranes was probably one of the reasons for membrane disruption and gelation [32].

Nowadays, we do not have sufficient conclusions to understand the specific mechanisms of transdermally applied metallic NPs. Actually, there is still debate as

to whether metallic NPs can penetrate the normal skin barrier. Additionally, the interactions of metallic NPs and skin cells remain controversial. These inconsistent results may be caused by several factors, such as different types of NPs and physicochemical characterizations, varying experimental approaches, and various cellular or animal models used in the experiments [33].

### *2.2.3 Gastrointestinal Exposure*

The ingestion of NPs can happen through contaminated foods or swallowing saliva or nasal fluids contaminated with environmental NPs. When nanoparticles have entered the buccal mucosa, they can impact on the physiological homeostasis of the human body in different ways. First of all, nanoparticles can directly strike the buccal epithelium. It was already showed that nano-$TiO_2$ particles have the potential to generate ROS and induce oxidative stress [34], which usually results in inflammation and/or cell death [35]. Second, free particles may pervade the epithelium and enter systemic circulation. It was demonstrated that nanoparticles accumulated in liver, spleen, lung, and kidney after oral administration [36].

NPs can permeate the digestive tract by direct ingestion [37], and dental prosthesis debris [38]. Nanoparticles can be easily absorbed by the digestive tract not only through the M-cells in the Peyer's patches and the isolated follicles of the intestinal associated lymphoid tissue, but also by transcytosis via enterocytes [39]. The primary components of the GIT include the mouth, esophagus, stomach, small intestine (duodenum, jejunum, ileum), and large intestine (cecum, ascending colon, transverse colon, descending colon, sigmoid colon, and rectum). The small intestine is the site of most nutrients digestion and this is the spot where absorption into the bloodstream of NPs would occur, especially in the segments of the jejunum and ileum [40].

The present available studies about the degree of $TiO_2$ particle uptake and absorption from the GIT into the blood circulation are not consistent, and may be species dependent, as studies in mice appear to differ from reported findings with $TiO_2$ exposures in rats and humans [41–44]. Other factors include the type of NPs, as well as important physicochemical characteristics including particle size, dispersibility, and charge. In general, the majority of biokinetic studies show that most of the ingested $TiO_2$ NPs are not absorbed into the bloodstream but instead are excreted from the GIT.

It is important to think about what happens to NPs contained in foods: will they remain in the intestine or will they move on into the body? The intestine should take up nutrition and protect the body from unwanted substances in the food. It is not known whether NPs are regarded as "unwanted substances" and excreted or not.

The hypothesis that nanomaterials will not remain there for indefinite periods is led by the rapid transit of material through the intestinal tract (on the order of hours), together with the continuous renewal of epithelium. The extent of particle absorption in the gastrointestinal (GI) tract is affected by size, surface chemistry and charge, length of administration, and dose [45].

Once ingested, NPs enter the stomach and are submitted to usual digestive processes that may afflict them. Meng et al. [46] have demonstrated that 23.5 nm Cu NPs consume the hydrogen ions in the stomach more quickly than microparticles, converting the Cu NPs into cupric ions whose toxicity is very high. NPs surviving on the gastric digestion can be absorbed in the enteric tract.

A consistent absorption was observed, with a systemic distribution to liver, spleen, blood, and bone marrow. Particles larger than 100 nm did not reach the bone marrow, and those larger than 300 nm were absent from blood. No particles were detected in heart or lung tissue. The uptake was mainly via M-cells (specialized phagocytic enterocytes) of the Peyer's patches, with translocation into the mesenteric lymph and then to systemic organs. A further possibility for intestinal uptake of NPs is via enterocytes [45]. In a study performed in mice orally administered with 4 and 58 nm gold NPs, Hillyer and Albrecht [47] showed the capture of gold NPs by the intestine, their passage through the blood and their translocation to the brain, lungs, heart, kidneys, intestine, stomach, liver, and spleen. The uptake occurred by persorption through holes created by extruding enterocytes. This effect was inversely proportional to the size of the NPs: the smaller the particle, the greater was the passage.

### *2.2.4 Interaction of NPs with Immune System*

NPs can interact with human immune system once they enter in the body [48]. Effects of NPs on this system can be of various types, in a range that spreads from a specific immune response to immunosuppression and autoimmunity, based mainly on size and chemical properties of NPs [49–52]. Effects can be also of indirect types due to NPs' capacity to change ionic homeostasis of a system. Currently there are no information in scientific literature of diseases caused by NPs, with only exception of contact dermatitis due to Pd NPs. Most studies were focused on effects of metal NPs (MeNPs) on immune system, and it was seen that innate immune system cells react to MeNPs in a similar pattern of how they react to pathogen microorganisms. Nanotubes administered intratracheally were found to produce dose-dependent lung lesions differently from carbon black [53], and multifocal pulmonary granuloma, effects different from those of quartz, carbon black, and graphite [54]. In some studies, it was demonstrated that subtoxic concentrations of ZnO NPs downregulated CD16 expression on NK-cells or AuNPs inhibited TLR-9 function in macrophages. In vitro, exposition of macrophages to MeNPs caused proinflammatory effects with increase of productions of various cytokines including IL8 and HSP70, mainly small NPs (< 5 nm of diameter). This effect was confirmed on human blood monocytes. CoNPs induced in peripheral blood lymphocytes and monocytes an increase in production of TNF-α and INF-γ and inhibition in production of IL-10 and IL-2 [55]. CoNPs in particular showed the capacity of activating immune system more strongly than other MeNPs. Two studies demonstrated that CoNPs have lower cytotoxicity than microparticles and ions, and also showed

that only CoNPs and microparticles had morphologic transforming potential [50, 56, 57]. Furthermore, Pd-NPs induce PBMC release of cytokines different from those released in presence of Pd ions [58, 59].

In conclusion it is clear that NPs can interact with immune system and can be relevant to increase in many diseases that in Western countries are increasing in number and social costs (allergies, autoimmunity, and cancers) despite today in literature no disease was clearly associated with NPs (except for allergic contact dermatitis). NPs can have a role in allergic sensitization because they can act like haptens and can elicit a Th2 response in lymphocyte population with the production of IgE antibodies by B cells [60]. In addition to direct sensibilization, NPs have the capacity to show also an adjuvant effect that may favor allergen sensitization. It was seen that the size of NPs may change the type of immune system response, with big NPs (>100 nm) more inclined to elicit a Th2 response, while little NPs (~50 nm) more inclined to elicit a Th1 response. Even chemical composition, dose, shape, and time of exposure can influence the types of responses to NPs [60].

## 2.3 Exposure Appraisal

The exposure appraisal should answer six primary questions: (1) How, when, and where does exposure occur? (2) Who is exposed? (3) How much exposure occurs? (4) How does exposure vary? (5) How uncertain are exposure estimates? and (6) What is the likelihood that exposure will occur?

The greatest exposure to NPs pertains to workers during production and transfer of the intermediate or final product to other handling steps. Exposure of the public to NPs can only occur through the product or release of NPs to the environment. Nevertheless, there does not exist to our knowledge a systematic approach related to the control of NP production and products. During a 2004 workshop in Brussels, experts of the European Commission suggested the development of a nomenclature for intermediate and finished engineered nanomaterials, assigning a universally recognized Chemical Abstract Service (CAS) Number to engineered NPs [61]. In particular some factors have to be studied and discussed to measure the exposure potential. Relevant factors are the extent of exposure (time and concentration), the uptake route (inhalation, transdermal, ingestion), and the probability of exposure. No systematic approach is currently available for measuring the probability of exposure related to NP production and handling processes. Oberdorster et al. [62] showed the substantial differences in mass concentrations and surface areas for particles: study showed that the use of mass concentration data alone is insufficient, and the number concentration and/or surface area need to be included.

Sampling of NPs is a great challenge. The sampling strategy should ensure that the particle collection methods represent as accurately as possible the real exposure at the site in question, and methods should be developed according to the size and nature of the particles under investigation. The separation of NPs from larger particles can only be reached at a relatively high pressure drop. Technologies to measure

some of these metrics for NPs in situ have been identified, for example to determine number concentration. However, these are not readily available, particularly in a form which may be used to measure personal exposure on a routine basis. The results of the measurements can confirm the suspected temporal and spatial variation in (number) concentration and aerosol size distribution. Anders et al. [63] reported the important role of serum proteins in modifying the ZnO nanocrystal surface resulting in the formation of considerably smaller agglomerates and stable NP dispersions.

Riediker et al. [64] concluded that there are major similarities about the effects that particles in wide size range can induce. Substantial differences can be seen in deposition, translocation, and clearance, especially for inhalation exposures.

In a review, Boccuni [65] reported selected techniques that can evidence the presence or absence of nanomaterials along with their comprehensive exposure measurement, that allow the quantification of NPs in the workplace. Further researches are needed to allow accurate quantification of personal exposure of workers. To raise the accuracy of risk assessment we need the development that describes the dispersion and transformation of NP and their agglomerates in the working environment. It is also crucial to set strategies and standard measurement methods to harmonize exposure data for risk assessment and to enable the development of safety standards.

## 2.4 Hazard Identification and Characterization

Health effect data on workers exposed to NPs are limited because of the incipient nature of the field, the relatively small number of workers potentially exposed to date for which the exposure has been sufficiently evaluated, and the lack of time for chronic disease to develop and be detected. Human data are derived from exposures to ultrafine and fine particles, which have been assessed in epidemiological air pollution studies and in studies of occupational cohorts exposed to mineral dusts, fibers, welding fumes, combustion products, and poorly soluble, low-toxicity particulates such as titanium dioxide and carbon black [35, 60, 66]. Many data, essentially related to exposure to engineered NPs, also are derived from animal studies [53, 54, 62, 67–71]. A strong positive correlation exists between the surface area, oxidative stress, and proinflammatory effects of NPs in the lung [35, 62]; however, the extrapolation of animal studies to humans needs a prudent evaluation.

Although the findings are not conclusive, various studies of engineered NPs in animals raise concerns about the existence and severity of hazards posed to exposed workers [72]. Possible adverse effects include the development of fibrosis and other pulmonary effects after short-term exposure to carbon nanotubes [62, 70, 71], the translocation of NPs to the brain via the olfactory nerve, the ability of NPs to translocate into the circulation, and the potential for NPs to activate platelets and enhance vascular thrombosis [73].

For poorly soluble, low-toxicity dusts such as titanium dioxide, smaller particles in the nanometer size range appear to cause an increase in risk for lung cancer in animals on the basis of particle size and surface area [62, 74–76].

There are few evidences in humans, as example in 2009 seven healthy women exposed to polyacrylate NPs in work place experienced pleural effusion and dyspnea, or a 22-year-old chemist who experienced throat congestion, flushing in the face, rhinitis, and erythema multiforme-like after exposition to dendrimers. Sneezing and contact allergic dermatitis were frequently reported in workers exposed to NPs [60].

None of these findings are conclusive about the nature and extent of the hazards, but they may be sufficient to support precautionary action. Ultimately, the significance of hazard information depends on the extent to which workers are exposed to the hazard [77].

It is evident that there are sufficient data in the literature to conclude that, from a risk assessment point of view and for some types of NP at least, it is not valid to rely entirely on toxicological findings from testing the component of an NP of interest in another physical form.

An approach to hazard identification and characterization for a chemical of interest could be the following: (a) If there are considerable available data in the literature, the hazardous properties of its nanoforms should be evaluated in a test battery. It must be reiterated that it is not scientifically valid to rely exclusively on the properties of the chemical in other physical forms for risk assessment purposes; (b) If NPs have very similar hazard properties to other physical forms, further work on hazard assessment on the NPs may not be necessary; (c) If the NP form has substantially unique properties and no information is available on its biological properties, suitable exposure methods should be used to evaluate their toxicity.

The question that needs to be addressed in this case is: what is the full package of tests that needs to be conducted? The selection of this test battery should be informed by knowledge of the chemical, physical, and biological properties, along with data on the same chemical in other physical forms. In vitro tests play an important role in this screening process; in principle, combined with information on the surface chemistry, these tests could provide an important early indicator of the differences or similarities in potential hazard between the NP form of a substance and other physicochemical forms. However, characterization of the uptake, distribution, deposition, and retention of NPs and the comparison with their larger sized counterparts may require an in vivo approach.

An interesting study was made in 2016 to assess the risk of oral exposure to titanium dioxide particles, and in this study the authors tried to establish some methods to characterize the risk [78]. Authors used two different approaches: (1) Traditional approach based on external exposure in humans and in dose levels in animals determining no adverse effects (NOAEL) or determining adverse effects (LOAEL); (2) Internal dose approach based on assessing internal dose levels using toxicokinetic data. It considers the organ concentration of a dose at which adverse effects appear and tissue accumulation of a substance over time.

The approaches to assess exposure have some critical issues, for example kinetic model used on internal dose approach is only partly mechanism-based, because

knowledge about kinetic of $TiO_2$ NPs is limited. Moreover, oral absorption is critical, because not all dose is absorbed and then redistributed to organs and no long-term studies are available today [78]. With traditional approach, $TiO_2$ NPs did not show adverse effects for liver and spleen, but potential risks could not be excluded for testes and ovaries, while potential risks for spleen, liver, testes, and ovaries were present with internal dose approach. The difference between these two approaches shows the importance to consider the toxicokinetic in assessing the risk, especially in the case of substances able to accumulate in human tissues. This aspect is particularly relevant in case of accumulation of a substance in a scenario of a lifelong exposure, in which it can reach considerable concentrations in various organs.

In conclusion, screening assessments of exposures to the more studied NPs could be conducted by developing toxicity benchmarks using the weight of evidence from studies of: (a) nanoscale forms in the toxicological and pharmacological literature; (b) fine-scale forms corrected for the proportionally greater surface area of nanoscale particles; (c) more toxic particles such as UFP; and (d) the toxicology and epidemiology of metal fumes. Uncertainties in such assessments will have to be considered given data limitations; however, collectively, the available studies are beginning to reveal important features necessary for initial risk assessments of specific NPs.

## 2.5 Risk Characterization and Management

Health effects and risk data on workers exposed to NPs are yet limited, despite some studies showing that exposure to NP may promote some adverse effects on organ, tissue, cellular, subcellular, and protein levels, as mentioned above. Moreover, the toxicity of chemicals, like NPs, depend on the dose, time of exposure, and route of administration. Risk assessment of nanomaterials is currently considered as a scientific challenge for stakeholders and there is an ongoing discussion on how to consider nanomaterials in well-proven risk assessment approaches which initially were developed for conventional chemicals. First requisite for evaluations of workers' exposure requires a comprehension of potential hazards as well as a reasonable exposure estimate, but in many circumstances the exposure assessment cannot be quantified, due to technical limitations of measurement (methodologies) either in the workplace or in the environment, and often times the exposure is so low that it cannot be measured. Therefore, often times the exposure evaluations require estimations quantified by product life cycle [40]. Due to the lack of available data on the risk characterization of different NPs, no generic conclusions are possible at this stage.

Consequently, each product and process that involves NPs must be considered separately in terms of worker safety during the manufacture of NPs, safety of consumers using products that contain NPs, safety of local populations due to chronic or acute release of NPs from manufacturing and/or processing facilities, potential human health risk for reexposure through the environment due to disposal or recycling of NP-dependent products.

In the absence of suitable hazard data, a precautionary approach should be adopted. Containment and control of potential exposures should pursue protection practices relevant to the activity of worker and commensurate with the available control measures. The control measures include elimination of the substance, replacement with a safer substance, use of PPE, and control of procedures for the use of the substance. In addition, it should also be noted that there is no reliable information on the effect of the simultaneous exposure to multiple forms of NPs. It would be appropriate to assume that the effects are additive, or there could be interactions between NPs and other stressors (either physical, chemical, or biological) which should be considered on a case-by-case basis.

The main source of information on the potential for adverse human health effects with NPs are the epidemiological and in vitro studies of airborne particles in ambient air. A wide variety of in vitro assays are available to assess cellular toxicity. In vitro assays are used in most studies to evaluate the cytotoxicity and biological responses of NPs [79]. Researchers often tend to implement comparatively simple in vitro test systems that are relatively easy to perform, control, and interpret. However, there is a need to develop validated in vitro assay systems for toxicity testing of an expanding range of NPs. In vitro methods can be precisely controlled; hence, they can provide more reproducible toxicity data than in vivo models but require higher standardization [80]. The studies have shown that smaller particles of low solubility (less than 1 μm) are substantially more toxic than larger particles. Furthermore, it has been found that as far as ambient air pollution with fine particles is concerned, there is a population subgroup (including individuals with severe chronic respiratory and heart disease) that is much more sensitive to the adverse effects than the public as a whole [81]. There is some evidence that NPs can have a different toxicity compared with larger particles of the same substance, for example, the different modulation of cytokine production by mononuclear cells exposed to $CoCl_2$, microparticles, and NPs [55]. Co microparticles showed a greater inhibitory effect compared with other Co forms. Equally CoNPs administered intratracheally were found to produce dose-dependent lung lesions differently from carbon black [82], and multifocal pulmonary granuloma, effects different from those of quartz, carbon black, and graphite [54]. Furthermore, there are differences in toxicity even between NPs in molecular or ionic form as highlighted in in vitro experiments. For example there is a significant increase of intracellular ROS in Pd(IV) exposed human PBMC cells, but not in Pd-NPs exposed cells; moreover, cells exposed to Pd(IV) ions showed a significant amplification of cell cycle arrest in the G0/G1 phase and a significant reduction in the GS and G2/M phases [3].

The conclusion from these studies is that NPs have a specific activity/toxicity and support the case for a separate/additional risk assessment of substances that are in NPs form. These differences may be attributable to the fact that they have a much greater surface area to weight ratio than larger particles and, as a consequence, they tend to be more chemically reactive and bind other substances to their surface more effectively. Because of the inverse relationship between particle size and surface area, it is imperative that dose–effect (or concentration–effect) relationships are established as a function of total surface area and/or number of particles, rather than

mass units. Furthermore, a comparison should be made between the effects of the conventional and the NP form, and further between NPs in molecular or ionic form.

The International Organization for Standardisation (ISO) has established a number of technical reports to provide a framework for risk assessment of nanotechnologies such as ISO/TR12855 and ISO/TR13121. Risk management includes: (a) the identification of the hazard (describe NPs, their applications, physicochemical profiles, hazard profiles, and exposure profile); (b) the evaluation of the risk based on the combination of type of hazard, mode, and time of exposure and potential risks; (c) control risk with the possibility of intervening at various levels (level 1: eliminating the NPs on request; level 2: replace the NPs with a safer product; level 3: reduce exposure to NPs through the use of PPE or particular production procedures); (d) deciding whether to continue the development and production of nano material (sharing of information with stakeholders; collect more security information); and (e) update the risk assessment process through regular reviews.

To better clarify, risk knowledge has been categorized into simple, complex, uncertain, or ambiguous, based upon whether the method of evaluation was scientific (evidence-based) or societal (value-based). Simple risks have clear cause–effect relationships for materials and their impact. Complex risk refers to the difficulty in identifying the causal links and their effects. There is insufficient knowledge about the cause and effect relationship. Uncertain risk knowledge refers to the incompleteness of knowledge, with the available knowledge relying on uncertain assumptions, assertions, and predictions. Ambiguous risk knowledge has variable interpretations, although it largely denotes a lack of proper understanding of the phenomena and their effects.

Today the particle size distribution is one of the main physicochemical characteristics most studied in toxicological studies on NPs; however, other important parameters in which to concentrate research are: surface, surface reactivity, solubility in water, agglomeration, chemical composition, morphology, particle number, and mass concentrations [83].

According to the above, nanotechnology products will require pre-market testing for health and environmental impact, life cycle assessment, and consideration of secondary risks. Therefore, further studies will have to develop appropriate methodologies for monitoring nanomaterials together with methods to reduce exposure.

## 2.6 Control Banding for Engineered Nanoparticles

The traditional approach to protecting workers' health is based on measuring the exposure to potentially hazardous agents. Measurements of worker exposures to these agents are typically compared to occupational exposure limits (OELs) to determine if existing control measures provide adequate protection. Reliance on this approach has become increasingly difficult due to the growing number of potentially hazardous materials in the workplace that do not have OELs. The large and rapidly growing number of types and structures of nanomaterials (e.g.,

nanoparticles, nanofibers, nanotubes) has presented a major challenge as it is impossible to perform toxicological evaluation on each nanomaterial prior to potential worker exposure. Control banding (CB) is a risk management strategy that has been used to identify and recommend exposure control measures to potentially hazardous substances for which toxicological information is limited. A number of different strategies have been proposed for using CB in workplaces where exposure to engineered nanomaterials can occur. At the base of the CB there is the awareness that there is really only a limited number of approaches for risk control and management, that had been developed by solutions previously used to control similar professional exposures: (1) potential hazard control, (2) engineering controls, (3) good professional hygiene practices, and (4) advices from qualified personnel. A basic principle for CB is the requirement for an easy-to-use method, even for non-expert, capable of giving consistent and accurate results, as a possible alternative to complicated sampling. Appropriate controls, based on hazard and exposure bands, must be correctly implemented and managed through periodic evaluations, verifying their correct and safe functioning through monitoring, in order to keep workers' exposures within acceptable limits.

Control banding does not replace experts in occupational safety and health area, nor it does eliminate the need for exposure monitoring.

In conclusion, the use of CB for reducing exposures to nanomaterials has the potential to be an effective risk management strategy when information is limited on the health risk to the nanomaterial and/or there is an absence of an OEL. However, there remains a lack of evidence to conclude that the use of CB can provide adequate exposure control in all work environments. Additional validation work is needed to provide more data to support the use of CB for the safe handling of nanomaterials.

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# Chapter 3
# Immune Toxicity of and Allergic Responses to Nanomaterials


**Yasuo Yoshioka, Toshiro Hirai, and Yasuo Tsutsumi**

**Abstract** Over the past decade, the remarkable development of nanomaterials and nanotechnology has led to their use in many applications. But as the uses of nanomaterials have increased, so have concerns regarding their potential adverse effects (that is, nanotoxicity) in humans and the environment. Because the body's immune systems are responsible for dealing with foreign substances, we likely should expect at least some interaction of nanomaterials with our immune systems with daily use of nanomaterials, and we must understand those interactions in order to use nanomaterials safely or to develop safer nanomaterials. In this review, we summarize recent advances in immunotoxicology studies of nanomaterials, especially (1) macrophage recognition of nanomaterials with particular emphasis on the effect of par-

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ticle size, and (2) in vivo responses after skin exposure to nanomaterials, including the onset or aggravation of allergy. In addition, we discuss challenges to further understanding the immune system–nanomaterial interaction, with the goal of increasing the safety of these compounds.

**Keywords** Allergy · Cytokine · Nanomaterial · Nanotoxicity · Macrophage · Skin

## 3.1 Introduction

Recent advances in nanotechnology have enabled the design and production of many engineered nanomaterials (i.e., materials with at least one dimension measuring 1–100 nm), including nanoparticles, nanofibers, and nanosheets. Due to their small size and large surface area, nanomaterials have unique physical and chemical properties, such as enhanced hardness, plasticity, conductivity, diffusivity, optical properties, and chemical reactivity, compared with larger materials, making nanomaterials attractive in a wide range of applications. More than 1800 diverse nanotechnology-based products have been used in a broad range of applications in the machinery and textile industries, cosmetics, and medicine.

As the use of nanomaterials increases, so have concerns regarding the safety of nanomaterials; these concerns regarding nanotoxicity have arisen due to two main reasons [1]. The first reason is that, compared with larger materials, nanomaterials have greater potential to travel through an organism because they can cross various biologic barriers [2], for example, the placenta [3] and blood−milk barrier [4]. The second key reason is that the biologic activity of a material typically increases as its particle size decreases: smaller materials occupy less volume, leading to increased surface area per unit mass and thus increased potential for biologic interaction. In accordance with these factors, both in vitro and in vivo studies as well as both acute and chronic experimental models have revealed previously unappreciated toxic effects of nanomaterials, including immunotoxicity, neurotoxicity, genotoxicity, and reproductive toxicity. In addition, concerns regarding the human health risks of engineered nanomaterials have emerged [5, 6]. Therefore, more information regarding nanotoxicity is warranted for utilizing the potential benefits of nanomaterials and to design safer nanomaterials.

Given that—like bacteria, viruses, and allergens—nanomaterials are foreign substances to the body, they likely would first encounter the immune system and consequently trigger various immune responses. Therefore, because nanomaterials probably initiate some sort of an immune response regardless of the route of exposure, the immunotoxicity of nanomaterials is one of the highest priority research interests in the nanotoxicity field [7, 8]. Two predominant scenarios of immunotoxicity are: (1) nanomaterials induce an inflammatory response that directly causes a health problem and (2) the immune response induced through exposure to nanomaterials modulates immune responses to other targets, consequently deranging

appropriate responses to targets (e.g., suppressing immune response to viruses) or aggravating ongoing health issues (e.g., exacerbating allergy). To prevent either situation, we need to understand the mechanisms through which our immune systems recognize nanomaterials and the various possible responses to this recognition. In this review, we overview current knowledge regarding immune recognition of nanomaterials and their hazards in vivo.

## 3.2 Nanomaterial-Recognizing Receptors on Macrophages

Professional phagocytes such as macrophages, dendritic cells, and neutrophils are primarily responsible for the elimination of foreign substances from the body. Therefore, these cells likely are similarly charged with recognizing nanomaterials. Once phagocytes find nanomaterials, the immune response induced probably depends on how they are recognized. For example, some nanomaterials might induce macrophage activation because they are recognized by immune-activating receptors and consequently trigger the production of inflammatory cytokines and chemokines, thereby recruiting numerous inflammatory immune cells and thus resulting in tissue damage. We begin this discussion by introducing reports regarding the receptors on macrophages that potentially recognize nanomaterials.

A wide variety of cell-surface molecules on macrophages have been shown to be receptors for pathogenic particles. For example, class A scavenger receptors, such as SR-A1 and MARCO, and class B scavenger receptors, such as SR-B1 and CD36, bind to bacteria and apoptotic cells [9], indicating that these molecules might be candidate cellular receptors of nanomaterials. In fact, polystyrene nanoparticles and $SiO_2$ nanoparticles bind to MARCO [10, 11]. SR-A1 and the mannose receptor CD206 contribute to the uptake of $SiO_2$ particles by primary human macrophages [12]. In addition, SR-B1 is a receptor for $SiO_2$ nanoparticles, and SR-B1-deficient macrophages neither internalized $SiO_2$ nanoparticles nor promoted inflammatory responses to $SiO_2$ nanoparticles. Furthermore, SR-B1 binds to $SiO_2$ nanoparticles but not $TiO_2$ nanoparticles [13], even though SR-A1 and MARCO are known to bind to both $SiO_2$ and $TiO_2$ particles [14].

Regarding their immunotoxicities, one characteristic point of nanomaterials needs to be remembered. Nanomaterials can be quite different in size, shape, and surface charge even when their core materials have the same chemical formula (Fig. 3.1). That is, even nanomaterials with the same name (e.g., $SiO_2$ nanoparticles) can behave differently depending on the specific product in which they are used, and the immune system may recognize and therefore respond to these formulations differently. Many recent studies including our own have shown that size, shape, and surface charge all influence the pro-inflammatory effects of nanomaterials [1, 15]. For example, when we compared the inflammatory effects of $SiO_2$ particles that differed in diameter (30–1000 nm), $SiO_2$ nanoparticles with diameters of 30 and 70 nm induced greater cytokine production by macrophages in vitro than did larger particles [16]. Furthermore, intraperitoneal injection of $SiO_2$ nanoparticles induced

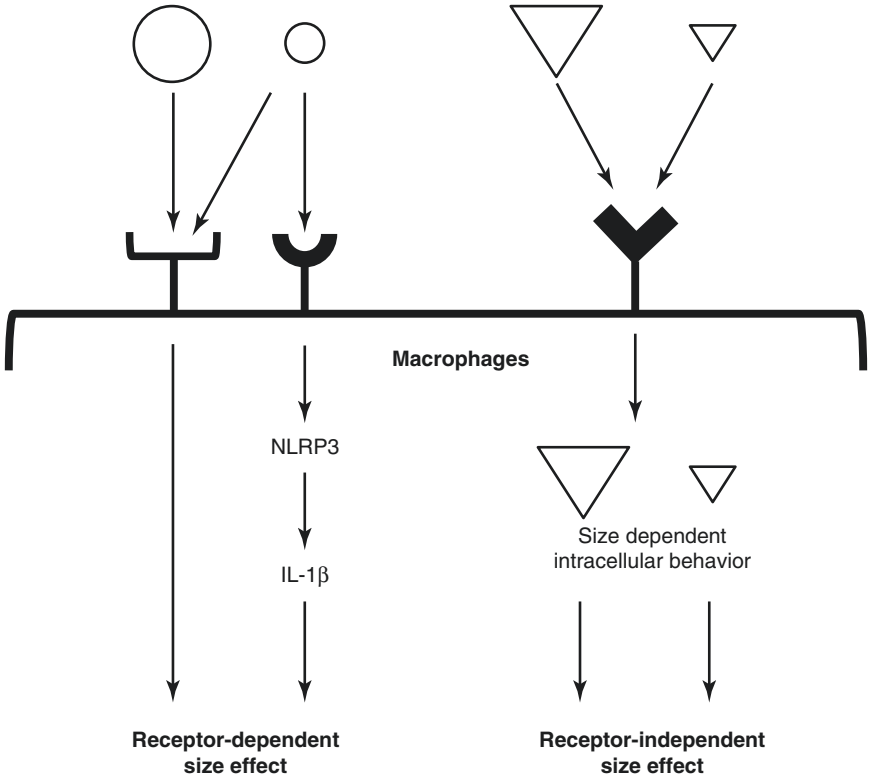


**Fig. 3.1** Several parameters contribute to the immunotoxicity of nanomaterials

stronger inflammatory responses with cytokine production than did larger particles, whereas surface modification of $SiO_2$ nanoparticles suppressed inflammatory responses. However, the mechanism underlying these differences (such as whether the recognizing receptor changes) largely remains to be clarified.

## 3.3 Size-Specific Effects of Nanomaterials on Their Recognition by Macrophages

We recently investigated the effect of the size of $SiO_2$ particles (diameter: 10, 30, 50, 70, 100, 300, and 1000 nm) on pro-inflammatory responses by a human macrophage cell line [17]. The secretion of IL-1β showed a bell-shaped distribution, where $SiO_2$ nanoparticles with a diameter of 50 nm had the greatest effect on secretion; $SiO_2$ particles with larger or smaller diameters had progressively less effect on IL-1β secretion by the cell line. Interestingly, SR-A1 contributed to IL-1β induction

and the cellular uptake of 50- and 100-nm $SiO_2$ nanoparticles but not their 10- and 1000-nm counterparts; this results suggest that only $SiO_2$ nanoparticles of specific sizes induce SR-A1-mediated inflammatory responses. In addition, after intravenous injection of mice with $SiO_2$ nanoparticles that ranged in size from 10 to 1000 nm [18], decreases in platelet count and increases in liver damage and lethal toxicity showed a simple correlation with decreasing particle size; in contrast, among the variously sized $SiO_2$ nanoparticles, those 50 nm in diameter induced the most severe hypothermia. These results show that the inflammatory responses induced by nanomaterials can be highly size-specific and that various nanomaterial-recognizing receptors critically regulate these responses. Several in vitro studies have shown that cellular uptake of nanomaterials is most efficient for those with a diameter with 50 nm, compared with larger and smaller nanomaterials [19, 20], thus supporting the concept of size-specific immunotoxicity. Taken together, differences in the recognition mechanism, such as the receptors involved, contribute to the size-associated effect on the pro-inflammatory response to nanomaterials (Fig. 3.1).

Macrophage subsets differ in the efficiency with which they uptake nanomaterials. For example, 300 nm particles are cleared more slowly in Th1-prone mice compared with Th2-prone mice, and M2 macrophages, which are induced by Th2 cytokines, take up nanoparticles more efficiently than M1 macrophages [21]. In addition, M2 polarization of macrophages promotes nanoparticle internalization [22], suggesting that global immune regulation and macrophage subsets should be considered in regard to the clearance and immunotoxicity of nanomaterials. However, whether the different responses by various macrophage subsets are due to the presence of different nanomaterial-recognizing receptors remains to be seen.

## 3.4 Potential Phagocytic Receptor Independent Size Effect of Nanoparticles

Among the inflammatory responses induced by nanomaterials, the NLRP3 inflammasome-mediated pro-inflammatory effects of nanomaterials, such as the production of IL-1β (a strong pro-inflammatory cytokine), are gaining particular attention because of their role in initiating inflammation [23]. The secretion of IL-1β is tightly regulated and involves at least two processes: the NF-κB-dependent synthesis of pro-IL-1β and the NLRP3 inflammasome (caspase 1)-dependent cleavage of pro-IL-1β for the secretion of mature IL-1β. NLRP3-deficient macrophages do not secrete IL-1β after their treatment with nanomaterials, indicating that activation of the NLRP3 inflammasome is indispensable for nanomaterial-induced IL-1β secretion [23]. Both $SiO_2$ and $TiO_2$ nanoparticles strongly activate macrophages to induce IL-1β secretion in vitro and promote pulmonary inflammation in vivo [24]. In addition, NADPH oxidase-dependent activation of the NLRP3 inflammasome is crucial for the lung fibrosis due to multiwalled carbon nanotubes [25]. It is thought that IL-1β from macrophages acts on pulmonary epithelial cells and fibroblasts,

stimulating these cells to produce various inflammatory cytokines, including TNF-α and IL-6, and resulting in massive inflammation in the lung [26]. Several studies have demonstrated that $K^+$ efflux, lysosomal stress, and reactive oxygen species are involved in the activation of the NLRP3 inflammasome by micro- and nanomaterials [23]. In addition, carbon black nanoparticles were shown to induce the programmed cell death designated pyroptosis, which is distinct from apoptosis, through the NLRP3 inflammasome [27]. We noted that 50 nm and 100 nm $SiO_2$ nanoparticles that are both acquired through scavenger receptor A1, while 50 nm achieve the greater activation of NLRP3 to induce production of IL-1β compared to 100 nm in an independent way of the amount of silica particles taken up by the cells, suggesting that only specific size of particles activate intracellular signaling that is somehow coupled with the receptors on phagocytic cells [17]. We also noted that differences in particle size could influence membrane trafficking of endosomal vesicles, thus suggesting that the size effect of nanomaterials can be induced in a receptor-independent manner [28] (Fig. 3.1). The fact that some crystals can induce lysosomal damage independently of phagocytic receptors, which activate NLRP3 to induce IL-1β, might support this hypothesis [29].

## 3.5 Immune Responses Due to Cutaneous Exposure to Nanomaterials

Because of the potential for inhalational exposure to nanomaterials in the workplace, in vivo studies have particularly focused on the pulmonary inflammatory responses to these compounds [30]. However, the increasing use of nanomaterial-containing skin care and other consumer products provides many opportunities for dermal exposure to nanomaterials [31]. For example, because their ultraviolet-protective properties are stronger than those of larger particles, ZnO and $TiO_2$ nanoparticles have been used in sunscreens. $SiO_2$ nanoparticles are used as an anti-setting agent in a wide variety of cosmetics, and Ag nanoparticles (because of their antimicrobial properties) are found in diverse consumer products, including clothing, antibacterial sprays, detergent, socks, and shoes.

The stratum corneum prevents the penetration of some foreign substances into the body, and, in general, healthy skin is considered to be impervious to all but small (<500 Da) lipophilic molecules [32]. Many studies have assessed the skin penetration of nanomaterials by using healthy and damaged skin samples in both in vitro and in vivo models [33]. These reports suggest that nanomaterials typically can penetrate deep into the epidermis of allergic or damaged skin, although nanomaterials have limited penetration into healthy skin.

Because irritant and allergic dermatitis are the most frequent health issues induced by cutaneous exposure to chemicals in humans [34, 35], the potential for nanomaterials to irritate and sensitize skin should be a primary focus of investigation. Polystyrene nanoparticles (diameter, 50 nm) and $TiO_2$ nanoparticles (primary

size, <25 nm) do not induce acute skin irritation after topical skin treatment in mice [36]. In addition, $SiO_2$ nanoparticles (approximate diameter, 100 nm) applied to the ear of mice causes only slight thickening of the ear [37]. These results suggest that many nanomaterials do not irritate healthy skin, probably because the penetration of nanoparticles into healthy skin is low. However, because ZnO nanoparticles reach the deep layers of allergic skin and modulate immune responses [38], we still need to consider the skin irritating effect of nanomaterials when exposure is combined with damaged skin.

Sensitization to a chemical refers to the induction of specific acquired immunity (i.e., via antibodies or T cells) to that compound. After sensitization, decreased doses of the induction agent can stimulate a full immune response; for this reason, the sensitization potential of nanomaterials should be evaluated. Other than a few reports showing that a fullerene derivative can induce specific antibodies only when conjugated with carrier proteins and immunized with a strong adjuvant [39], no evidence clearly indicates that nanomaterials themselves can induce acquired immunity to themselves. However, metal ions always accompany metal nanoparticles; these ions can function as immunogenic haptens and thus might induce metal allergy. In this regard, we showed that immunizing mice by using LPS with metal nanoparticles such as Ag nanoparticles or Ni nanoparticles, but not with metal ions, can sensitize them to the metal and induce metal allergy [40]. This result does not mean that metal nanoparticles are immunogenic but rather that metal nanoparticles might induce sensitization. We are daily exposed cutaneously to metallic ions released from jewelry and clothes, and recent reports have indicated the possibility that metal nanoparticles are naturally and spontaneously generated from metal ions through chemical or photochemical reduction [41]. Therefore, in addition to synthesized metal nanomaterials, naturally occurring metal nanoparticles should be considered as possible sensitizing agents.

Because nanomaterials can stimulate the production of pro-inflammatory cytokines, it is also possible that nanomaterials act as a kind of adjuvant to modulate immune responses to other targets. For example, repeated topical application of ZnO in a mouse model of atopic dermatitis suppressed local skin inflammation but induced systemic IgE levels [38]. In another study, skin painting of $SiO_2$ nanoparticles combined with 2,4-dinitrofluorobenzene (DNFB), a known skin sensitizer, exacerbated DNFB-induced ear thickness and lymphocyte proliferation [37]. In contrast, 30-nm $SiO_2$ nanoparticles interacted with house dust mites allergens to generate allergens–$SiO_2$ agglomerates; cutaneous exposure of mice to these agglomerates induced reduced levels of house dust mite allergens-specific IgG but equivalent levels of IgE compared with those after single exposure to house dust mite allergens [42]. Because allergen-specific IgG can act as a blocking antibody to suppress an IgE-mediated allergic response [43], the IgE-biased immune response resulted in more severe anaphylaxis in the agglomerate-treated mice [42]. Notably, the immune-modulating effect of $SiO_2$ nanoparticles occurred only when mice were co-exposed to $SiO_2$ nanoparticles and house dust mite allergens as agglomerates but not when they were exposed to either allergens separately or when co-exposed but in low-agglomerating conditions. Together, these findings indicate that the

adjuvanticity of nanomaterials as well as their ability to interact with environmental allergens are important to consider in regard to the safe topical application of nanomaterials.

## 3.6 Future Prospects and Conclusion

Numerous challenges remain to fully understanding nanomaterial-induced immunotoxicity. First, the precise molecular mechanisms of nanomaterial-induced immunotoxicity have yet to be fully determined. When nanomaterials enter the body, they typically become coated with various kinds of proteins (the so-called protein corona). The components of the protein corona influence the pathway of cellular uptake [44–46], suggesting the importance of elucidating the relationship between the protein corona and immunotoxicity. In addition, the interaction of nanomaterials with commensal bacteria on the skin and the indirect effects of nanomaterials on host immune systems need to be elucidated, because the microbiota is important for cutaneous immune homeostasis. Furthermore, nanomaterials induce not only immune activation but also immune suppression [47, 48], which is largely underinvestigated. Second, many reports have addressed the nanotoxicity of single nanomaterials, but only a few studies address the combined toxicity and the synergistic toxicity of multiple nanomaterials [49]. Because we likely are exposed simultaneously to a wide variety of nanomaterials, it is important to assess the combined toxicity of multiple nanomaterials. Finally, attention should be given to nanotoxicity not only in experimental animals but also in humans. The human population is concurrently exposed to multiple nanomaterials and chemicals at much lower doses than those routinely examined in animal toxicity studies. In addition, the human immune system differs in several important ways from that in mice [50]. However, the effects of any interaction between such compounds on their individual or combined toxicity are virtually unknown, suggesting that a model system to predict immunotoxicity in humans needs to be developed [51]. These future studies will promote the safer and more useful applications of nanomaterials in our daily life. In addition, this information will facilitate the design of not only nanomaterials that lack immunotoxicity but also nanomaterials that are capable of modulating immune responses as desired.

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# Chapter 4
# Inflammation and Environmental (Ultrafine) Nanoparticles


**Francesca Larese Filon**

**Abstract** Environmental nanoparticles or ultrafine particles (UFPs) are defined by their aerodynamic size <100 nm. They are either emitted directly or formed from precursor gases: in urban areas UFPs exposure originates from many combustion processes, mainly by motor vehicles. Due to the higher surface/volume ratio UFPs are biologically more reactive than larger particles because they can enter deeply into the lung, reaching the alveoli and internal organs through blood circulation.

We systematically searched in Medical Database PUBMED (MEDLINE) for eligible studies investigating inflammatory effects of environmental nanoparticles.

Data available in literature demonstrated a higher inflammatory response associated to exposure to UFPs: in in-vitro studies, in animal and human epidemiological studies. Target organs resulted lung and cardiovascular system and effects are demonstrated mainly in patients with chronic respiratory and cardiovascular diseases.

In general, exposure to UFPs resulted more effective than exposure to fine particles, suggesting a specific role of UFPs, probably related to their small site that permits higher penetration into lung and translocation in blood circulation. Moreover, toxic substances, such as polycyclic aromatic hydrocarbons that can be present in UFPs can enhance inflammatory but also carcinogenic effects.

**Keywords** Ultrafine particles · Nanoparticles · Environment · Toxicity · Epidemiology

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## 4.1 Introduction

Environmental nanoparticles or ultrafine particles (UFPs) are defined by their aerodynamic size <100 nm. They are either emitted directly or formed from precursor gases: in urban areas UFPs exposure originates from many combustion processes, mainly by motor vehicles (diesel fuel, gasoline, and even compressed natural gas) [1]. UFPs can originate also from industrial emissions, heating systems, incinerators, but also from natural sources such as hot volcanic lava, ocean spray, fires, and any kind of smoke. Combustion processes, if not complete, lead to the formation of particles of 15–30 nm in diameter. These primary particles, composed of elemental carbon aggregate into larger particles (60–100 nm): diesel exhaust particles surfaces can absorb chemicals, such as polycyclic aromatic hydrocarbons (PAH), aliphatic hydrocarbons, quinones, transition metals and others [2].

Due to the higher surface/volume ratio UFPs are biologically more reactive than larger particles [3, 4]: they can enter deeply into the lung, reaching the alveoli and penetrate the biological membranes. In vitro studies demonstrated their capability to translocate into blood circulation, overcome the placental barrier and diffuse into all organ systems including heart, brain, kidney, liver, and nervous system [5–7]. Toxicological studies suggest that UFPs can exert a higher toxicity per mass compared to larger particles due to the high surface reactivity and leaching of toxic substances. However, toxicological and epidemiological data are still lacking and their inflammatory effects need to be deeply analyzed.

It is well known that air pollution is responsible for 3.7 million premature deaths each year [8] with the increase of cardiorespiratory morbidity and mortality [9–11], but the contribution of UFPs to these effects is not completely defined. World Health Organization (WHO) has concluded that there is considerable evidence of the toxicological effects of UFPs on human health [12, 13]; however, epidemiological evidence linking UFPs exposure to human health is much more limited.

UFPs are probably the main mediators of particulate matter toxicity due to the greater penetration efficacy into the respiratory system and to the higher translocation rates from the airways into the blood circulation [3, 14]. Moreover, due to their high organic chemical content, they provide proportionally more redox cycling chemicals than larger particles [15].

Ohlwein et al. in 2019 [16] reviewed the epidemiological evidence on health effects specifically associated to UFPs finding an increase of short-term effects on inflammation, autonomic tone, and blood pressure. However, the evidence was inconsistent or insufficient on mortality and morbidity or on long-term effects. This is in accord with HEI in 2013 [5] that stated “the current database of experimental and epidemiologic studies does not support strong and consistent conclusions about the independent effects of UFPs on human health.”

The aim of our study was to investigate the inflammatory properties of UFPs, to understand better their potential to cause health effects such as the increase of cardiovascular and respiratory morbidity and mortality [5].

## 4.2 Material and Methods

We systematically searched in Medical Database PUBMED (MEDLINE) for eligible studies investigating inflammatory effects of environmental nanoparticles using the following terms: ultrafine [All Fields] AND particles [All Fields] AND ("inflammation" [MeSH Terms] OR "inflammation" [All Fields]) OR ("environment" [MeSH Terms] OR "environment" [All Fields] OR "environmental" [All Fields]) AND ("nanoparticles" [MeSH Terms] OR "nanoparticles" [All Fields]) AND ("inflammation" [MeSH Terms] OR "inflammation" [All Fields]). The search retrieved 882 papers from 2008 to 24.02.2020. Five hundred and fifty were excluded leaving an overall number of 332 original references were considered for further evaluation. Fifty-three were finally evaluated for this review.

## 4.3 Results and Discussion

### *4.3.1 In Vitro Studies*

Many studies analyzed in vitro effects of UFPs. Water suspension of UFPs caused inflammatory, cytotoxic, and genotoxic responses [17–20] in cell lines and fine particles PM2.5 induced oxidative stress, aryl hydrocarbon receptor-mediated gene expression, inflammatory responses, DNA damage, changes in the cell cycle, and autophagy in human bronchial and lung cells [21–23]. Šimečková P et al. in 2019 [24] demonstrated induction of gene markers GREM1, EGR1, GDF15 and reduction of SOX9 in lung epithelial cells exposed to PM0.5 as sign of toxicity in lung epithelial cells. Recently, Sotty et al. in 2020 [25] studied inflammatory cytokines (e.g., tumor necrosis factor-alpha, TNFα; interleukine-1 beta, IL-1β; interleukine-6, IL-6; interleukine-8, IL-8), chemokines (e.g., monocyte chemoattractant protein 1, MCP-1, and regulated upon activation normal T cell expressed, RANTES), and growth factors (e.g., granulocyte macrophage-colony stimulating factor, GM-CSF, and transforming growth factor-alpha, TGFα). They demonstrated highest effects of UFPs versus PM 2.5 in causing inflammation with highest sensitivity of asthma—and notably COPD—diseased human bronchial epithelial versus normal human bronchial epithelial cells.

Xia et al. in 2018 [26] using an in vitro cell culture assays with lung-derived antigen-presenting cells and allergen specific T cell and in vivo mouse models of allergic airway inflammation demonstrated that UFPs exacerbate allergic airway inflammation by promoting a Jag1-Notch 4-dependent interaction between alveolar macrophages and allergen-specific T cells, leading to augmented Th cell differentiation.

UFPs are able to inhibit phagocytosis by enhancing their interaction with the alveolar epithelium. Moreover, particles with a high surface area to mass ratio are

able to adsorb potentially toxic organic chemicals or metals, increasing their capability to be a source of reactive oxygen species (ROS) [27].

### *4.3.2 Data on Animals*

Many studies demonstrated in animal models the pro-inflammatory effects after exposure to UFPs. Farina et al. [28] compared the acute and subacute effects of diesel exhaust particles on bronchoalveolar lavage fluid, lung and heart parenchyma in mice. They found an increase of markers of cytotoxicity, oxidative stress, and inflammation both in respiratory and cardiovascular systems. Diesel exhaust particles exposure was more harmful. Data obtained indicated that the chemical composition of UFPs is the key to the differential stress response for the higher concentrations of PAHs on diesel exhaust particles as compared to biomass burning-derived particles. On the contrary, the necrotic damage caused by both kind of particles could be specifically related to their physical nature.

Mice exposed intranasally to UFPs according to acute and repeated exposure protocols presented more particle-laden macrophages and greater chronic inflammation compared to fine particles-exposed mice [29].

In addition to the number of particles, it has been also shown that particle size plays an important role in their immunotoxicity; however, data available is only for silica, that presents a specific toxicological profile [30]. Smaller particles are eliminated less efficiently by macrophages and remain for an extended period of time in lungs, which may promote steady recruitment of more macrophages and induction of chronic inflammation [30].

Studying data on allergic response on mice, Ryclik [31] found that in utero UFPs exposure at a level close to the WHO recommended PM guideline suppressed an early immune response to house dust mites allergen, likely predisposing neonates to respiratory infection and altering long-term pulmonary health, with suppression of Th2- and/or Th17-driven inflammation (asthma promotion) and suppressing effector T cells (increased susceptibility to respiratory infection). However, data are not homogeneous and findings from the previous studies vary considerably, demonstrating either increased airway inflammation and airway hyperreactivity indicative of an asthmatic phenotype [32–34], no effect [35], or even protection from airway inflammation in response to allergen challenge [36].

Other data demonstrated a role for the cardiovascular system. Morales-Rubio et al. [37] found that in utero UFPs exposure induced intrauterine oxidative damage and inflammation and stimulated programming and activation of angiotensin II type I receptors (AT1R) and angiotensin-converting enzyme (ACE), which resulted in increased blood pressure in the male offspring.

### *4.3.3 Data on Humans*

UFP particles can cause lung inflammation when inhaled and Strak et al. [38] found a relationship between exhaled nitric oxides (FeNO) and exposure to particle number concentration together with $NO_2$ and $NO_x$ after accounting for other pollutants such as particulate matter. More recently, Clifford S et al. [39], studied lung inflammation and exposure to particulate matter in 655 Australian children finding that ultrafine particle number concentration was positively associated with an increase in C-reactive protein (CRP) (1.188-fold change per 1000 UFP $cm^{-3}$ day/day (95% CI 1.077–1.299)) and an increase in FeNO among atopic participants (1.054-fold change per 1000 UFP $cm^{-3}$ day/day (95% CI 1.005–1.106)) without effects on asthma or other respiratory diseases or symptoms. This is consistent with the known propensity of UFPs to penetrate deep into the lung and circulatory system. The authors found some evidence that the atopic individuals were more susceptible than nonatopic individuals to experiencing cough and wheeze in association with higher ultrafine particle number concentration exposures [39].

In a review of literature [40] Heinzerling et al. concluded that in single-pollutant models, UFPs were associated with incident wheezing, current asthma, lower spirometric values, and asthma-related emergency department visits among children. Also, higher exhaled nitric oxide levels were positively correlated with UFPs dose among children with asthma or allergy to house dust mites in one study. However, multivariate models accounting for potential co-pollutant confounding yielded no statistically significant results. Although evidence for a relationship between UFPs and children's respiratory is accumulating, the literature remains inconclusive. Interpretation of existing data is constrained by study heterogeneity, limited accounting for UFPs spatial variation, and lack of significant findings from multipollutant models.

The increase in 24-hour UFPs exposure was related to higher FeNO in all children, particularly in those with persistent respiratory symptoms, and most specifically in children with asthma-like symptoms [41].

Also Buonanno et al. [42] reported, in 103 Italian children (healthy and asthmatic/atopic) aged 8–11 years, a crude positive correlation between UFPs (personal measurements) and FeNO only in subgroups of asthmatics, and to a lesser extent, in children allergic to house dust mites. Habre et al. [43] studied adults with mild and moderate asthma exposed to UFP near airport, finding an increase in IL-6 as marker of inflammation, an elevated soluble tumor necrosis factor receptor type II (s-TNFrII) with a decrease in Forced Expiratory Volume in 1 s. They did not observe any association with exhaled nitric oxide.

The cardiovascular inflammatory route was studied by Delfino et al. [44] finding "quasi-UFPs" (particles with aerodynamic diameter < 0.25 μm) to be significantly associated with the inflammation markers IL-6 and soluble TNF-α. In 54 participants UFPs exposure was associated with a complex series of metabolic variations

related to antioxidant pathways, in vivo generation of reactive oxygen species, and processes critical to endothelial function [45]. Comparison of the metabolite levels between the low and high-exposure groups identified five metabolites significantly different: elevated methionine sulfoxide and cystine are consistent with increased oxidative stress with activation of pro-inflammatory cytokines, regulate early events in atherosclerosis and is associated with cardiovascular disease (CVD) and endothelial function. MetSO is the oxidation product of methionine with ROS and a recognized marker of oxidative stress. Changes in arginine, aspartate, and glutamine suggest alterations to critical processes for endothelial function. Changes in NO production result in disruption to vascular homeostasis, and have been implicated in a wide range of pathological states.

Cardiovascular effects have also been reported especially in individuals with existing metabolic or cardiovascular conditions. Lag 4-day particle numbers was associated with total and cardiorespiratory mortality in Germany [46]. Thrombogenic effects and platelet activation were seen in patients with coronary heart disease [47]. An increase in pulse wave velocity and augmentation index was seen in individuals with chronic obstructive pulmonary disease [48] and immediate changes in heart rate variability were found in diabetics or people with impaired glucose metabolism [49].

Delfino et al. in 2008 [50], studying 29 elderly subjects with coronary artery disease found an increase of IL-6 and TNFrII in relation to outdoor particle number concentration. These associations were stronger for exposure to smaller particles [51].

The inflammatory role of UFPs was demonstrated also in volunteers exposed to spark generated carbon black ultrafine particles with ozone [52] that caused increase in plasma club protein 16, the number of sputum cells, the number and percent of sputum neutrophils, and sputum interleukin 6 and matrix metalloproteinase 9. However, limited effect was found without ozone exposure and no effect on cardiovascular biomarkers was found. Corlin et al. in 2018 [53] observed that exposure to particle number concentration was associated with increase in measures of CVD risk markers, especially among certain subpopulations.

## 4.4 Conclusions

Data available in literature demonstrated a higher inflammatory response associated to exposure to UFPs. Lung and cardiovascular system must be considered the target organs and effects are demonstrated mainly in patients with chronic respiratory and CVD. However, epidemiologic studies are still insufficient and many times do not consider multi-exposure pathway.

In general, exposure to UFPs resulted more effective than exposure to fine particles, suggesting a specific role of UFPs, probably related to their small site that permits higher penetration into lung and translocation in blood circulation. Moreover, toxic substances, such as PAH that can be present in UFPs, can enhance inflammatory and carcinogenic effects reaching deeper levels into lung and other internal organs. The actual evidence, albeit not conclusive suggests a specific inflammatory role of UFPs.

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# Chapter 5
# Monitoring Nanomaterials in the Workplace


**Adrienne C. Eastlake, Luca Fontana, and Ivo Iavicoli**

**Abstract** Increased engineered nanomaterial production, combined with widespread use and worldwide distribution, have increased the likelihood of occupational exposure. Considering that engineered nanomaterials have additional toxicological concerns relative to their larger material forms, there exists a clear need to develop, implement, and apply an adequate strategy for occupational risk assessment and management. Unfortunately, a thorough evaluation of pertinent engineered nanomaterial properties cannot be obtained using a single instrument or analytical technique. Therefore, it is recommended that the collection and characterization of engineered nanomaterials should be performed via a multifaceted approach involving the use of multiple complementary sampling tools and analytical methods.

**Keywords** Exposure assessment · Engineered nanomaterials · Occupational exposure · OECD three-tiered approach · NIOSH Nanomaterial Exposure Assessment Technique (NEAT 2.0) · Personal exposure

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## 5.1 Background

In recent years, the unique physicochemical properties of engineered nanomaterials (ENMs) have been widely exploited in numerous industrial and commercial sectors to improve the effectiveness of myriad consumer products and industrial applications [1]. Increased ENM production combined with widespread use and worldwide distribution have increased the likelihood of occupational exposure to ENMs. Although human exposure to ENMs may take place in any stage of a material's life cycle (from their synthesis and integration in the laboratory to their release during use or disposal of ENM-containing products), such exposures are more likely to occur in industrial facilities and/or research laboratories where ENMs are produced or handled in large quantities or over long periods of time [2]. Also, considering that ENMs have additional toxicological concerns relative to their larger material forms [3–13], there exists a clear need to develop, implement, and apply an adequate strategy for the occupational risk assessment and management of ENMs [14].

ENMs are generally considered chemicals in spite of the extraordinary classification challenges they create due to the diversity of sizes, shapes, chemical composition, and morphologies they can assume. Consequently, principles of ENM risk assessment and management traditionally used for chemicals may also be applicable to ENMs. The gold standard for chemical risk assessment and management was established by the US National Academy of Sciences [15]. According to this paradigm, the risk assessment process is based on four critical steps including hazard identification, dose–response assessment, exposure assessment, and risk characterization. Unfortunately, the application of this model to ENMs is especially challenging given limited toxicology data and few occupational exposure limits (OELs).

For example, occupational ENM monitoring can be conducted by assessing different metrics such as mass, number, and/or surface area concentration [16]. However, currently there is no international consensus on the most adequate metrics to be measured [17], although it has been suggested that ENM toxicity is closely related to surface area and number concentration, rather than mass dose [3, 18]. Moreover, the ability of ENMs to induce adverse effects has been associated with several intrinsic physical and chemical characteristics such as size, shape, and chemical composition [3, 9, 11, 19, 20]. Therefore, the development and implementation of an appropriate sampling strategy should directly measure different concentration-related metrics or, at a minimum, provide sufficient characterization of ENM physicochemical properties properties to allow for an accurate estimation of these dose metrics from mass-based measures of ENMs [21]. Unfortunately, a thorough evaluation of pertinent ENM properties cannot be obtained using a single instrument or analytical technique. Rather, collection and characterization of ENMs should be performed via a multifaceted approach involving the use of multiple complementary sampling tools and analytical methods [22, 23].

In the forthcoming sections, important occupational exposure assessment strategies are described to provide practical information useful for determining and characterizing ENM occupational exposure levels.

## 5.2 Organization for Economic Co-operation and Development

The Organization for Economic Co-operation and Development (OECD) is an intergovernmental organization with representatives from 34 countries that coordinate policies, areas of mutual concern, and work together to address international problems. Much of this work is performed through expert working groups and committees organized around topics of shared interest. The OECD Working Party on Manufactured Nanomaterials (WPMN) was established in 2005 to evaluate the safety of nanomaterials. A steering committee was formed to address the potential for human health and safety implications and to work toward creating a science-based and internationally harmonized standard [24].

## 5.3 OECD Three-Tiered Approach

The OECD WPMN performed a systematic comparison of 14 different published nanomaterial-specific exposure and measurement approaches (for use in the absence of OELs) and compared the similarities and key differences between these approaches [25]. This review indicated that most of the reviewed documents made use of a tiered assessment strategy. In addition, analysis of the reviewed approaches indicated that, to be valuable, an approach should be cost effective, based on current measurement methods, able to discern the ENM of interest, and capable of providing comparable results. The WPMN collated all information and created a three-tiered approach to form a comprehensive and consistent method to address gaps in the 14 methods reviewed. A summary of the OECD tiered method is provided in Fig. 5.1.

### *5.3.1 Tier One*

#### 5.3.1.1 Information Gathering

The aim of tier one is to gather as much information as possible about the occupational workplace under evaluation, such as processes involved and materials in use. All data gathered are then evaluated to determine if additional assessment work should be performed. Tier one can involve a walk-through of the facility to visually confirm the potential for specific tasks or processes to generate emissions, or it may consist of carefully reviewing policies and procedures related to activities involving the production or handling of ENMs. In addition, thorough characterization of materials used in the workplace (ENMs in particular) should be performed using a suite of laboratory analytical approaches as mentioned earlier. Information on the

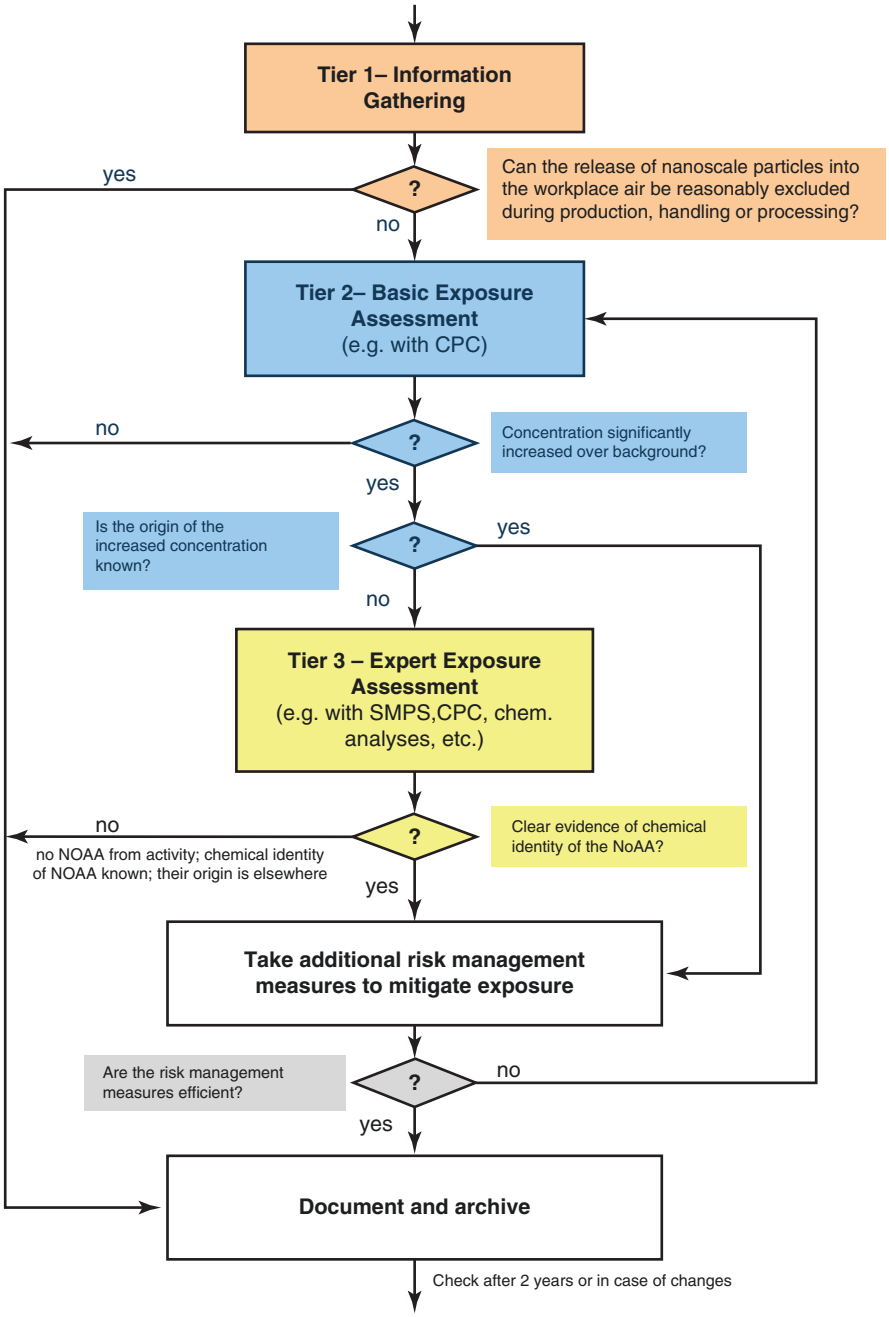


**Fig. 5.1** OECD tiered approach flowchart. Reprinted from 'Harmonized tiered approach to measure and assess the potential exposure to airborne emissions of engineered nano-objects and their agglomerates and aggregates at workplaces'. Series on the Safety of Manufactured Nanomaterials,' OECD. 2015, No. 55. ENV/JM/MONO(2015)19

hazard potential of a particular ENM should be evaluated. Risk management or control banding tools that allow for data collection and evaluation can be helpful at this point, e.g., Control Banding Nanotool, NanoSafer, and Stoffenmanager Nano [26–29]. If the hazard potential is high (i.e., exposure to a low concentration could lead to health effects), then tier two or tier three should be considered.

Following is a list of the minimum information typically required for effective use of risk management or control banding tools:

- Workplace information, such as type of workplace, processes, materials, production volume, and the presence of exposure control measures (such as general or local exhaust ventilation)
- ENM(s) of interest, information to include: particle structure, particle size, aspect ratio, and composition (such as powder or solid)
- Workplace activities, such as processes and tasks performed, processing of composites, presence of other processes in the workplace that could potentially effect measurement methods, and the presence or absence of ventilation

Once all data are collected and analyzed in tier one, it should be determined if there is the potential for the release of ENMs in the work environment. If the possibility for release of ENMs exists, then it is recommended to pursue tier two exposure assessment measurements.

## *5.3.2 Tier Two*

### 5.3.2.1 Exposure Assessment

The aim of tier two is to determine whether an exposure to ENMs may occur. This aim is completed by making use of portable field equipment and knowledge of the material and processes gathered during tier one. As no single commercial instrument is currently capable of providing all information needed to adequately identify specific ENMs, multiple direct reading instruments (DRIs), such as condensation particle counters (CPC) or optical particle counters (OPC), are used in concert with off-line, collection-based sampling (e.g., filter-based collection). Off-line sampling media can be analyzed using electron microscopy to determine number concentration, composition, and morphological. Information gathered during tier one is essential to guide the planning and execution of the exposure assessment. It is important to select and use DRIs that are capable of measuring the ENM of interest. Tier one data will also provide input as to potential emission sources, sampling locations, and the duration of sampling required.

Background sampling should be performed to help separate process-related emissions from emissions attributable to ambient environmental conditions (e.g., nearby vehicle exhaust, neighboring industrial emissions, kitchen areas). As DRIs are unable to differentiate between the ENM of interest and naturally occurring and incidental sources, it is essential that concurrent background sampling is performed

to compare with any other sampling. Instead of making a recommendation as to how a background sample should be performed, the OECD method instead references several general methods recommended in the reviewed approaches. Some of these methods include: (1) measuring before and after processing or handling of ENMs (time variance approach); (2) measuring simultaneously in an area not affected by the processing or handling (spatial variance approach); (3) measuring in the same area where the ENM is handled or processed, but when no ENM is present; (4) or a combination of any of the above.

When DRIs are used in data log mode, it is important to note tasks and processes that take place over the entire duration of the operation evaluated, even events that might seem insignificant. This applies to DRI data collected to monitor both the processes/tasks and the background. During analysis of the data collected, any change in number or concentration can then be linked to specific activities, tasks, or processes that may have contributed to that change (i.e., a decrease based on local exhaust ventilation or an increase due to benchtop agitation/handling of a dry powder).

Based on input from subject matter experts, a minimum of 45 min of sampling is recommended for both the assessment and background [25, 30]. If possible, sampling during a specific task in addition to assessing full-shift will provide an understanding of the changes in aerosolized materials throughout the day. When sampling is complete, fluctuations in DRI data should be compared among sampling locations throughout the day. When the data indicate stable particle number concentrations for a contiguous duration, the mean and standard deviation for that stable concentration should be calculated and noted. The standard deviation for the background should be of the same order of magnitude or smaller than those obtained from the processes. If the standard deviation from the process is larger than that of the background, then it is recommended to subtract the average background from the process concentrations. If the difference of the concentrations between background and process data is more than three times the standard deviation for the background, then a tier three investigation should be conducted [30].

Data reporting requirements for tier two include:

- Instruments and metrics used
- Information on
  - Emission sources
  - Potential confounding factors (such as forklifts or motors)
  - Workplace activities performed throughout the day or process
- Concentration of DRI data reported over time
  - Analysis should indicate if process concentration is significant relative to background.
  - Trends should be evaluated and compared to workplace activity documentation.
- Off-line analysis data can be used to augment DRI data
  - Electron microscopy for ENM morphology and energy dispersive X-ray analysis for chemical identification

If tier two data indicate the location of an ENM concentration increased (exceeding three times the background standard deviation), then risk management actions should be pursued. These actions may include, for example, the installation and use of local exhaust ventilation. The effectiveness of any risk management action should be verified by repeating all tier two sampling and analyses to verify a decrease in exposure potential.

## *5.3.3 Tier Three*

### 5.3.3.1 Expert Exposure Assessment

The aim of tier three is to build on the information gathered in both tier one and tier two, by determining if the potential for ENM exposure exists or if additional risk management actions need to be taken. In tier three, all appropriate exposure assessment techniques, equipment, and samplers should be used to identify the potential for occupational exposure.

In tier three, measurement methods may include instruments that are not easily operated or portable. These instruments may include, but are not limited to the following: surface area monitors, diffusion chargers, electrical mobility analyzers, and aerodynamic particle sizers. These instruments may require expert experience in order to use, analyze, and interpret the data obtained. As in tier two, DRI data must be collected in concert with off-line analysis to determine number concentration, composition, and morphological characteristics or mass concentration. Off-line analyses can also include mass analysis or collection and interpretation of surface wipe samples. Information gathered during tier one is essential to guide the overall planning and performance of the exposure assessment.

Data reporting requirements for tier three are the same as indicated for tier two. Additional DRIs are used in tier three, which may require additional data analysis and focus on particle sizes and ranges.

Data analysis requirements are indicated below:

- The average, maximum, and minimum data should be provided for the particle spectrum in addition to the particle size range (i.e., <100 nm or 1–400 nm).
  - This should include background and any other area locations sampled.
- If similar data were collected by different instruments, then any variability between instruments should be taken into consideration.
- When data are logged over a period of time, it is important to note every workplace event that may have caused an increase or decrease and interpret the data within the appropriate context.

If tier three data indicate an ENM concentration increase over background, then risk management actions should be taken in accordance with the hierarchy of controls. The effectiveness of any risk management action should be verified by

repeating all tier two and tier three sampling and analyses to verify a decrease in exposure potential.

## 5.4 United States National Institute for Occupational Safety and Health (NIOSH)

The NIOSH is the United States federal agency that conducts research and provides guidance and recommendations on occupational injury and illness. Since 2004, the NIOSH Nanotechnology Research Center has been performing research to:

- Increase the understanding of ENM worker hazards and health risks
- Identify and fill research gaps regarding ENM hazards
- Create and provide ENM guidance materials to inform a wide variety of audiences on hazards, risks, and appropriate risk management strategies
- Perform epidemiologic studies on ENM workers
- Assess and promote national and international risk management guidance

As part of ENM exposure assessment research, the NIOSH field team has performed over 120 exposure assessments since 2006. By collecting field data in a variety of facilities on many different ENMs, the NIOSH field team has been able to create a method that is both adaptable for a variety of facility types and flexible enough to be used for different types of materials.

## 5.5 United States NIOSH Approach: Nanomaterial Exposure Assessment Technique (NEAT 2.0)

The Nanomaterial Emission Assessment Technique (NEAT) was first published in 2010 to assist occupational safety and health specialists with the identification and measurement of ENMs in the workplace [31, 32]. In addition to the method, data were published on 16 field assessments that used the method [32, 33]. NEAT was included in the OECD review of tiered approaches. The original method focused on the use of DRIs to detect emissions from short-term tasks or processes. The collection of off-line filter-based samples was used, but the data obtained from these samples could not be compared to any existing ENM-specific OELs as they were not taken over a full-shift or in the worker's personal breathing zone (PBZ) (defined as a 30 cm hemisphere around mouth and nose). In addition, at this time, OELs did not exist for most ENMs. NEAT did not address the potential for the following: fluctuation of DRI data because of incidental or intermittent background particles; or, extended exposure to ENMS such as full-shift or performing multiple ENM tasks.

Based on ongoing NIOSH field team research, it was determined that the methods described in the NEAT method were focused on *emissions* as opposed to a comprehensive *exposure* assessment. Therefore, as the knowledge, experience, and

**Table 5.1** Components of the Nanomaterial Exposure Assessment Technique (NEAT 2.0)

| Collect basic workplace information | Design and implement the sampling plan | Risk assessment | Risk management |
|---|---|---|---|
| Work flows, staffing, and tasks<br>Materials used<br>Safety data sheets<br>Literature review<br>Anticipate and recognize hazards<br>Other indicators of potential exposure situations | Full-shift and task-based integrated filter sampling for elemental mass and microscopy characterization.<br>Direct reading instruments<br>Evaluate ventilation and engineering controls | Evaluation of data:<br>Background<br>Engineering Controls<br>Worker Practices<br>Develop strategies to mitigate exposure potential based on results and utilizing the hierarchy of controls.<br>Communicate potential occupational risks | Confirmation of continued risk control<br>Additional measurements or controls may be required |

Reprinted from 'Refinement of the Nanoparticle Emission Assessment Technique into the Nanomaterial Exposure Assessment Technique (NEAT 2.0),' Eastlake AC, Beaucham C, Martinez KF, Dahm MM, Sparks C, Hodson LL & Geraci CL. (2016) Journal of Occupational and Environmental Hygiene, 13:9, 708–717

measurement techniques progressed, it became possible to revise the emission-centered technique to focus on exposure assessment [4]. The Nanomaterial Exposure Assessment Technique (NEAT 2.0) is a series of codependent elements that are used to perform a comprehensive exposure assessment to characterize the potential for worker exposure to ENMs as opposed to focusing on task and process emissions [4]. A summary of the components of the NIOSH NEAT 2.0 is provided in Table 5.1.

The key component of NEAT 2.0 is the use of tandem off-line filter-based sampling. It is recommended that one of these samples be analyzed for mass and the other with electron microscopy. These samples are collected on filter media consistent with the type and composition of the ENM of interest. These samples are collected in the workers' PBZ, area(s) close to the task or processes evaluated, and in a background (far field) area. The selected background area should be away from the task or processes evaluated and on a different ventilation system. PBZ samples can be collected full-shift for comparison with any existing OELs or shorter durations, such as for identifying exposures specific to a particular task [5, 6]. It should be noted that there are still relatively few OELs available for ENMs.

For three nanoparticles—titanium dioxide ($TiO_2$), carbon nanotubes (CNTs), and carbon nanofibers (CNFs)—NIOSH has completed a risk assessment and provided risk management guidelines, including detailed sampling and analysis guidance and recommended exposure limits (RELs), which are believed to be protective over a working lifetime [5, 6]. As of this writing NIOSH has a proposed REL for silver nanomaterials [7]. These RELs are expressed as the respirable fraction of mass per unit volume, over a full work shift:

- Ultrafine $TiO_2$: REL = 300 micrograms per cubic meter ($\mu g/m^3$)
- Carbon nanotubes (CNTs) and carbon nanofibers (CNFs): REL = 1.0 $\mu g/m^3$ as elemental carbon
- Silver nanomaterials: REL = 0.9 $\mu g/m^3$

Comparing nanomaterial exposure levels to the OELs for larger forms of the material may not properly protect workers as studies have determined that ENMs may be more toxic than their larger material forms [3, 5–13, 20, 34]. Electron microscopy analysis should be used to confirm the presence of an ENM by matching its physicochemical characteristics in a collected field sample with its characteristics in a known bulk sample. As existing analytical methods for elemental mass may not be specific to the ENM of interest, modifications to the collection process may need to be performed to obtain results (such as maximizing flow rates to collect sufficient mass).

DRIs are used to determine variations in number, mass concentration, and/or approximate size range of particles. As not all instruments are capable of determining the presence of all types of particles (such as due to high aspect ratio), this method recommends the use of a suite of DRIs together at the same locations where filter-based samples are collected (such as work process area and background). These instruments are used in data-log mode and, if accurate notes are taken detailing worker processes throughout the day, can provide insight into specific worker activities or tasks that contribute to an increase or decrease in particle concentrations or counts. These instruments typically include, but are not limited to, the following: (1) CPC; and/or (2) OPC.

### *5.5.1 Collect Basic Workplace Information*

Initial characterization of the worksite consists of obtaining information on the work processes used, the workers, and the ENM of interest. Information can be obtained through a walk-through of the facility and interviews with workers. Current literature along with safety data sheets should be reviewed to determine safety and health data. It should be noted that information on many ENM-specific safety data sheets may not be accurate as they may provide information about the larger or bulk form of the material instead of information specific to the ENM, or they may lack critical information [35, 36]. Data on the ENM should be obtained such as physical aspects (e.g., size, shape, coatings) and state during use (e.g., slurry, dry powder, or composite).

The number of workers, the type of processes performed, and the workflow should be documented. Process flow diagrams should be reviewed, if available. In addition, existing ventilation systems and exposure control devices should be documented. All data should be evaluated to determine the potential for exposure hazards and emissions. If the potential for exposure exists, then a sampling plan should be designed and implemented.

### *5.5.2 Design and Implement the Sampling Plan*

Based on data obtained in the initial worksite characterization, a sampling plan should be organized. The plan should focus on both task-based and full-shift

samples collected in both the surrounding area and PBZ to determine worker exposure. Tandem filter-based samples should be collected to allow for both mass and electron microscopy analysis. An array of DRIs can be used to support data provided by the filter-based samples. Surface wipe sampling can also be used to verify the spread of materials throughout the facility and to verify housekeeping practices are effective. Following the hierarchy of controls, both general and engineering control ventilation, administrative controls, and the use of any personal protective equipment (PPE) should be evaluated and documented.

### *5.5.3 Risk Assessment*

Results of filter-based mass data should be compared with corresponding ENM occupational exposure limits, if available. For ENMs, such as carbon nanotubes and fibers that may include incidental materials that contribute to the mass results, such as carbon emitted from engines or combustion processes, it is important to subtract the mass of the background samples from other representative samples in order to determine the exposure potential of the ENMs. This is not necessary for ENMs that do not have environmental contributions, such as nanosilver or titanium dioxide. It is important to note that OELs for bulk or larger materials may not protect workers handling the same material in the nanoscale size range. Electron microscopy results can confirm the presence of the ENM in the location sampled. DRI data do not identify the specific type of particle (or ENM), but can document changes in particle number or concentration throughout the day. When these data are analyzed and compared with documentation of task and worker activities, they can indicate the potential for ENM release from specific tasks/processes or the effectiveness of ventilation or engineering controls. Recommendations for the use of specific engineering controls or changes in work practices should take into account all data obtained. NIOSH supports use of the hierarchy of controls and recommends the use of engineering and administrative controls before the use of PPE. PPE is the least preferred control method because it transfers the responsibility for personal safety from the employer to the employee, and there is considerable variability from one individual to the next in the use and fit of the PPE.

### *5.5.4 Risk Management*

Once any recommended changes in work practices or engineering controls are implemented, it is recommended that subsequent sampling efforts be performed to confirm that the changes actually decrease the exposure potential as anticipated. Additional sampling should be performed annually or whenever changes are made to the process.

## 5.6 Other Sampling Techniques

Exposure assessments require the collection of information sufficient to determine the extent to which a worker is exposed to a particular chemical or condition during workplace activities [15]. To obtain data that most accurately represent exposure conditions, such assessments should involve the use of personal measurement devices that are able "to breathe together with the worker," which ensures sampling of the environmental air within the worker's PBZ [37]. Currently, comprehensive ENM exposure assessments require the use of multiple DRIs that can be impractical for personal sampling and allow only for a static measurement at a predetermined sampling position (usually located in an area near a suspected source of ENM emission) [18, 38]. Further, the expense of the most advanced characterization instruments often limits multiplexed sampling, which can be essential for distinguishing ENM emissions from background conditions.

As a result of these analytical limitations, most ENM occupational exposure literature data are provided by studies that use various combinations of DRIs designed to stitch together a more integrated picture of a particular exposure scenario (i.e., CPC, OPC, scanning mobility particle sizer, electrical low pressure impactor, micro-orifice uniform deposit impactor, diffusion chargers) [2, 16]. However, although a suite of DRIs may be placed as close as physically possible to the breathing zone of selected workers, they do not represent personal sampling. The limitations of fixed DRI sampling are especially apparent when workers move within and through the designated work environment. In some cases, the worker may move away from a DRI's static sampling position, which can lead to the mischaracterization of a particular exposure scenario [37]. Recently, innovative samplers and monitors have been developed to overcome the limitations of static instrument positioning and allow for evaluation of individual exposure to airborne ENMs [37]. The use of these portable, small, and lightweight devices could represent an important step forward in the field of ENM exposure assessment, especially considering that both the OECD three-tiered approach and NEAT 2.0 recommend the use of both portable equipment and filter-based sampling.

### *5.6.1 Personal Monitors*

Personal monitors are real-time devices that collect data on airborne ENM levels by measuring lung deposited surface area (LDSA) or particle number concentrations with high time resolution. Currently, five different monitors are available commercially. They are: (1) the Miniature Diffusion Size Classifier DiSCmini (Testo, Titisee-Neustadt, Germany, identical with miniDiSC); (2) the Aerasense NanoTracer (Oxility, Eindhoven, the Netherlands); (3) the Partector (Naneos, Windisch, Switzerland); (4) the Personal Ultrafine Particle Counter (PUFP C100 and C200, Enmont, New Richmond, OH; USA); and (5) the MicroAeth AE51 (AethLabs, San Francisco, CA, USA) [18, 38–46].

DiSCmini, NanoTracer, and Partector exploit the principle of unipolar diffusion charging to calculate the LDSA. Briefly, sampled particles are charged using a unipolar diffusion charger, which allows for the measurement of induced current. The induced current is directly proportional to the LDSA concentration [46]. In addition, the DiSCmini and NanoTracer are also capable of estimating the particle number concentration and the average particle diameter [18, 38]. The PUFP C100 and C200 models are water-based CPCs that measure particle number concentrations, while the MicroAeth AE51 is a portable aethalometer that is capable of measuring black carbon concentration [18, 38].

Some studies have used personal monitors to quantify occupational ENM levels and their effectiveness or applicability in routine environmental monitoring practices has been tested in several laboratory studies [2, 18, 38, 44]. In general, the accuracy and comparability of LDSA concentration measurements conducted with the DiSCmini, NanoTracer, Partector, and MicroAeth AE51 personal monitors is in the range of ±30%. The accuracy of particle number concentrations determined by diffusion chargers can be lower since this metric is inferred by assuming parameters of the particle size distribution [18, 38, 45–48]. Although the accuracy of particle concentration measurements obtained from personal monitors falls short of more robust stationary reference instruments, the tradeoff is worth considering given that the data obtained in a worker's PBZ may provide a more realistic estimate of ENM inhalation exposure.

### *5.6.2 Personal Samplers*

Personal samplers are instruments that collect particles using a substrate such as a filter or flat surface. Here, the emphasis is on collection and preservation of ENMs rather than their immediate detection and quantification. Substrates can be removed from personal sampling devices and characterized using sophisticated analytical techniques such as inductively coupled plasma mass spectrometry (ICP-MS); electron microscopic (scanning electron microscopy or transmission electron microscopy with chemical detectors) or Raman spectroscopy analyses to obtain information (mass, chemical composition, size, shape). Collectively, these techniques can provide a wealth of information about the ENM of interest. Several filter-based personal samplers are available: (1) the NanoBadge (Nano Inspect, Alcen group, Paris, France and French Alternative Energies and Atomic Energy Commission CEA, Grenoble, France); (2) the Nanoparticle Respiratory Deposition sampler (NRD, Zefon International, Ocala, FL, USA); (3) the handheld electrostatic precipitator (ESPnano, Spokane, WA, USA); (4) the Partector TEM (Naneos particle solutions GmbH, Windisch, Switzerland); (5) the Thermal Precipitator Sampler (TPS, RJ Lee Group, Monroeville, PA, USA); (6) the personal sampling system Personengetragenes Gefahrstoff-Probenahmesystem (PGP) (GSA Messgerätebau GmbH, Ratingen, Germany); and (7) a filtration badge and Raman spectrograph (StatPeel Switzerland) [2, 49–53].

In general, these instruments consist of a particle size-selective inlet, a filter cassette/net/grid, and a personal pump. Although they may not all be specific for nano-sized particles, their use may still be helpful in efforts to thoroughly characterize ENMs, particularly under real-world exposure scenarios. Currently, little information is available regarding the comparability of personal nanoparticle samplers to each other or to standard techniques [18, 38]. Additionally, these samplers usually use a low flow rate. Based on the subsequent analytical technique that has been chosen to characterize the sample, a low flow rate may require a long duration sample to obtain adequate sample for analytical detection. Alternatively, individual aerosol particle analysis is sensitive to oversaturation of the filter or substrate surface. When oversaturation occurs, attached or overlapping particles may confound results. Therefore, the use of a personal sampler device for ENMs requires consideration of the ENM particle number concentration, as well as the rate and duration of sampling [18]. Even under highly controlled situations, some trial and error may be necessary to adjust sampling variables to achieve optimal results. For some personal samplers, such as the partector TEM or the ESPnano, the instrument is capable of suggesting an optimal sampling duration to the operator.

## 5.7 Conclusions

Both the OECD tiered approach and NEAT 2.0 methods have considered the knowledge and contributions of many experts. Both methods rely on pre-assessment and final confirmation steps, but differ in recommended approaches. Within OECD, discussion regarding exposure assessment is based on the collection of airborne data from DRIs with the Tier 3 investigation triggered when the difference of the concentrations between background and process data is more than three times the standard deviation for the background. However, there is currently no consensus method on how to statistically analyze and report DRI data [54, 55]. The collection of filter-based samples is mentioned, but is not indicated as a key part of the assessment. In NEAT 2.0, integrated filter-based sampling is the key step in the exposure assessment process. Subsequent analysis of these samples is used to confirm the presence of the ENM of interest. In addition, both surface contamination and dermal exposures are noted. As DRIs are unable to effectively identify the presence or type of ENMs, they are used to support the integrated filter-based results, identify emission sources, and verify the efficacy of engineering controls. Although these methods may look similar, they are not (Table 5.2). OECD is a tiered approach, which takes the user through a stepwise process to perform both an exposure assessment and a complete risk evaluation. NEAT 2.0 is not a tiered approach, but leads the user through different codependent elements that support a comprehensive exposure assessment.

Overall, the data obtained using NEAT 2.0 may support a tiered approach to risk assessment. Given the diversity of ENM types and exposure scenarios, it is highly unlikely that a single instrument or technique will ever be capable of providing all

**Table 5.2** Comparison between the OECD tiered approach and NEAT 2.0

| OECD tiered approach | NEAT 2.0 |
|---|---|
| Tier 1—Pre-assessment information gathering<br>Tier 2—Basic exposure assessment (area particle counters and EM samples)<br>Tier 3—Personal breathing zone samples, mass samples additional aerosol samples<br>Confirmation | Pre-assessment prioritization<br>Exposure Sampling (particle counters, EM samples, mass samples in personal breathing zone and area. Sometimes expanded into additional aerosol samples. Focus on integrated sampling with use of DRIs to identify emission sources.<br>Risk Management Summary<br>Confirmation |

of the data needed for an adequate risk assessment. Further, new ENMs are introduced to workplaces and commerce with increasing frequency. Fortunately, new tools and analytical techniques are being developed to address challenges that ENMs pose workplace safety. Looking ahead, a critical role of the occupational safety professional will be to maintain awareness of current knowledge and recommended strategies regarding the identification and management of emerging workplace ENM risks.

## 5.8 Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention and of the other Institutions where the authors work. Mention of company or product does not constitute endorsement by NIOSH, CDC.

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# Chapter 6
# Immunotoxicity of Nanoparticles


**Claudia Petrarca, Rocco Mangifesta, and Luca Di Giampaolo**

**Abstract** In this chapter, we review the latest and main research findings on anthropic nanoparticles (NPs) from the immune-cytotoxicological perspective aiming at defining the intrinsic chemical-physical and the extrinsic (acquired by the interaction with the environment) characteristics of the nano-systems to which the immune system appears to be unresponsive, tolerant, or anergic. The hereby outlined presumptive and speculative determinants of immune compatibility of nanoparticulate matters, although based on experimental data, represent a basic information for the design of new biocompatible nanomatters; finally, they represent an evaluation tool for potential immune outcome in professionally involved workers and may be important to enhance the exertion of risk assessment.

**Keywords** Nanoparticle · Nanomaterial · Immune · Disease · Incidental · Risk · Exposure · Monodisperse · Aggregate · Allergy · Autoimmunity · Sustainability · Workplace

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## 6.1 Introduction

All industry sectors are developing, producing, and applying nanomaterials, a variety of new supplies made of NPs, each showing remarkable and unique chemical-physical properties for advanced technologies. Actually, a continuously broad and complex assortment of substances are conveniently named "nanoparticles" by scientists and producers as a whole; nonetheless they have in common only the nanometric dimensional scale (1 dimension ≤100 nm and aspect ratio < 3:1, at least). However, the interaction between NPs and biological systems is unlikely constrained by that dimensional boundary; moreover, nanomaterials of same composition interact differentially with the environments and organisms. So far, the level of environmental contamination is hardly ascertainable and the standards for risk assessment not established yet; and biomarkers for biomonitoring nanomaterial workers need validation [1]. Nonetheless, nanomaterials affect almost all areas of science and technology offering advantages nowadays not renounceable. Still, the nanotech innovation, wich is a priority in current business plans, cannot be separated from the precautionary principle to approach substances of unassessed or unpredictable effects on human health. Regrettably, based on their nominal size, the existing protective equipment, that can retain particles not smaller than or equal to 300 nm (0.3 μm), are of unknown efficacy for NPs. Moreover, the risk of incidental exposure cannot be ruled out for nanomatters massively produced. Hence, nanomaterials have begun to be investigated as potential harm for humans, after their production was already ongoing. Earliest studies were carried out using high quantity of uncharacterized NPs, unlikely to be representative of the exposure level for humans, and involved limited groups of subjects [2, 3]. Further research on the potential effects of NPs had toxicity as the focus. However, there might be physicochemical features of NPs unable to elicit, at least under certain conditions (physiologic, expositive, environmental and so on) and even worth to the immune system. Indeed, the need for physicochemical characterization of NPs has been greatly prompted by human purposes, pointing to demonstrate their safety; essential for the completion of such assessment is the response of the immune system, given its central role in the acceptance or rejection of xenobiotics. To this aim, bioassays in vitro and in vivo could be performed as screening approach of development and production of NPs [4], even for those not strictly designed for human applications, as a precautionary approach. Our previous review describes the fate of metallic NPs after the encounter with the cells of innate immunity and discussed the possible effects of MeNPs, for instance cytotoxicity, genotoxicity, and modulation of membrane receptors, gene expression and cytokine production; such effects cannot be a priori considered to negatively affect health and could eventually be worth as vaccines, in antitumor immunity and autoimmunity control; nonetheless, workers professionally exposed to high levels of NPs should be monitored for immunotoxicity [5, 6]. The chance that NPs might assist, exacerbate, or directly induce immunotoxicity and immune-mediated pathologies is suggested by recent investigations in cellular and animal models. Moreover, few human reports suggest that NPs might

(directly or indirectly) cause, favor, or exacerbate preexisting diseases mediated by the immune system, allergic and autoimmune. These studies are heterogeneous and not easily reproduceable, unless they are designed for therapy; even so, the real plausibility of the observed experimental effects is uncertain as there are only a few reported cases of allergy onset or exacerbation in workers exposed to NPs. However, a comprehensive knowledge of the role of NPs in inducing immune-related toxicities is compulsory for timely and proper prevention of invalidating diseases that might determine forthcoming removal from workplace, job loss, and compensation. Meanwhile, the assessment of the circumstances affecting activation or quiescence of the immune system at the encounter with NPs is auspicial to implement both risk assessment and preventive measures for nanosafety at workplace. However, even if well characterized for the physical-chemical facet, NPs might assume a different look to the host immune system when surrounded by aqueous biologic protein-containing matrixes/vehicles/media, because they could be promptly surrounded by biofilms and their properties be profoundly altered. An experimental study conducted by our group started to corroborate the concept that, beside the full physical–chemical characterization of the original nanomatter, its fate after interaction with a surrounding milieu is crucial to forecast the effects it could produce in biological systems [7]. Another study addressed this point showing that, differently from air and skin, protein inside the body fluids can soon surround encountered NPs (forming the so-called protein coronas) and positively or negatively modulate the subsequent immunological cascade; in fact, NP–protein aggregates could interact with the innate immune system and antigen-presenting cells, dendritic cells (DCs), macrophages, and monocytes [8]. Furthermore, biophilic coatings of NPs intended for human use (drugs, cosmetics, food) might also react/adsorb proteins and other classes of biomolecules. Therefore, it would be important to establish or predict the biological performance of spontaneous biofilm-coated NPs, which might be of foremost value for the safety of anthropic NPs.

## 6.2 Metallic Nanoparticles Immunocytotoxicity in Rodent Model Strains

Due to their characteristic physical, chemical, and optical properties, metallic and metal oxide NPs are attractive tools for use in a wide range of applications intentionally meant for humans, as preservatives, pharmaceutics, and cosmetics. Therefore, the assessment of biocompatibility with living cells of these NPs is demanding. In particular, as already described for cobalt-NPs (Co-NPs) in vitro, an inflammatory process can be elicited by those types of NPs but being inflammation a modulator mechanism both in health and disease, an in vivo safe outcome might occur depending on the reciprocal mode of interaction of NPs with the immune cells. Our group performed the earliest studies on NP–biosystems interaction and effects using murine 3T3 fibroblast cell line in vitro, a standardized cell model for

toxicology assays. Diverse cobalt forms, Co-NPs, Co-microparticles (Co-MPs), and ions ($Co^{2+}$) assessed physically and chemically, were purposely radiolabeled to be tracked inside the cell. All forms of Co resulted internalized, but massive amount of Co was found inside the nucleus and mitochondria of cells exposed to NPs, perhaps the compounds that were found to be released exclusively from NPs [9]. In a following study, we showed that low dose of each Co form affects distinctively the cDNA expression profile: $Co^{2+}$ ions downregulate the pathways involved in mitochondrial function, MPs and NPs, respectively, upregulate those promoting carcinogenesis or inflammation [10]. Next, the ionic compounds were found more cytotoxic than particles, and forms released from particles resulted to lie in between. Instead, cobalt could induce morphological transformation only in particulate forms (MPs > NPs) likely by inducing oxidative stress, since *foci* formation inhibited by an antioxidant agent. Thus, not only released compounds, but also the physical properties of Co contribute to the observed effects [11]. Deduced from these studies, the role of ion release is an additional factor to be taken into account for toxicity evaluation of NPs containing metals [10, 11].

A comparative study on sanitizing NPs of silver (Ag-NPs), zinc oxide (ZnO-NPs), and titanium dioxide ($TiO_2$-NPs) has shown that composition and chemical stability are relevant characteristics determining immune-cytotoxicity of the airways. In fact, a single intratracheal (i.t.) instillation produced oxidative stress and dysregulation of the cytokine network (rat, bronchoalveolar lavage fluid) as well as reduction of alveolar macrophages phagocytic function, in a dose-dependent manner, being ZnO-NPs the most cytotoxic [12]. Interestingly, 20 nm Ag-NPs (6 mg/kg bw/day i.v., 28 days) cause reduction of thymus weight, NK cell activity, IFN-γ production, and functional immune response, i.e., decrement of T cell-dependent antibody response (TDAR) [13]. Larger uptake of well-dispersed single Ag-NPs (100 μg/ml) appears to explain the higher cytotoxic effect observed in exposed IL-2-dependent T lymphoblastoid cells, compared to aggregate-forming fullerene C60; also, short-term exposure to subtoxic Ag-NPs causes overexpression of IL-2 receptor, potentially leading to T-cell dysfunction [14]. Acute exposure to ZnO-NPs causes death of immune cells in vitro and concomitant increased levels protein marker of autophagic vacuoles (LC3A), the structures devoted to get rid of damaged subcellular structures. Hence, $Zn^{2+}$ ions dissolving from NPs are suspected to cause unmanageable increase of intracellular ROS, since chelation or uptake inhibition of ions and specific molecular blockers of autophagy and ROS can abrogate those effects. Also, ZnO-NPs lead to death of splenocytes in intranasally (i.n.) exposed mice [15]. Further, positively charged 20 nm NPs induced higher cytotoxicity of murine macrophages (Raw 264.7), compared to the larger (100 nm) and negatively charged NPs, respectively. Next, in orally administered mice (750 mg/kg/day dose, 2 weeks), NK cells were suppressed and maturation of T cells was reduced; nitric oxide (NO) production in vitro by splenocytes diminished, but cell-mediated immune response was not affected. Consistently, serum levels of pro-inflammatory (IL-1β, TNF-α), anti-inflammatory (IL-10), and Th1 cytokines (IFN-γ and IL-12p70) were significantly suppressed. Hence, size and charge of ZnO-NPs are relevant to in vitro and in vivo immunosuppression [16]. An inflammatory response was also detected in human blood cells exposed to this type of NPs [17].

The toxicity of quantum dots (QDs) of CdSe/ZnS was analysed using macrophages and lymphocytes in vitro and in BALB/c mice. Macrophages treated with 1.25 or 2.5 nM QDs exhibited decreased cell viability, increased levels of ROS and apoptosis, altered phagocytic ability, and decreased release of TNF-α and IL-6 when stimulated with lipopolysaccharide (LPS). In contrast, QDs-exposed lymphocytes showed enhanced cell viability, increased release of TNF-α and IL-6, when exposed to a lymphocyte activator (CpG-ODN), and decreased transformation ability treatment in response to LPS. In vivo, following the injection of QDs, they were gathered and persisted in major immune organs for more than 42 days, peritoneal macrophages phagocytic activity; however, ex vivo lymphocytes showed lower viability, disproportion of the subsets, higher release of TNF-α and IL-6, and lower transformation ability in response to LPS. These findings suggest immunosuppressive role of QDs and increase in susceptibility to pathogen-elicited diseases [18]. ZnO-NPs were also evaluated for the effects on thymus and spleen after oral intake in young male Wistar albino rats, compared to a control group. NPs intake was associated with increased blood leucocyte count and significant decrease of total antioxidant capacity and anti-inflammatory cytokine levels (IL-4 and IL-10); signs of spleen and thymus damage were detected at both biochemical and histological levels, as well as increase of the pro-inflammatory cytokines IL-1β, TNF-α, and INF-γ. The analysis of gene expression resulted in upregulation of those genes taking part in immunomodulation (CD3, CD11b, heme oxygenase-1) and inflammation (TLR4, TLR6). These findings suggest that the observed toxicity on spleen and thymus are likely a consequence of the activation of oxidative/inflammatory pathways [19]. Instillation (i.t.) or inhalation of ZnO-NPs exacerbate (LPS-induced) innate immunity-mediated pulmonary inflammation, in hypersusceptible animals in vivo; repetitive pulmonary exposure has aggravating effects on Th2-mediated allergic inflammation; very small NP exacerbates emphysematous pulmonary inflammation, concomitantly with enhanced local proinflammatory molecules. Hence, ZnO-NP exposure may synergistically facilitate pathological pulmonary inflammation via both innate and adaptive immunological impairment [20]. Aluminum (Al)-based NPs are inhalable emerging pollutants. Four types of them, two rod-type (short or long aspect ratios) and two spherical (with or without -OH groups), cause toxicity at pulmonary level 13 weeks after a single i.t. instillation. In particular, the high aspect ratio is linked to biopersistence and the presence of -OH groups is associated with lower biostability and highest sub-chronic immunotoxicity [21].

## 6.3 Nanoparticles for Human Use and Tested for Immunotoxicity in Rodent Strains

Engineered NPs intended as carriers for drug or antigen delivery, imaging and vaccine adjuvants are required to be assessed for their safety profile. Compatibility with the immune system is the crucial end-point that is investigated mainly by in vivo tests in rodents nowadays. These studies provide clues on how NPs might provoke

unintended toxic effects through exposure modalities not conceivable in humans, such i.t. or injective administration of single high dose. Nevertheless, those experimental findings point out at physical–chemical characteristics that could be intentionally conferred/provided to NPs in order to reduce their immunotoxicity or, reversely, to be avoided if able to elicit detrimental immune responses. Lipid-based NPs (LNPs) can vehicle drugs to specific tissues/cells, protecting them from immune response or, as adjuvants, stimulating it; however, they provoke immunotoxic effects upon interaction with subsets of leukocytes [22]. Prolonged circulation of nanodrugs in vivo can be conferred by polymeric coatings that limit the uptake by the immune organs and, thus, allow their accumulation. However, comparative studies in vitro and in vivo showed that zwitterionic PCB (polycarboxybetaine) and nonionic PEG (polyethyleneglycol) could induce the expression of cytokines [23], indicating highly repetitive monomers and polarized electrical charges as determinants of immunotoxicity, in spite of overall hydrophilicity. The polymer PELGE of different diameters have been examined for sub-chronic toxicity and immunotoxicity in dose–effect experiment in rats: the larger particles (200 nm) did not produce evident signs of immunotoxicity after 28 days of continuous intravenous (i.v.) administration; contrarywise, the smaller NPs (50 nm) were associated with an increased organ coefficient and histopathological changes of the spleen, increased serum IgM and IgG levels, alterations in blood lymphocyte subpopulations, and enhanced expression of spleen IFN-γ [24]. Proinflammatory cytokines are also detectable in liver and serum after the administration in vivo of polyethylenimine (PEI) likely by inducing high oxidative stress and NLRP3-inflammasome activation, determining increased phagocytosis by peritoneal macrophages and increased spleen weight [25]. Polylactic acid (PLA) is a biodegradable polymer; however, the size of the degradation products depends on the type of medium which, in turn, affects cytotoxicity: in fact, two different PLA particles, non-nanosized (A) and nanosized (B) dissolved in RPMI culture medium, to form 100 nm NPs with neutral zeta potential, show similar low cytotoxicity toward peripheral blood mononuclear cells (PBMCs). Otherwise, in DMEM medium, PLA-A resulted to form smaller NPs, compared to PLA-B, and were more cytotoxic to cells in vitro, likely via induction of ROS [26]. Hydrophilic carbohydrate shells can lessen the immunotoxicity observed for polymeric hydrophobic NPs both in vitro and in vivo, as measured by their secreted cytokines' pattern and level [27]. Antiviral drug was demonstrated to be immunologically and hematologically inert as solid drug NPs (SDNs) compared to the aqueous form in primary healthy PBMC, a cell model suitable for the evaluation of NP's safety [28]. Also, dextran-coated ferrite NPs (DFNPs, <25 nm) do not produce any immunological reactions in vitro and in vivo [29]. Mesoporous silica NPs (MSNs, ordered pores) are rapidly and efficiently taken up into the endosomal compartment by primary APCs antigen-presenting cells of mouse spleen; however, MSNs did not affect cells viability, even in relevant concentrations, and induce low expression of activation markers and release of pro-inflammatory cytokines (IL-6, IL-12 and IL-1β). Nonetheless MSNs, compared to the more cytotoxic and pro-inflammatory colloidal silica NPs (Col-NPs, amorphous), caused dysregulation of the spleen, although both types of NPs were i.p. administered for 4 weeks (2, 20, and 50 mg/kg/

day) [30]. MSNs caused histological alteration and weight increase of liver and spleen, increase of splenocytes counts, increase of serum IgM/IgG, and altered lymphocyte subpopulations, not observed in the control mice (Col-NPs) [31]. Further, MSNs are taken up by murine macrophages in vitro and induce activation and pro-inflammatory cytokines (TNF-α, IL-1β) that are sensibly lowered by end-capping MSNs with proteins [32]. A similar approach was applied to minimize the immunotoxicity of hydrophobic ordered mesoporous carbon NPs (MCNs), modified with PVP or PEG (90 nm) to increase zeta-potential and dispersion; still, original and modified MCNs promoted the differentiation and maturation of DCs in vitro, induced apoptosis of T cells and caused the reduction of secreted TNF-α and IL-6 levels; also, all MCN types deposited in the lungs, although without histopathologic meaning [33]. In addition, the synergistic effects with toxins and bacteria in the environment and the role of contaminant surfactants of synthesis should be considered [34]. High level of iron is found in liver, spleen, and thymus 13 weeks after a single i.v. shot of iron oxide NPs (FeONPs) (2 and 4 mg/Kg). Moreover, FeONPs induced significant increase of leukocytes and neutrophils, secretion of IL-8 and lactate dehydrogenase, stimulation of chemotaxis-related proteins and reduction of those involved in antigen presentation [35]. A single i.t. instillation of FeNPs (1, 2, and 4 mg/kg) was given to parent mice and their offspring were evaluated for immune-toxic signs 28 days after having received one single dose (4 mg/kg) through the same route. At the maximal dose, the surviving parental mice showed iron accumulation in the ovary and the testis and enhanced expression of major histocompatibility complex (MHC)-II; in offspring mice, beside the increased mortality and the significant hematological and biochemical changes, particularly in females, the immune response resulted differentially polarized in between genders at the middle dose, suggesting that non-observed adverse effect level (NOAEL) for reproduction and development may be inferior to 2 mg/kg, and that female are more sensitive [36]. Another form of iron, intended for i.v. administration in hyperthermia management, is dextran-stabilized iron oxide-NPs (DIONPs), that have been studied in vitro using *human* peripheral whole blood in vitro, at increasing concentrations (0.008–1 mg/ml). DIONPs significantly inhibited the proliferation of mitogen PHA-stimulated T lymphocytes and the expression of cytokines' mRNA. Conversely, in vivo, the systemic administration of DIONPs boosted the proliferation of PHA-stimulated splenic lymphocytes and the secretion of IL-1β, suggesting that DIONPs maintain integrity or undergo degradation depending on the type of interaction with the milieu and that, consequently, the immune response could result variably affected [37]. Some formulations of injectable iron oxide NPs (IONPs) impair mitochondria, induce oxidative stress, and alter the membrane potential of in vitro primary human T cells, that lower the cytokine production and proliferation (mitogen-activated); since T cell lines could not recapitulate those toxic effects, case-specific mechanistic investigation should be performed with primary cells [38]. Porous NPs with core/shell structure $\gamma$-$Fe_2O_3$/$SiO_2$-$NH_2$ (13 nm) localized in the cell cytoplasm and extracellular space of proliferating human PBMCs, with minimal T and B cell proliferative responses (0.12–75 μg/cm$^2$, 24–48-72 h). However, IL-6 and IL-8, and GM-CSF were more highly secreted by

mitogen-stimulated cells (3, 15 and 75 μg/cm$^2$, 48 h), while TNF-α and IFN-γ were not. In monocytes and granulocytes, MNPs significantly enhanced the respiratory burst but not the phagocytic activity (75 μg/cm$^2$, 48 h) [39]. Size and tri-dimensional shape (globular vs planar vs linear) influence the immunological recognition of nucleic acid-NPs (NANPs) by phagocytic cells in primary PBMCs but they are immunologically inactive toward IFN production by plasmacytoid DCs [40]. The effect of electrical charge on immunotoxicity has been evaluated by comparing the colloidal silicon dioxide NPs (Si-NPs) of two sizes (20 nm and 100 nm) and positively charged (modified with L-arginine addition) or not 20 nm ones. In female C57BL/6 mice, after 14 days of oral Si-NPs administration (750 mg/kg/day) the small-sized Si-NPs showed the strongest in vivo immunosuppression on the proliferative capacity of lymphocytes, killing activity of NK cells and inhibition of pro-inflammatory cytokines secretion (IL-1β, IL-12, IL-6, TNF-α, IFN-γ) [41]. Negatively charged $TiO_2$-NPs (20 nm, BSA-functionalized) cause higher immunotoxicity, consisting of decrease in the number of murine macrophage cell line in vitro, oxidative stress response (increase of ROS and NO), disruption of the mitochondrial membrane potential and increase of pro-apoptotic factors, compared to untreated control; furthermore, several Toll-like receptors (TLRs), concurrently activated with oxidative stress proteins, appeared mechanistically involved in immunotoxicity [42]. Biocompatibility and hydrophilicity of adenosine 5′-monophosphate (AMP) is shown to be useful to abrogate the immunotoxicity of innovative NPs, without hampering their hi-tech properties; for instance, AMP surface-coated organic quantum dots (QDs), although still able to trigger a weak response by macrophages in vitro and accumulate in immune organs, do not cause inflammation nor histopathological anomalies as the original QDs [43]. Cytotoxicity and immunotoxicity of polymeric nanomaterials, such as copolymeric micelles [poly(acrylamidoethylamine)-block-poly(DL-lactide), PAEA90-b-PDLLA40], can be efficiently attenuated crosslinking PAEA with polyethyleneglycol (PEG) and reducing the accessibility of biomolecules to the core, an effect that is increased accordingly with the extent of the crosslinking [44]. Recently, antimicrobial Ag-silica nanorattles have shown to be taken up through an active phagocytic process by DCs of the immune system but not affecting their viability nor inducing unwanted immunological effects [45]. Immunotoxicity of nanosized PPE micelles can be lowered by providing the micelles surface with zwitterionic charge, as shown in mouse macrophages as lack of detection of secreted cytokines [46]. In a similar approach for reducing toxicity and improving properties of $TiO_2$NPs as UV filter, our group synthetized, characterized, and tested $TiO_2$-MSNs (4.4 nm $TiO_2$ inside 5 nm pores of MSNs) in relation to $TiO_2$NPs (commercially available) and void mesoporous silica ($SiO_2$) NPs (MSNs) (100 nm). MSNs are considered as nontoxic at the occupational exposure levels; nevertheless, potentially dangerous organic molecules can be formed due to their photocatalytic and hydrophilic properties. $TiO_2$-NPs (4.4 nm) were synthetized with improved UV properties for human use. The aim of this study was to assess the possible toxicity of $TiO_2$-MSNs, compared with the MSN alone and $TiO_2$-NPs, on several cytoimmunological biomarkers of

toxicity. As a cellular in vitro model, we used human PBMCs, either resting or activated, were exposed in vitro/ex vivo to $TiO_2$-MSN (4.4 nm $TiO_2$ into MSN pores) and to "void" MSN (100 nm) or $TiO_2$-NPs (21 nm). Exposed cells were characterized for: cell viability/apoptosis, ROS, nuclear morphology, cytokines secretion. $TiO_2$-MSNs suspensions—causing detectable biologic alterations of PBMCs at the lowest doses—were characterized for particles size by NPs tracking analysis (NTA). We found that the viability of activated lymphocytes was significantly reduced at high doses (50 and 100 μg/ml) of all NPs. All NPs induced apoptosis, but only nano-$TiO_2$ (alone or assembled into MSN particles) induced ROS. MSNs down-modulated all cytokines, except for IL-1β (upmodulated). Nano-$TiO_2$ induced IL-10, and inhibited IL-6 and the other cytokines, except for IL-1β (unchanged). $TiO_2$-MSNs showed immunostimulatory properties as almost all cytokines were upregulated, except for IFN-γ and IL-2 (reduced) and IL-6 (unchanged). These findings suggest that either nano-$TiO_2$ or void MSNs are more cytotoxic for primary huPBMC, compared to novel $TiO_2$-MSNs, likely throughout higher induction of ROS; moreover, they resulted immunosuppressive and pro-inflammatory, respectively. Instead, the exposure to $TiO_2$-MSN was associated with lower ROS and cytotoxicity but, nevertheless, pro-fibrotic and pro-allergenic cytokines were more strongly released in the culture medium by huPBMC. Our data suggest that unwanted and unpredictable immune-cytotoxic activity might arise in presumed healthier NPs based only on technical improvement (personal data, in press).

## 6.4 Experimental Data from Human Ex Vivo Primary PBMC and Established Cell Lines In Vitro

The use of particles from micro- to nanoscale provides benefits to diverse scientific fields and among those are Co-NPs of great interest both in industry and in life-science but under-investigated with regard to toxicology. At this we conducted a study aimed at evaluating in vitro the potential interference of Co-NPs on the production of several cytokines (IL-2, IL-4, IL-6, IL-10, IFN-γ, and TNF-α) by PBMCs, comparing their effects to those of Co micro-Ps and Co(II) ions. Cells were cultured with escalating concentrations ($10^{-5}$, $10^{-6}$, and $10^{-7}$ M) of Co-NPs (or microparticles or $CoCl_2$ or nothing). Co microparticles showed a greater inhibitory effect compared to other Co forms (at any concentration tested and toward all cytokines), whereas Co solutions selectively inhibited IL-2, IL-10, and TNF-α, at maximal concentration. CoNPs induced increase of TNF-α and IFN-γ release and inhibition of IL-10 and IL-2. These findings recall the cytokine pattern detected in the experimental and clinical autoimmunity; on this basis, we argued that immune endpoints ought to be considered in subjects exposed to CoNPs [47]. In a new study, synthetic and fully characterized palladium NPs (Pd-NPs) were used as model of those emitted from catalytic converters. Since this palladium had been previously proved to be a skin sensitizer, we analyzed the cytokine profile of PBMCs drawn from healthy

nonatopic female donors (n = 8) at baseline and after incubation with Pd-NPs (5–10 nm) or Pd(IV) ions: NPs inhibited secreted TNF-α and IL-17, whereas ions inhibited secreted IL-10 and IL-17. In LPS-stimulated cultures, release of IFN-γ, TNF-α, IL-10, and IL-17 was inhibited by ions, whereas NPs enhanced secreted IFN-γ and inhibited TNF-α and IL-17 release. In conclusion, Pd ions act as cytokines' inhibitors, whereas Pd-NPs modulate the pattern of secreted cytokines toward that characterizing delayed allergic reactions; interestingly, this finding is in accordance with the rise of cases of allergic contact dermatitis to Pd in increasingly polluted urban environments (by automotive Pd-NPs) [48]. The assessment of Ag-NPs (< 100 nm) immunotoxicity was performed in vitro using three different target cells, human peripheral whole blood and enriched monocytes, and THP-1 cells. Exposed to Ag-NPs alone or in combination with cytokines and immune stimulators, all cell models responded producing higher dose-related proinflammatory cytokines IL-8 and TNF-α; moreover, Ag-NPs boosted the humoral response, since cells produced more IFN-γ and IL-4; finally, the inhibitory cytokine IL-10 was reduced [49]. Possible toxicity of palladium NPs (Pd-NPs) is of concern as they are released in the urban environment through catalytic engines and in many occupational settings. We previously studied the toxicity of Pd-NPs at high dose and, next, at low subtoxic doses. In particular, we have exposed in vitro normal human PBMCs entering mitotic division to Pd-NPs or to Pd(IV) ions: as findings, Pd(IV) exposed cells showed significant increase of intracellular ROS, while those exposed to Pd-NPs did not. TEM revealed accumulation of lipid droplets and vacuoles of autophagy and mitophagy, which appeared more conspicuous in cells exposed to Pd(IV) ions than to Pd-NPs. Also, cell cycle alterations (G0/G1-phase block) were mostly found in cells exposed to ions. These results suggest that ions, as such or released by NPs, are the true inducers of (acute) Pd toxicity [50]. Poly(lactic-co-glycolic acid)-NPs (PLGA-NPs) are biomaterials studied as tunable adjuvants for antitumor immunotherapy, being able to gain positive and negative electrical charge. Regardless of the charge, PLGA-NPs are rapidly engulfed by human primary DCs but determine different phenotypes and cytokine secretion profiles. PLGA-PEO(polylactic-co-glycolic acid)-NPs were tested on human peripheral whole blood or PBMCs (0.12 and 75 μg/cm$^2$) displays significant time-dependent decrease in the proliferation rate. Low noncytotoxic concentrations (0.12 and 3 μg/cm$^2$) displayed moderate suppression of proliferative activity of T cells and B cells; middle doses (3 and 15 μg/cm$^2$, 4 h) moderately reduced cytotoxicity of natural killer (NK) cells (92%); the low-dosed cultures (0.12 μg/cm$^2$) showed stimulated phagocytic activity and respiratory burst [51]. Another study showed that Ag-NPs cause inhibition of mitogen-induced PBMC proliferation likely for membrane-mediated effects following interaction; still, particles/liberated ions could bound up by the serum proteins and have acted on the cells before cell division started to be detectable [52]. Among the studies described above, we may consider that of our group on biofuel products of combustion, passively inhaled by humans. Diesel combustion is the major source of ultrafine particles (UFP), comprising a nanosized fraction, in the urban living environment. Recently, biomass-derived fuel is being used more as a substitute of the conventional fossil petroleum-derived for its environment

sustainability, based on lower amounts of conventional pollutants released. Nevertheless, our research group found that new and potentially harmful contaminants can be released by biofuel combustion engines, since they emit a significantly higher amount of sub-10 nm NPs, compared to conventional fuels. For toxicological tests in vitro, human keratinocytes (HaCaT) and alveolar epithelial (A549) were exposed alternatively to the sub-10 nm fractions, obtained from diesel or biodiesel fuels, dispersed in culture medium. Interestingly, the sub-10 nm fraction extracted from biodiesel exerted a stronger cytotoxic effect on keratinocytes than on alveolar epithelial cells, suggesting that the dermal route of exposure might be the most sensitive; moreover, keratinocytes produced significantly higher levels of secreted cytokines and chemokines involved in inflammation, angiogenesis, and cell proliferation. Notably, in that regard, the <10 nm fractions were far more active than the fraction containing >10 nm particles. This study of ours showing differential size-related inflammatory responses and the disregarded harmfulness of the sub-10 nm, raises the question of the potential impact on human health of novel materials considered environmentally friendly, released thoughtlessly but potentially reacting with chemical and physical agents to form other NPs of unknown behavior [53]. All data obtained on immunocytotoxicity the above described studies are listed in Table 6.1.

## 6.5 Pollutant NPs Immunotoxicity in Wild Animals and Aquatic Organisms

Immunotoxic effects of anthropogenic NPs are emerging from studies in vivo on aquatic and terrestrial animals since they are detected as contaminants of the natural environment. Among these, metal-oxide and carbon-based NPs were described to cause sublethal effects in the immune system of fish and marine invertebrates; in particular, phagocytes were unable to process them and, consequently to frustrated phagocytosis, lysosomal vacuoles resulted damaged and ineffective against pathogens [54]. Whole body exposure of freshwater mussel *Elliptio complanata* was exposed in vivo to Ag-NPs of two different sizes (20 and 80 nm) for 13 weeks at increasing doses (0, 0.8, 4, or 20 μg/l) and parallelly to $Ag^+$ ions (as $AgNO_3$), as control. The highest bioconcentration of $Ag^+$ in soft tissue was observed for the ionic form, suggesting a role for released ions and also for (lower) accumulation of Ag from Ag-NPs; the bioaccumulation was slightly higher for 20-nm Ag-NPs. Both sizes induced phagocytosis and decreased hemocytes cytotoxicity in vitro. Aggregation of NPs was observed, suggestive of a role in bioavailability and immunotoxicity of silver NPs [55]. In the mussel *Mytilus galloprovincialis*, after in vivo exposure (3–6-12 h) to dimensionally heterogeneous Ag-NPs (<50 nm; <100 nm), blocking or not the endocytic pathways with specific agents, inflammation of digestive gland was maximal with the <100 nm preparation, but no conclusions could be drawn on the relevance of size and time [56]. Next, a sublethal dose in vivo (100 μg/l)

**Table 6.1** Human ex vivo primary PBMCs and in vitro normal cell lines responses to NPs exposure

| Nanoparticle | Diameter | Controls | Time/mode of exposure | Cell model | Human donor | Immunotoxic effects | Determinants of toxicity | Reference |
|---|---|---|---|---|---|---|---|---|
| Cobalt (Co) | 3.5 nm (+aggregates >100 nm) | Co-microP $Co^{2+}$ ions | Hours-days/ Protein-containing aqueous medium | PBMC | Healthy | Mitochondrial dysfunction | Release of ions | [47] |
| Palladium (Pd) | 8.8 nm | Pd(IV) ions | | PBMC | Atopic (female) | Secretion of autoimmunity-related cytokines | Release of ions | [48] |
| Palladium (Pd) | 8.8 nm | Pd(IV) ions | | PBMC | Healthy | Cell cycle disturbance of PHA-activated lymphocytes | Release of ions | [50] |
| Silver (Ag) | <100 nm variable | w/o AgNP | | THP-1 PBMC Monocytes (PBMC) | Healthy | Immunostimulatory > IL-8 > humoral response | Release of ions Protein corona | [49] |
| PLGA-PEO | <100 nm Variable | PLGA | | PBMC | Healthy | DC cytokines alteration, inhibition of NK cytotoxicity, inhibition of T cell proliferation | Negative or positive charge | [51] |
| Silver (SNP) | 66 ± 2.7 nm | 28 ± 1.5 nm Gold (GNP)/ w/o NP | | Lymphocytes | Healthy | Inhibition of mitogen-induced proliferative responses and viability | Interaction with cell membrane and related component ions (?) | [52] |
| Combustion products *from (bio)fuel engine* | Sub-10 nm fraction | >10 nm fraction | | Keratinocytes Alveolar epithelium | Healthy | Cytokines alterations | Sub-10 nm size | [53] |

was associated with toxic effect in immune cells, regardless of the time, the greater being caused by the smaller Ag-NPs; therefore, immunotoxicity was determined by size-dependent clathrin-mediated endocytosis, since by blocking that pathway the hemocytes were more sensitive to the larger Ag-NPs, while they were not by using a different blocker or a carrier [57]. The bivalve species *Tegillarca granosa*, suitable as human food, was studied to elucidate the true impact of NPs release in the environment using $TiO_2$-NPs exposure at environmental realistic concentrations of 10 and 100 μg/L: the hemocyte counts and phagocytic activity were significantly reduced after 30 days of exposure, as well as the expressions of genes encoding Pattern Recognition Receptors (PPRs) and immune-related molecules, suggesting a lower sensitivity to pathogens [58]. The exposure of this clam to metallic oxide-(ZnO-, $Fe_2O_3$-, CuO-) NPs and multi-walled carbon nanotube (MWCNT) resulted in cytotoxicity (reduced total counts, cell viability and altered cell composition) and inhibition of phagocytic activities involving ROS formation and expression of immune- and neurotransmitter-related genes, consistently with alteration of the neurotransmitters level in vivo [59]. Moreover, $TiO_2$-NPs affect the immune responses at gene expression level, synergically with 17β-estradiol. Immunotoxicity of CuO-NPs and $CuSO_4$-NPs, contaminants of water and soil in India, have been investigated comparatively for their potential immunotoxicity in the earthworm *Metaphire posthuma* living in moist soil. Coelomocytes of this earthworm were used to analyze immune-related parameters (total count, phagocytic response), generation of cytotoxic molecules, the activities of various enzymes and total protein, under the exposures of 100–500-1000 mg of either type of NPs, per kg of soil, for 7 and 14 days. Coelomocyte counts significantly decreased, with maximal diminution under the highest dose treatment after 14 days, for both types of NPs that caused defective innate immunity in all conditions for both NPs [60]. In recent years, the use of zebrafish (*Danio rerio*) adult and embryos is rising as an established animal model system for NPs toxicity assessment and suitable to study immunotoxicity of different metal and metal oxide NPs [61]. Ag-NPs are suspected to represent one of the ecotoxicological risks on aquatic organisms; for this, Ag-NPs' fate, bioavailability, and effects have been studied in juvenile rainbow trout: fishes were exposed for 96 h to Ag-NPs (40 μg/L) or ions $Ag^+$ ($AgNO_3$, 4 μg/L) in 10-times diluted municipal wastewater; then, Ag concentrations were measured both on water samples and fish tissues (liver and gills). In wastewater, Ag-NPs appeared as small non-charged aggregates (11.7 ± 1.4 nm), inducing morphological modifications of gills without bioaccumulation. Instead, dissolved $Ag^+$ was found bioavailable in diluted effluent wastewater and induced oxidative stress in gills and increased significantly metallothionein levels in the liver. Both Ag forms were found in liver and induced immunosuppression and inflammation. Hence, Ag produce harmful effects in fish in any form; also, Ag-NPs in wastewater are bioavailable, despite they form larger aggregates [62]. Next, monodispersed Ag-NPs (20 nm citrate dispersion, pH 3.0) at low-to-high range concentrations have been tested for immunotoxicity on the functional activity of leukocytes of the cetacean Bottlenose Dolphins, *Tursiops truncatus*. Ag-NPs acted differently on leukocytes or PBMC, producing cytomorphological alterations and localizing at different sites in the cytoplasm. Likely,

time- and dose-dependent cytotoxicity (and apoptosis) are linked to the monodispersed status of Ag-NPs (at high concentration, 10/50 μg/ml). Sublethal doses (as in our study on Pd-NPs, 0.1, 1 μg/ml) negatively affect the functional activities of cPMNs (phagocytosis and respiratory burst) and cPBMCs (proliferative activity) [63]. However, monodispersed NPs might not be existing in real conditions. In conclusion, most NP types present in the aquatic environment display sublethal effects on the immune system: cell mediated immunity and phagocytic cells are the primary target of NP immunotoxicity, characterized by lysosomal destabilization, frustrated phagocytosis, and dysfunction of phagocytes; the humoral immunity is minorly target of direct immunotoxicity, but plays a role in NPs dissemination through the body and presentation to the phagocytic cells [54].

## 6.6 Role of Agglomerates/Aggregates on NPs Immunotoxicity

More recently, the role of aggregation/agglomeration status of nanomaterials is considered relevant for a proper assessment of their toxicological burden. In fact, if NPs would aggregate (in air) to form particles larger than 300 nm, they could be captured by the filters available today. Nonetheless, Ag-NPs in wastewater are bioavailable to rainbow trout despite they form aggregates [62]. Respiratory toxicity and immunotoxicity of C60 fullerene aggregates were evaluated in mice and rats following nose-only inhalation, for 13 weeks. Immunotoxicity of C60 aggregates (50 nm [nano-C60] and 1 μm [micro-C60] diameter) was tested in exposure concentrations allowing mass-based and surface area-based comparisons (nano-C60: 0.5 and 2 mg/m$^3$ (0.033 and 0.112 m$^2$/m$^3$); micro-C60: 2, 15 and 30 mg/m$^3$ (0.011, 0.084 and 0.167 m$^2$/m$^3$). Inflammatory responses, dependent on the concentration, were observed in the lungs; in particular, increase in monocyte chemoattractant protein (MCP)-1 and macrophage inflammatory protein (MIP)-1α was detected in bronchoalveolar lavage fluid (BALF), where cell immunophenotype changes were observed too. Notably, based on surface-area exposures, the worst effects were observed for micro-C60 than for nano-C60 [64]. The relevance of surface area in the immunotoxicity potential of NPs was observed also by our group: we performed a study using natural asbestos owing well-defined mineralogy and stoichiometry that was tested as model nanosized material. Asbestos are major materials of occupational relevance and health concern for being an underhand inducer of several invalidating diseases (pleural plaques, asbestosis, malignant mesothelioma, as known). In fact, asbestos is not unique in those respect but includes various crystals of different stoichiometry. In our study, the nanofibers used to address this issue were found to be crystals of crocidolite, described by the chemical formula ($ABNa_2C(Fe^{2+}2.5\ Mg0.5)CFe^{3+}2\ T(Si_8O_{22})W(OH)_2$), and organized into one crystal structure. In this study, although the nanofibers were ultrasonically dispersed into serum-containing cell culture liquid medium, were on silica-wafers from solutions at increasing mass

concentration (0.1–100 mg/l) and then were counted and identified as single or aggregate fibers. At low concentration (0.1 and 1 mg/l), the nanofibers appeared to be monodispersed, whereas by increasing the concentration (5–10 mg/l) nanofibers appear increasingly agglomerating. In vitro tests, on lung epithelial cells, performed using the same nanofibers-containing dispersions used in structural assessments showed that the single-nanofibers were less cytotoxic. Importantly, since the mineralogy of single fibers were invariable, this decreasing rate of cytotoxicity was to be addressed to the increasing amount of agglomerated fibers. Hence, single versus agglomerated fiber population is a factor that cannot be neglected in defining the final adverse effects of asbestos. The analytical protocol proposed here is valuable for any aero-dispersed dust, in polluted environments, as well as in the interpretation of experimental studies [65].

## 6.7 Human Immune Diseases Potentially Linked to Nanoparticle Exposure

The potential of engineered NPs to prompt allergic diseases has been reviewed in our previous editorial work (Springer Science+Business Media Singapore 2017 1 T. Otsuki et al. (eds.), *Allergy and Immunotoxicology in Occupational Health*, Current Topics in Environmental Health and Preventive Medicine, DOI https://doi.org/10.1007/978-981-10-0351-6_1) and listed as Chap. 3 by Petrarca C. et al. Certain NPs interact with proteins and, hence, modify their antigenicity and elicit altered immune responses and even autoimmunity; other NPs may induce allergic sensitization. In vitro studies demonstrated that NPs can modulate cytokine production toward Th1 (Pl, Pd, Ni, Co) or Th2 (Ti, mw and sw carbon nanotubes) production patterns. Some NPs have been linked to allergic sensitization either acting as adjuvant, promoting specific pattern of cytokines, antibody and cells that favor allergic sensitization to environmental allergens, or as haptens inducing antigen-specific IgE production. The available data suggest that engineered metallic NPs may contribute to pro-inflammatory disease processes in the lung, particularly allergy, through the elicitation of oxidative stress [66].

## 6.8 A New Viewpoint for the Design of Low-Risk Anthropic Nanoparticles

Based on the data reviewed in the present chapter, a set of features that are associated to low immunotoxicity of anthropic NPs are reasoned that might be considered both for design presumptively safe new nanoproducts and, ultimately, provide some clues for the arduous assessment of occupational risk and prevention of potential damage caused by exposure to nanoparticulate matter in manufacturing workers.

Although the data derive from various groups of investigators and are obviously not based on human cohorts, when analyzed comparatively and collectively they are strongly suggestive that there would be a "safe zone" for NP production at industrial level. Indeed, NPs have been regarded as the asbestos of the new millennium for their unpredictable behavior which outflows the toxicological profiles classically depicted for non-nanosized materials. Nevertheless, they have been largely produced and employed daily in activities and tools since about two decades. Moreover, nano-compounds with no apparent in vitro or in vivo toxicity may still trigger various components of the immune system and lead to serious adverse reactions. Therefore, immunotoxicity appears to be one major biological compartment to be analyzed for the assessment of NPs ongoing pharmaceutical release process [67]. Analogously, such an approach could be adopted for any kind of nanosized item in research and development. Observational and experimental data exposed in this chapter are summarized in Table 6.1 to orienting prevention measures for workers dealing with nanomaterials. Clearly, any actions taken to reduce, limit, antagonize, shield, control, neutralize the determinants of NPs immune(cyto)toxic effects, both at environmental and organism level, are auspicial for professionally involved subjects: for instance, the control of polymer chemistry and supramolecular assembly provides a great opportunity for the construction of biocompatible (therapeutic) NPs. However, the sources of data collected regarding immunotoxicities of nanomaterials are diverse, and experiments are usually conducted using different assays under specific conditions. Cytokines are useful biomarkers for predicting the in vivo behavior and immunotoxicity of NPs. For example, the release of the cytokine IL-1β is considered an in vitro biomarker of immunotoxicity (NLRP3-mediated inflammasome activation) for intrinsically adjuvant NPs, such as liposomes and polymer-based used in vaccines to provide adaptive immunity [68]. Confounding factors and potentiating cofactors might affect NPs involvement in the onset and progression of immune system-mediated diseases; one of these might likely be the inner circadian rhythm regulating various immune functions (such as leukocyte numbers, activity, and cytokine secretion, which might affect susceptibility to infections) and supposed to control the 24 h pattern of symptoms of immunological and allergic ailments [69]. Notably, data on most of the engineered metallic NPs have been obtained in laboratory rodents via a non-inhalation route of exposure (systemic i.v. or i.p., local at airways i.t. or mucosa p.o.), daily during few weeks (2–4-8), to high or low doses of a large set of NPs (1–100 nm). Experiments in rodents enlightened that ions leaked from metal-based NPs contribute to their immunotoxicity by causing oxidative stress to mitochondria, and that NPs aggregation and agglomeration with proteins are mitigating phenomena. Studies on wild organisms are based on more realistic exposure doses of NPs in the aquatic environment and sustaining the relevance of oxidative stress and damage to innate immunity cells, perhaps again involving ion formation for water and soil polluting partially aggregated AgNP. Nevertheless, the data obtained clearly indicate that interaction with protein-containing aqueous media and the release of (known and unknown) ionic forms of the nanosized metals and metal oxides should be maximally limited. Data from human target cells ex vivo/in vitro, although with the limits of the short time of exposure in liquid media, converge to the "ion relevance" for metallic NPs and to the "aggregate influence" on the

immunotoxic effects of metallic and metal oxide NPs. Human cell studies suggest that the analysis of cytokine patterns released by (autologous) cells ex vivo could allow evaluation and monitoring of NPs immunotoxicity reflecting the activation or suppression of specific functional subsets (effector, memory, inhibitory, inflammatory, profibrotic, etc.). Purposely built-up NPs for humans have been more extensively tested aiming at defining the determinants of immune hiddenness or, undesirably for large-scale produced and released NPs, immune stimulation depending on the intended end-use. Both branches of investigation provide useful data, some overlapping those obtained with other approaches: (a) the immunotoxicity of nanomaterials is concentration and dose dependent; (b) the synthesis of degradable NPs is essential to decrease toxicity; (c) cross-linking minimizes the release of free polymeric chains and maintains high stability of the NPs, thereby lowering their immunotoxicity; (d) lowering the amine density of cationic polymers lowers the cytotoxicity of the NPs and immunotoxicity; (e) neutral NPs usually have the lowest immunotoxicity, compared to polar and charged NPs; (f) morphology, dimension, and surface chemistry influence on the ability of nanomaterials to interact with the various components of the biological system and to modulate the immune system [70, 71].

## 6.9 Conclusions

More studies are needed to clarify the central issues of environmental and internal exposures and, beyond, find out the mechanisms of cell entry and the transduction pathways activated by NPs from outside the cell. The formation of mixtures with other environmental toxicants should be ruled out or taken into consideration. Full chemical-physical characterization of the NPs of interest is demanding as well as the definition of their fate when put in close proximity with human primary cells in biological matrixes. More similar to occupational exposure, long-term and low-dose setups should be the preferred experimental setting. However, speculative and observational studies as the ones presented here could achieve the highest relevance if health data and retrospectively analyzable biomaterials would be gathered from workers and their family members and children. Meanwhile, tailoring the properties of nanomaterials on the basis of the available data appear to be the most practicable way for risk assessment and prevention of harm potentially linked to NPs exposure at work.

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# Chapter 7
# Occupational Respiratory Allergic Diseases: Occupational Asthma


**Sasho Stoleski**

**Abstract** Work-related asthma (WRA) is the most common work-related lung disease, while occupational asthma (OA) is the most frequent occupational lung disease in developed countries in the last three decades. Due to specific occupational exposure, WRA is classified into OA and work-exacerbated asthma (WEA). Furthermore, OA, according the pathogenic mechanisms involved in its development, is classified into allergic and nonallergic OA. Allergic OA can be caused by IgE-mediated and IgE-independent immunological mechanisms. This chapter reviews epidemiological and etiopathogenetic characteristics, current diagnostic approach, treatment, and preventive measures, as well as dilemmas associated with different types of OA.

**Keywords** Work-related asthma · Occupational asthma · Occupational exposure · Allergic occupational asthma · IgE-mediated allergic occupational asthma · IgE-independent occupational allergic asthma

## 7.1 Work-Related Asthma

Occupational exposures may cause new-onset asthma in a healthy subject, aggravate preexisting asthma in a symptomatic individual or reactivate asthma in an asymptomatic individual [1]. WRA or work-attributable asthma is a form of asthma caused or triggered by specific agents and/or conditions at the workplace. This form

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**Table 7.1** Etiopathogenetic classification of OA

| Work-related asthma | | |
|---|---|---|
| Asthma caused by work—Occupational asthma (OA) | | Asthma exacerbated by work—Work-exacerbated asthma (WEA) |
| Allergic OA (with latency period)—sensitizer induced OA | | |
| IgE-mediated | Non-IgE-mediated | |
| Non-allergic OA (without latency) | | |
| Single exposure—reactive dysfunction airway syndrome RADS | Multiple exposures—irritant induced OA | |

of asthma is the most common lung disease in Europe and the United States within last three decades and covers 20–30% of all asthma cases in adults [2–4].

WRA is not unique and homogeneous entity, and includes several different types of asthma associated with workplace having different pathogenic mechanisms. In the asthma consensus that has been adopted over the past two decades, individual forms of WRA have been precisely defined in order to improve their diagnosis, treatment, and prevention [5]. Differentiating the various forms of WRA, being difficult sometimes is of particular importance having in mind their various medico-legal implications [6]. According to the etiopathogenetic mechanisms, WRA is classified as OA and WEA (Table 7.1). WEA (work-aggravated asthma or work-aggravated asthma symptoms) is defined as preexisting or new-onset asthma, allergic or nonallergic, worsened by nonspecific stimuli from the work environment (respiratory irritants, cold and dry air, physical exertion, etc.) [7]. Since occupational exposure is not a direct and unique cause of this entity, WEA is not included in the List of Occupational Diseases in the countries worldwide, and this form of asthma does not have the legal implications of OA.

## 7.2 Definition and Classification of OA

OA is defined as new-onset asthma caused by agents and/or conditions attributable to a particular workplace environment and not by stimuli encountered outside the workplace [1, 8, 9].

According to the etiopathogenetic mechanisms involved in its occurrence, OA is classified as allergic OA, OA with latency period or sensitizer-induced OA, and nonallergic OA, OA without latency period or irritant-induced OA [7, 10].

**Key Notes**
Classification of occupational asthma (OA):

- Allergic OA, OA with latency period or sensitizer induced OA (IgE-mediated and IgE-independent).
- Nonallergic OA, OA without latency period or irritant induced OA (one or several episodes of exposure).

OA is an etiological, not a nosological entity, because according to its pathohistological and pathophysiological features, clinical manifestations and therapeutic modalities it is not different from asthma with nonoccupational etiology. The two types of OA are included in the List of Occupational Diseases of the Republic of North Macedonia (R. N. Macedonia), since 2004, labeled as allergic asthma caused by inhalation of substances appropriately identified as allergy triggers and related to the type of work (304.06 A), and asthma caused by substances, scientifically proven as triggers of irritation associated with the workplace (304.06 B). Workers having OA who have been diagnosed according to the conditions and criteria listed in the List of Occupational Diseases have the compensation rights specified by the actual legislation [11].

## 7.3 Epidemiology

According to the results of several studies, OA is about 5–20% of all asthma cases in adults. The incidence of OA is higher in developed countries than in developing countries (13–20% versus 5–6% of all adult asthma cases) [12]. The average annual OA incidence in developing countries is 2/100,000, while in the Scandinavian countries it is much higher, and 18 new cases per 100,000 adults are registered annually [13]. The annual incidence of OA in R. N. Macedonia in the period 2005–2014 ranged from 1.8/100,000 in 2013 to 2.8/100,000 in 2006 [14].

Data from several studies on the prevalence of allergic OA show that it affects 1–3% of workers in the food industry, 3–5% of workers in the wood industry exposed to western red cedar dust, 7–9% of bakers and workers from the food industry working with flour, 3–30% of workers working with laboratory animals, 5–30% of workers in the automotive industry that use isocyanates, about 46% of workers exposed to salts of platinum, and even 66% of workers involved in the production of detergents, and exposed to proteolytic enzymes [15, 16].

According to the results of allergic OA studies in the R. N. Macedonia, its prevalence is 1.6% in tea processors, 5.19% among the grinders of grain, 5.7% among the rice processing workers, 6.2% in the leather industry workers, and 2.3% and 1.3% in crop and dairy farmers, respectively [17, 18].

Considering the different representation of individual industrial and commercial branches in different countries, there are different prevalence rates of OA caused by certain occupational agents. In the 1990s the most common allergic OA in Britain was caused by isocyanates, in Finland allergic OA caused by animal epithelium, and in Italy, OA caused by latex [19]. The highest rates of new cases of allergic OA in R. N. Macedonia in the period 2004–2015 were registered among grain and flour workers, cleaners in private and public buildings, textile workers, and farmers [14].

Unlike the allergic OA, the epidemiological characteristics of the nonallergic OA are less known. It is estimated that about 10% of all OA cases are due to the nonallergic OA. Research conducted in G. Britain suggests the prevalence of reactive dysfunction airway syndrome (RADS) in a series of occupational inhalation incidents of about 9% [19].

## 7.4 Risk Factors

The occurrence of OA is due to the interaction of the individual endogenous factors with the environmental factors. According to research results, risk factors for OA development are the type and intensity of occupational exposure, genetic factors, atopy, nonspecific bronchial hyperresponsiveness, occupational rhinitis, preexisting asthma, and smoking [20].

**Specific Occupational Exposure: Occupational Allergens and Irritants** OA can be caused by numerous workplace allergens and irritants.

Exposure to occupational allergens with different allergenic potential together with exposure intensity (relation dose/response) is known risk factors for OA development. So far, more than 300 sensitizing agents have been identified from the working environment with potential to cause allergic OA through various pathogenic mechanisms [8, 21, 22]. Depending on the molecular weight, workplace sensitizers are classified as high-molecular-weight and low-molecular-weight. High-molecular-weight agents (HMW) are complete allergens with a size of at least six molecules of glucose. These are usually proteins, polysaccharides and peptides of vegetable, animal and synthetic origin [22].

The most important HMW agents causing allergic OA are [22]:

- Flour
- Cotton, linen, and silk
- Secretions and excrements from laboratory and domestic animals
- Proteolytic enzymes
- Latex

The risk of developing allergic OA caused by HMW occupational sensitizers exists in:

- Bakers and grain millers
- Textile workers
- Health, veterinary, and laboratory workers
- Detergent manufacturers
- Crop and dairy farmers
- Leather and shoemaking workers
- Workers in the production and processing of rubber
- Processors of coffee, tea, and spices

Low-molecular-weight agents (LMW) are incomplete allergens, i.e., haptens, and become complete allergens by binding to tissue proteins. LMW occupational sensitizers have different chemical structure.

The most important LMW agents causing allergic OA are [22]:

- Isocyanates (toluene diisocyanate, methane diphenyldiisocyanate, hexamethylene diisocyanate)
- Anhydrides (epoxy resins, colophony, phthalic acid)

- Acrylates
- Aldehydes
- Metals and their salts (platinum, nickel, chromium)
- Wooden dust (western red cedar, mahogany)
- Drugs (antibiotics, analgetics, anesthetics) and others

The risk of developing allergic OA caused by LMW occupational sensitizers exists in:

- Workers in the production of paints and varnishes
- Automotive industry workers (spray painters)
- Pharmaceutical industry workers
- Metal industry workers
- Chemical industry workers
- Electronic industry workers
- Wood industry workers
- Workers in the production and processing of plastics

Table 7.2 gives an overview of occupations and allergens proven to be causal factors for allergic asthma development.

The intensity of the exposure, i.e., the dose–response ratio is a proven risk factor for the occurrence of allergic sensitization, allergic occupational rhinitis (OR), and allergic OA. Thus, results of the studies indicate that IgE-mediated allergic sensitization does not occur at occupational exposure of wheat flour allergens in ambient air concentrations in the working environment below 0.5 mg/m$^3$, i.e., at concentrations of latex allergens lower than 0.6 ng/m$^3$ or at concentrations of allergens in laboratory rat's urine lower than 0.7 μ/m$^3$ [23–25].

Nonallergic OA occurs after one or several exposure episodes to very high concentrations of respiratory irritants in the form of gases, vapors, or aerosols. According to the mechanisms of occurrence, the nonallergic OA includes several entities. New-onset asthma caused by an acute inhalation incident is called reactive airway dysfunction syndrome (RADS), which is the best-defined type of nonallergic OA. New-onset asthma caused by several exposure episodes to very high levels of respiratory irritants is designated as non-RADS irritant-induced OA [8].

The most important respiratory irritants causing the nonallergic OA are: chlorine and chlorine compounds, ammonia, nitrogen oxides, sulfur dioxide, sulfur hydrogen, ozone, welding fumes, fire fumes, and diesel particles [20].

The risk of nonallergic OA occurrence, that is, OA caused by occupational irritants, exists in:

- Petrochemical industry workers
- Firefighters
- Traffic workers
- Construction workers
- Miners
- Cleaners (domestic, private, and public buildings)
- Chemical industry workers, etc.

**Table 7.2** Job positions and occupational allergens proven as causal factors for development of allergic OA

| Occupation | Agent/hazard |
|---|---|
| Exposure to high-molecular-weight agents | |
| Textile workers | Cotton, silk, linen, synthetics |
| Grain millers, bakers, farmers | Flour, grain dust |
| Agricultural workers, coffee, tea and spices processors, cooks | Vegetable pollens, molds |
| Laboratory workers, veterinary workers, farmers, leather workers | Secretions and excrements from laboratory and domestic animals |
| Detergent manufacturers, food processors | Proteolytic enzymes |
| Health and veterinary workers | Latex |
| Exposure to low-molecular-weight agents | |
| Cleaners (private and public buildings) | Quaternary ammonium compounds, formaldehyde, glutaraldehyde, chlorine compounds (chloramine-T, chlorohexidine, hexachlorophene) |
| Workers in the automotive industry (spray painters), workers in rubber production, workers in the production of paints and varnishes | Isocyanates |
| Workers in the production and processing of plastics, workers in the production of epoxy resins | Anhydrides (trimellitic acid, phthalic acid) |
| Metal industry workers | Metals (chrome, nickel, platinum) |
| Workers in the electronic industry | Colophony (abiotic acid) |
| Workers in the wood industry | Dust from western red cedar (plicatic acid) |
| Workers in the chemical industry, workers in the production of plastics | Acrylates |
| Hairdressers | Persulfates |
| Workers in pharmaceutical industry | Medicines |
| Health workers | Formaldehyde, glutaraldehyde |

**Genetic Factors** The role of the genetic factors in the onset of allergic OA is the subject of multiple studies, but the results obtained are still modest. It is considered that the genetic predisposition, i.e., the increased risk of the appearance of allergic OA is due to gene polymorphism. HLA class II molecules are characterized by pronounced polymorphism, and variations in their protein structure determine the specific epitopes of T2-helper lymphocytes (CD4+ lymphocytes) in certain individuals [8]. However, the current knowledge of the association of allergic OA with HLA class II does not refer to the possibility of identifying persons predisposed to the appearance of an allergic OA prior to employment in certain jobs.

**Atopy** Atopy, defined as personal and familiar predisposition for IgE antibodies production as a response to low dose allergen exposure, mostly proteins, and

development of typical symptoms of asthma, rhinoconjunctivitis, or eczema/dermatitis is a risk factor for development of IgE-mediated allergic OA, while its role in the development of IgE-independent allergic OA is less clear [26].

**Bronchial Hyperresponsiveness** Bronchial hyperresponsiveness (BHR), defined as an excessive bronchoconstriction response to various physical, chemical, and pharmacological stimuli, is considered a risk factor for the occurrence of allergic OA, regardless of the pathogenetic mechanisms [27].

**Occupational Rhinitis** OR is a proven risk factor for the occurrence of allergic OA caused by both HMW and some LMW agents. The allergic OR usually precedes the development of an allergic OA, with the risk of asthma being highest in the first year after the onset of clinical manifestation of rhinitis. Rarely, OR may occur at the same time or after the onset of OA symptoms [28].

**Preexisting Asthma** Preexisting asthma is considered a risk factor for the emergence of IgE-mediated allergic OA [27].

**Cigarette Smoking** The results of studies that investigate the association between active and passive smoking and the occurrence of allergic OA are controversial [29].

## 7.5 Pathogenesis

Allergic OA occurs after a certain period of occupational exposure (latency period) in which sensitization of the specific occupational allergen and the development of chronic airway inflammation occurs. Symptoms of allergic OA in more than 50% of cases occur in the second or third year after starting of work and start of specific job exposure. Symptoms of allergic OA occur in more than 80% of cases within the first 10 years of employment at a given job position.

Allergic sensitization of the occupational allergen may be IgE-mediated (IgE-mediated allergic OA) or it is an allergic OA where IgE-mediated mechanisms cannot be proven, and that is the case of IgE-independent or non-IgE-mediated allergic OA.

IgE-mediated OA is caused by HMW agents and some LMW agents (platinum, anhydrides, some medicines, etc.). It occurs after a latency period after beginning of occupational exposure, and the results of several studies indicate that in most cases sensitization occurs to a single workplace allergen [30].

The pathogenetic mechanisms of IgE-mediated OA are identical to the mechanisms of nonoccupational allergic asthma, i.e., IgE-mediated OA is taken as the prototype for the occurrence of allergic asthma as well. Chronic inflammation involves many inflammatory cells and more than 100 inflammatory mediators that cause multiple airway effects, of which the most important are bronchoconstriction, mucus hypersecretion, mucosal edema, and the activation of the sensory nerves.

According to current knowledge, the mechanism of occurrence and maintenance of chronic inflammation consists of cascade activation of inflammatory cells and release of mediators ("allergic cascade"). The most important inflammatory cells are: T2-helper lymphocytes, eosinophils, mast cells, dendritic cells, B lymphocytes, and macrophages [31].

The allergic cascade is initiated by Type I immune reaction (early or immediate reaction) in which the antigen is the occupational allergen to whom allergic sensitization has been developed, and the antibody is the specific IgE. Mastocytes are thought to have a major role in causing acute symptoms (early asthma reaction), while eosinophils have a primary role for late asthma reaction, i.e., chronic inflammation due to which the underlying pathophysiological phenomena of asthma are developed (variable and reversible airway obstruction and BHR).

The most important inflammatory mediators in the IgE-mediated allergic cascade are: cytokines, chemokines, lipid and peptide mediators, and growth factors. Cytokines, such as interleukin-4, interleukin-5, interleukin-13, etc., orchestrate inflammation, and chemokines (eotaxin-1, eotaxin-2, eotaxin-3, etc.) selectively recruit inflammatory cells from circulation into the airway wall. Lipid mediators (leukotrienes, prostaglandins, etc.) and peptide mediators (histamine, eosinophilic cationic protein, eosinophilic basic protein, quinines, etc.) cause the effects of smooth muscles, blood vessels, and mucous glands, while growth factors (growth factor of fibroblasts, growth factor of endothelial cells, etc.) stimulate the proliferation of fibroblasts by collagen production and angiogenesis [25].

Most of the LMW occupational agents cause IgE-independent allergic OA, and the pathogenic mechanisms of this type of allergic OA are less well-known than those of the IgE-mediated OA.

In vivo and in vitro tests for specific IgE within this type of allergic OA are negative. LMW agents usually cause a late asthmatic reaction, and sometimes a double, early, and late asthmatic reaction (e.g., a biphasic reaction in isocyanate asthma). The emergence and maintenance of IgE-independent chronic airway inflammation is due to an insufficiently defined reaction with the possible participation of IgG antibodies and cellular immunity, as well as nonimmune mechanisms of the type of neurogenic inflammation by activation of the non-adrenergic non-cholinergic system, release of asthmogenic cytokines, chemokines, and mediators from the damaged epithelium, etc. The role of specific T2-helper lymphocytes in the pathogenesis of IgE-independent allergic OA is still not clear [32].

The pathogenetic mechanisms of the nonallergic OA are still insufficiently known. In this type of OA the symptoms occur immediately, that is, up to 24 h after inhalation of high doses of irritants in the working environment, which is why this type of OA is still called OA without a latency period. It is assumed that respiratory irritants in excessive concentrations can cause reflex bronchoconstriction by stimulating sensory C-fibers in the airway through local (neuropeptide) and central (vagal) mechanisms followed by neutrophil influx, and later mononuclear cells in the airways wall. Furthermore, possible etiopathogenic factors include epithelial damage with the subsequent release of asthmogenic cytokines and mediators, and complement activation [33].

## 7.6 Pathohistological Pattern

The pathohistological finding of chronic eosinophilic inflammation which involves large and small airways (chronic eosinophilic desquamant bronchitis) is characteristic of all asthma types. Chronic airway inflammation in asthma is characterized by desquamation of the epithelium, wall inflammation predominantly by T2-helper lymphocytes, eosinophils and mast cells, enlargement of the muscular mass, vascular congestion, mucosal hypertrophy, gout cells hyperplasia, and characteristic subepithelial fibrosis. Inflammatory changes lead to wall thickening and significant reduction of the airway's lumen, i.e., their remodeling [33].

Investigations of the bronchoalveolar lavage and biopsy material suggest that pathohistological changes in the airway wall in individuals with allergic OA do not differ from those in subjects with nonoccupational allergic asthma. There is also no difference in pathohistological changes in the airway wall in individuals with IgE-mediated and IgE-independent allergic OA. The pathohistological finding in the reactive dysfunctional airway syndrome is characterized by a lower count of eosinophils and T2-helper lymphocytes in the airway inflammatory infiltrate compared to allergic OA, as well as with more pronounced desquamation of the epithelium and subepithelial fibrosis [15, 25].

## 7.7 Clinical Presentation

The allergic OA is characterized by a latency period from the beginning of the current job exposure to the appearance of asthma symptoms (mostly dry cough, dyspnea, wheezing, and chest tightness).

In IgE-mediated asthma, e.g., baker's asthma, the onset of early (immediately after the start of the shift and during working hours) and late symptoms (4–12 h after the end of shift) is characteristic. The appearance of late symptoms only (symptoms after the end of the shift or at night after the work-day) is often encountered in the IgE-independent allergic OA, for example in isocyanate asthma (asthma in spray painters). Nasal (sneezing, itching, rhinorrhea, and nasal obstruction) and conjunctival symptoms (redness, itching, and tearing) occur in both types of allergic OA, more commonly in IgE-mediated allergic OA. Both types of allergic OA are characterized by the cessation or improvement of symptoms over weekends, sick leave, and annual vacations, with the possible loss in reversibility of symptoms while away from work in persons with long-term occupational exposure. As with OR, the onset of symptoms can be triggered by numerous nonoccupational physical and chemical agents (temperature amplitudes, dry and cold air, respiratory irritants) [34, 35].

The most common causes of acute exacerbations, as in nonoccupational asthma are: acute respiratory infections, exposure to workplace or environmental irritants, use of medicines (aspirin and other nonsteroidal anti-inflammatory agents, β-blockers), gastroesophageal reflux, and emotional stress.

The nonallergic OA is clinically manifested by the onset symptoms in the first 24 h after the acute inhalation incident and their persistence over the coming months and years. The causes of acute exacerbations in this type of OA do not differ from those of the allergic OA [36].

The degree of OA is determined according to the recommendations of the Global Initiative for Asthma (GINA), as well as the severity of nonoccupational asthma. It is classified as an intermittent, mild persistent, moderately severe persistent and severe persistent OA according to the frequency of day and night symptoms, results of lung functional tests and the frequency and severity of acute exacerbations [37].

## 7.8 Diagnosis and Differential Diagnosis

The diagnosis of certain work-related asthma types is a complex process that requires a serious and detailed approach not only because of the therapeutic effects, but also because of the different legal implications. The diagnosis of work-related asthma should be considered in all cases of new onset or worsened adult asthma. Given the lack of a single precise and reliable method in diagnosing individual forms of work-related asthma, this is achieved by combining several methods, that is, by fulfilling certain diagnostic criteria. Table 7.3 presents the diagnostic criteria for epidemiological and clinical diagnosis of various types of work-related asthma by the American College of Chest Physicians (ACCP) [38].

The diagnostic procedure in the allergic OA consists of a asthma diagnosis and proving the causal relationship between the disease and specific occupational exposure through the work history data, evaluation of the workplace, results of allergological tests, result of a specific bronchoprovocation test with the suspected occupational sensitizer, and the results of the exposure and elimination tests with serial peak expiratory flow (PEF) monitoring, serial determination of the BHR degree, and serial measurements of inflammatory markers [38].

**Table 7.3** Diagnostic criteria for epidemiological and clinical diagnosis of work-related asthma

| |
|---|
| A. Asthma diagnosis |
| B. Onset of asthma symptoms after starting certain job |
| C. Association between asthma symptoms and work |
| D. One or more of the following criteria: |
| 1. Occupational exposure to agent or process that may cause OA |
| 2. Positive exposure and elimination test by serial spirometry or serial PEF monitoring |
| 3. Positive exposure and elimination test by serial NBPT |
| 4. Positive SBPT to a specific workplace agent |
| 5. Onset of asthma with clear association to symptomatic exposure to workplace irritant |
| ***Allergic OA*** |
| Epidemiological diagnosis: A + B + C + D1 or D2 or D3 or D4 |
| Clinical diagnosis: A + B + C + D2 or D3 or D4 |
| Probable OA: A + B + C + D1 |
| ***Nonallergic OA:*** A + B + C + D5 |
| ***Work-exacerbated asthma:*** A + C |

**The diagnosis of asthma** is set by the standard diagnostic procedure, i.e., by verifying the asthma symptoms and proving the variable and reversible airway obstruction and presence of BHR [20].

**The working history** provides data on the conditions and characteristics of the current workplace of the patient/worker, i.e., for its working activities, the characteristics of the specific occupational exposure (type, duration, intensity), possible changes in the work process, and previous jobs.

**Evaluation of the workplace** provides data that qualitatively and quantitatively determine the specific occupational exposure. A great number of occupational sensitizers typical for certain occupations and industrial processes are known, but, on the other hand, allergic sensitization can be due to an unknown occupational sensitizing agent or to the complex mixtures that occur in certain technological processes [39].

**Allergological tests** in OA diagnostics, as well as in diagnosis of OR, have limited relevance. Most LMW agents cause IgE-independent allergic OA, whose pathogenic mechanisms are insufficiently known, and allergological investigations (determination of IgG and its subpopulations) are not routinely performed. On the other hand, even in cases of allergy testing (skin prick tests with occupational allergens and determination of specific IgE) in IgE-mediated allergic OA, false-positive and false-negative results may occur, especially when using poorly standardized or insufficiently purified preparations of occupational allergens, sensitization of unknown occupational allergens, etc. The results of studies performed to assess the risk of allergic OA in certain workplaces indicate a much more sustained data if occupational-specific risk is considered, than for a specific substance-specific risk, given the huge and ever-growing number of potential occupational sensitizers, as well as difficult, and sometimes even impossible, proving of all agent-specific risks [40].

**Specific bronchoprovocation test (SBPT) or specific inhalation challenge (SIC) test** with the suspected occupational allergen is a "gold standard" in the diagnosis of allergic OA. Despite the fact that it is used since the 1960s and the progress that has been made in the performance, there are a lot of limitations of its routine use for diagnosis of allergic OA. Namely, SBPT is performed according to a complicated protocol; its performance requires a qualified staff and technical requirements; significant adverse effects may occur; the method is not yet sufficiently standardized; preparations of occupational allergens are often non-standardized; performing in laboratory conditions cannot reconstruct the working environment, and false-positive and false-negative results are possible. Indications for the use of SBPT are cases with legal implications that cannot be solved in any other way, cases where an employee with an allergic OA refuses to leave the workplace, as well as research on identification of new occupational sensitizers.

SBPT is carried out in reference institutions with technical and personnel capabilities in conditions of strict control and adherence to the protocol. The test can also be performed within the subject's workplace, but with a high risk of adverse events such as severe or life-threatening allergic reactions. The process of testing takes 2–3 days with a series of inhalations of slow-growing concentrations or duration of inhalation of the suspected occupational allergen and monitoring of the spirometric

parameters. The test is considered positive when $FEV_1$ value is reduced by at least 20% compared to the baseline.

The negative SBPT does not exclude the existence of an allergic OA due to the possibility of obtaining false-negative results (provocation with the wrong occupational agent, inability to reconstruct the working conditions, etc.). False-positive results are also possible, as in the cases of irritation effects by very high concentrations of the provoking agent [40, 41].

Diagnosis of allergic OA in everyday practice is performed with **exposure and elimination tests**. In most cases, the diagnosis is set or excluded by performing one test, while in insufficiently clear cases the tests are combined or repeated. The diagnosis of allergic OA with these tests is based on detection of difference in the intensity of chronic airway inflammation determined by functional-diagnostic or laboratory methods under conditions of subject's exposure to the occupational agent causing the illness (exposure), and in conditions when the subject is not exposed to the same agent (elimination). In the allergic OA, the intensity of chronic inflammation is significantly increased during the exposure period compared to the elimination period, while significant difference in the intensity of chronic inflammation within the two examined periods is not observed in the case of work-exacerbated asthma.

According to actual recommendations, exposure and elimination tests with serial PEF monitoring, serial determination of the BHR level with nonspecific bronchoprovocation test (NBPT) and serial determination of inflammatory markers of asthma are most relevant [39].

With the **serial peak expiratory flow rate measurements, i.e., serial PEF monitoring** changes in the intensity of chronic inflammation during exposure and elimination of the occupational allergen that caused the disease are detected, by determining changes in airway obstruction variability. In allergic OA, the average daily variation of peak expiratory flow during the exposure period to the causal agent is significantly higher than those registered during the absence from the workplace (Fig. 7.1).

From the beginning of the 1980s, the exposure and elimination test with the serial PEF monitoring is routinely used in diagnostics of allergic OA, determining the asthma degree, and its relation to the level of exposure. According to the currently recommended protocol, the serial PEF monitoring is performed with at least four measurements per day for two working weeks (exposure) and two weeks' absence from work (elimination).

The sensitivity and specificity of this test with respect to SBPT are between 80 and 90%. Namely, according to the results obtained from the research in this field, in the case of correctly performed and interpreted exposure and elimination test with serial PEF monitoring the percentage of false-positive results is less than 10%, while the percentage of false-negative results is in the range 20–25%. By increasing the number of measurements during the day, i.e., by performing them every 2 h in the exposure and elimination periods and by statistical analysis of the data obtained with the variant analysis method (ANOVA), the sensitivity and specificity of the test reach 100% when compared to the results obtained with SBPT [42].

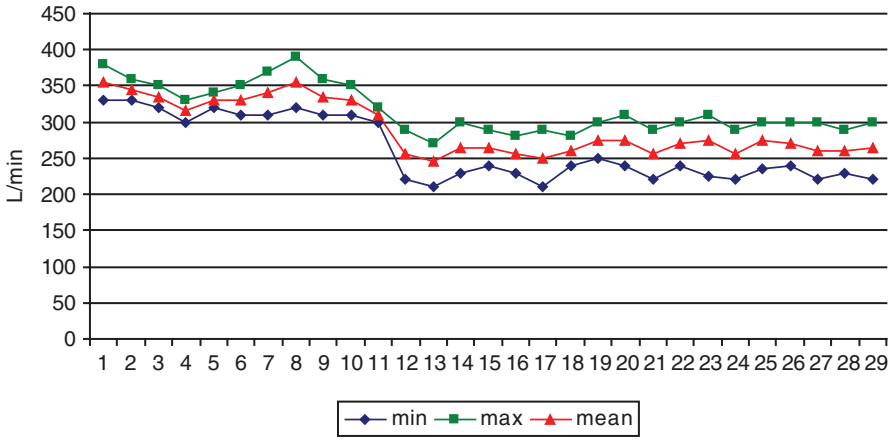


**Fig. 7.1** Positive exposure and elimination test by serial PEF—monitoring in crop farmer with allergic OA. Minimum, maximum and average PEF values in elimination [1–15] and exposure [16–30] period. Average daily PEF variations in the elimination period—12.5%, average daily PEF variations in the exposure period—25.6%. Average daily PEF value in exposure period—230 L/min, average daily PEF value in elimination period—315 L/min. (Source: Institute of Occupational Health of R. N. Macedonia—Skopje)

**Serial NBPT with histamine/methacholine** detects changes in the intensity of nonspecific BHR during the exposure periods of the occupational allergen causing the disease and its elimination while absent from work. The allergic OA is characterized by a significant difference in the intensity of nonspecific BHR during exposure of the causal agent in relation to its intensity when the subject is not exposed to the same agent. The test consists of conduction of NBPT with histamine or methacholine during the period when the subject goes to work and repeating it while he is absent from work for at least two weeks. The test is considered to be positive in cases of an increase in the degree of nonspecific BHR during the exposure period compared to the elimination period [40].

The exposure and elimination test with serial NBPT in the diagnosis of allergic OA is primarily indicated in addition to the exposure and elimination test with a serial PEF monitoring in cases where this test does not produce results that confirm or exclude the diagnosis of the disease. By combining these two tests, in most cases, even unclear ones, the diagnosis is established (OA or WEA).

By **serial determination of inflammatory markers in the biological material**, the difference in concentrations of chronic airway inflammation markers (usually eosinophilic cationic protein) in the blood, sputum, or urine during the exposure period to the causal agent is detected in comparison with their concentration in the biological material at a time when the subject is not exposed to it. These are still not well-standardized tests for conduction and interpretation, due to which they are not applied in the routine diagnosis of allergic OA.

**Key Notes**
Diagnostic procedures for evaluation of allergic asthma causal relationship with specific occupational exposure:

- Work history and workplace evaluation and risk assessment
- Allergological tests
- Specific bronchoprovocation test
- Exposure and elimination tests
  (serial PEF monitoring, serial NBPT)

The diagnosis of nonallergic OA in a person with diagnosed asthma is based on the fulfillment of the following criteria: documented absence of preexisting chronic lung disease, occupational exposure to high concentrations of gas, smoke or vapors with an irritant effect on the respiratory system, onset of symptoms within 24 h after a documented inhalation incident at the workplace, the symptoms have the character of asthma symptoms (cough, dyspnea, wheezing, chest tightness), duration (persistence) at least three months, preserved lung function parameters or presence of ventilatory insufficiency of obstructive type, positive NBPT or bronchodilator test and excluded other lung and extra-lung diseases with similar symptoms [43].

**The differential diagnosis** of allergic OA is a matter of distinguishing with other lung diseases caused or exacerbated by occupational exposure, such as: hypersensitivity pneumonitis and other occupational diseases of the lung interstitium, chronic obstructive pulmonary disease (COPD), bronchiectasis, etc. In addition, consideration should be given to extrapulmonary disorders in the workplace, such as chronic rhinosinusitis, laryngeal dysfunction, left heart failure, etc. The differential diagnosis of nonallergic OA is done with the acute irritation respiratory syndrome, the syndrome of multiple chemical sensitivity and others [44].

In some cases it is quite difficult to differentiate the allergic OA from work-exacerbated asthma, so in these cases, as previously mentioned, it is recommended to combine exposure and elimination tests and/or repeat them. It is particularly difficult to differentiate these two forms of asthma in relation to work in cases of newly reported disease related to occupational exposure to allergens that can be encountered also in the living environment. Such are: cat/dog fur allergens among workers in the veterinary service, pollen from linden, birch, wormwood, elder, etc. among tea-processors, cereal plants pollen among employees in the milling and food industry, etc. On the other hand, certain allergens, until recently present only in the work environment, become widely present in the environment by incorporating them in products for general use (e.g., isocyanates in household adhesives, detergents within enzymes, etc.) [39, 40].

## 7.9 Treatment

The main objectives of OA treatment, as well as the treatment of nonoccupational asthma, are to achieve and maintain disease control without or with minimal side effects from the drugs administered. The treatment consists of measures for occupational exposure control and pharmacotherapy [45].

**The control of the specific occupational exposure** is the basic measure in the treatment of allergic OA, and consists in termination the exposure, i.e., replacing the job position, immediately after the diagnosis is established. Exposure termination should be early, complete, and definitive because further exposure even to very low concentrations of the occupational sensitizer can cause a worsening of the disease course of the disease and significant pulmonary function decline.

Namely, there are no safe concentrations of the occupational allergen in which the patient/worker is sensitized, that is, the symptoms of OA can be stimulated even by the smallest concentrations of that allergen [46].

Improvement of the disease and its severity occurs usually after 2 years of exposure termination. Early beginning of inhaled corticosteroids (ICS) use significantly improves the disease course. Furthermore, when transferring the worker to another job position, care must be taken into account about the possible cross-sensitivity of the substances to which he/she is exposed in the new position with the occupational allergen to which he/she is sensitized, if the allergen is known or identified.

The exposure termination, that is, the change of workplace and/or job position, is not a crucial element in the treatment of the nonallergic OA. Patients with this form of work-related asthma may remain in the same workplace by improving workplace conditions (reduction in the degree of occupational exposure) and optimizing the pharmacological treatment [47, 48].

**Pharmacological treatment** of OA does not differ with the treatment of nonoccupational asthma, and consists of chronic asthma treatment and treatment of acute exacerbations. As with OR, pharmacotherapy does not represent an alternative to control measures on specific occupational exposure.

In chronic treatment, two groups of drugs are basic, anti-inflammatory (preventive agents or controllers) and bronchodilator drugs (symptomatic and relievers). The most important anti-inflammatory drugs for the chronic treatment of asthma are ICS, anti-leukotrienes and cromolins, while the most important symptomatic drugs are short- and long-acting β2-agonists and theophylline. The individualized and stepwise approach according to the extent of the achieved control of the disease (controlled, partially controlled and uncontrolled asthma) is an optimal way of successful treatment of the disease. The treatment of exacerbations, in addition to the regular administration of anti-inflammatory drugs, consists of repeated application of bronchodilators and the use of systemic corticosteroids [45, 46].

**Key Notes**
Treatment of allergic OA:

– Termination of specific occupational exposure (early, complete, and definitive)
– Pharmacological treatment

## 7.10 Disease Course and Prognosis

There is no any documented case of cured allergic OA. Symptoms of the disease and BHR persist for a long time after the termination of the specific occupational exposure, that is, the results of the studies indicate that the symptoms of the disease after an interruption of exposure persist in more than 50% of the patients, and the BHR in more than 70% of the patients persists to the end of their life. Early diagnosis, early termination of exposure, and early introduction of ICS in treatment are key factors for a better course and prognosis of the disease.

The results of the studies also indicate long-term persistence of symptoms and BHR and in nonallergic OA, and the main factors influencing the course of the disease are the intensity and duration of exposure to irritants that cause the disease.

Asthma symptoms and BHR persist for a long time after the termination of the specific occupational exposure. Namely, studies in this field indicate that the symptoms of the disease after occupational exposure termination persist in more than 50% of the patients, and BHR in more than 70% of the patients persists to the end of their life. Early diagnosis, early termination of exposure, and early introduction of inhaled corticosteroids in the asthma treatment are key factors for a better course and prognosis of the disease [47].

Research also indicates long-term persistence of symptoms and BHR in nonallergic OA as well, and the main factors influencing the course of the disease are duration and intensity of exposure to irritants that caused the disease [45].

## 7.11 Prevention

Preventive measures are essential for OA, since the disease, similar to other occupational diseases, is potentially preventable. Preventive approach to the disease implies active collaboration between employers, workers, and occupational health services.

**Primary prevention** consists of measures for occupational exposure control, that is, for maintaining the concentrations of occupational sensitizers and irritants to the lowest possible level.

The most important technological and technical preventive measures for collective protection in relation to OA prevention are:

- Workplace risk assessment of every job position performed due to the current legislation by identifying occupational sensitizers and irritants and their effects on respiratory health of workers
- Elimination of known sensitizers out of the production process and their substitution with substances with lower allergenic potential
- Avoidance of chemical substances whose molecules have long side chains in the technological process (it is considered that they determine the allergen potential of the substance)
- Improvement of general and local ventilation in the working environment
- Sealing and automation of the technological process
- Modification of the technological process
- Regular ambient monitoring

Technical preventive measures for personal protection (respirators with filters, gas masks, etc.) play an important role in cases where collective protection measures cannot provide adequate occupational exposure control. Particular importance should be given to the worker's training for proper use of personal protective equipment, regular maintenance of personal protective equipment, as well as the control of their properness.

Informing workers about their exposure to occupational sensitizers and irritants during work, as well as their potential effects on the respiratory system through adequate education is of great importance in the primary prevention of OA. Also, promotion of a healthy lifestyle and healthy living habits of workers plays a major role in the disease prevention.

Preemployment detection of subjects with atopy and BHR prior to placing them at job positions at risk for OA development is not recommended given their relatively high prevalence in the general population (30–40% for atopy, or 10–20% for BHR). The results of the studies indicate a low positive predictive value of atopy in relation to the occurrence of OA in subjects employed in different occupations (5–15%). According to the recommendations of the British Occupational Health Research Foundation (BOHRF), the presence of poorly discriminating factors such as atopy, BHR, individual or familial history of asthma, smoking, and HLA phenotype should not be reasons for negative professional orientation and selection [49].

**Secondary prevention** of OA consists of measures and activities for early disease detection and early intervention in its course, thus preventing the disease's deterioration and permanent lung function impairment. This could be achieved through regular preventive (periodical) health examinations of workers with specific occupational exposure that are performed within regular time intervals and according to a certain defined protocol (standardized questionnaires, lung functional monitoring, allergological tests, evaluation of nonspecific BHR, etc.) [45, 50].

**Tertiary prevention** consists of measures and procedures for treatment and rehabilitation of subjects with clinically manifest disease [45, 46].

## 7.12 Conclusion

OA is the most common occupational lung disease globally in the last few decades. According to expert estimates, OA is due to about one-fifth of all adult asthma cases indicating that the disease is an extremely important public health problem worldwide. According to the mechanism of occurrence, OA can be allergic and nonallergic, with the allergic form more common than the nonallergic disease type. Although significant progress has been made in the knowledge of pathogenetic mechanisms, diagnosis, treatment, and prevention of disease in recent decades, in some of these areas there are still some insufficiently clear and controversial aspects and they are subject to current and future intensive research. Improving the control of occupational exposure, improving the existing diagnostic methods, and introducing new methods with higher sensitivity and specificity, as well as the improvement and alignment of all elements of disease management are the most important OA-related challenges in the upcoming period.

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# Chapter 8
# Occupational Respiratory Allergic Diseases: Occupational Rhinitis


**Sasho Stoleski**

**Abstract** Occupational rhinitis (OR) is an insufficiently investigated and underdiagnosed entity with a frequency that is thought to be 2–4 times higher than the frequency of occupational asthma (OA). The classification of OR, as well as the classification of OA, is based on its etiopathogenetic mechanisms. OR can be caused by immune mechanisms (IgE-mediated and IgE-independent allergic OR) and nonimmune mechanisms (nonallergic OR). This chapter presents the epidemiological and etiopathogenetic characteristics of allergic OR, its relationship to allergic OA, and the current diagnostic approach, treatment, and recommended preventive activities.

**Keywords** Occupational rhinitis · Occupational allergic rhinitis · IgE-mediated allergic occupational rhinitis · IgE-independent allergic occupational rhinitis · Occupational allergic asthma

## 8.1 Work-Related Rhinitis

Work-related rhinitis is a form of rhinitis that is caused or induced by specific agents and/or workplace conditions. According to the ethiopathogenetic mechanisms involved in its occurrence, that is, whether specific agents and conditions in the

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workplace cause the disease or are triggers of its symptoms, the work-related rhinitis is classified as an OR and rhinitis exacerbated at work or work-exacerbated rhinitis (WER).

WER or work-aggravated rhinitis is a preexistent or new-onset rhinitis (allergic or nonallergic) whose symptoms are exacerbated by nonspecific stimuli from the work environment (respiratory irritants, temperature amplitudes, cold and dry air, physical strain) [1].

## 8.2 Definition and Classification of Occupational Rhinitis

OR is so far an insufficiently investigated clinical entity and for its definition there is still no accepted clear consensus by international and national associations. According to the current guide of the European Academy of Allergology and Clinical Immunology (EAACI), which summarizes the results of previous research in this field, and based on its pathophysiological and clinical features OR is defined as an inflammatory nasal disease characterized by intermittent or persistent symptoms (sneezing, itching, rhinorrhea, and nasal congestion) and/or variable reduction of airflow through the nose and/or hypersecretion of the nose that are due to reasons and conditions specific to the specific workplace, not to stimuli outside it. Considering its frequency, the impact on the quality of life of patients and the relationship with OA, the OR is a significant disease in the occupational pathology of the respiratory system [2].

Analogously to the classification of OA, which is a much better investigative entity, the OR according to the ethiopathogenic mechanisms of its occurrence is classified as an allergic and nonallergic OR. Allergic OR, depending on the immune mechanisms involved in its occurrence, is classified as IgE-mediated allergic OR and IgE-independent allergic OR (Table 8.1) [3].

**Table 8.1** Ethiopathogenetic classification of OR

| Work-related rhinitis | | |
|---|---|---|
| Rhinitis caused by work—Occupational Rhinitis (OR) | | Rhinitis exacerbated by work—Work-Exacerbated Rhinitis (WER) |
| Allergic OR (with latency period)—sensitizer induced OR | | |
| IgE-mediated | Non-IgE-mediated | |
| Nonallergic OR (without latency) | | |
| Single exposure—RUDS | Multiple exposures—Irritant induced OR | |

OR is included in the List of Occupational Diseases of the Republic of North Macedonia (R.N. Macedonia) since 2004, as an "*Allergic rhinitis caused by substances appropriately identified as triggers of allergy and associated with the type of work* (304.07)."

## 8.3 Epidemiology

Despite the fact that it is a common disease, so far few studies have been performed, both in the general population and in workers in certain occupations, which explore the incidence and the prevalence of OR. Similar to OA, OR is considered a disease that is pretty much sub-diagnosed.

According to the results of these studies, the prevalence of OR in the general population is 2–4 times higher than the OA prevalence, i.e., ranging from 4 to 20%. The results of these studies suggest that the prevalence of allergic OR caused by HMW agents in different occupations ranges from 2 to 87%, while the prevalence of allergic OR caused by LMW agents is 3–48% [3]. According to the data from the Finnish Occupational Disease Registry for the period 1986–1991, the risk of allergic OR was the highest in bakers, food processors, furriers, livestock farmers, veterinarians, electricians and electronic industry workers, and workers in shipbuilding [4].

According to study carried out by the team of the Institute of Occupational Health of R. N. Macedonia, among workers from different occupational settings in Skopje in the mid-1990s, the prevalence of OR among all respondents was 10.1%, being the highest among the workers in the pharmaceutical industry (15.5%). On the other hand, according to the research of adult respondents from six cities in the R. N. Macedonia in 2003, the OR prevalence was 7.6%, and the most important risk factors for its occurrence are dust, moisture, and chemical substances from the working environment [5, 6].

As with OA, it is considered that the prevalence of allergic OR is 2–3 times higher than the prevalence of the nonallergic OR. Unlike the allergic OA, it is estimated that the prevalence of allergic OR caused by HMW agents is higher than that of allergic OR caused by LMW agents.

The socioeconomic impact of the OR, especially direct and indirect costs caused by this disease are not known, but given its frequency, they are certainly significant. On the other hand, both the impaired quality of life and the reduced productivity caused by the disease are of great importance for patients with OR.

## 8.4 Risk Factors

According to the results of few small-scale studies, risk factors for the occurrence of OR are the type and intensity of occupational exposure, atopy, smoking, and nonspecific bronchial hyperresponsiveness. Unlike in OA, genetic risk factors for the occurrence of OR have not been investigated in any study yet.

**Specific Occupational Exposure** OR can be caused by multiple workplace allergens and irritants. The same occupational allergens that can cause allergic OA in exposed workers can also cause OR. According to the molecular weight, occupational

allergens that may induce OR in exposed workers are classified as HMW and LMW agents.

HMW agents are usually glycoproteins of vegetable or animal origin. The most important HMW agents that can cause OR in the exposed workers are flour, cotton, linen and silk, latex, secretions and excrements from domestic and laboratory animals, proteolytic enzymes by detergents.

LMW agents are low molecular weight chemicals that act as haptens, and become complete allergens after being absorbed in the body and tied to the tissue proteins. The most important LMW allergens that can cause OR in exposed workers are: isocyanates, anhydrides, acrylates, metals and their salts, wood dust, and drugs.

High-risk occupations for the occurrence of allergic OR caused by HMW and LMW occupational allergens are shown in Table 7.2.

The intensity of specific occupational exposure is also a risk factor for the occurrence of allergic OR. Many research results in the field indicate a clear dose–response relation in regard to occurrence of allergic sensitization, allergic OR, and allergic OA.

Occupational irritants that can cause nonallergic OR are the same that cause nonallergic OA, i.e., chlorine and chlorine compounds, nitrogen oxides, ammonia, formaldehyde, and ozone [7].

**Atopy** Atopy is a proven risk factor for allergic sensitization to HMW occupational agents. The study results that examine the role of atopy as a risk factor in the occurrence of allergic OR caused by LMW occupational agents are controversial.

**Smoking** The link between smoking and the occurrence of sensitization to occupational allergens, OR and OA, despite more research carried out in recent decades, remains unclear.

**Nonspecific Bronchial Hyperresponsiveness** According to the results of a few studies, the role of nonspecific hyperresponsiveness as a risk factor in the occurrence of allergic OR cannot be excluded.

## 8.5 Pathogenesis

Allergic OR occurs after the sensitization of a high or low molecular agent in the working environment, and the symptoms occur when the sensitized worker is recontacted with the occupational allergen. According to research results, skin and rhinoconjunctival symptoms in most cases occur in the first two years after a specific occupational exposure, while asthma symptoms in most cases occur in the second and third year of exposure. The link between allergic sensitization and clinically manifested disease is complex, and data show that allergic OR occurs in about half of sensitized workers.

IgE-mediated allergic sensitization occurs in allergic OR caused by HMW occupational allergens and some LMW occupational allergens (platinum salts, anhydrides, etc.). The allergic reaction in this type of allergic OR consists of an immediate or early stage (type I) reaction in which mast cells have a dominant role, and of late stage reaction in which eosinophils play a dominant role. IgE-mediated sensitization in this type of allergic OR is proven by a positive skin prick tests to occupational allergens, and/or a positive specific IgE by in vitro allergological tests.

Allergic sensitization to most of the LMW occupational agents is not IgE mediated; therefore, allergic OR caused by these agents is called IgE-independent or non-IgE-mediated allergic OR. Skin prick tests and specific IgE to occupational allergens in this type of allergic OR are negative. The mechanism of allergic sensitization is not known; it may include IgG and/or cellular immune mechanisms, while the early (immediate) phase within the allergic reaction is absent.

Nonallergic OR or irritant-induced OR is caused by nonimmune mechanisms, i.e., it occurs after one or several episodes of exposure to very high levels of workplace respiratory irritants. Intermittent or persistent nasal symptoms in this case occur immediately after the inhalation incident at the workplace, i.e., there is no latency period between exposure and the appearance of clinically manifested symptoms. Analogously to RADS in nonallergic OA, the nonallergic OR that occurs after a single episode of exposure to excessively high concentration of occupational irritants is called reactive upper airways dysfunction syndrome (RUDS). The pathogenetic mechanisms of the nonallergic OR are unknown, and the causal relationship of the disease with certain job position is proven by the timeline association between the exposure to unusually high concentrations of workplace irritants and the occurrence of nasal symptoms and/or other objective clinical signs of the disease [8].

## 8.6 Relationship Between Occupational Rhinitis and Occupational Asthma

According to research results, patients with OA in a number of cases have associated OR, and nasal symptoms usually precede the onset of respiratory symptoms. The concept of united airway disease, that is, the concept of two clinical manifestations of a single disease, is confirmed in the field of occupational allergic airway diseases. Namely, as in the case of nonoccupational allergic rhinitis and nonoccupational allergic asthma, allergic OR is a risk factor for the development of allergic OA, but the percentage of patients with OR who develop OA is not known. According to the data from the Finnish Occupational Disease Registry, the risk of OA is approximately five times higher in patients with allergic OR compared to those who do not have this disease, and according to the results of other studies, the percentage of patients with allergic OA that appeared after allergic OR ranges from 20 to 80% [9, 10].

**Key Notes**
Occupational rhinitis is a risk factor for development of occupational asthma.
The percent of subjects with occupational rhinitis who will develop occupational asthma is not known.

The relationship between allergic OR and allergic OA is expressed in patients with allergic OR caused by both HMW and LMW occupational agents, while the severity of symptoms is greater in patients with allergic OR caused by HMW agents. According to the results of a study by Malo et al., nasal symptoms are registered in about 90% of patients with allergic OA caused by HMW occupational agents. Survey of workers engaged in fodder production shows that in the period of 11 years allergic OA has developed in 36.7% of workers with allergic OA and in only 5.2% of workers who did not have allergic OR [11].

Major risk factors for the occurrence of allergic OA in patients with allergic OR are the existence of nonspecific bronchial hyperresponsiveness, as well as the persistence of nasal symptoms and the longer duration of OR. Association between OR and OA is also established in nonallergic forms of these diseases, but this link is much less explored [12].

## 8.7 Clinical Presentation

Allergic and nonallergic OR are clinically presented by intermittent or persistent nasal symptoms, i.e., sneezing, itching, rhinorrhea, and nasal congestion. In most cases, nasal symptoms are associated with conjunctival symptoms (redness, itching, and tearing of the eyes). In patients with longer disease duration, especially those with persistent symptoms, the occurrence of olfactory dysfunction (hyposmia or anosmia), sinusitis and sinonasal polyposis, as well as sleeping disorders, is common. The occurrence of respiratory symptoms (cough, dyspnea, wheezing, and chest tightness) suggests OA development.

In patients with allergic OR, there is a characteristic association between symptoms and workplace, i.e., the exposure of the agent that has caused the disease. Nasal symptoms occur or become more prominent when the patient is at the workplace, while they are lost or decreased by intensity during periods of absence from work (weekends, annual vacations, etc.). Allergic OR caused by HMW occupational allergens has characteristic clinical presentation of symptoms, i.e., sneezing, itching, and rhinorrhea immediately after starting the shift (immediately after allergen exposure), while nasal congestion occurs after several hours of work or after the completion of the work shift. This characteristic appearance of symptoms is rarely found in allergic OR caused by LMW occupational allergens [13].

In patients with nonallergic OR, nasal symptoms occur immediately after the workplace inhalation incident, followed by their intermittent or persistent appearance. The occurrence of nasal symptoms, both in patients with allergic OR, as well as with nonallergic OR may be induced by numerous nonoccupational physical and chemical agents (changes in ambient temperature, dry and cold air, respiratory irritants).

Classifications of the severity of OR do not differ from those of nonoccupational allergic rhinitis. According to the classification of the Working Group on Allergic Rhinitis and its impact on asthma (ARIA) based on daily activities and sleeping characteristics, the rhinitis is classified into mild and moderate/severe. According to the current document for rhinosinusitis and nasal polyps by the EAACI, based on the visual analog scale (VAS) rhinitis is classified as mild, moderate, and severe [14].

## 8.8 Diagnosis and Differential Diagnosis

The diagnosis of OR is based on anamnesis and work history data, as well as ENT examination, allergological tests, functional examinations, and the nasal provocation test. Due to the frequent association of OR with OA, the possible coexistence of associated OA is also evaluated within the diagnostic procedure.

**Anamnesis and Work History** The anamnesis provides data on the type of symptoms and their characteristics, i.e., their appearance, duration, intermittence or persistence, severity, inducing or worsening factors, and their relationship to the patient's workplace.

The work history provides data on the conditions and characteristics of the current workplace, i.e., for its working activities, characteristics of the specific occupational exposure (type, duration, and intensity), possible changes in the work process, previous job positions, etc.

Although the basic step in the diagnostic procedure, anamnesis, and work history is not sufficient to establish the diagnosis of OR.

**Nasal Examination** With the ENT examination, that is, with the frontal rhinoscopy and nasal endoscopy, data on the condition of the nasal mucosa and nasal passages, as well as possible pathological conditions (nasal septum deviation, nasal polyps) are obtained.

**Allergological Tests** Evidence of IgE-mediated allergic sensitization is performed by skin prick tests to occupational allergens and/or the determination of specific serum IgE levels. As previously mentioned, these tests can detect sensitization in patients with allergic OR caused by HMW and a small number of LMW occupational allergens. The negative finding of allergy testing does not exclude the diagnosis of OR. On the other hand, allergic sensitization is a condition, not a disease, that is, at least half of the sensitized persons remain asymptomatic throughout their

lives. Also, a major problem in the diagnosis of allergic sensitization in occupational allergies is the nonexistence of standardized allergenic extracts from numerous occupational allergens which has an impact on the accuracy of the results obtained and the occurrence of adverse reactions.

**Functional Examinations** The most commonly performed test of the nasal function in subjects with OR are: rhinomanometry, acoustic rhinometry, measurement of the peak nasal inspiratory flow, measurement the degree of inflammation of the nasal mucosa, and the detection of nonspecific nasal hyperreactivity [15].

Anterior or frontal rhinometry is a method that determines the flow and resistance of the air flow through the nasal passages. It is a method that is easily performed without big effort and cooperation of the examinant. The posterior rhinometry collects the same data, but it is performed by placing an oral catheter in the pharynx, which requires greater cooperation of the examinant.

Acoustic rhinometry measures the airflow through the nasal passages by reflection of sound waves. The method is noninvasive, reproducible, and does not require greater cooperation from the examinant.

Measurement of the peak nasal inspiratory flow (PNIF) is a simple and cheap method for assessing the condition of the air flow through the nasal passages whose results are correlated with the results of the anterior rhinomanometry. Serial measurement of the peak nasal inspiratory flow can be performed in periods of exposure and elimination (period of working and absence from work) to the occupational allergen, but the method is not yet standardized and validated.

The degree of nasal mucosa inflammation can be assessed by determining the levels of inflammatory cells and mediators in the nasal secretion. It can be also assessed by determining the concentration of nasal nitric oxide, but this method in the diagnosis of allergic OR is not yet standardized and validated.

Nonspecific nasal hyperreactivity is a more intense nasal response (sneezing, nasal secretion, and/or nasal congestion) of certain pharmacological or physical stimuli (histamine, methacholine, cold and dry air). Unlike nonspecific bronchial hyperresponsiveness in patients with allergic OA, nasal hyperreactivity is not found in all patients with allergic OR, and therefore has limited importance in the diagnosis of allergic OR.

**Nasal Provocation Test** The nasal provocation test (NPT) is considered a gold standard in the diagnosis of allergic OR, although the test is still non-standardized. The NPT can be performed in an outpatient laboratory or at the workplace, and is based on determining the nasal response (symptom-score, changes in the nasal flow determined by rhinomanometry, acoustic rhinometry or by measuring the peak nasal expiratory flow or, by detecting changes in the concentration of inflammatory markers) following the application of an occupational allergen that is considered to have caused the allergic OR. Given that NPT can induce an early (immediate) and late nasal response, according to the EAACI recommendations, the monitoring of the nasal response is performed within 5, 10, 15, 20, 30, 45, and 60 min after the application of the allergenic extract, and then every hour for the next 10 h.

The most important limitations of NPT are different criteria for positive response and for completion of the test that are not yet standardized and fully validated, together with the nonexistence of standardized allergenic extracts for many occupational allergens. False-positive results are obtained with hyperreactivity of the nasal mucosa due to recent allergen or irritant exposure or due to rhinosinusitis, while false-negative results are obtained when performing NPT with an occupational allergen that does not cause the disease, longer absence from work, and in patients receiving intranasal corticosteroids at the time of the test. Adverse reactions that may occur during NPT include: induction of a severe nasal response and/or an asthmatic attack. Contraindications for the test are: pregnancy, current infectious rhinitis or sinonasal surgery, atrophic rhinitis, and associated severe asthma [16].

**Evaluation of Accompanying OA** Evaluation of a possible associated OA is performed by the diagnostic procedure explained in the part about Occupational Asthma.

**Diagnostic Algorithm** The diagnostic algorithm for allergic OR consists of three steps [10, 17].

The first step is taking an anamnesis with work history and ENT examination. If there is a doubt about the occupational etiology of the rhinitis, its causality with the workplace should be confirmed by objective methods.

The second step involves evaluating the patient's sensitization to occupational allergens with in vivo or in vitro allergological tests. Convincing anamnesis and work history together with positive allergy tests suggest a likely allergic OR.

The third step consists of an objective evaluation of the cause–effect association of the rhinitis with the workplace by performing the NPT. Positive NPT confirms the diagnosis of allergic OR. In case of a negative NPT result in a patient with anamnesis data highly susceptible to allergic OR, the relationship of the disease with the workplace is determined by evaluating the symptoms, nasal flow, and/or measuring inflammatory markers during periods of exposure and elimination to occupational allergens.

**Key Notes**
Diagnostic procedures for evaluation of cause–effect association of allergic rhinitis with specific occupational exposure:

- Work history and workplace assessment
- Allergological tests
- Nasal provocation test
- Tests of exposure and elimination
  (not as a routine diagnostics)

The diagnosis of the nonallergic OR is based on proving the temporal association between exposure to unusually high levels of workplace irritants and the occurrence of nasal symptoms and/or other signs of the disease, meanwhile excluding the allergic OR.

**Differential Diagnosis** The differential diagnosis of OR consists in the exclusion of other forms of chronic rhinitis (vasomotor, hormonal, infectious, medicamentous, etc.).

## 8.9 Treatment

The primary goal of the OR treatment is elimination or minimization of symptoms and their impact on the quality of patient's life, and prevention of OA development, and it should be performed with the cooperation of a specialist in occupational medicine, an ENT specialist, and a pulmoalergologist. The treatment consists of measures for occupational exposure control, pharmacotherapy, and immunotherapy [18].

**Control of specific occupational exposure** is an essential measure in the treatment of allergic OR [19, 20].

Considering the disease nature, the optimal measure is termination of the specific occupational exposure, that is, change of the job position of a subject with OR with some other workplace where he/she will not be exposed to the occupational allergen that is considered to be a cause for allergic OR. On the other hand, due to the high frequency of the disease, as well as due to the significant socioeconomic implications that most often are caused by changing the workplace, in numerous workers with allergic OR alternative of exposure termination is staying at the same job position but with reduction in the degree of occupational exposure. Exposure termination is recommended for subjects who are at high risk of developing allergic OA (associated nonspecific bronchial hyperresponsiveness, severe persistent allergic OR, and/or long-term exposure duration) and for people working at workplaces where the occupational exposure degree cannot be reduced.

Reducing the degree of occupational exposure is a reasonable alternative to exposure termination in cases where it can be reduced by applying technical and technological preventive measures for collective health protection (automation, hermetization, improvement of general and local ventilation, changes in the technological process etc.) and technical measures for personal health protection (application of respirators with dust filter or gas masks for protection against harmful gases, fumes, vapors, and fogs).

The results of a few studies show that in most of the patients there is no withdrawal of nasal symptoms, neither in cases of exposure termination, nor in cases of its degree reduction. It is not known at this time whether allergic OR can lead to persistent damage in the nasal function.

**Pharmacotherapy** of the allergic OR, according to current recommendations based on evidence-based medicine, consists of the use of nonsedating antihistamines, intranasal corticosteroids, and leukotriene antagonists. The effectiveness of anti-IgE antibodies in the treatment of allergic OR is still unknown. Pharmacotherapy is not an alternative to preventive measures for control of specific occupational exposure.

**Immunotherapy** in the treatment of allergic OR has limited use due to the non-existence of standardized allergenic extracts of most occupational allergens. According to research results, the application of specific immunotherapy was effective in treating allergic OR among bakers sensitized to wheat allergens and in laboratory workers sensitized to allergens from the laboratory rats' urine and latex allergens [21].

**Key Notes**
Treatment of allergic occupational rhinitis:

- Termination of specific occupational exposure (in cases with a high risk for OA development) or reduction of the exposure degree
- Pharmacological treatment
- Immunotherapy

## 8.10 Prevention

Prevention of OR consists of measures for primary, secondary, and tertiary prevention.

**Primary prevention** consists of measures for specific occupational exposure control.

Thus, the research results indicate a significant reduction in the incidence of allergic OR in healthcare workers by replacing latex gloves with latex-free gloves, as well as workers in the production of detergents with a reduction in the occupational exposure level. As with the OA, the detection of atopic subjects by preemployment medical screening at high-risk job positions is not recommended having in mind its high prevalence in the general population [22].

**Secondary prevention** consists in the implementation of measures for early detection of OR and early intervention in its course, and is carried out with the regular periodical preventive medical examinations of workers. Evaluation of nasal symptoms within the periodical medical examinations is performed by a questionnaire, and additional allergological and functional examinations in the subjects suspected of OR. Regular periodical medical examinations in the first five years of employment at a certain workplace is of particular importance for early detection of OR, since in most cases sensitization to occupational allergens and the onset of symptoms occur mainly within this time frame.

**Tertiary prevention** involves measures and procedures for treatment and rehabilitation of clinically manifested OR. The possibility of OA development should be carefully evaluated in each patient with OR [22].

## 8.11 Conclusion

Although OR is considered to be the most common occupational respiratory disease in the last few decades worldwide (with estimated frequency 2–4 times higher than the frequency of OA), this clinical entity remains insufficiently investigated and known in terms of its epidemiology, pathogenesis, diagnosis, treatment, and prevention. The definition of OR still does not have a generally accepted consensus, and its classification is based on the current OA classification, as common accompanying disease. Similar to OA, OR according to the mechanism of its occurrence may be allergic and nonallergic, but data about the frequency and pathogenetic mechanisms of its different types are still insufficient. Improvement in occupational exposure the control, standardizing the existing diagnostic methods and their wider implementation in everyday work, introducing new diagnostic methods with higher sensitivity and specificity, as well as improving all elements of the disease management are the most important challenges associated with OR in the upcoming period.

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# Chapter 9
# Occupational Skin Diseases


**Dragan Mijakoski**

**Abstract** Occupational skin diseases (OSD) represent 10–40% of all registered occupational diseases in most European countries, mostly comprising contact dermatitis, contact urticaria, and skin cancer as the most important OSD. Occupational contact dermatitis (OCD) is one of the most important occupational diseases, in general, and it is frequently present in dermatology practice. This clinical entity can be manifested as an effect of irritant or allergic reaction to certain hazards that are present at the workplace. The identification of concrete etiological occupational factor could be performed by skin tests with specific workplace allergens. The cessation of occupational exposure is the first and the most important step in the management of OCD. Occupational skin cancer (OSC) (basal cell carcinoma, squamous cell carcinoma, malignant melanoma) is a frequent malignant neoplasm and the most important etiological factor is occupational exposure to ultraviolet (UV) radiation, along with arsenic, polycyclic aromatic hydrocarbons, ionizing radiation, and trauma. The prevention of OSC should involve specific technical/organizational measures, personal protective measures, preventive medical examinations of workers, and educational activities.

**Keywords** Occupational skin diseases · Contact dermatitis · Contact urticaria · Skin cancer · Workplace hazards · Irritant or allergic skin reaction · Skin (patch) tests · UV radiation · Prevention · Occupational health

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## 9.1 Introduction

The skin is our communication with the environment and it plays a key role as a barrier to hazards, both environmental and occupational, entering the human organism. Many job activities are related to specific workplace exposures and numerous occupational hazards can lead to disruption of the skin barrier, with subsequent development of occupational dermatoses. But, it is noteworthy that several individual (genetic) factors could influence the outcome.

Exposure at the workplace is responsible for a wide range of skin problems [1]. Occupational skin diseases (OSD) are among the top three registered occupational diseases (OD) in Europe [2]. Occupational contact dermatitis (OCD), occupational contact urticaria, and occupational skin cancer (OSC) are the most important occupational dermatoses. Other OSD involve skin infections, hair follicle disorders, pigmentation disorders (post-inflammatory hyperpigmentation and acquired leukoderma), and other skin diseases, such as: scleroderma, Raynaud phenomenon, and telangiectasias.

***Occupational skin infections*** could be clinically manifested as a consequence of workplace exposure to bacteria (Erysipelothrix in slaughterhouse workers, butchers, fishermen, aquarium workers, farmers, and veterinarians), fungi (Sporotrichosis in gardeners, florists, peasants, housewives, hunters, miners, fishermen, and veterinarians), viruses (warts in butchers, milker's nodules or nodus mulgentium in milkers and other animal handlers caused by the Paravaccinia virus; orf or ecthyma contagiosum in shepherds, goatherds, veterinarians caused by a Parapox virus) or parasites (Cheyletiellosis in veterinarians or laboratory personnel).

***Occupational hair follicle disorders*** could have a clinical presentation of folliculitis (caused by motor oil in mechanics) and chloracne (induced by tar derivatives and halogen-containing compounds). Occupational acne is represented by comedos, papules, and pustules caused mostly by industrial oils and greases. Car mechanics and maintenance workers are at the highest risk for developing these forms of occupational dermatoses.

## 9.2 Occupational Contact Dermatitis

**Definition** *Contact dermatitis* is an inflammatory skin reaction caused by direct contact with noxious agents in the environment [3]. It is caused by external factors, particularly substances, interacting with the skin [4].

*Occupational contact dermatitis* is defined as "local inflammatory process of the skin caused as a direct effect of specific occupational agents" [5]. OCD is a skin condition caused by work-related exposures [4]. OCD is an exogenous eczema caused by the interaction of the skin with chemical, biological, or physical agents found in the work environment [6].

**Classification of OCD** The same classification of contact dermatitis should be used for both occupational and nonoccupational type of the disease as recommended by relevant sources (American Academy of Dermatology [7, 8]; European Society of Contact Dermatitis [9]).

Contact dermatitis is divided into two broad categories based on different pathogenic mechanisms: irritant and allergic contact dermatitis. Furthermore, unknown and/or contributive etiologic and pathogenic factors lead to additional clinical forms of OCD and therefore it is classified as:

1. Occupational allergic contact dermatitis
2. Occupational irritant contact dermatitis (acute and chronic form)
3. Photoallergic and phototoxic contact dermatitis
4. Contact urticaria

**Epidemiology** OSD represent 10–40% of all registered OD in most European countries, mostly comprising contact dermatitis, contact urticaria, and skin cancer [10, 11]. Together with musculoskeletal diseases, neurologic diseases, lung diseases, and diseases of the sensory organs, OSD are found to be amongst the most frequently notified OD in Europe [12, 13].

Contact dermatitis is frequent clinical entity and it is present in 2–4% of adult population [14]. Contact dermatitis accounts for about 90% of all occupational dermatoses and in 80% of the cases, it will impair a worker's most important tool, the hands [6]. About 40% of all dermatitis cases are caused by occupational agents, and OCD is a predominant clinical entity among all OD (20–70%). Irritant contact dermatitis (ICD) represents the most common type of contact dermatitis and accounts for 80–95% of all cases, and occupational allergic contact dermatitis represents 5–20%, depending on the data source, workplace characteristics and specificities, and workplace agents [1, 4, 6, 14]. The average incidence rate of registered OCD is about 0.5–1.9 cases per 1000 full-time workers per year, with a significant social and economic impact [10, 11].

The real prevalence of OCD is usually not clear because workers with minor skin changes do not visit, at all, the family physician, dermatologist, or allergist, nor report the skin problem to the responsible occupational medicine specialist. Additionally, some of the workers with more severe clinical features are initially treated, some of them are, unfortunately, mistreated by primary care physicians, and only a part of them are referred to dermatologist and/or allergist. Therefore, it is of crucial importance for the worker and his clinical condition, to raise awareness about prevention, diagnosis, and treatment of OCD and other occupational and work-related skin diseases in physicians who take care of these patients (occupational medicine specialists, primary care physicians, dermatologists, and allergists) [1].

It is evident that national registries of all OD, including OSD, are often incomplete due to high under-diagnosing and under-reporting [11]. Additionally, the assessment of OSD in European countries is not homogeneous, mainly because of differences between the health systems across countries [13]. It should be noted that OSD patients do not differ with regard to their disease across Europe. Hence, they

should be treated and assessed in the same way, based on scientific evidence-based criteria. However, only few countries in Europe have established recommendations for the diagnosis and management of OSD [15].

On the other hand, the definitions of work-related skin disease (WRSD) and OSD are different in different countries. Mainly, it is established that WRSD are caused or worsened by an occupational activity, while OSD need to fulfill additional legal criteria, which differ from country to country [13]. The definition of occupational dermatoses given by the American Medical Association is well known: "all dermatologic conditions where it can be demonstrated that the work is its fundamental cause or a contributing factor to it" [16, 17]. In 1983, at the Xth Ibero-Latin American Congress of Dermatology, occupational dermatoses were defined as "any affection of the skin, mucous or skin adnexa directly or indirectly caused, conditioned, maintained or worsened by anything that is used in professional activity or exists in the work environment" [11].

As a conclusion, there is no official, best reliable, and applicable international definition. The definition of OD and, specifically, the definition of OSD are much more complex because they have to include medical and legal criteria, as well as political aspects of the issue [11].

However, the terms "work-related disease" and "occupational disease" are differently defined. "Work-related diseases" are defined as diseases that have multiple causes, and one of that causes is certain factor from the working environment. Work-related diseases have multiple causes, where factors in the working environment may play a role, together with other risk factors, in the development of such diseases [18, 19]. Work-related factors could influence the course, the outcome, and complications of the disease. Work-related diseases include diseases with solid scientific evidence concerning a possible occupational origin which may, however, not fulfill all given criteria for recognition of an OD according to the official list of ODs. On the contrary, OD could be defined from medical or legal aspect. From medical aspect, they are defined as diseases that are contracted primarily as a result of an exposure to risk factors arising from work activity [18–20]. From a legal aspect, according to the actual Law on Pension and Invalid Insurance, OD are defined as diseases caused by long-term direct influence of work process and work conditions on the work ability of the insured person [20].

Most European countries have an ILO/EU recommendation-based List of ODs [11, 20, 21]. Only a few of them have an "open" List of ODs. All OD lists depend on the national legal system and on how the OD recognition process is formally implemented in the given country. Hence, European countries have different criteria to recognize and compensate ODs.

### *9.2.1 Etiology*

***Irritant contact dermatitis* is the most frequent OSD, accounting almost 80% of all OCD cases. Direct cytotoxic effect of the suspect agent (irritant) on the cells of the epidermis and dermis causes the clinical manifestation of ICD. Irritating**

**hazards are mainly chemical agents, but they also may be represented by mineral or vegetal particles that abrade or get imbedded in the skin [1, 6].**

Irritant substances are able to damage the natural barrier function of the skin. Occupational irritants are represented by strongly acidic or alkaline substances (e.g., wet cement and other corrosive materials) and chemicals like oils, detergents, shampoos, cleaning agents, dust, and fiberglass. It is important to notice that corrosive substances begin to cause damage as soon as they contact not only the skin, but also eyes, respiratory tract, or gastrointestinal system. Additionally, physical exposures, such as drying the skin with paper towels and exposure to heat and friction can also irritate the skin [4, 22–24].

The irritants, according to the mechanism of action, are divided into two groups: *immediate (corrosive) irritants* (substances which generate corrosion or chemical burns within minutes to hours of a single exposure), and *cumulative irritants* (substances with weaker effects that are manifested after repeated application). There are individual differences between workers in the clinical manifestation of irritation. But, in conditions of sufficient exposure to the agent and high enough concentration of the irritant, every person is prone to the development of ICD. Destruction of epidermal and dermal cells, changes in epidermal barrier, transepidermal water loss, and inflammation secondary to non-immunologic release of vasoactive peptides and pro-inflammatory cytokines result in visible skin changes. The main complaints encompass pain, burning sensation, and itching [1, 6].

***Allergic contact dermatitis (ACD)*** caused by a cell-mediated immune reaction, represents 20% of all OCD cases. It is manifested as a result of an action of chemical or biological agents that are otherwise not effective in a majority of individuals. Etiological agents—contact sensitizers are widely present everywhere around us. They are most frequently small molecules that are lipophilic. Many occupations are characterized by exposure of workers to substances (allergens or sensitizers) that have the potential to cause an allergic reaction [1, 4]. There are specific legislative acts that regulate the concentration of sensitizers in products. For example, Australian Approved Criteria for Classifying Hazardous Substances, if a sensitizer is present in the product in a concentration equal to or more than 1%, that sensitizer must be listed on a material safety data sheet (MSDS) and identified with the designated risk phrases, R43 "May cause sensitization by skin contact" [4, 25]. However, in particular cases, concentrations of sensitizer less than 1% could provoke ACD in sensitized worker.

These contact sensitizers could interact with antigen-presenting cells (APCs) in the epidermis (Langerhans cells) and dermis (dermal dendritic cells) after penetrating stratum corneum. Since they are haptens (incomplete antigens), contact allergens have to be processed by APCs (after their internalization in APCs). After that, haptens are bound to proteins of the major histocompatibility complex (MHC). The process of reexpression results in the expression of complete antigens at the APCs surface. Then, changed APCs migrate to local lymph nodes and present the new allergens (haptens + MHC proteins) to naive T cells. T lymphocytes are then proliferated (clonal proliferation) and differentiated into cluster of differentiation (CD) 4 and CD8 effector, suppressor, and memory T cells [1, 14].

Contact sensitizers are, firstly, represented by *general allergens*, such as nickel and chromium compounds, aniline dyes, topic medications, perfumes, and other

substances. General population could be exposed to these aforementioned general sensitizers. Additionally, the contact allergy could be caused by substances which are characteristic for specific workplace and process of work. These workplace allergens are defined as *occupational contact sensitizers*. The group of occupational contact sensitizers includes about 3000 agents and their number continuously increases due to new technologies of work. Occupational contact allergens are mainly substances with low molecular weight and small molecule, i.e., haptens that easily penetrate through skin. Both inorganic (metals and their compounds) and organic (natural and synthetic) substances could act as occupational contact sensitizers [14].

### *9.2.2 Pathogenesis*

The development of OCD depends on several factors, which are mainly related to the normal structural and functional integrity of the skin. The skin enables normal values of vital chemicals and nutrients in the body and it provides both a barrier against dangerous substances from entering the body and a shield from the harmful effects of UV radiation emitted by the sun.

The protective function of the skin is based on the protective skin surface biofilm, skin pH, secretions from the sweat and sebaceous glands, as well as the outermost portion of the epidermis (stratum corneum), which is relatively waterproof and, when undamaged, prevents most bacteria, viruses, and other foreign substances from entering the body. Additional protection is provided by melanin that is produced by melanocytes. The primary function of melanin is to filter out UV radiation from sunlight. Langerhans cells from the epidermis help to detect foreign substances and defend the body against infections. However, protective function of the skin is complemented by the dermis that is a thick layer of fibrous and elastic tissue (made mostly of collagen, with a small but important component of elastin) that gives the skin its flexibility and strength. The sweat glands produce sweat, composed of water, salt, and other chemicals, and it helps to cool the body. On the other hand, the sebaceous glands secrete sebum, oil that keeps the skin moist and soft and acts as a barrier against foreign substances. Most deeply in the skin, there lies a layer of fat that helps insulate the body from heat and cold, provides protective padding, and serves as an energy storage area [14, 26].

Taking into account the wide specter of different protective possibilities of the skin, the manifestation of noxious effects of workplace hazards on the skin, is dependent of several conditions. First of all, the protective function of the skin has to be impaired. Different inflammatory processes, changes in skin structure and function, such as injuries, macerations, and hyperemia, oxidative stress mechanisms, as well as altered immunological reactivity of the worker have an important role in the development of clinically manifested skin diseases [14]. The development of occupational dermatitis could follow one of the two basic mechanisms: nonallergic or allergic.

1. *Nonallergic mechanism* is represented by different types of reactions, such as irritant, mechanic, toxic, or actinic. Irritant reaction develops as an effect of more severe inflammation. This inflammation is produced by the pro-inflammatory cytokines, released by skin cells (mainly by keratinocytes), most frequently due to chemical stimuli. The activation of innate immunity without previous sensitization leads to irritant reaction, which differentiates this type of contact dermatitis from the allergic one. The irritant reaction causes three main pathological features: disruption of the skin barrier, changes in epidermal cells, and release of pro-inflammatory cytokines.

Taking into consideration the aforementioned processes in the skin, the solvents could cause skin irritation because they act as removers of the essential lipids from the skin. The loss of lipids leads to increased transepidermal water loss, and the skin becomes more sensitive to direct effects of other workplace hazards.

On the other hand, microtraumatic injuries could also lead to an irritant skin reaction (e.g., fiberglass leading to skin microtraumatic injuries and itching). Physical irritants (e.g., friction, abrasion, and occlusion) and detergents can cause more severe ICD in the context of simultaneous action comparing to their individual action.

The pathogenesis of nonallergic contact dermatitis involves different cells, including epidermal cells, fibroblasts, endothelial cells, and different types of leucocytes. It is important to notice that their interaction is controlled by many different cytokines and lipid mediators. However, the keratinocytes have the most important role in the initiation and amplification of inflammatory skin reactions through release of cytokines and consecutive response to these substances. Epidermal keratinocytes could release the following cytokines: inflammatory cytokines, such as interleukin (IL) 1, and tumor necrosis factor alpha (TNF-alpha), chemotactic cytokines (IL-8, IL-10), growth stimulating cytokines (IL-6, IL-7, IL-15, granulocyte-macrophage colony-stimulating factor (GM-CSF), transforming growth factor alpha (TGF-alpha)), as well as cytokines that regulate the humoral immunity (IL-10, IL-12, IL-18). Intracellular adhesive molecule 1 has an additional effect in stimulating the infiltration of epidermis with leucocytes during skin inflammatory reactions.

Workers with positive history of atopic dermatitis have susceptibility to develop nonallergic hand contact dermatitis. The polymorphism of the filaggrin gene (FLG), leading to decreased filaggrin production, could affect the skin barrier and that condition is a predisposing factor for the development of atopic dermatitis. Null alleles of FLG are associated with an increased susceptibility to chronic ICD [14, 27–30].

2. *Allergic reaction* involves the sensitizing mechanism in the action of different workplace agents. The most frequent pathogenic process within this type of skin reaction is contact allergy. Contact allergy is initiated by contact sensitizing substances (complete allergens or haptens) and it is realized by T-lymphocytes and specific epidermal cells—Langerhans cells. Langerhans cells represent a population of tissue-resident macrophages that form a network of cells across the epidermis of the skin, but which have the ability to migrate from the epidermis to draining lymph nodes [31].

Langerhans cells transport the antigens to regional lymph nodes where they present to naive T cells, make an interaction with CD4+ T helper lymphocytes, and initiate adaptive skin immune responses [32]. Then a cascade of actions starts and it results in a delayed hypersensitivity reaction accompanied by inflammatory process. The naïve T cells that have recognized an antigen start to proliferate and differentiate into antigen-specific effector and memory T cells that circulate in the blood and lymphatic system.

Sensitizing substances have to be lipophilic and to have low molecular weight (<1000D) in order to effectively penetrate the stratum corneum barrier, which is the outermost layer of the epidermis, and it is water impermeable. In skin (contact) allergy, the most prevalent form of immunotoxicity in humans, low molecular weight organic substances that are chemically reactive (chemical sensitizers) bind to skin proteins, and then immunogenic neoantigens are produced (process known as haptenization). The hapten–protein conjugates are most often formed by reactions between electrophilic haptens and nucleophilic side chains such as cysteines (thiols) and lysines (primary amines) in skin proteins [33].

However, not all sensitizing chemicals are directly reactive, but require previous activation. Prehaptens are chemical agents that are not activated by the proteins of the skin and require chemical transformation by oxidative derivatization by ambient or air oxidation to form hydroperoxide (e.g., fragrance materials and dyes used in hair coloring, such as para-phenylenediamine) [34]. On the other hand, a prohapten is a chemical which by itself is non- or low-sensitizing but that is transformed into a hapten in the skin (bioactivation) usually via enzyme catalysis. It is not always possible to know whether a particular allergen that is not directly reactive acts as a prehapten or as a prohapten, or both, because air oxidation and bioactivation can often give the same product [35].

Haptens could also activate Toll-like receptors (TLR) and innate immunity. TLRs are single, transmembrane proteins (receptors) that have a significant role in the innate immune system. They are present on macrophages and dendritic cells in order to recognize diverse pathogen-associated molecules from bacteria, fungi, parasites, and viruses. After recognition by TLRs, the immune cell responses are activated. TLRs could initiate both innate and adaptive immune responses aimed to fight infection. The innate immunity provides immediate protection that is relatively nonspecific, but potentially damaging to healthy tissue when long lasting, while the adaptive immunity gives specific antibody-secreting B cells and cytotoxic T cells [36].

The recognition of haptens or haptenated proteins results in releasing of different pro-inflammatory mediators leading to activation of dendritic cells (Langerhans cells within the epidermis). The role of the innate immunity in the development of contact dermatitis is clear when taking into consideration that some chemical substances could act as both irritants (causing skin inflammation after primary, single exposure) and contact sensitizers. The inflammation that is induced by haptens or haptenated proteins leads to accumulation of T cells at the site of the skin contact with allergen. Pro-inflammatory cytokines released by T cells are causing cell damage and cell death that is manifested by the signs and symptoms of contact dermatitis [37].

During occupational exposure to specific workplace allergens, allergic pathogenic mechanisms could develop either *immediate (type I) hypersensitivity reaction with a humoral immune response by Immunoglobulin E (IgE) antibodies and involvement of eosinophils and basophils* or *delayed (type IV) hypersensitivity reaction with cellular response by involvement of sensitized T lymphocytes and their mediators—lymphokines*. The cytokines have an important role because they regulate auxiliary adhesion molecules as well as intracellular adhesion molecule 1. It is supposed that IL-8 is the most important cytokine in skin contact allergy. Additionally, CD4+ chemokine receptor (CCR) 10+ memory T cells remain present in the tissue even after the cessation of occupational exposure and after complete remission of the clinical manifestation.

The existing skin diseases, skin damages, certain drugs, as well exposure to UV radiation could induce and boost contact allergy. The development of clinical manifestation of occupational contact allergy is strictly dependent on primary contact with allergen and subsequent development of sensitization.

Many workers with OCD to nickel have damaged form of filaggrin gene. Filaggrin helps aggregation of cytoskeletal proteins and when it is absent, the skin barrier is impaired.

### *9.2.3 Clinical Presentation*

In 90% of the cases, OCD is clinically manifested as eczema. Acute forms are presented by erythematous, edematous, and urticarial changes that are pruritic. Vesicles and bullae are not unusual manifestations. Subacute forms also demonstrate erythema and edema, but erosions, crusting, and desquamation replace vesicles and bullae. Finally, chronic cases are defined by dry and rough skin, fissure, pigmentation, and increased thickness (lichenification) [1].

1. ***ACD*** that is characterized by a delayed type immune response (type IV hypersensitivity reaction) and caused by a contact sensitization could be manifested by a variety of clinical signs and symptoms. Inflammatory reaction with erythematic skin changes, papules, and vesicles, associated with erosions, edema, and itching is a predominant type of ACD clinical feature. Lichenified pruritic plaques may indicate a chronic form of the condition. Recurrent ACD episodes lead to classical clinical presentation of eczema with thickened, rough, and dry skin that is locally pigmented with presented excoriations and lichenifications. The inflammatory process continues with subsequent development of crusts, fissures, skin cracking, and secondary infections. It is important to notice that skin changes have an unclear border from the surrounding healthy skin and they are most frequently localized to the area of contact (at the exposed body parts), but ACD skin changes also have a propensity to spread to more distant sites [14, 38].

*The primary location of skin changes and the type of contact sensitizer* could influence the specifics of clinical presentation in occupational settings. The hands are the most frequently affected body parts in occupational ACD, while the skin changes

of forearms, upper arms, lower legs, calves, and feet depend upon the usage of personal protective equipment, the type of contact sensitizers as well as their potency to penetrate the clothes or to get on the skin and to express their action. The face, which is mainly changed in form of swelling of the eyelids, could be affected through direct contact with contact sensitizers or by dirty hands. However, sometimes the primary location denotes the occupation of the worker (for example, skin changes of the ears caused by the contact with headphones in telephone operators, or due to occupational contact with certain materials, such as formaldehyde, rubber, and plastics). Different contact sensitizers that are present at the workplace could also, very frequently, act in the context of nonoccupational exposure (for example, nickel or chromium), while others are dominantly related to occupational exposure (for example, epoxy resins). These considerations have to be taken into account during the verification of the occupational etiology of ACD. The aforementioned skin lesions could be manifested either after several weeks of exposure to sensitizing substance or, more frequently, after repetitive contacts with workplace hazard during several decades of work and they are not manifested in all exposed individuals [14, 39].

*Over 3000 contact sensitizing substances are listed as possible causes of this type of OCD and practically there is no workplace without the presence of some of them.* Certain job sectors are related to specific sensitizing substances and the occurrence of OCD is characteristic for the workers employed in these sectors. In the agriculture, the pesticides are found; in metal and metal-processing industry—chromium, nickel, and cobalt compounds, or lubricating oils; in the construction industry—chromium compounds and organic solvents; in the plastics industry—acrylates, phthalates, and formaldehyde; in the chemical industry—a wide spectrum of chemical substances; in the wood industry—resins, dyes, adhesives, varnishes, and organic solvents; in the textile and leather industry—dyes and chromium compounds; in the healthcare sector—antibiotics, disinfectants, antiseptics, and latex (the main integral part of gloves) [14].

2. ***ICD*** is represented by an inflammatory process and subsequent irritant reaction that damages the protective skin layer and skin barrier. In ICD the skin is affected by physical factors (e.g., friction), environmental factors (e.g., cold), overexposure to water, or chemical hazards (e.g., acids, alkalis, detergents, and solvents). Irritants remove oils and moisture from the outer layer of the skin. Therefore, chemicals could penetrate deeply in the skin tissue, causing further inflammation. There are two clinical forms of ICD: acute and chronic [14, 40].

– The ***acute ICD*** is usually manifested after short-term, intensive skin exposure to strong chemical irritants, such as: strong acids and alkalis, concentrated solutions of sodium hypochlorite, isothiazolinone, fungicide chlorothalonil, aliphatic amine epoxy catalysts, detergents, or shampoos. Strong irritants can also cause immediate burns after contact with skin. On the other hand, repetitive contacts with irritants could cause dry and reddish skin, but tissue necrosis is not excluded. The evolution of skin changes into fissures and skin cracking is frequently seen and hands are the typical primary localization of these features. The most frequently affected workers are the construction ones that are exposed to lime and cement, hairdressers in contact with shampoos and hair dyes, as well as healthcare workers due to exposure to

soaps and disinfectants. After the cessation of contact, skin changes are quickly withdrawn [1, 14].

– The ***chronic form of ICD*** is manifested as a result of chronic skin irritation due to long-term and repeated exposure to chemical irritants. Clinical features of chronic ICD are similar to the skin changes that are present in allergic contact dermatitis, but former ones have certain specifics. They are strictly limited to the place of contact and have clear border from the normal unaffected skin. Additionally, they are manifested in every exposed person; after a cessation of the contact they persist for a certain amount of time and they are withdrawn only after longer period of interrupted exposure. Skin changes are mainly present on the hands, fingers, and between fingers [14].

This type of OCD could affect workers from every industrial sector and every profession. It is well established that the same substance could act as both irritating and sensitizing agent. Pesticides in agriculture; different chemicals in chemical industry; lime and cement in construction industry; acids, alkalis, oils, and emulsions in mechanical industry; and medications and disinfectants in healthcare represent some examples of chemical acting as both irritants and sensitizers. In addition to chemicals, sand particles, sawdust, metal filings, or plastic may cause mechanical irritation on the exposed skin. Some plants, such as philodendrons and daffodils contain high levels of oxalic acid that causes dermatitis in gardeners [1, 14].

3. ***Photoallergic and phototoxic contact dermatitis***

– *Photoallergic contact dermatitis* is a distinct form of contact dermatitis that occurs as a result of photosensitization, when a photosensitizing substance causes a delayed-type hypersensitivity cutaneous reaction only after being exposed to the solar UV radiation. Clinical presentation is similar to that of ACD with a polymorphism of changes of the skin that is exposed to solar radiation. Different chemicals are defined as photosensitizing substances, such as: some medications (e.g., sulfonamides); quinine that is found in compounds for hair dying or it is used as preservative in refreshing drinks; dimethylthiourea found in photocopy paper, and other chemicals. The increased workplace risk is detected in occupations where there is present skin exposure to some of the photosensitizing substances, accompanied by exposure to the sun.

– *Phototoxic contact dermatitis* is referred as a phototoxic reaction that may occur when certain chemicals are applied to the skin and subsequently exposed to the solar or UV radiation. This type of contact dermatitis develops due to the interaction between UV radiation and certain substances. The substances involved, known as phototoxic substances, poses photodynamic effect and they intensify the skin reaction to solar radiation. Direct tissue damage caused by light activation of the phototoxic substance is responsible for the development of phototoxic contact dermatitis, while photoallergic reactions are caused by cell-mediated immune response (the antigen is the light-activated photosensitizing substance). In phototoxic dermatitis, clinical presentation is similar to that of ICD and the signs and symptoms include: redness of the skin and its pigmentation, as well as edema, vesicles, and bullae. The described skin changes could be manifested in every exposed person, even after the first contact. The prerequisite for the skin reaction is that the phototoxic substance has to be present in high enough concentration and it has to act

long enough period of time. Some drugs (e.g., tetracyclines and barbiturates), tar and its derivatives, certain dyes (eosin and acridine), some preservatives in textile and wood industry have a potential to act as phototoxic substances. The increased workplace risk is detected in occupations where there is present skin contact and skin absorption to some of the phototoxic substances, accompanied by exposure to the sun [14, 39, 40].

4. ***Contact urticaria*** has to be analyzed as a distinct clinical entity. It is a transient and localized skin redness and swelling that develops immediately after the contact with certain substance. Contact urticaria has to be distinguished from OCD since the skin reaction is in the form of urticas that are developed after exposure to different agents at the workplace. The pathogenesis of the disease could include: allergic mechanism (through I type hypersensitivity reaction), nonallergic mechanism (through direct release of vasoactive substances, such as histamine), or by unidentified mechanism. Within the allergic mechanism, the contact with a sensitizing substance causes the clinical manifestation. As an example, we can mention the contacts with organic solvents, many medications (most commonly antibiotics), natural rubber latex, some metals (e.g., nickel), parabens, short chain alcohols, raw meat, fish, and vegetables, serum, and saliva. Nonallergic mechanism mainly occurs due to contact with nicotinic acid, cobalt, balsam of Peru, benzoic acid, sorbic acid used as a preservative in many foods. Unknown (unidentified) mechanism is manifested during occupational exposure to ammonium persulfate (hair blanching agent), and potassium ferricyanide (photographic chemical). The aforementioned substances indicate many different workplaces and occupations with an increased risk for the development of occupational contact urticaria (workers in pharmaceutical industry, health care, hairdressers, as well as photographers) [14, 39].

### *9.2.4 Diagnosis and Differential Diagnosis*

Contact dermatitis, contact urticaria, and skin cancer are the most common WRSD and OSD and, therefore, it is necessary to have a correct etiological diagnosis in order to successfully treat and prevent skin condition. The data obtained through careful personal, medical, and specific work history, a thorough physical examination, as well as workplace risk (exposure) assessment are the prerequisites in determining the occupational character of the disease. Especially important are the data concerning the occurrence of skin changes and determined correlation of occupational exposure with localization of skin lesions and their evolution is mandatory. The exclusion of nonoccupational exposure (at home, during hobbies, medication intake, personal habits, and use, outside the workplace, of cosmetics, protective moisturizers, and topical medicaments) has a significant role in the diagnosis of OCD. Additionally, the data about the time of occurrence of skin lesions, the length of the occupational exposure, the previous condition of the skin, as well as information about the evolution of lesions are also needed during the diagnostic process. Particular attention has to be put on the usage of preventive measures (especially personal protective equipment, their type, and character). Diagnostic procedure has

to be upgraded with information on workplace eco-technological process, potential contact sensitizers at the workplace, and objective data about chemicals with relevant work-related epidermal and dermal contact. Workplace visits are recommended in order to identify relevant exposure of workers and perform a complete assessment since in more than 80% of cases with occupational ACD workplace exposure assessment has contributed to a correct diagnosis [1, 11, 14].

***Clinical presentation*** with the occurrence of characteristic skin changes and their specific location are recognized as key elements in the diagnostics of OCD. During physical examination, it is important to note the severity and distribution of lesions and their interference with worker's functioning. Entire skin has to be examined, not only the sites presented by the patient, because distant parts of the skin may be affected by a primary OD or other nonoccupational skin diseases, such as atopic dermatitis or psoriasis. Photographs taken by the patients or their physicians could be useful in diagnostics and follow-up of the evolution of the disease. It is also important to note the effects of various treatments, holidays, and periods of sick leave [1, 11, 14].

***The exposure-elimination test*** provides dynamic follow-up of the skin changes that are related to occupational exposure. In clear occupational etiology of the skin disease, the cessation of workplace exposure will result in the improvement of clinical features with a possible recovery of skin lesions. During the exposure-elimination test, it is recommended to stop occupational exposure for 2 weeks during which the patient has to be out of the workplace for those 2 weeks (period of elimination). After that, the patient returns at the workplace for additional 2 weeks (period of exposure). During the period of exposure, the clinical manifestation will be worsened and intensified and the possibilities for the recovery of skin lesions will be minimized [14].

#### 9.2.4.1 Skin Tests

Tests for the evaluation of skin reactivity—application of standard and specific occupational allergens by skin (prick) test for the assessment of type I (immediate) hypersensitivity reaction and by epicutaneous (patch) test for the determination of the presence of contact allergy—type IV (delayed) hypersensitivity reaction.

Allergy evaluation in OCD has to include epicutaneous (patch) testing according to the European Society of Contact Dermatitis (ESCD) guidelines [3]. Patch testing is indicated in all cases with work-related relapsing or persisting (>3 months) contact dermatitis [11]. The usage of epicutaneous (patch) tests has high cost-effective value and enables etiological diagnosis of the determined contact dermatitis.

The patch test is performed by applying allergens under occlusion on the skin under standardized conditions [3]. The most frequently used is the TRUE test. During the test, a standard series of the most common contact allergens is applied and it determines the relevant etiological causes of the allergy in more than a half of the patients. In occupational allergy, besides the standard battery of tests and allergens, additional occupational allergens are used in accordance with the workplace exposure in concrete patient. But, the TRUE test does not contain some allergens

that could be frequent causes of ACD (e.g., acrylates, pesticides, chemicals used in the production of rubber, plastics, dyes, or photographic materials). For the purposes of testing allergy to these substances, Finn chambers, allergEAZE chambers or IQ Chamber patch test could be used. During the photopatch test, in order to diagnose photoallergic CD, the substances are applied in duplicate sets—the first one with 5 J/cm$^2$ UV-A radiation, and the other one without UV radiation [3, 14].

When the working diagnosis is likely to be an ACD, it has to be taken into consideration that for the aims of more precise definition of contact allergy, particularly in the cases of suspect occupational contact allergy, it is always important to use additional testing substances. The selection of specific contact sensitizers will be based upon the characteristics of the actual workplace, profile and occupation of the worker, as well as known specific risk substances in the context of occupational exposure. The usage and interpretation (including false-positive and false-negative reactions) of epicutaneous tests with occupational contact allergens are identical with the principles of work with general contact allergens (according to the ESCD guidelines) [3, 14]. It is noteworthy that the assessment of patch tests has to be performed not only after 48 h, but it has to be repeated after 72 h and even after 1 week following the application of the suspected etiological agent. The positive epicutaneous test with specific occupational contact allergens confirms the occupational contact sensitization and enables identification of the potential etiological factor for the development of occupational ACD [14].

The clinical relevance of positive patch tests is determined in the light of previous and present workplace exposures. Positive reactions without actual clinical manifestation are important in terms of detecting former unknown exposures. If contact allergy tests with strongly suspected occupational substances are negative, the possible changes in composition of working materials or inappropriately low concentrations of the allergens in the working materials, which have to be diluted for patch testing, has to be taken into consideration [3, 11].

In workers in which the test was slightly positive (+) to a certain substance, it is recommended to use the repeat open application test (ROAT), developed by Hannuksela and Salo. Within this test, the suspected substance has to be applied two times a day (on the volar aspect of the forearm near the antecubital fossa) for up to 2 weeks (but sometimes for up to 4 weeks). After several days of application if contact dermatitis develops, then even slightly positive patch test reaction is clinically relevant [14].

Immediate hypersensitivity testing (skin prick testing or prick-prick testing) are recommended in addition to patch testing, in immediate contact reactions (protein contact dermatitis or contact urticaria) and also in (hand) dermatitis where immediate reactions can contribute to the clinical manifestation [3].

***In vitro immunological tests***, that define cellular immune response, could be beneficial within diagnostics of OCD.

***Chemical tests—analyses*** are practical and important in the determination of suspect potential or new unknown allergens in certain materials or objects. As an example we can mention dimethylglyoxime test (used to determine the presence of nickel in a metal object) or other chemical analyses for the identification of formaldehyde, cobalt, chromium, or other substances.

***Skin biopsy*** could be beneficial in the exclusion of certain pathological conditions, such as psoriasis or skin lymphoma, but it often do not ensure precise diagnosis and therefore it is not used routinely.

***Differential diagnosis*** in OCD involves exclusion of other forms of contact dermatitis with nonoccupational etiology, as well as exclusion of other dermatoses of different etiology and similar clinical presentation or location of skin lesions (candidiasis, psoriasis, seborrheic dermatitis, scabies, Herpes simplex infection, lichen planus, drug reactions, and other skin changes).

### *9.2.5 Treatment*

The treatment of OCD follows the principles of treatment of the same nonoccupational dermatosis. However, the basic key rule in occupational medicine is the cessation of workplace exposure. The avoidance of the etiological factors (e.g., irritants and allergens) at the workplace and control of occupational exposure by technical and/or organizational measures is essential. The interruption of the contact with workplace sensitizing/irritant substance, particularly during early stages of the disease, could be very beneficial for the evolution and prognosis of the skin lesions. In each case of OCD, the possibility for nonoccupational exposure as well as the probability for the development of cross-sensitization to similar chemical substances has to be taken into account [11, 14].

### *9.2.6 Prevention*

***Primary prevention*** of OCD includes control of occupational exposure with qualitative and quantitative workplace risk assessment. That process should be reviewed and updated regularly. It is necessary to implement technical-technological preventive measures, such as work process automation and hermetization and substitution of potentially sensitizing substances with other safer materials. The milestones in delivering primary prevention are: adequate personal protective equipment, since they can also be source of contact sensitizers (for example, rubber gloves) and correct use of substances for personal hygiene. Priority has to be given to the education and training of the exposed workers. In addition to legislation, continuous surveillance is needed to identify new work-related risks [11, 14].

***Secondary prevention*** involves rigorous health control of exposed workers through preventive medical examinations, as well as timely cessation of exposure. These principles have to be implemented during occupational orientation and selection (before the employment of workers on the specific workplaces).

***Tertiary prevention*** includes treatment and rehabilitation of persons with OCD.

***Medico-Legal Aspects*** Occupational allergic skin diseases, including OCD, are included in the List of Occupational Diseases (Official Gazzete of RM, No 88/04) with legal conditions and criteria for the verification of the occupational character of these diseases.

## 9.3 Occupational Skin Cancer

OSD could be differentiated according to the morphology and severity of the skin lesions. Workplace exposure to certain physical, chemical, or biological hazards could result in very slight skin changes, such as skin erythema to very complex and serious changes, as a skin cancer [41].

Skin cancer is a frequent malignant neoplasm, and it usually has an epithelial origin (basal cell carcinoma—BCC and squamous cell carcinoma—SCC) and low malignancy potential. Much less frequent, but with far more higher malignancy potential is malignant melanoma that stems from melanocytes. Occupational skin cancer is a frequent skin disease and the most important etiological factor is occupational exposure to UV radiation. OSC is characteristic for occupations with workplace tasks that are performed outdoors, such as construction, agriculture, farming, sports, communal hygiene, and other outdoor workers. Occupational skin carcinogens, other than UV, are ionizing radiation and different chemical carcinogens (polycyclic aromatic hydrocarbons, oil derivatives, and arsenic) [20]. Studies have shown that workers exposed to UV radiation have almost double risk of developing SCC, and 43% higher risk of developing BCC than nonexposed workers [42–44].

### *9.3.1 Types of OSC and Precancerous Lesions*

***Non-melanocytic skin cancers (NMSC)*** involve: BCC, SCC, and rare soft tissue sarcomas. BCC is the most prevalent skin carcinoma in Caucasians (75–80% of all NMSC, and 10 times more frequent than in non-White populations). BCC is a malignant tumor of the basal cells of the epidermis, which can invade through adjacent tissues. It develops on sun-exposed areas (usually on the face), grows slowly and has little tendency to metastasize. Risk factors for the development of BCC include intermittent sunlight exposure (the most important), genetic factors, exposure to ionizing radiation, tar, lack of enzymes required to repair the DNA (e.g., xeroderma pigmentosum) and immunosuppression. SCC represents 20–25% of reported NMSC and it is the most common NMSC in non-Caucasians. SCC is a malignant tumor of keratinocytes. The predilection sites are hands and legs and it can metastasize. Predisposing factors include chronic sun exposure, occupational exposure to carcinogens, such as arsenic and polycyclic aromatic hydrocarbons (PAH), previous scars and burns, workplace heat exposure, immunosuppression, as well as genetic dermatosis (e.g., albinism, and xeroderma pigmentosum). NMSC mainly occur on the head and neck, while most of the melanomas develop on the trunk and limbs [41, 45, 46].

***Malignant melanoma*** is rarer than NMSC, but it is an aggressive skin cancer that arises from skin melanocytes, the pigment cells of the epidermis. Melanoma mortality has shown increasing trend in the last decades, but less rapidly than the incidence (among the ten most frequent cancers in Australia, Europe, and North America). Associations with occupation, diet, and hormonal factors, apart from exposure to solar radiation as a major cause for the increase in the incidence, are not strongly conclusive. Predisposing factors for the development of melanoma are skin phenotypes I and II, White-skinned populations, familial history of melanoma, history of previous melanoma, typical nevus and/or > 1 atypical nevi, certain gene mutations, sun exposure, previous sun burns, other dermatoses (e.g., xeroderma pigmentosum), immunosuppression, and other malignancies [41, 45, 46]. Outdoor workers with repeated episodes of severe sunburn might be at risk of malignant melanoma.

***Premalignant conditions*** could occur in patients exposed to a carcinogen and they may develop into a malignancy. *Actinic keratoses* may be both occupational and nonoccupational, induced by continuous exposure to UV radiation and characterized by abnormal proliferation of keratinocytes. They are usually found on sun-exposed sites (dorsal parts of the hands, the forearms, the face, and the scalp). *Tar keratoses* ("warts") are related to exposure to coal tar, pitch, shale oil, and products of the distillation of coal. They are small, brownish, round- or oval-shaped plaques with flat surface (smooth or warty) and they commonly appear on the hand's dorsum, lower arms, and face. *Arsenic keratosis* occurs as a precancerous lesion related to chronic arsenicosis. This lesion is specific to the inducing carcinogen (arsenic exposure) and it is potentially developed into malignancy (SCC or multiple BCC). Typically it is manifested by multiple yellowish punctate papules on the palms of the hands and soles of the feet. *Bowen's disease* (intra-epidermal carcinoma), typically associated with arsenic ingestion, is an in situ SCC with a potential to be transformed into SCC. The predilection sites involve sun-exposed parts of the body (head, neck, lower leg, and arms). Erythematous solitary or multiple, irregular, well-defined plaques, and topped with scales or crust are pathognomonic finding. *Keratoacanthoma* refers to an epithelial tumor with rapid growth (dome shaped nodule with a central keratinous plug), found in sun-exposed areas and related to UV and tar exposure. It is histopathologically similar with SCC, with a tendency to undergo spontaneous regression. Finally, *lentigo maligna* is a proliferation of melanocytes that may develop into malignant melanoma. This pigmented macule occurs at the sun-exposed locations.

### *9.3.2 Etiopathogenesis of OSC*

The natural sources of ***arsenic*** are the ores with zinc, lead, copper, and iron. It is used in the glass production, the making of microchips and semiconductors, and the production of insecticides and herbicides. Occupations at risk for occupational exposure to arsenic involve mining, smelting, farming, pesticides production, and agriculture. Arsenic and its compounds are carcinogenic and mutagenic; they enter the body through inhalation and ingestion. Arsenic has a potential to induce

chromosome mutation and seems to act as cocarcinogen with UV radiation. Its carcinogenic effects mainly target keratinocytes and epidermal stem cells causing arsenic keratoses that tend to progress into SCC and BCC.

***Polycyclic aromatic hydrocarbons (PAH)*** are constituted of two or more benzene rings, such as: such as benz (a) pyrene and dibenz (a, h) anthracene. PAHs are found in coal tar products and oil (e.g., coal tar pitches, coke oven emissions, soot, cutting and lubricating oils, anthracene, fuel and diesel oils, crude paraffin, and asphalt). Industries with occupational exposure to PAHs include: aluminum reduction workers, coal gasification workers, coke oven workers, locomotive engineers, road pavers and highway maintenance workers, shale oil workers, steel and iron foundries, as well as tool fitters and setters.

***UV radiation***, together with visible light and infrared radiation comprise the optic radiation. The source of natural UV radiation is the sunlight, and occupational exposure to artificial UV radiation can be found during welding process, in the food industry, and healthcare. UV radiation causes mutations in the p53 tumor-suppressor gene and it is carcinogenic through direct cell damage (DNA mutation) or by indirect mechanism (T lymphocyte suppression). It is well known that ***ionizing radiation*** has a potential to cause SCC, BCC, and premalignant skin changes. It may cause DNA damage that can induce carcinogenesis (mutation, chromosome aberration, and genomic instability). ***Trauma*** (especially burns resulting in a scar) may be occasionally followed by a skin cancer development (usually BCC).

### *9.3.3 Diagnosis and Treatment of OSC*

Actinic keratosis, SCC, BCC, or melanoma can be recognized as OSD only in some European countries. The diagnosis of OSC includes usual dermatological procedures (clinical examination, dermoscopy, skin biopsy, and dermatopathology). The treatment should apply national and international guidelines [11, 13].

### *9.3.4 Prevention of OSC*

The preventive strategy should involve:

- ***Technical/organizational measures*** (e.g., roofing of permanent outdoor working places, use of mobile sun shades, provision of shaded places for breaks, avoidance of UV exposure during midday, replacement of carcinogenic materials with noncarcinogenic ones)
- ***Personal protective measures*** (e.g., sunglasses, appropriate clothing and headgears, sunscreens with very high, broad-spectrum, photostable filters for both UVB and UVA, use of protective shields on equipment, protective clothing and hygienic measures)

- ***Preventive medical examinations of workers*** (preemployment and periodical checkups with particular attention to skin status),
- ***Educational activities*** (specific skin protection workshops and individual counseling for workers, employers, and healthcare professionals)
- ***Research activities*** (to foster research in the field of occupational skin diseases, occupational carcinogens, skin cancer, workplace UV exposure, climate changes and health and safety of outdoor workers, particularly by funding and developing occupational cohorts that will enhance evidence base for the identification of health risks and safe and healthy preventive strategies and policies) [47]

## 9.4 Conclusion

OSD, the most frequently represented by OCD, contact urticaria, and OSC, are highly preventable. While the prevention of OSD is a top-priority problem in European countries, diagnosis, prevention, treatment, and compensation of OSD is still organized very differently in different countries. The diagnosis of these clinical entities should be based on the identification of occupational etiological factor while the cessation of occupational exposure is first and the most important step in the management of OSD. The treatment of OSD does not, in principle, differ from the treatment applied in the same nonoccupational skin disease. The prevention of OSD should be achieved by a risk management process that has to be based on a proper workplace risk assessment. Appropriate technical/organizational measures, personal protective measures, preventive medical examinations of workers, and educational and research activities should complete primary, secondary, and tertiary prevention of OSD and they have to be based on the current theoretical and practical findings and recommendations.

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# Chapter 10
# Expanding Concept of Immune Reconstitution Inflammatory Syndrome: A New View Regarding How the Immune System Fights Exogenous Pathogens


**Yumi Aoyama and Tetsuo Shiohara**

**Abstract** Increased occurrence of opportunistic infections has been reported coincident with restoration of host CD4+ T cell number and reactivity: rapid immunological recovery from an immunosuppressive state can be associated with an exuberant inflammatory response detrimental to the host and worsening clinical manifestations of opportunistic infections, which has been referred to as immune reconstitution inflammatory syndrome (IRIS). Despite the considerable information that has accumulated during the past few decades, the concept of IRIS has not been widely appreciated yet in many clinicians. Elucidation of the events leading to the development of opportunistic infections could be key to our understanding of IRIS. Although, upon occurrence of opportunistic infections, withdrawal of immunosuppression in patients on immunosuppressive therapy appears to be intuitively logical, it should also be recognized that withdrawal of immunosuppressive agents, when abruptly and rapidly, has been shown to predispose IRIS. The principle of therapies for IRIS is achieving a fine balance between host immune responses and infectious agents, but not eradication of the latter. In this review, we describe how often IRIS could be involved in a variety of severe illness, ranging from severe drug eruptions to sarcoidosis. IRIS is eminently treatable with clear benefits from prompt recognition and appropriate management. We also provide evidence for the clinical usefulness of applying this concept to a variety of immunosuppressive therapy-related opportunistic infections and describe how to improve the condition with an otherwise life-threatening prognosis.

**Keywords** Autoimmune bullous disease · Cytomegalovirus · Herpes simplex virus · Immune reconstitution inflammatory syndrome · Sarcoidosis

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## 10.1 Introduction

Traditionally, latent infections with herpesviruses, such as herpes simplex virus (HSV) and Epstein–Barr virus (EBV), have been thought to become reactivated upon immune suppression, which support the view that continuous immune surveillance is needed to control viral infections: for instance, cytomegalovirus (CMV) disease has been believed to occur only if the T cell response is compromised. Although there is a growing acceptance that immune cells, especially T cells, play a protective role against viral infections, the alternative possibility is that the amplification of an immune response that is primarily protective under either normal or noninflammatory and non-pathological conditions may paradoxically be harmful if given rapidly or abruptly. In this regard, we have to recognize that immune responses constantly occurring for tissue homeostatic purposes may be different in the extent and kinetics from those under stressful conditions, in which increased amounts of co-stimulatory signals might be present. These considerations suggest that reactivations of herpesviruses would occur not only in an immunosuppressive setting but also in the setting that restoration of host immunity occurs rapidly and abruptly. In support of this notion, increased occurrence of opportunistic infections have been reported coincident with restoration of host CD 4+ T cell number and reactivity: rapid immunological recovery from an immunosuppressive state and an imbalance characterized by either suboptimum or excessive expression of immune responses could become associated with an exuberant inflammatory response detrimental to the host and worsening clinical manifestations of opportunistic infections, which has been referred to as immune reconstitution inflammatory syndrome (IRIS) [1, 2, 3, 4]. Although IRIS has been most frequently reported in patients with HIV infection starting antiretroviral therapy (ART), evidence is accumulating that inflammatory responses analogous to IRIS also occur after abrupt reduction or discontinuation of potent immunosuppressive therapy, such as corticosteroids and TNF-α inhibitors [5, 6]. Thus, the spectrum of IRIS has expanded since its initial description in the setting of HIV infection (Table 10.1). We will review evidence for the clinical

**Table 10.1** Clinical illness consistent with IRIS in a non-HIV setting

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| 1. *Mycobacterium avium complex* infection |
| 2. Tuberculosis |
| 3. Cryptococcosis |
| 4. Herpes simplex |
| 5. Eczema herpeticum |
| 6. Herpes zoster |
| 7. Hepatitis C virus infection |
| 8. Cytomegalovirus infection |
| 9. Kaposi sarcoma |
| 10. Graves' disease |
| 11. Hashimoto disease |
| 12. DiHS/DRESS |
| 13. Rheumatoid vasculitis |
| 14. DPP-4i-associated bullous pemphigoid |

usefulness of applying this concept to a variety of immunosuppressive therapy-related opportunistic infections and describe how to improve the condition with an otherwise life-threatening prognosis.

## 10.2 The Diagnosis of IRIS

IRIS is a syndrome with a common clinical phenotype arising from diverse pathways operating variably in individual patients. Accordingly, the symptoms of IRIS range from a self-limited mild disease to a severely ill, life-threatening disease [6]. IRIS usually occurs as a paradoxical deterioration of clinical symptoms suggestive of infectious disease in association with recovery of immune responses after initiating ART in patients with HIV infection. In the non-HIV setting, exacerbation of inflammatory responses or paradoxical development of infectious disease such as CMV disease have been reported to occur upon reduction or discontinuation of potent immunosuppressive agents, such as systemic corticosteroids and TNF-α inhibitors [5, 6, 7, 8]. Importantly, because clinical symptoms suggestive of opportunistic infections, which occur, upon reduction of potent immunosuppressive agents, would be considered as the result of immunosuppressive therapy, discontinuation of the immunosuppressive agents could be intuitively rational. Unfortunately, however, this decision without adequate thought would lead to exacerbation of IRIS, while ignoring the fact that abrupt discontinuation of immunosuppressive agents may lead to IRIS. Thus, non-HIV-IRIS, previously thought to be rare, has become clear to be perhaps more under-recognized than rare.

Unfortunately, no uniform definition of IRIS exists, but the diagnosis of IRIS usually requires the following criteria [1]: a paradoxical deterioration in clinical symptoms consistent with an infectious or inflammatory condition temporally related to ART initiation or reduction/withdrawal of immunosuppressive agents [2]; a decrease in viral loads associated with an increase in CD4+ T cell counts [3]; clinical symptoms not explained by a newly acquired infection but explained by the expected clinical course of a previously recognized infectious agents, or by side effects of therapy; and [4] any event occurring after ART initiation or after reduction or discontinuation of immunosuppressive agents [1]. The rapid increase in CD4+ T cell numbers is more likely to be due to redistribution of this population to the circulation rather than preferential cell proliferation. Not only the frequency but also functions of CD4+ T cells can be also restored to a clinically relevant degree after starting ART therapy; however, in non-HIV setting, it remains undetermined whether such rapid recovery of CD4+ T cells would occur upon reduction/withdrawal of immunosuppressive agents. In this regard, previous studies suggested that a high baseline CD4+ T cell count is protective against developing IRIS while lower CD4+ T cell counts are predictive of IRIS development [9, 10]. The role of CD4+ CD25+ Foxp3+ regulatory T cells (Tregs) in the pathogenesis of IRIS is controversial. Although an impaired Treg function could be theoretically involved in the excessive inflammation typically observed in IRIS, no evidence is presently

available to indicate the role of Tregs. Although IL-17 levels were reportedly increased in those who developed IRIS [11], there was no associated decrease in the frequencies of Tregs. Recent studies have demonstrated an increase in the frequency of Tregs in various types of IRIS, including mycobacterial-IRIS and cryptococcal-IRIS [12, 13], although this finding has not been confirmed in other studies.

IRIS can be divided into two broad categories, unmasking and paradoxical. Unmasking IRIS occurs when a previously unknown opportunistic pathogen is present for which a patient showed negative tests but the tests became positive upon ART initiation with concomitant development of symptoms suggestive of the infection. In contrast, paradoxical IRIS occurs when a disease has been previously diagnosed and the patient received treatment prior to ART initiation with the patient experiencing symptoms suggestive of infection [9]. Although there may be some differences depending on the target pathogens between unmasking and paradoxical IRIS, excessive cytokine production could represent at least one common mechanism for IRIS events, regardless of target pathogens.

## 10.3 Expanding Concept of IRIS

Herpesviruses have long been suggested to have an etiological role in various inflammatory and autoimmune diseases. How, then, can the etiological role be confirmed? According to Koch's postulates, the virus in question should be detected in most if not all patients; however, in the diseased lesions the virus is not necessarily detected at increased frequency: thus, the absence of viral DNA in the lesions cannot necessarily be taken as proof against causation of the disease. The virus may trigger the earlier phases of the disease but may be excluded from the lesions due to exuberant immune responses to the pathogen. Thus, no detection of viral genomes at the time of clinical onset of IRIS can be explained by assuming that virus clearance may take place upon reconstitution of a valid immune response. An intriguing that has received little attention so far is that restoration of host immunity may have been adverse sequelae, particularly when it occurs abruptly and rapidly. Recently, the development of IRIS has also been observed in lymphopenic and neutropenic patients after withdrawal of anticancer drugs [3, 14].

The concept of IRIS could be applied to increase the efficiency of antiviral and antitumor therapy. Samsouk et al. previously described an interesting case of a renal transplant recipient who acquired posttransplant hepatitis C virus (HCV) infection with progressive liver disease but subsequently experienced spontaneous viral clearance after removal of immunosuppression [15]. In this case, increases in CD4+ and CD8+ T cell counts correlated with the disappearance of HCV RNA [15]. This procedure could also be available for the treatment purpose. Restoration of the immune system after rapid withdrawal of immunosuppression would paradoxically serve to enhance antiviral or antitumor responses that may be otherwise insufficient for viral or tumor clearance. We have recently demonstrated that rapid withdrawal of topical corticosteroids sufficiently enhanced antitumor efficacy of imiquimod

(IMQ) [16]. Despite great potential of IMQ in the treatment of neoplasms, the efficacy has been limited to a given disease setting where tumors are small in size and in low-risk location in patients who will not undergo other well-established therapies [17]. These findings suggested the necessity for adjunct therapy applicable to IMQ therapy. Because the increased frequency of CD4+ Foxp3+ Tregs within the tumor environment can permit tumor cells to escape host immune resistance [18], enhancement of tumor immunotherapy by eliminating such tumor-associated Tregs has been well studied [19]. Because our previous study on IMQ monotherapy showed that Tregs occurred in significantly lower frequencies in the dermal infiltrates of Bowen's disease lesions before treatment in patients who eventually revealed a complete response (CR) than those in patients with a partial response (PR) [16]. We hypothesized that decreasing recruitment of Tregs at the tumor site prior to IMQ therapy may have therapeutic value. In our sequential therapy, immediately after cessation of typical corticosteroids, patients were begun on IMQ therapy. Our study clearly demonstrated that Tregs were more profoundly deleted from the tumor lesions by a 2-week treatment with topical potent corticosteroids rather than CD8+ T cells and, upon starting IMQ therapy, CD8+ T cells were recruited to the lesions more rapidly than Tregs [16]. Because a delay in starting IMQ therapy after abrupt cessation of topical corticosteroids reduced the efficacy of the sequential therapy, delayed timing of the switch from corticosteroids to IMQ therapy after abrupt cessation of topical corticosteroids reduced the efficacy of the sequential therapy. Starting IMQ therapy at a time when Tregs were the lowest in frequency could be essential for achieving more robust immune responses to tumor cells. CR of Bowen's disease occurred at 8 weeks in all patients treated with the sequential therapy ($n = 7$), while CR was only observed in three of the eight Bowen's disease patients treated with IMQ monotherapy [16]. Similarly, rapid steroid withdrawal in hepatitis C virus-positive kidney transplant recipients was shown to be effective in clearing the otherwise difficult-to-treat virus at that time [20]. Thus, reconstitution of a valid immune responses against previously unrecognized viruses would reduce viral loads on the one hand, but cause tissue damage at sites of subclinical infection that had not been clinically recognized before a reduction or withdrawal of immunosuppressive therapy on the other. Thus, we have to recognize that although host immunity is crucial in the eradication of pathogens such as viruses, immunological recovery occurring after a reduction or withdrawal of immunosuppressive agents may contribute toward worsening disease expression.

## 10.4 Severe Drug Eruption as Another Manifestation of IRIS

Drug-induced hypersensitivity syndrome (DiHS)/drug reaction with eosinophilia and systemic symptoms (DRESS) is life-threatening multiorgan system reaction caused by a limited number of drugs (Fig. 10.1): they include carbamazepine, phenytoin, phenobarbital, zonisamide, allopurinol, dapsone, salazosulfapyridine, and mexiletine [21, 22, 23]. This syndrome has several unique clinical features that

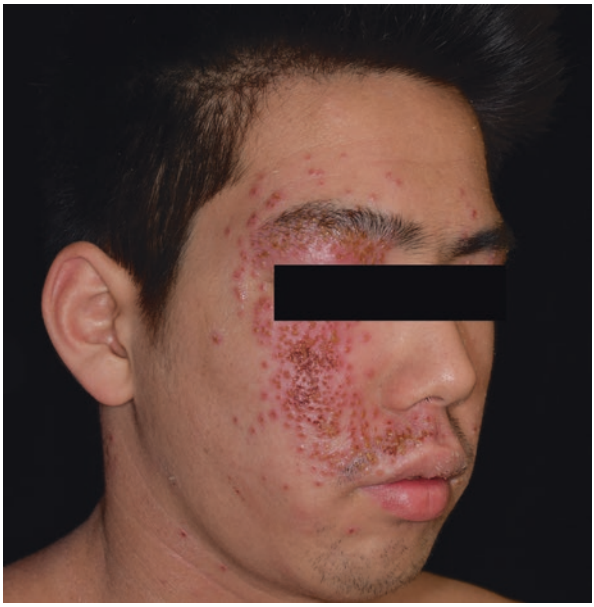

**Fig. 10.1** Clinical manifestation of a patient with drug-induced hypersensitivity syndrome (DiHS)/drug reaction with eosinophilia and systemic symptoms (DRESS) caused by carbamazepine. Macular erythema and pustular plaques developed on the entire body. Facial edema with lymphadenopathy is one of the typical clinical manifestations of DiHS/DRESS

cannot be solely explained by drug antigen-driven oligoclonal expansions of drug-specific T cells, which have also been implicated in the pathogenesis of other drug eruptions. They include a paradoxical deterioration of clinical symptoms, frequent flare-ups, and a stepwise development of several organ system failures after withdrawal of the causative drugs [21, 24]. Importantly, several herpesviruses including herpesvirus-6 (HHV-6), HHV-7, EBV, and CMV can be reactivated during the course of this syndrome in a sequential order as in graft-versus-host disease [25]. However, several questions have been raised as to why viral reactivations could only be detected 2–3 weeks after onset, why paradoxical deterioration of clinical symptoms was detected after withdrawal of the causative drug, and why DIHS/DRESS patients showed unexplained cross-reactivity to multiple drugs with structures totally different from the original causative drugs despite a very limited number of drugs responsible for initiating this syndrome. These observations could be explained by assuming that rapid restoration of pathogen-specific immunity would reduce viral loads at onset of DiHS/DRESS, thereby rendering them undetectable in the blood.

At present, comprehensive explanations of why a paradoxical deterioration of clinical symptoms is often observed after withdrawal of the causative drug in DiHS/DRESS are unavailable, but some clues may come from previous reports suggesting that causative drugs shown to induce DiHS/DRESS have in common intrinsic properties to potentially cause immunosuppression [26, 27]. In view of properties of these causative drugs, withdrawal of the causative drug could be associated with rapid restoration of virus-specific cellular and humoral responses that would reduce

viral loads but cause tissue damage. Thus, clinical symptoms of DiHS/DRESS are likely to be mediated by rapidly restored antiviral immune responses.

## 10.5 Severe Skin Superinfections as a Manifestation of IRIS in Atopic Dermatitis

Atopic dermatitis (AD) patients run a higher risk of developing severe skin superinfections with a number of viruses including HSV, *Molluscum contagiosum* virus, and vaccinia virus: after the causative virus they are named, eczema herpeticum (EH), eczema molluscatum, and eczema vaccinatum, respectively. EH caused by an extensive disseminated cutaneous infection with HSV 1 or 2 in the most commonly recognized viral complication in AD patients [28, 29]. EH can present in a primary form or a recurrent form and the primary infection is generally considered to be more severe with greater cutaneous involvement, lymphadenopathy, and fever [30]. Patients with recurrent HSV infections, however, often develop disseminated vesicular lesions accompanied by systemic symptoms (Fig. 10.2), such as fever, malaise, and lymphadenopathy, findings indistinguishable from the primary infection. The eruption is most frequently located on the face, neck, and the upper part of chest, forearms, and wrists while milder cases have lesions limited to the head and neck [30]. The vesicles rapidly evolve to pustules or dry out, forming crusts [31]. The vesiculopustular lesions tend to occur in areas where the skin has been most severely affected by the underlying skin disease in the primary form. Primary infections are thought to directly spread to a diseased cutaneous region by dissemination or autoinoculation. However, given that even recurrent EH lesions often occur in the previously affected site, the dissemination of vesiculopustular lesions is likely not true autoinoculation derived from the original infection site but may represent reactivation from viral latency at the site. Thus, it is difficult to distinguish between a primary infection and reactivation on clinical grounds alone without the aid of serologic assays. These findings, together with the observation that severe, untreated AD lesions have EH develop more easily than patients with well-controlled disease [29], suggest that AD patients with uncontrolled eczematous lesions are at greater risk for the development of this viral complication. Consistent with this view, Wollenberg et al. reported that the majority of the patients with EH had not received any corticosteroid therapy in the 4 weeks before onset of EH [29], which suggests the possibility that EH may occur as a result of rebounding immune responses after withdrawal of topical corticosteroids, namely topical IRIS. Because severe recurrence of EH can take place in some AD patients who often interrupt topical treatment with corticosteroids, prophylactic acyclovir or valacyclovir can be a therapeutic option that may reduce recurrence.

EH occurs almost exclusively in patients with AD, particularly in those who fail to control skin lesions. The reason of why patients with AD or those with a history of AD are at great risk for the development of EH remains unknown. AD patients

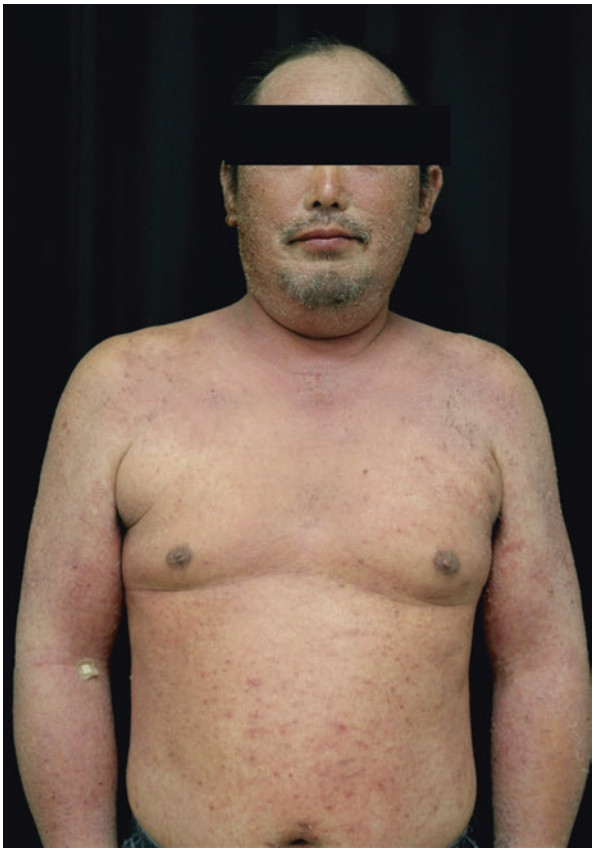

**Fig. 10.2** Clinical manifestation of eczema herpeticum (EH) due to recurrent herpes simplex virus infection in a patient with atopic dermatitis (AD) occurring upon withdrawal of topical corticosteroids. Discreate small, umbilicated vesicular lesions with crusts are preferentially seen in the right side of the face

with EH had a significantly higher frequency of CD4+ FoxP3+ Tregs in peripheral blood mononuclear cells (PBMC) at onset as compared with that after resolution and that in DiHS/DRESS$^{-}$. The increased Tregs at onset of EH exhibited phenotype characteristics similar to those in healthy controls. After resolution of EH, the frequencies of FoxP3+ Tregs decreased to values similar to those in healthy controls [32], as shown in DiHS/DRESS [27]. These results suggest that expanded Tregs were contracted upon clinical resolution and that EH can be only aborted by a timely decrease in Treg cell frequencies. These results indicate that a timely decrease in Treg cell frequency may help prevent the further development of EH.

A likely interpretation of these observations is that an abrupt shift of host immune responses from an immunosuppressive state to a robust pathogenic inflammatory state, IRIS, would occur upon withdrawal of topical immunosuppressive agents, such as corticosteroids in AD patients, which could be manifested as an exacerbation of clinical symptoms long before onset of EH. Probably, this shift would allow latent HSV to be reactivated in an uncontrolled fashion. According to this scenario, expansions of Tregs initially required for preventing excessive pathogenic inflammation as a result of withdrawal of topical corticosteroids could in turn contribute

to HSV reactivation, resulting in the initiation and progression of EH. These data propose a dual role of Tregs, either beneficial or harmful, by ameliorating the tissue-damaging effects of antiviral immune responses at the site of inflammation depending on how and when they are expanded. This scenario provides a potential explanation for why apparently disparate data on the role of Tregs in the pathogenesis of AD have been reported: this is due in part to a neglect in previous studies of evaluating the effects of Tregs on HSV reactivation.

## 10.6 Granulomatous Reactions as a Manifestation of IRIS

Sarcoidosis is a chronic inflammatory disorder characterized by the noncaseating epithelioid granuloma in a variety of tissues, including the lung, skin (Fig. 10.3), and liver [33]. Noncaseating granulomas develop in a number of infectious disease and in delayed hypersensitivity reactions to exogenous antigens such as silicone and

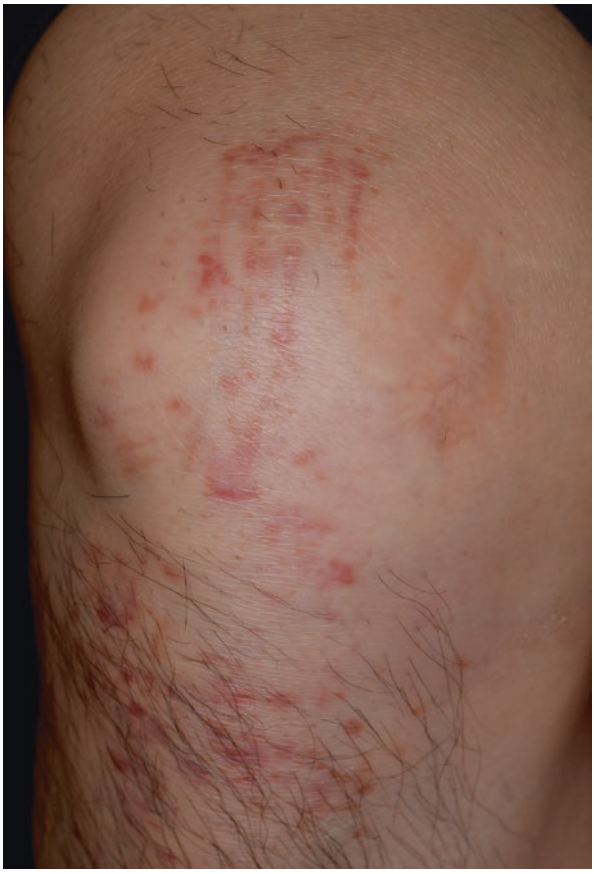

**Fig. 10.3** Papular sarcoidosis on the knees. Multiple infiltrated reddish papules developed on the scars resulting from trauma

beryllium [34]. Thus, any agents to which they had all been exposed might be the trigger: they include viruses, pathogens, chemicals, and atmospheric pollutants. Although definitive identification and proof of such agents are still lacking, it has been hypothesized that the disease can be initiated when a genetically susceptible host is exposed to a specific environmental antigen(s) [35]. Inactive lesions may be quiescent for years that become infiltrated with some triggers and develop apparent sarcoid lesions. The time between primary exposure to agents and onset of sarcoidosis can vary from a month to several years [36, 37]. Thus, this condition should be recognized as an occupational disorder as well as the result of past incidental cuts or abrasions, which result in the development of granulomas [38]. According to a traditional view, the development of sarcoidosis requires at least three major events: exposure to antigen; accumulation of CD4+ T cells in the antigen-exposed sites; and antigen-driven overexuberant activation of CD4+ T cells, which occurs within the target organs [39]. The paradox that despite extensive inflammation anergy develops as indicated by suppression of the immune response to tuberculin [40] could be reconciled by the observations that Tregs expand in active sarcoidosis lesions [41]. In view of the observations that IL-2 plays an important role in the maintenance of mature Tregs [42] and that IL-2 consumption by Tregs plays an essential role in Treg suppressor function by causing death of activated CD4+ T cells due to IL-2 deprivation [43], the onset of sarcoidosis is likely to be triggered by a rapid recovery of immune responses in which IL-2 can be abundantly produced. Importantly, Tregs accumulated in the lesions suppress early stages of granuloma formation but have no positive influence on sarcoidosis lesions [44]. Sarcoidosis-associated Tregs are memory phenotype Tregs and much more prone to apoptosis than naïve Tregs, corresponding to "exhausted" Tregs with reduced in vitro survival potential [45]. However, it has also been demonstrated that Tregs and Th17 cells mutually influence the pathogenesis of sarcoidosis and that an imbalance between Tregs and Th17 cells is associated with pulmonary sarcoidosis relapse after corticosteroid withdrawal. Interestingly, in analogy with sarcoidosis, the acute phase of DiHS/DRESS is characterized by expansions of fully functional Tregs but followed by a progressive loss of Treg function [46]: clinical resolution of DiHS/DRESS is accompanied by a gradual shift from Tregs to Th17 cell development [47].

These findings suggest similarities in the pathogenesis between sarcoidosis and DiHS/DRESS. Indeed, a previous study suggested that sarcoidosis is another manifestation of IRIS in HIV-infected patients, because active sarcoidosis developed after being started on ART [48]. Consistent with this view, we and others demonstrated that granulomatous dermatitis was observed in the cutaneous lesions of DiHS/DRESS [49, 50], suggesting that both diseases have some similar underlying pathogenesis in common. Indeed, interstitial granulomatous dermatitis occurring in patients receiving the different agents with TNF-α inhibitory activity has been reported [51]. Although they may occur during or after immunosuppressive therapy, it should be kept in mind that rapid reduction or withdrawal of immunosuppressive agents might predispose patients to IRIS. Thus, corticosteroids might be appropriate as part of early intervention but could be potentially harmful to the host if given later.

## 10.7 Treatment-Refractory Cases as a Manifestation of IRIS

Dipeptidyl peptidase 4 (DPP-4) is the enzyme which is present in liver, kidney, and intestine and act by rapid cleavage of the N-terminal dipeptides of glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), known as incretin effect [52]. DPP-4 inhibitors (DPP-4i) thus can enhance endogenous secretion of both GLP-1 and GIP with food intake, leading to insulin secretion and to the reduction of glucagon secretion [53]. Based on the effects, DPP-4i have been used worldwide as a first- or second-line pharmacological therapeutic option for type 2 diabetes [53, 54]. DPP-4 is also known as a T cell activation antigen, CD26, and is highly expressed on activated T cells, particularly Th1 and Th17 cells [55]. Recent reports also have demonstrated a significant alteration in circulating Tregs and Th17 cells in diabetic patients treated with DPP-4i as compared with those not treated with them [56]. These findings suggest that diabetic patients treated with DPP-4i could be in an immunosuppressive state; and thus we could hypothesize that IRIS develops during or after withdrawal of DPP-4i given their immunosuppressive properties. To date, however, no case of IRIS during or after treatment with DPP-4i has been reported. We have recently experienced a case who developed esophageal ulcers and bullae in the trunk suggestive of mucous membrane pemphigoid (MMP) during and after treatment with DPP-4i as a manifestation of IRIS, and subsequent flaring of his disease after withdrawal of DPP-4i and pulsed systemic corticosteroids, respectively, as described below.

A 71-year-old man with a history of type 2 diabetes presented to our hospital with a 3-week history of multiple bullae of the oral cavity, lips, and around his eyes, and pruritic bullae over the trunk and extremities (Fig. 10.4). He was treated with linagliptin for 5 years. He was initially diagnosed as drug eruption or viral exanthema and linagliptin was immediately withdrawn. A week later, virological examinations revealed a positive CMV antigenemia. Immediately intravenous ganciclovir (GCV) was started, his pruritic erythema and erosions were gradually decreased, and GCV was withdrawn. However, 1 week later, he showed pronounced exacerbation of bullae and erosions on the oral cavity. The biopsies obtained from the oral mucosa and trunk showed subepidermal bullae with a sparse lymphocytic infiltrate. Direct immunofluorescence (DIF) of the lesion showed a linear deposition of IgG and C3 along the basement membrane zone (BMZ). Results of the bullous pemphigoid (BP) autoantibody panel found a slightly elevated anti-BP180NC16a IgG antibody levels (13.3 U/ml; normal <9.0) and presence of autoantibody against laminin332. He was diagnosed as DPP-4i-associated MMP. Although oral prednisone, 40 mg daily, was initiated, his oral erosions were resistant to systemic corticosteroid therapy. Therefore, pulsed prednisone, 500 mg for 3 days, was initiated. Although some improvement was transiently seen, this was followed by rapid deterioration. His esophagogastroduodenoscopy (EGD) was remarkable for patch esophageal ulcerations suspected as CMV infection. Virological examinations revealed a positive CMV antigenemia, confirming reactivation of CMV presenting as severe oral lesions and esophagitis. Subsequent immunohistochemical

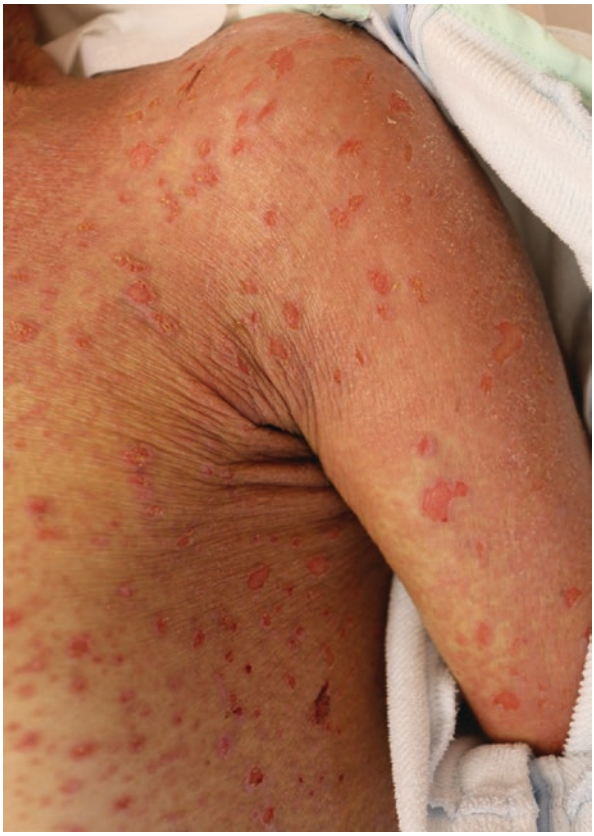

**Fig. 10.4** Skin manifestation developed in patient with mucous membrane pemphigoid (MMP). Multiple erosions with itchy diffuse erythema are seen in the trunk and extremities

examination of a biopsy from the pharynx revealed multiple cells with CMV inclusions and CMV antigen expression, thus confirming histopathological diagnosis of CMV disease. The patient immediately started receiving intravenous ganciclovir (GCV). One week after initiation of GCV therapy, improvement of his oral lesions was detected. Because the CMV antigenemia results became negative, GCV was withdrawn and virological examination revealed again a positive CMV antigenemia. After initiating GCV therapy in addition to prednisone, bullae, and erosions completely resolved.

This case was unique in that two episodes of CMV reactivation developed immediately after withdrawal of DPP-4i and pulse prednisone, respectively. These findings suggest that CMV ulcers (or esophagitis) occurred as a manifestation of IRIS. This view is supported by the finding that a rapid increase in CD4+ T cell counts was noted after withdrawal of DPP-4i and pulsed prednisone. Previous our studies suggested that CMV reactivation/disease develops 2–3 days after tapering corticosteroids in a non-HIV setting, such as BP and DIHS/DRESS [57, 58]. After pulsed prednisone, a rapid and great reduction of prednisone dose is necessarily needed, thereby increasing the risk of IRIS, especially CMV-IRIS [58]. In

considering the finding that most of CMV-IRIS occurred over a predictable time course, usually several days after withdrawal or tapering of systemic prednisone, CMV ulcers (esophagitis) in our patient is likely to develop as a manifestation of IRIS. Five days after withdrawal of DPP-4i, sudden exacerbation of clinical symptoms consistent with the diagnosis of MMP was associated with the detection of CMV antigenemia but not a significant rise in autoantibody (autoAb) titers, suggesting that CMV reactivation could be involved in the pathogenesis of a subset of DPP-4i-associated BP/MMP presenting as a manifestation of IRIS.

This case prompted us to investigate whether CMV reactivation as a manifestation of IRIS could occur coincident with a rapid immune recovery in patients with refractory autoimmune bullous disease (AIBD), such as BP and pemphigus vulgaris (PV). We retrospectively analyzed patients with refractory AIBD who developed CMV reactivation during immunosuppressive therapy. We identified several risk factors involved in CMV-IRIS in our case series. Risk factors included withdrawal of DPP-4i. pulsed prednisone and cyclophosphamide and a large reduction of prednisone. Although CMV reactivation occurring in patients on immunosuppressive therapy was traditionally thought to be merely an epiphenomenon of the underlying immunosuppression in AIBD, we found that CMV reactivation occurs upon a rapid immune recovery rather than in the nadir of immunosuppression and significantly affects patient outcomes.

In AIBD, especially PV, mucosal involvement is not limited only to oral cavity but extends to the esophagus or other gastrointestinal tissues as well: EGD is one of the most commonly performed endoscopic procedures. Our findings emphasize the importance of recognizing esophageal and pulmonary involvement in AIBD patients with CMV reactivation as serious complications of AIBD.

## 10.8 Refractory Rheumatoid Vasculitis Complicated by CMV Reactivation

Rheumatoid vasculitis (RV) is a rare and typically late complication of rheumatoid arthritis (RA). Severe RV is poorly managed by current anti-inflammatory drugs including systemic corticosteroids: thus, management of RV is largely empirical due to lack of randomized control studies. Many sporadic case reports document localized infections caused by opportunistic microorganisms in patients with RV treated with immunosuppressive agents [59]. The prevailing dogma shaped by clinical observations depicts impairment of immune responses as the inducers of such opportunistic infections. Recent studies, however, have revised this dogma and suggest that opportunistic infections may paradoxically occur upon a rapid restoration of immune responses. Consistent with this view, immunosuppressive therapy-refractory cases tend to show more severe inflammation. Therapy-refractory RV and CMV vasculitis share many clinical symptoms, both leading to severe ulcers making it difficult to distinguish them clinically. When patients have an apparent flare

that cannot be explained by an increase in inflammatory parameter and RA symptoms, CMV-IRIS should be suspected and a more thorough clinical evaluation of CMV reactivation should be done. To identify CMV reactivation, repetitive diagnostic measures with taking of tissue samples and CMV-specific immunohistochemical staining of the tissue samples are necessary. In addition, detection of CMV antigen and CMV DNA in the blood could further improve sensitivity of detecting CMV reactivation.

## 10.9 Management of IRIS

Not to misconstrue this unique manifestation of IRIS as a relapse of disease or as failure of therapy, clinicians need to be made aware of this entity. We also have to recognize that many antifungal agents have immunomodulatory effects while having antifungal effects [60]. Statins, in addition to their potent lipid-lowering effects, have been shown to have the ability to modulate inflammatory responses [5], suggesting that their abrupt withdrawal may induce IRIS, as shown in DPP-4i-associated BP/MMP. Thus, CMV reactivation as a manifestation of IRIS can occur during or after therapy with immune checkpoint inhibitors [61]. Patients with inflammatory bowel disease have been shown to have an increased risk for CMV reactivation. Secondary autoimmune disease including acquired hemophilia A (AHA) can also develop in BP patients receiving DPP-4i upon cessation of DPP-4i and withdrawal of systemic corticosteroids in association with epitope spreading [62].

Although corticosteroids are most frequently used immunosuppressive agents as treatment of IRIS, there have not been clear guidelines for how patients with IRIS should be treated with corticosteroids. Because a mild form of IRIS responds well to specific treatment alone for the underlying pathogens, anti-inflammatory therapies such as corticosteroids are not generally required for the mild form: the opportunistic infection as a manifestation of IRIS would resolve spontaneously without the use of immunosuppressive therapy in a mild form. Although discontinuation or reduction of immunosuppressive therapy upon diagnosis of opportunistic infection seems to be intuitively rational, abrupt reduction of immunosuppressive therapy in some cases would result in rebound of pathogenic inflammatory responses, thereby causing a severe form of IRIS. In a severe form of IRIS, anti-inflammatory therapy in addition to antimicrobial therapies is absolutely needed to ameliorate clinical symptoms arising from rebound of pathogenic inflammatory responses. Although systemic corticosteroids gave promising results in terms of ameliorating vigorous restoration of immune responses to pathogens when combined with antimicrobial therapies, drug dose should be reduced gradually even upon resolution of clinical manifestations. Thus, the use of systemic corticosteroids might be appropriate as part of early management but could be potentially detrimental to the host by increasing the risk of disease progression to full manifestations of IRIS upon the withdrawal or reductions.

The principles of therapies for IRIS is achieving a fine balance between host immune responses and infectious agents, but not eradication of the latter. A slower

rate of decreases after the initiation of immunosuppressive therapy would be essential for maintaining the balance the fine balance. In DiHS/DRESS, we recommend that systemic corticosteroids be initiated at a sufficient dose of 40–60 mg per day and be followed by a gradual dose reduction of prednisone at least over >8 weeks. Tapering more gradually over a prolonged period (5–10 mg/2 weeks) is recommended to achieve the optimum therapeutic results [58]. Although many physicians believe that 5 mg of prednisone is a physiological dose that may not have important harms, a reduction of 5 mg prednisone in a patient who received low doses (5–10 mg daily) of prednisone for years or months would have a strong impact on a rapid immune recovery given the cumulative effects on the immune system. Thus, these findings clearly suggest the importance of the cumulative effects of the previous past use of systemic corticosteroids on flare-ups of the disease upon a reduction of systemic corticosteroids. Indeed, we previously reported an interesting case in which long-lasting contact dermatitis was shifted to the clinical phenotype to TEN-like symptoms upon a reduction of 5 mg of prednisone [63].

Although pulsed prednisone has often been used for conventional treatment-refractory cases with severe drug eruptions such as DiHS/DRESS and AIBD, previous reported cases including our own [58, 59, 64, 65] suggest that the use of pulsed prednisone, although beneficial in the short-term outcomes, may be related to later development of CMV reactivation. We reasoned that a large reduction of prednisone doses needed immediately after pulsed prednisone may paradoxically induce a rapid recovery of immune responses that could in turn contribute to the development of CMV reactivation as an additional manifestation of IRIS. Our retrospective cohort study clearly demonstrated that fatal complications occurring in the late stage of DiHS/DRESS could be preventable with anti-CMV therapy: in support of this possibility, a delay in initiating anti-CMV therapy after the first detection of CMV reactivation was likely to reduce efficacy; cessation of anti-CMV therapy was temporarily associated the development of CMV disease or complications [58], and not only CMV-related but also—unrelated complications resolved during anti-CMV therapy and their treatment resurgence soon after cessation of anti-CMV therapy [58]. These results could be interpreted as an indication that anti-CMV therapy may have been also effective at curtailing the risk of other herpesvirus-related complications [58]. Indeed, anti-CMV therapy has been reported to exert beneficial anti-EBV or anti-HHV-6 effects [66, 67]. Thus, we could suggest that anti-CMV therapy, only when combined with sufficient anti-inflammatory therapy, has direct or indirect efficacy against other members of the herpesvirus family.

## 10.10 Conclusion

Despite the considerable information that has accumulated during the past decades, the concept of IRIS has not been widely appreciated yet in many clinicians. Prompt recognition of the concept of IRIS could help clinicians to determine the optimal therapeutic strategy for the individual patient with opportunistic infections. With

increasing evidence of the involvement of IRIS in opportunistic infections, therapeutic paradigm is shifting from mere immunosuppression to the prevention of rapid and abrupt immune recovery. A more sophisticated understanding of the temporal and reactive sequence of immunological changes occurring during the acute and later resolution phases of severe inflammatory disease would promote a more logical basis for the management.

**Compliance with Ethical Standards**
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# Chapter 11
# Workplace Risk Assessment in Occupational Allergology


**Dragan Mijakoski and Sasho Stoleski**

**Abstract** Identification, control, and monitoring of workplace risks has a main aim of eliminating or reducing specific occupational risks and this process is realized within the activities of prevention of adverse health effects and work ability impairment in exposed workers. The workplace analysis is based on the identification and evaluation of occupational hazards and dangers in the work environment. The quantitative process that provides a numerical assessment of the association between exposure, dose, and effects is defined as a risk analysis. Workplace risk analysis consists of three consecutive phases of action: risk assessment, risk management, and risk communication. According to the recommended methodology risk assessment is implemented in five steps. During the risk management phase, responsible persons are identified for the implementation of each of the necessary activities within the proposed measures. Finally, workplace risk communication is implemented as an act of conveying or transmitting information between parties about the risks identified at the workplace.

**Keywords** Workplace · Hazard · Danger · Occupational risk · Risk assessment · Occupational allergy

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## 11.1 Introduction

One of the most important objectives of occupational medicine is protection and promotion of the health, safety, and well-being of the workers, and the basic activities are focused on creating conditions for a healthy and safe workplace and working environment. The monitoring and analysis of working conditions are among the basic specific tasks of the occupational medicine, with the ultimate goal of realizing the policy of "healthy worker at a healthy workplace" [1].

The process of identification, control, and monitoring of workplace risks has a main aim of eliminating or reducing specific occupational risks and this process is realized within the activities of prevention of adverse health effects and work ability impairment in exposed workers. At the workplace, the employee is exposed to the influence of a wide variety of factors, including different allergens and irritants that determine the working environment and workplace conditions.

In this context, the workplace is defined as a set of tasks and activities for which the worker has qualifications, knowledge, and skills and which characterize the type of work performed by the employee [2, 3]. Additionally, the working environment is represented by a summary of all technical-technological, physical, chemical, biological, psychosocial, and ergonomic factors to which the human being is exposed during his/her work activities. On the other hand, according to the International Labour Organization (ILO), working conditions refer to different topics and issues, such as work organization and working time (e.g., hours of work), remuneration, or physical conditions and job demands that are present at the workplace [4]. The conditions and hazards in the working environment have to be assessed by specific measures and procedures and they have to be compared with the recommended and recognized standards and norms in the field of health and safety at work, especially with the standards that are determined by the national legislation.

Workplace exposure assessment or workplace risk assessment, mainly, constitutes a part of primary prevention. Primary prevention is based upon the control of workplace exposure. It involves all measures and procedures that are employed at the workplace in order to assess the conditions and hazards in the working environment. Primary prevention, also, includes all technical/technological and organizational measures, as well as personal protective equipment. Going further, workplace risk assessment is a key instrument for setting a diagnosis of occupational and work-related diseases. It is widely used especially in the detection of the relationship between workplace hazards (e.g., irritants or sensitizing substances) and disease (e.g., skin or respiratory disease) [5–7] as well as determination of the occupational etiology of certain diseases. The assessment of workplace risk and the analysis of occupational exposure, together with properly taken medical and occupational history will help occupational medicine specialist in establishing the occupational etiology of the allergic, respiratory, or skin disease.

## 11.2 Analysis of the Workplace

### *11.2.1 The Assessment of Workplace Conditions*

The assessment of the workplace conditions starts with obtaining information about the basic data for the company and the performed inspection and evaluation at the workplace. Workplace inspections are recommended, because for example, more than 80% of the cases with occupational allergic contact dermatitis have been correctly verified as occupational thanks to workplace exposure assessment (workplace visit and assessment of product labels) [5, 8, 9].

This process, which includes several different activities, requires a multidisciplinary and intersectoral approach by directly engaging certified occupational health services with specialists in occupational medicine. In the multidisciplinary team, apart from physicians (occupational medicine specialists and specialists in other medical specialties like otorhinolaryngologists, ophthalmologists, and psychiatrists) and different competent experts from various fields (e.g., psychologists, safety at work engineers, social workers, pedagogues, or economists), employers and workers' representatives have an extremely important role.

In accordance with the recommendation of the International Labour Organization for Occupational Health Services No. 171 (ILO Occupational Health Services Recommendation No.171), the analysis of the workplace includes several elements [10]:

- Identification and evaluation of the environmental factors which may affect the workers' health
- Assessment of conditions of occupational hygiene and factors in the organization of work which may give rise to risks for the health of workers
- Assessment of collective and personal protective equipment
- Assessment where appropriate exposure of workers to hazardous agents by valid and generally accepted monitoring methods
- Assessment of control systems designed to eliminate or reduce exposure

### *11.2.2 Occupational Hazards and Dangers*

The workplace analysis is based on the identification and evaluation of occupational hazards and dangers in the work environment. For each workplace, a register of occupational hazards is made. Occupational hazards, i.e., harmful occupational agents are usually classified as physical, chemical, biological, psychosocial, and ergonomic.

***Physical occupational hazards*** include: microclimate factors (temperature, humidity, thermal radiation, and airflow), atmospheric factors (heat, cold, etc.),

noise, vibration, atmospheric pressure, ionizing radiation, nonionizing radiation, and other physical hazards.

***Chemical occupational hazards*** are all inorganic and organic chemical substances present in the working environment in various forms and aggregate states (aerosols, smoke, vapors, gases, particulate matter, and liquids) that can adversely affect the health of occupationally exposed workers.

***Biological occupational hazards*** are all living organisms or substances produced by living organisms that have the ability to cause disease, injury, or any other adverse effect on exposed workers. These include: microorganisms (viruses, bacteria, or fungi), plants, animals, their secretions, excrements, parts of organisms, products, as well as other biological hazards.

***Psychosocial hazards*** are various stress factors, a result of the interaction between working conditions, individual factors, and situational factors. Stress factors arise from work conditions and work organization or by the demands of the workplace, and affect health of the exposed workers, workplace productivity, and their communications with the environment.

***Ergonomic factors*** should ensure adaptation to working environment, machines, tools, and devices according to the physical and psychological characteristics of worker. They become harmful agents when the elements of the ergo-system (worker, machine, working environment) are not in optimal relationship, i.e., there is an inadequate ergonomic design at the workplace, which is detrimental to the health of the exposed workers, as well as on the efficiency and productivity of labor.

For each of the identified occupational hazards and dangers it is necessary to assess the characteristics of exposure (level, intensity, and duration) and to estimate the possible risk of health damage in the exposed workers. Recognizing, identifying, and grouping the hazards and dangers in the workplace and working environment are basic steps in the workplace analysis, and, above all, in the workplace risk assessment, i.e., they represent an important element in the evaluation of the health and safety at work for every job position.

In accordance with the current legislation in the Republic of North Macedonia in this domain (Rulebook on the procedure for preparation of a safety statement, its contents, as well as the data for workplace risk assessment, Official Gazette of the Republic of North Macedonia No. 02/09), which is harmonized with the EU regulation, all elements in this process are legally defined [11].

"Risk assessment" is the systematic recording and assessment of all factors in the work process in order to determine the possible types of dangers and hazards in the workplace and the working environment that can cause adverse health effects, injures at work, occupational diseases, and work-related illnesses. The risk assessment and the measures determined by the employer are carried out in accordance with the regulations on safety and health at work, in order to ensure elimination of dangers and hazards in the workplace and the working environment, i.e., eliminating or reducing the risk to an acceptable degree that prevents injuries at work, health damage, or illness of the employee.

### *11.2.3 Occupational Exposure: Monitoring and Evaluation*

The analysis of workplace and working environment conditions includes monitoring of exposure to occupational hazards and dangers, as well as monitoring of adverse exposure effects on the health of exposed workers. Monitoring and evaluation include:

- Ambient monitoring—is a qualitative and quantitative determination of the chemical and biological hazards and measurement of intensity of physical hazards at the workplace and in the immediate working environment of exposed workers.
- Personal exposure monitoring—is the determination of specific occupational exposure for each worker individually or at the group level to specific occupational hazard to which the worker workers are exposed.
- Biological monitoring—is a qualitative and quantitative measurement of the chemical hazards or their metabolites, i.e., microbiological identification of biological hazards in the biological material (blood, urine, sputum, expired air, hair, nails, breast, or milk) of occupationally exposed subjects.
- Psychological hazards, i.e., work-related stress and its manifestations (mobbing, burnout syndrome), are evaluated by questionnaires and interviewing.

The workplace analysis also provides a health assessment of the work environment by comparing the measured exposure to specific occupational hazards at the workplace, with the established standards of occupational exposure. These standards are defined as "permissible levels" or "exposure limits" and are derived from numerous studies that analyze the correlation between exposures and caused adverse health effects. This implies that the measured exposure, which is close to or above the defined limit values for certain occupational hazards accepted in national regulations, is risky for the health of the exposed workers, in accordance with the defined criteria. These standards become part of the legislation in accordance with the adopted national laws and practice, for example, Maximum Allowable Concentrations (MACs) in Germany, Permissible Exposure Limits (PELs) in the United States and others.

In our country, within the framework of harmonization of the national legislation with the EU in the field of health and safety at work, new bylaws are expected to be adopted to define the standards for the acceptable exposure limits in the working environment (2000/39/EC on indicative occupational exposure limits, 90/394/EEC on carcinogens, 2000/54/EEC on biological agents).

The workplace analysis provides the necessary data on the effects of the conducted preventive and control actions in the enterprise, but at the same time determines the priorities for intervention in the working environment in order to protect, improve, and promote the working environment and the health of the employees.

## 11.3 Workplace Risk Assessment

The workplace analysis provides adequate data on the conditions in the working environment, with the identification and evaluation of occupational hazards that, in conditions of occupational exposure, could adversely affect the health, well-being, and work ability of the workers. The likelihood of the occurrence of potentially harmful effects on health of workers is obtained by the workplace risk assessment [2, 3, 12].

### *11.3.1 Risk and Workplace Risk*

The ***risk*** refers to a quantitatively expressed measure of the chance or probability that, as a consequence of an exposure, potentially harmful effects on health will be manifested or the person will be harmed. That is the expected frequency of occurrence of harmful health effects, illness, or death as a consequence of exposure to harmful factors in the working and living environment [2, 3, 13].

***Workplace risk***, however, is defined as the chance or probability of manifesting harmful effects on the health of workers, which are a consequence of the occupational hazards, found in the working environment, or a consequence of the methods of work performance [2, 3].

The quantitative process that provides a numerical assessment of the association between exposure, dose, and effects is defined as a ***risk analysis***. The main aim of workplace risk analysis is to protect workers' health, safety, well-being, and his/her work ability. It helps to minimize the possibility of the workers to be being affected or harmed due to workplace activities [2, 3, 14].

Workplace risk analysis consists of three consecutive phases of action:

- Risk assessment
- Risk management
- Risk communication

***Risk assessment*** has evolved into a key element of the prevention of occupational diseases, work-related diseases, and injuries at work. That is the initial phase of workplace risk analysis and it is the first step toward systematic management of occupational safety and health. The risk assessment is, in fact, a process of evaluating the risks to the safety and health of workers arising from the hazards and dangers at the workplace. It refers to a process of systematic studying of all aspects of the work and it addresses the following issues [2]:

- Which elements of the workplace have the potential to cause injury or damage
- Whether workplace hazards and dangers can be eliminated or reduced
- Which preventive or protective measures are applied or which preventive or protective measures should be applied in order to control or reduce the risks to the safety and health of workers

The promotion of health and safety at work is, in fact, based on the assessment of the workplace risk of injuries and health disorders in exposed workers, as well as on the development and implementation of preventive measures aimed at controlling occupational risks.

For the purposes of workplace risk assessment, the European Agency for Safety and Health at Work (EU-OSHA) from Bilbao, within the framework of the Risk Assessment Campaign (2008–2009), has recommended a methodology that is implemented in five steps [14]:

1. Collecting information
2. Identifying hazards
3. Assessing risk arising from hazards (estimating probability and severity of consequences and deciding whether the risk is tolerable)
4. Planning actions to eliminate or reduce risk and reviewing assessment
5. Documenting risk assessment

## *11.3.2 Collecting Information*

During the ***first step (Collecting information)*** it is necessary to obtain information about: the location of the workplace; workers and vulnerable groups of workers (pregnant women, young or disabled workers); work equipment, materials, work processes; work tasks (how and for how long); the already identified hazards and their sources; the potential consequences of the present hazards; the use of protection measures; accidents, injuries at work, occupational and work-related diseases and other manifestations of impaired health; as well as legal and other requirements at the workplace [2, 14].

### 11.3.2.1 Collecting Information for Occupational Allergens and Irritants

The risks arising from occupational allergens and irritants have to be included in the workplace risk assessment. Namely, already identified occupational irritants and sensitizing substances have to be recognized in order to conduct subsequent risk assessment. In occupational allergology, special focus has to be put on the recognition of allergies, respiratory and skin disease in exposed workers. All occupational, work-related, and nonoccupational diseases have to be noticed. These data could be obtained through preventive medical examinations of workers and they have to be stated within workplace risk assessment. This process also has to include a precise evaluation of the ability of workers with certain health impairments (e.g., contact dermatitis or COPD) to continue to work in the context of exposure to different workplace hazards. Workplace visits are recommended and the results of measurements of noxious or hazardous factors at the workplace could be useful.

Workplace allergens and irritants could be identified by using product labels and Material Safety Data Sheet (MSDS). But, in practice, many MSDS are incomplete and they are not giving the comprehensive picture of all allergens and irritants in a product [5, 15]. In other cases, some allergens are specific substances and materials and they are usually not listed on the MSDS (e.g., wood dust, animal fur, enzymes, as well as other substances or materials). Available workplace check lists could be used in the identification of those materials.

### *11.3.3 Identifying Occupational Hazards*

In ***identifying occupational hazards***, i.e., implementing the second step of the risk assessment at the workplace, the multidisciplinary team is using general and specific checklists of hazards as well as lists that are specific for different sectors. Those checklists have to cover the most frequent occupational exposures, but also nonoccupational ones. Namely, the contacts with chemicals outside of the workplace could bias both verification of occupational allergic diseases and the procedure of workplace risk assessment at all. Within the checklist, it has to be marked whether a certain hazard exists at the workplace.

#### 11.3.3.1 Identifying Occupational Allergens and Irritants

Apart from other hazards (e.g., moving parts of machines; ultraviolet radiation (UVR), infrared radiation (IRR), laser, and microwave radiation; or biological hazards), general checklist usually includes chemical substances (including dust/allergens/irritants). The specific checklist on chemical substances includes different items about the use of hazardous chemical substances (classified as very toxic, toxic, harmful, corrosive, irritant, sensitizing, carcinogenic, mutagenic, toxic to reproduction, explosive, oxidizing, extremely flammable, highly flammable, or flammable), proper labeling of hazardous chemicals, or about the proper provision of collective and personal protective equipment for the workplaces where the chemicals are used. In practice, there are also checklists that are specific for different sectors (e.g., for construction industry including items on the use of suitable protective measures to prevent exposure to dust/allergens/irritants—wood, cement, silica; for food processing industry with questions about direct contact with raw materials and/or materials of animal or plant origin; for woodworking, items about the evaluation of the quality of workplace air, usage or respiratory protection while spraying, and regular training of workers about handling dangerous chemicals; for agriculture, questions about the correct storage of pesticides or about the implementation of safe system for working with dry substances (grain, fertilizers, sand)) [14].

When conducting workplace risk assessment, the team has to take into consideration that, for example, irritating factors at the workplace may enhance the impact of allergens (e.g., welding fume or diesel exhaust may increase the airways'

reactivity to allergens; frequent use of water may weaken the skin's barrier function, leading to enhanced development of contact dermatitis). The evaluation always has to be performed for the context of working environment and workplace that is characterized by a mixture of hazards and dangers to which the workers are exposed to.

For each identified hazard at the workplace, it is necessary to evaluate the routes of ***exposure*** of workers (e.g., through skin contact, respiratory system or by gastrointestinal system) that are performing their job tasks at that specific workplace. After that, the ***dose–response relationship*** has to be analyzed.

### *11.3.4 Assessing Risk Arising from Hazards, Including Workplace Allergens and Irritants*

The ***third step or assessing risk arising from hazards*** involves the use of matrices for each identified hazard in order to estimate the probability and severity of consequences (negative health effects in the form of occupational and work-related diseases or injuries at work, as well as work ability impairment) and deciding whether the risk is tolerable. The application of those matrices, i.e., by including two elements:

1. Probability of occurrence (highly improbable, probable, or highly probable) and
2. Severity of consequences that may be caused by the corresponding hazard (moderate, medium, or extreme harm)

It is assessed whether the workplace risk is small, medium, or high. This step ends with the conclusion (the procedure defined as ***risk characterization***) whether the risk is acceptable (small, medium) or unacceptable (high).

### *11.3.5 Planning Measures and Activities to Eliminate, Prevent, or Reduce the Risk*

After assessing the workplace risk for each hazard and its characterization, the fourth step is aimed at ***planning measures and activities to eliminate, prevent, or reduce the risk*** to the smallest possible level with a subsequent ***audit of the assessment***. At the same time, during this ***risk management*** phase, responsible persons are identified for the implementation of each of the necessary activities within the proposed measures.

Preventive and protection measures are implemented according to a certain list of priorities, starting from the elimination of hazard/risk, minimizing hazard/risk through organizational measures, minimizing hazard/risk by collective preventive measures, to reducing the risk by the application of appropriate personal protective equipment.

When selecting preventive measures and determining the level of risk control, the so-called ALARP principle (As Low As Reasonable Practicable) is implemented, which requires the risk level to be reduced to a "rationally viable (manageable) limit." The adequacy of the applied or proposed measures is assessed and monitored by comparing with examples of good practice or through a cost/benefit analysis.

#### 11.3.5.1 Measures and Activities to Reduce the Risk Caused by Allergens and Irritants

The preventive measures which can be used to reduce the risk of chemical substances (whether allergens or irritants), involve:

- Substituting toxic with less toxic substances
- Elimination of carcinogenic substances
- Providing Material Safety Data Sheets for all hazardous chemical substances
- Installing appropriate collective protection equipment
- Ensuring personal protective equipment for all workers
- Providing local exhaust ventilation at the workplaces with high concentration of chemicals
- Ensuring regular medical examinations of workers exposed to chemical substances, and/or
- Regular education and training of workers

Some of the additional preventive measures that can be implemented in woodworking industry to reduce the risk caused by allergens and irritants are the following or similar:

- Regular cleaning of the workplace
- Regular checking and cleaning of the exhaust ventilation system
- Using automated and hermetic systems for applying coatings and adhesives
- Educating workers about healthy lifestyle, and/or
- Providing adequate work–rest schedule and breaks

### *11.3.6 Documenting the Assessment of Risks Arising from Workplace Hazards, Including Allergens and Irritants*

The last step in the process of workplace risk assessment practically refers to ***documenting risk assessment***. All activities are recorded by application of appropriate forms containing: general data (name of the company and address, name of the workplace, names of the persons working at that workplace, date of the assessment, names of the persons who have conducted the assessment); list of the identified hazards and dangers; already used preventive/protective measures to reduce the risk of the identified hazards and hazards; results and conclusion of risk assessment and

further planned activities for the reduction of workplace risk. The results of the risk assessment are useful for designing and implementing programs and interventions aimed to reducing risk as well as for evaluation and monitoring of the changes resulting from such programs.

### *11.3.7 Communication of Risks Arising from Workplace Hazards, Including Allergens and Irritants*

The process of risk analysis has to include ***risk communication*** activities as a final step. That is a purposeful exchange of information about risks between interested parties. Workplace risk communication is the act of conveying or transmitting information between parties about the risks identified at the workplace [16]. Within the field of occupational health and safety, interested parties include government, enterprises, trade unions, employers and workers, organizations of employers and employees, safety at work engineers, specialists in occupational medicine, scientists, and professional organizations. The risk communication means have to be usable (easy to be read, understood and remembered) and useful (to be relevant and helpful). It is also important to use appropriate frame of the risk communication in order to achieve influence on the intentions to follow safe practice and safe behavior. In that context, risk communication could be formulated in either "negative" (e.g., negative consequences of not performing a safe working practice) or "positive" (e.g., positive consequences of performing a safe working practice) meaning [17].

Risk communication is aimed to: raise awareness, encourage protective behavior, inform to build up knowledge on hazards and risks, improve relationships (build trust, cooperation, networks), and involve key actors in decision-making. It engages many techniques and activities ranging from media and social media communications, mobile phone apps, organization of meetings, workshops, courses, and seminars, preparation and distribution of flyers and brochures [18].

## 11.4 Conclusion

The risks arising from occupational allergens and irritants have to be included in the workplace risk assessment. Special focus has to be put on the recognition of allergies, respiratory and skin disease in exposed workers. Different checklists are used in identifying workplace chemical substances (including allergens and irritants). Preventive and protection measures to reduce risks arising from workplace allergens and irritants have to be implemented according to a certain list of priorities. The communication of risks arising from workplace allergens and irritants should contribute to building workplace preventive culture, reducing incidence of respiratory, skin and other occupational allergic diseases, and promoting workplace health and safety.

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# Chapter 12
# Pesticide and Immunotoxicology


**Tomoki Fukuyama and Risako Tajiki-Nishino**

**Abstract** Several types of pesticides such as organophosphates, phenoxyacetic acid, and carbamate have a high risk of affecting human immune system, causing immune suppression, allergies, and autoimmune diseases. However, immunotoxicology of pesticides has not been characterized well so far. Therefore, this chapter will address the immunotoxic response including immunosuppression, hypersensitivity, and autoimmune diseases to environmental chemicals such as pesticide, herbicide, and fungicide. In addition, current detection method for immunotoxic substances is not sensitive and optimized enough to look for the events associated with pesticide exposure, whereas general toxicity, reproductive toxicity, neurotoxicity, and genotoxicity are basically well detected throughout the international guidelines issued by Organization for Economic Cooperation and Development (OECD) or United States Environmental Protection Agency (EPA). Recently, our group has developed several immunotoxicology tests particularly for immunosuppression and respiratory sensitization both in vivo and in vitro. This chapter will also focus on newly developed testing method to detect the immunotoxic event associated with pesticide exposure.

**Keyword** Pesticide · Immunosuppression · Allergy · Autoimmune disease

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## 12.1 Introduction

Exposure to environmental agents can compromise numerous immunological functions. In the United States alone, 20,000 pesticide products are on the market, and 1 billion pounds of active ingredients are applied annually for agricultural, industrial, and residential pest control [1]. Current immunotoxicological analyses can evaluate the potential adverse effects of several types of pesticides, such as organophosphorus and organochlorine, on host immune mechanisms. Many of these effects can lead to harmful changes in host responses, including increased susceptibility to infectious diseases and tumorigenesis, the induction of hypersensitivity reactions, or an increased incidence of autoimmune disease [2].

Immunotoxicological testing has emerged in this decade as an important adjunct to routine safety evaluations of environmental chemicals, and has been incorporated into the guidelines issued by several regulatory authorities, including the Environmental Protection Agency, Food and Drug Administration, and the International Conference on Harmonization. The most common immunotoxicology guidance documents recommend T-dependent antigen response tests primarily because this assay represents a comprehensive evaluation of immune function based on an assessment of various components of the immune system involved in an antigen-specific antibody response [3]. However, original 28-day exposure protocol is time-consuming, costly, and may lead to immunotoxic drug resistance. Therefore, our research group recently developed a new immunotoxicity testing by focusing on short-term exposure protocol in mice [4, 5]. We will introduce the outline of our findings in this issue.

Pesticide-induced allergies are also focused as a part of immunotoxicity nowadays. Allergy is defined as an excessive immune response to xenobiotic. In particular, contact dermatitis is the prevalent occupational diseases in farmers since there are a lot of opportunities for transdermal exposure to the pesticide [6]. Previous studies identified that several pesticides such as Alachlor and carbamates (maneb, carbofuran, carbaryl) induce the allergic contact dermatitis [7, 8]. Asthma-like disease is also induced by inhaled pesticide such as 2,4-D (herbicide) and tetramethrin which is pyrethroid [9, 10].

Several animal models have been tested to identify chemical allergy [11, 12]. However, although environmental chemical allergens tend to have weak, minimal immunogenicity, these methods have focused on the detection of strong allergic reactions. Recently, the local lymph node assay was developed initially as an alternative approach to hazard identification [13]. It has now been evaluated extensively and validated formally [13, 14] because of having high sensitivity and convenience of use. However, there is no official test method being fully capable of assessment of respiratory sensitizers. In this issue, we will introduce our newly developed detection method of environmental-chemical-related respiratory hypersensitivity in mice.

Recently, several pesticides, including Hexachlorobenzene and Malathion, have been identified as a pesticide which induces or aggravates the autoimmune disease

in a mouse model [15, 16]. The mechanism of autoimmunity by pesticide is thought to change in the antigens associated with self or in the recognition of self by pesticide [17]. In a similar fashion, our group recently reported that immunosuppressive chemicals are indirectly involved in the aggravation of chronic allergy including atopic dermatitis and asthma [18, 19]. Current chapter will also describe our recent findings regarding immune enhancement induced by indirect exposure to pesticide.

## 12.2 Immunosuppression Induced by Pesticide

It is well known that exposure to several types of pesticides such as organochlorine, organophosphate, and organotin compounds, influence the human immune system and play roles in dysregulating immune functions [20–22]. Actually, previous studies have indicated that organochlorine pesticides such as dichlorodiphenyltrichloroethane (DDT) and lindane, as well as organophosphate pesticides such as parathion, malathion, and diazinon, suppress humoral immune response to T-dependent antigens [23–25]. Our group recently focused on the acquired immunotoxicity induced by Methoxychlor (MXC), an organochlorine pesticide p,p′-methoxy derivative of DDT, that has relatively low toxicity in mammals and lower bioaccumulation and higher degradability than DDT in the environment [26, 27]. However, immunosuppressive effects of MXC were still uncertain at that moment. Therefore, the immunosuppressive effects of MXC was evaluated in mice by the T cell-dependent antibody response to sheep red blood cells (SRBC) with plaque-forming cell (PFC) assay and enzyme-linked immunosorbent assay (ELISA) methods and by conducting phenotype analysis of splenic lymphocytes and histopathological analysis of the spleen [28]. Our findings indicated that oral administration of 30, 100, or 300 mg/kg MXC for 1 week significantly reduced IgM PFC response to SRBC and serum anti-SRBC IgM antibody titer. Comparable reductions were seen in splenic T cell populations, cellularity in the PALS (T cell area), and germinal center development in spleen. Thus, a relationship between a suppressive change in IgM PFC responses and decrease in the splenic T cell population and the T cell area of PALS is implied as a result of short-term repeated MXC exposure. T cell function, especially for helper T cells, has also been suggested as a target of immunotoxicity via the suppression of antibody production [29–31].

The original guidelines of immunotoxicology basically recommend using repeated doses, 28-day exposures, and adult animals to detect immunotoxicity caused by environmental chemicals. However, long-term exposure is time-consuming, costly, and may lead to immunotoxic drug resistance because the immune system is highly sensitive to the toxic effects of several types of chemicals [5]; such a change would distort estimates of advanced immunotoxicity. Therefore, new short-term exposure protocols are required to detect immunosuppression.

In the first stage of our studies, we developed a short-term method (administration via oral gavage for 3 days) for detecting thymocyte apoptosis induced by MXC [5, 32]. The thymus is responsible for the maturation and differentiation of most

peripheral T-lymphocytes [33]. It has long been known to be vulnerable to atrophy associated with exposure to a variety of substances, including hormones, immunosuppressive pharmaceuticals, and environmental chemicals [34–38]. Our findings indicated that MXC induced prominent increases in several parameters indicative of induced thymocyte apoptosis, including Annexin $V^{-}FITC^{+}$ cells, caspase 3/7, caspase 8, and caspase 9 activities, and DNA fragmentation, suggesting that short-term exposure has the potential to detect the immunosuppression caused by chemicals present in the environment.

Secondary, we aimed to develop a new short-term immunotoxicology protocol using several immunologic endpoints that tested short-term exposure to MXC, parathion, and the agricultural insecticide synergist piperonyl butoxide. Our results showed that MXC, parathion, and piperonyl butoxide each could reduce the anti-SRBC IgM response (SRBC-specific IgM levels in serum and the IgM PFC response to SRBC in splenocyte), and the numbers of IgM- and germinal center-positive B-lymphocytes in the spleen of mice. Our protocol detected pesticide-induced immunotoxic responses, such as increased apoptosis in lymphocytes in vitro, decreased antigen-specific IgM responses, and decreased IgM- and germinal center-positive B-lymphocyte counts. Additional studies to confirm these results should be expanded to include other parallel changes in cellular function that can occur in response to chemical exposure, as well as immunologic or histologic markers.

## 12.3 Several Types of Allergy Induced by Pesticide

Pesticides that have diffused into ambient air have a high risk of causing contact dermatitis, allergic rhinitis, and bronchial asthma [39–43]. Previous studies actually identified that Alachlor and carbamates induce the allergic contact dermatitis [6, 7]. Although aerial application of pesticides as a major source of exposure has been restricted in Europe and the United States in recent years, the ease of aerial spraying has still led to its wide use on many farm products in many countries [41]. Therefore, there is a need for protocols for the treatment and detection of dermal or respiratory allergic diseases triggered by pesticides that have diffused into the environment.

Several detection methods have been developed to identify chemicals that trigger skin sensitization such as guinea pig maximization test and Buehler test [11, 12]. Recently, the local lymph node assay (LLNA) was developed initially as an alternative approach to hazard identification [13, 14]. This assay measures proliferation of the draining lymph node cells and it comprises a sensitizing phase only. However, these methods have focused on the detection of strong allergic reactions, whereas environmental chemical allergens (such as diffused pesticides) tend to have weak or

minimal immunogenicity. Moreover, there is no official test method being fully capable of assessment of respiratory sensitizers. Therefore, new protocols are needed to detect particularly in respiratory allergies caused by weakly immunogenic and low-dose allergens.

In our first step of studies, we developed an improved model of the LLNA that uses long-term dermal sensitization followed by dermal challenge to clear up detection of weakly immunogenic and low-doses allergic reactions by pesticide [44]. After topically sensitizing BALB/c mice (9 times in 3 weeks) and topical challenging them with well-known Th2 type sensitizers (trimellitic anhydride (TMA) and toluene diisocyanate (TDI)), we assayed their auricular lymph nodes (LNs) for number of lymphocytes, surface antigen expression of B cells, and local cytokine production, and measured antigen-specific serum IgE levels. TMA and TDI induced marked increases in levels of antigen-specific serum IgE and of Th2 cytokines produced by ex vivo restimulated lymph node cells. Both chemicals induced significant increases in number of lymphocytes and surface antigen expression of B cells.

In the next step, we attempt long-term dermal sensitization followed by a low-dose intratracheal challenge to evaluate sensitization by the well-known respiratory sensitizers TMA and TDI [45]. TMA induced marked increases in antigen-specific IgE levels in both serum and bronchoalveolar lavage fluid (BALF) (see Fig. 12.1), proliferation of eosinophils and chemokines in BALF, and proliferation of Th2 cytokines in restimulated LN cells. TDI induced marked increases in levels of cytokines produced by restimulated LN cells. Our protocol thus detected respiratory allergic responses to low-molecular-weight chemicals and may be useful for detecting environmental chemical-related respiratory allergy.

In the final stage of our studies, we examined the allergic reactions caused by several types of pesticides using our long-term sensitization method in conjunction with a local lymph node assay [9]. The chemicals used in the study were the 2,4-dichlorophenoxyacetic acid pesticide (2,4-D), the organophosphorus pesticide BRP, and the carbamate pesticide furathiocarb. Our results indicated that upregulation of eosinophil proliferation, chemokine levels, and Th2 cytokine production in 2,4-D-treated groups being associated with a subsequent increase in IgE production in the serum and BALF (see Fig. 12.2). On the other hand, BRP or furathiocarb treatment induced MHC-class-II-molecule expression and Th1 cytokine production, but there was almost no eosinophil or Th2 cytokine production in the respiratory challenge protocol, and this lack of reaction was associated with the lack of increase in IgE production observed in the serum and BALF analyses. These results suggest that 2,4-D is a respiratory allergen and the others are contact allergens. Furthermore, our original protocol demonstrated that analysis of BALF and lung-associated LNs made it possible to detect and classify a chemically induced allergy by its type for further validation.

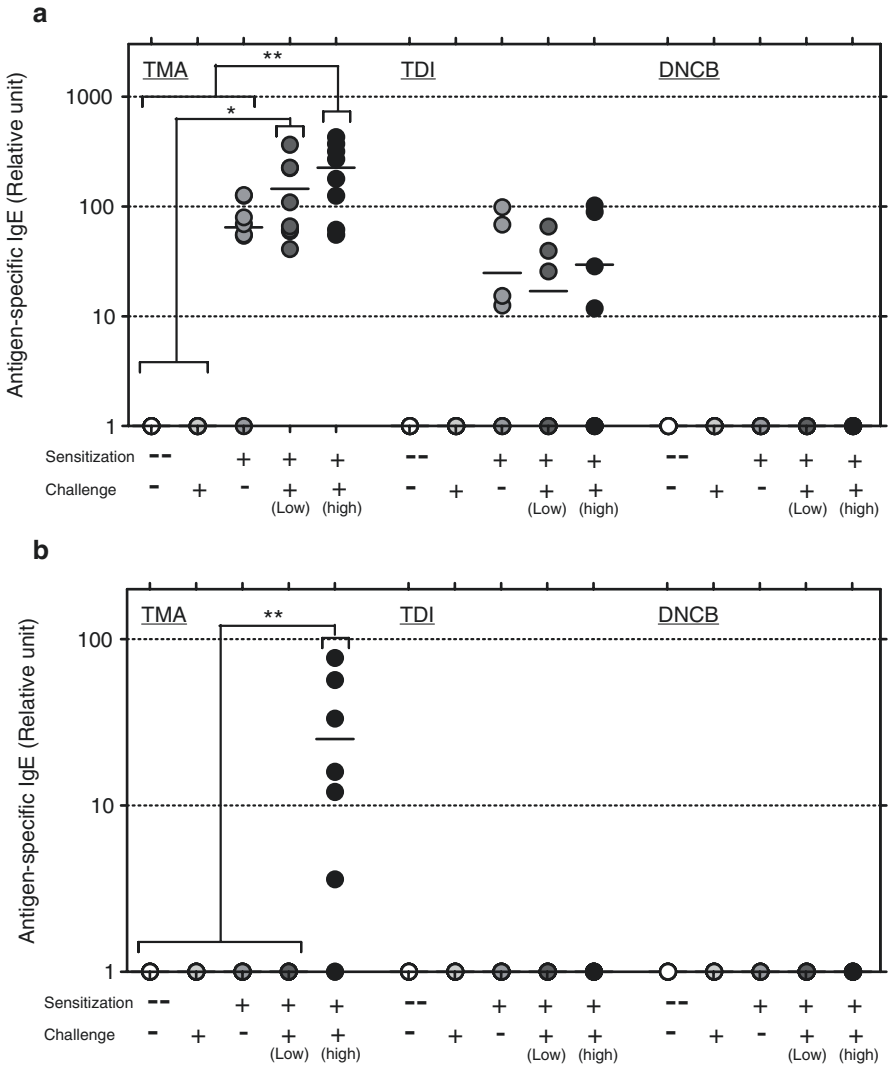


**Fig. 12.1** Antigen-specific IgE levels in mouse serum (**a**) and BALF (**b**) isolated 1 day after challenge with test solution. Results are expressed as mean (titer) ± S.D. Statistical significance is marked by asterisks: * for $P < 0.05$, ** for $P < 0.01$ (Tukey's *t*-test)

## 12.4 Pesticide Induced Indirect Immune Disorders

Recent investigations suggested that pesticide causes not only direct damages of the immune system, but also indirect damages since many kinds of cells are intricately involved in immune system. Actually, current evidence suggests that environmental chemicals including pesticides may increase the potency of allergens and thereby play a role in the development of allergic diseases [46, 47].

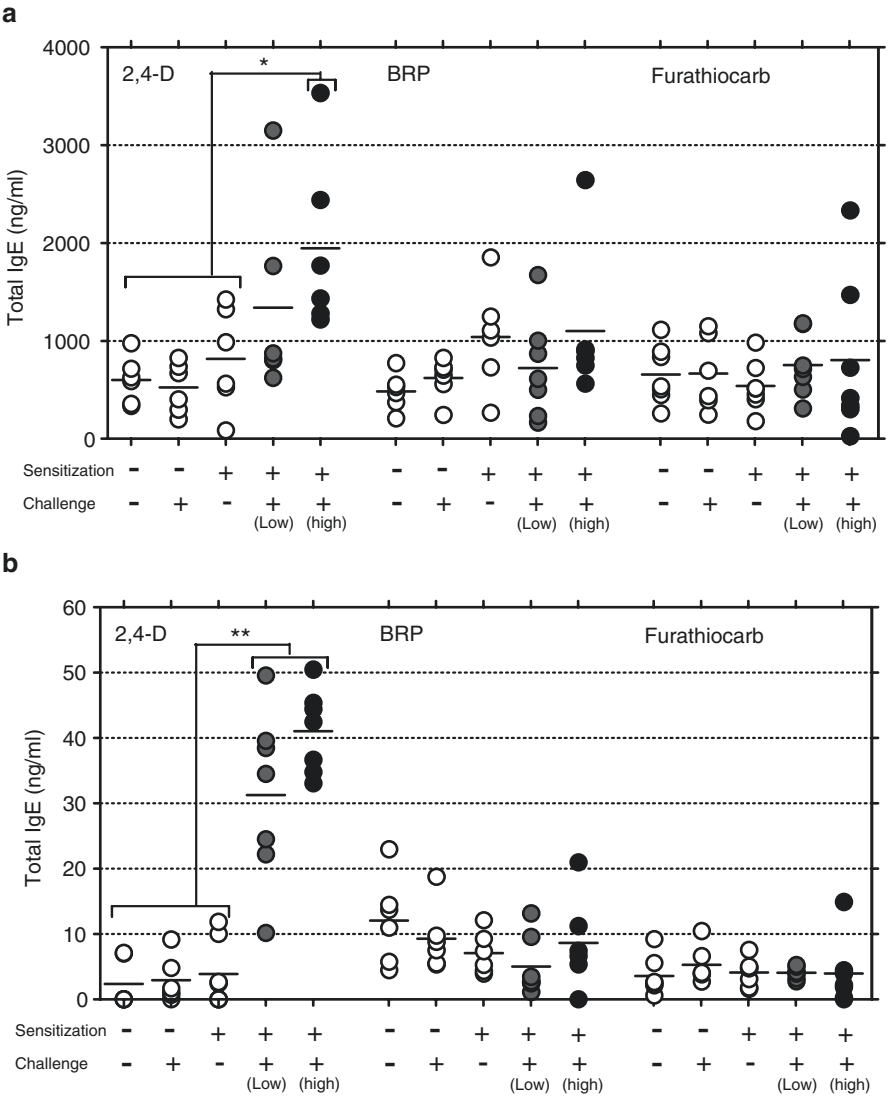


**Fig. 12.2** Total IgE levels in mouse serum (**a**) and BALF (**b**) isolated 1 day after challenge with test solution or solvent alone in respiratory challenge protocol. Figure shows individual values of the IgE level (ng/ml) and bars are expressed as mean (ng/ml). Statistical significance is marked by asterisks: ** for $P < 0.01$ (Tukey's *t*-test). $n = 6–7$ per group

Previously, our research group examined the relationship between immune disorders and the immunosuppression induced by immunosuppressive pesticides. We focused on the modulation of allergic potential by parathion, MXC, 2,4-D-butyl, and benzoic acid fungicide eugenol, as detected by an LLNA. Parathion and MXC are immunosuppressive chemicals, and 2,4-D-butyl and eugenol are contact allergens. Although parathion and MXC played roles in suppressing immune functions,

we demonstrated conflicting results in studies where T-lymphocyte-mediated allergic reactions were induced by prior oral exposure to MXC or parathion. Our findings clearly indicated that skin sensitization potential by 2,4-D-butyl or eugenol were significantly worse in parathion- and MXC-pretreated groups [48, 49].

In order to understand this paradox, we further examined whether prior oral administration of parathion or MXC during immature life stage affect the development of atopic dermatitis and asthma in the mature life stage. Our results demonstrated that prior exposure to parathion or MXC can modulate immune functions and increase the severity of atopic dermatitis and allergic asthma in mice. Prior exposure of parathion or MXC induced ear thickness, IgE level in serum, T and B cells counts in lymph node, and pro-inflammatory cytokine productions [18, 19] (see Figs. 12.3 and 12.4). In contrast, there were no changes, or some suppression,

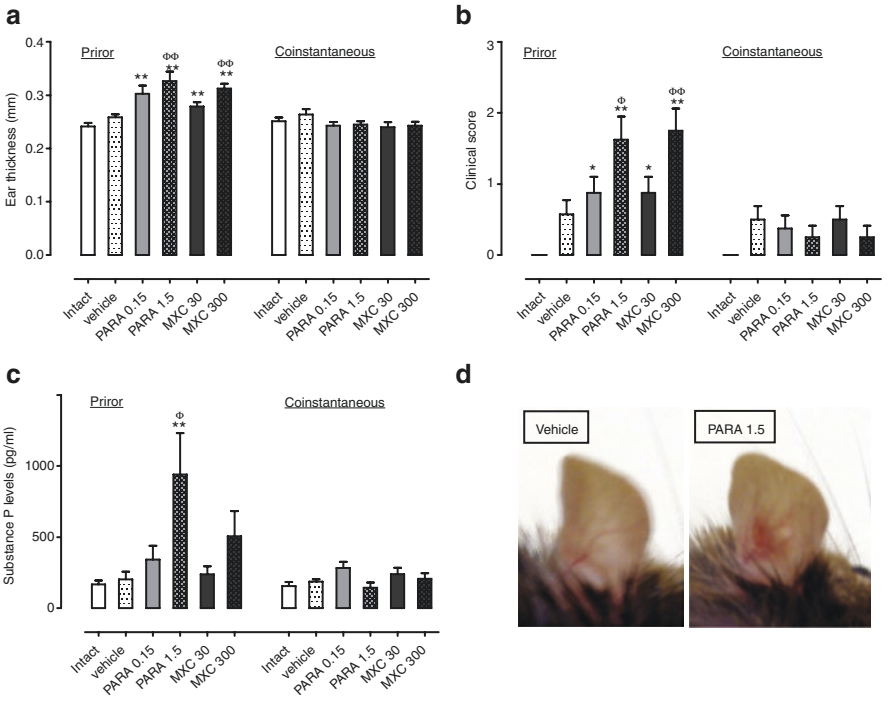


**Fig. 12.3** Ear thickness, clinical symptoms, and serum levels of substance P of mice in the two experimental protocols. (**a**) Ear thickness, (**b**) clinical symptoms and (**c**) serum levels of substance P of mice sensitized and challenged with Df with prior or coinstantaneous exposure to nothing (Intact), vehicle, parathion or methoxychlor. Ear thickness values are expressed as mean (mm) ± SD ($n = 8$ per group). Clinical scores are expressed as mean ± SD ($n = 8$ per group). Substance P levels are expressed as mean (pg/ml) ± SD ($n = 8$ per group). Statistical significance is indicated by asterisks: $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the intact group (Dunnett's multiple comparison test); $^{\Phi}P < 0.05$ and $^{\Phi\Phi}P < 0.01$ compared with the vehicle control group (Dunnett's multiple comparison test). (**d**) Representative clinical features of mice, from the prior exposure protocol after the 4 weeks oral exposure, to vehicle or methoxychlor (300 mg/kg). *PARA* parathion, *MXC* methoxychlor

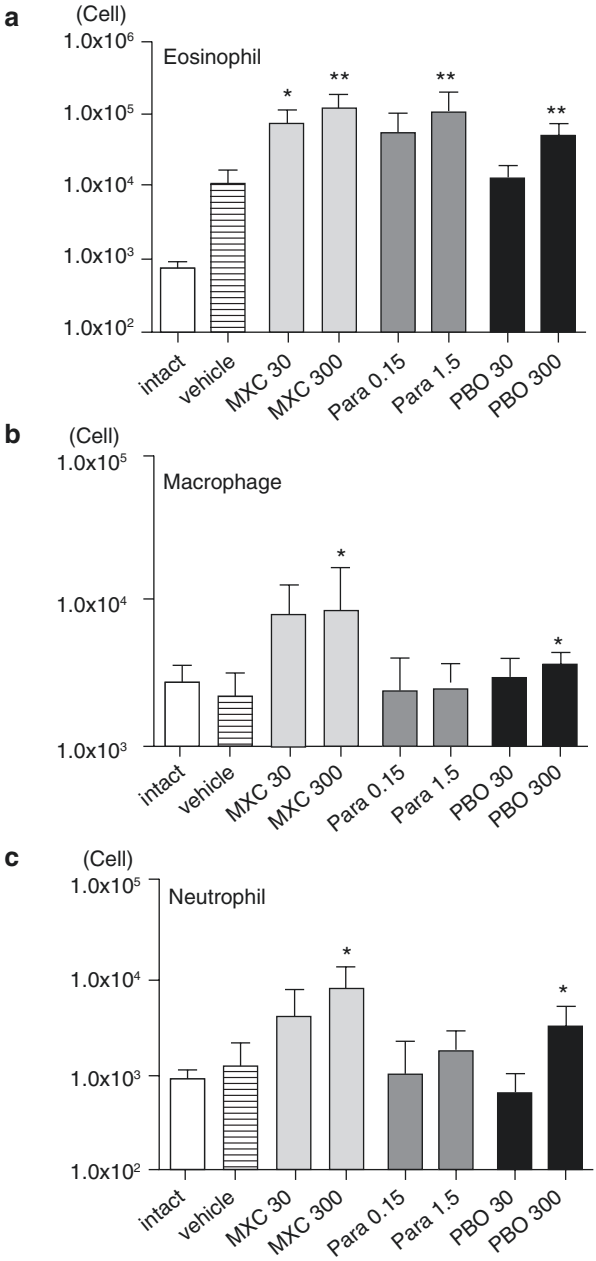


**Fig. 12.4** Eosinophil (**a**), macrophage (**b**) and neutrophil (**c**) counts in BALF. Designations of treatments are as in Fig. 12.2. Cell counts are expressed as mean ± SD ($n = 8$ per group). Statistical significance of the differences from the vehicle control group is indicated by asterisks ($^{*}P < 0.05$ and $^{**}P < 0.01$; Dunnett's multiple comparison test)

in groups coinstantaneously exposed to immunosuppressive environmental chemicals. These findings of upregulation and downregulation suggest that parathion and methoxychlor do not act as self-antigens. Interestingly, prior exposure to immunosuppressive pesticides induce a decrease in the number of regulatory T cells, which may influence this process [50], suggesting that immunosuppressive pesticides act

on preventing central tolerance of autoreactive T or B cells and/or altering gene expression.

Several studies linking environmental chemicals with immune disorders have noted the estrogenic character of those chemicals [51–53]. MXC is known to have estrogenic effect and estrogenic compound is reported to induce the immune disorders by binding estrogen receptor [54]. However, our observation that prior exposure to parathion, which is not a hormone disruptor, increased all the parameters we measured suggests that the increase in atopic dermatitis or asthma severity is not due to hormonal effects. To our knowledge, this is the first study to demonstrate the relationship between immune disorders and the non-estrogenic environmental chemicals.

Although several studies have shown that some environmental chemicals lead to autoimmune disease [15, 16], the relationship between chronic allergic diseases and the breakdown of immune regulation induced by exposure to environmental chemicals is not fully understood. Our report suggests directions for further investigation into mechanisms of atopic dermatitis caused by environmental chemicals.

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# Chapter 13
# Clinical Evaluation of Plasma Decoy Receptor 3 Levels in Silicosis

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**Abstract** Silicosis (SIL) is known to complicate various autoimmune diseases such as rheumatoid arthritis and systemic sclerosis (SSc). To investigate the immunological alterations in SIL, plasma decoy receptor 3 (DcR3) levels were measured. Additionally, correlation studies, multiple regression analysis, and factor analysis were performed using various clinical parameters including respiratory and exposure items, and immunological parameters such as cytokine levels and titers of various autoantibodies detected in SIL subjects. Although actual DcR3 values in SIL and SSc subjects were higher than those in HV, since age was the confounding factor, there were no significant differences. However, in terms of the role of

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DcR3 in SIL, positive correlations were found between DcR3 and TGF-β or soluble IL-2 receptor (sIL-2R). Multiple regression analysis showed a close and positive relation in SIL between DcR3 and G-CSF, and TGF-β and CENP-B antibodies. Finally, factor analysis indicated that DcR3 values were related to ANA and ANCA-antibodies, as well as G-CSF and IL-6. These data suggested that DcR3 could potentially be utilized as a representative marker of immunological dysfunction in SIL. Further studies are required to explore the cellular and molecular roles of DcR3, and to evaluate the clinical efficacy of utilizing DcR3 measurements for the early detection of complicated autoimmune diseases in SIL patients.

**Keywords** Silicosis · Autoimmune diseases · Systemic sclerosis · Decoy receptor 3

## 13.1 Introduction

Silicosis is known to be caused by the inhalation of crystalline silica particles, and typically affects workers in the mining, sandblasting, quarry, ceramic, and foundry industries, in addition to grinders, stone cutters, refractory brick workers, tombstone workers, and pottery workers [1–5] as shown in Fig. 13.1a. Silicosis is a progressive lung fibrosis condition. The important cellular and molecular mechanisms involved in the development of silicosis are thought to include activation of NACHT, LRR, and PYD domains-containing protein 3 (NALP3)-inflammasome in alveolar macrophages, which recognize silica particles as foreign, and signal the release of interleukin (IL)-1β and IL-18 which results in fibrosis surrounding the area at which the silica particles have accumulated [6–8]. These fibrotic changes lead to the development of small nodules which can be radiologically detected and sometimes can grow to more than 1 cm in diameter [2, 9–12]. Pulmonary complications of silicosis include lung tuberculosis, chronic bronchitis and airflow limitation (indistinguishable from that caused by smoking), nontuberculous mycobacterium infection, fungal lung infection, compensatory emphysema, and pneumothorax [1–5]. More recently, lung cancer has also been defined as being caused by silica exposure, and the International Agency for Research on Cancer (IARC) has included crystalline silica as a Group 1 carcinogen [13] (Fig. 13.1a).

In addition to various pathological changes in the lung, silicosis is known to complicate various autoimmune diseases such as rheumatoid arthritis (known as Caplan's syndrome), systemic sclerosis (SSc), systemic lupus erythematosus (SLE), and antineutrophil cytoplasmic antibody (ANCA)-related vasculitis/nephritis [14–21] as shown in Fig. 13.1a. The causative mechanism responsible for the silica-induced dysregulation of autoimmunity is thought to involve an adjuvant effect of silica particles to present relatively small molecules as self-antigens to be acted on by antigen-recognizing cells [22].

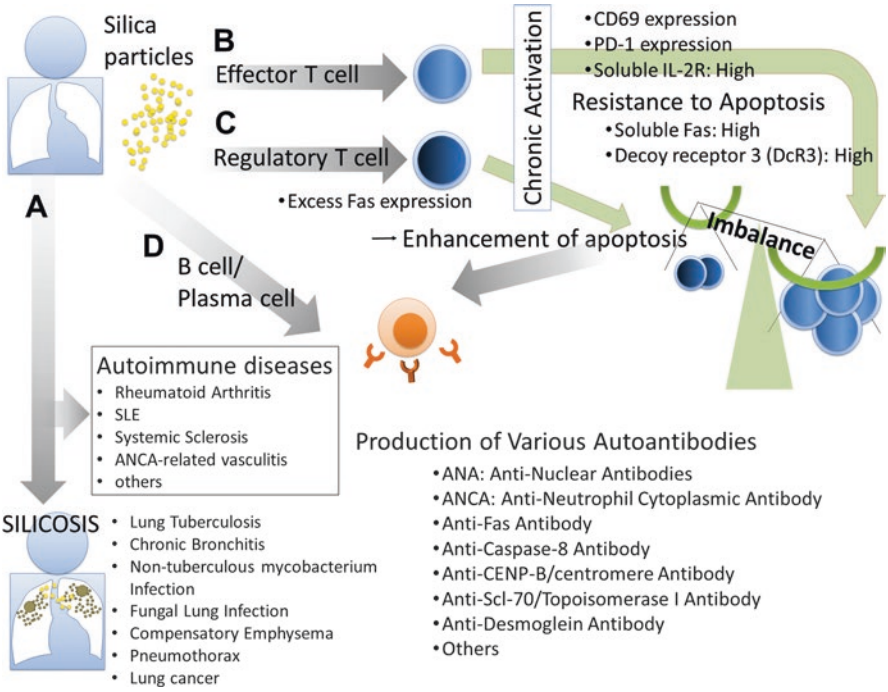


**Fig. 13.1** (**a**) Silicosis patients suffering from lung fibrosis and pulmonary complications as well as associated autoimmune diseases. (**b**) The effects of silica particles on effector T cells comprise evidence of chronic activation such as CD69 expression, PD-1 expression and higher serum soluble interleukin-2 receptor concentration, and resistance to apoptosis such as higher serum soluble Fas molecules and higher expression of decoy receptor 3 (DcR3) in peripheral blood mononuclear cells. (**c**) The effects of silica particles on regulatory T cells (Treg) revealed excess expression of Fas molecules causing early loss of Treg. These outcomes resulted in an imbalance of effector T cell dominance relative to Treg cells. (**d**) Various autoantibodies were detected in silicosis patients

However, recent investigations have indicated that silica particles may activate and increase the survival of responder T helper (rTh) cells (Fig. 13.1b), as well as CD4, CD25 and forkhead box P3 (FoxP3; a transcription factor) positive regulatory T (Treg) cells by facilitating progression to CD95/Fas-mediated apoptosis (Fig. 13.1c). These effects on rTh and Treg cells induce an imbalance (increase and decrease, respectively) in these two types of T helper subpopulations, thereby increasing the possible occurrence of autoimmune diseases [23–25]. In particular with regard to Treg cells, surface Fas molecules are expressed in excess by chronic exposure to silica particles and results in enhancement of Fas-mediated apoptosis in Treg cells [23] as shown in Fig. 13.1c.

Various molecules in the serum of silicosis (SIL) patients such as soluble Fas (sFas) [26], soluble Fas-ligand [27], and soluble IL-2 receptor (sIL-2R) [28] have been investigated and previously reported (Fig. 13.1b). Additionally, sFas expression in peripheral blood mononuclear cells (PBMCs) derived from SIL patients was

higher compared to cells derived from healthy volunteers (HV) [29, 30]. Furthermore, mRNA expression of decoy receptor 3 (DcR3) was also higher in PBMCs derived from SIL patients compared to HV [31]. DcR3 was initially identified in lung and colon cancer cells, and found to prevent cancer cells from inhibiting the binding between Trail-receptor, expressed in cancer cells, and Trail, secreted by tumor-attacking immune cells as a decoy receptor [32, 33]. Regarding rTh activation and survival, sFas and DcR3 seem to act similarly to prevent Fas- and Trail-induced apoptosis of rTh. This condition may lead to increased survival of these cells, including that of self-antigen reacting T cells, and therefore increased susceptibility to autoimmune diseases [24, 25, 30](Fig.13.1b).

Additionally, our previous investigations identified various autoantibodies in silicosis patients as shown in Fig. 13.1d [34–38]. The physiological roles of these autoantibodies in silicosis patients in addition to the mechanistic changes of rTh and Treg have been described in our previous reviews [39–43].

Recently, the measurement of DcR3 was achieved using an ELISA assay and elevated serum DcR3 levels were reported with some autoimmune diseases [44–50]. Thus, we present and compare the plasma concentration of DcR3 in SIL patients with that of HV and SSc subjects. Additionally, other parameters related to abnormality of autoimmunity, as well as respiratory and exposure conditions, are analyzed with respect to the clinical role of DcR3 in SIL.

## 13.2 Methods for Determination of Serum DcR3 Levels in Silicosis

### *13.2.1 Subjects*

All subjects were Japanese. Twenty SIL subjects (age: average ± standard deviation (SD) = 74.9 ± 5.4, 19 males and 1 female), 19 HV (age 44.8 ± 8.6, 9 males and 10 females) and 25 SSc subjects (age 62.3 ± 12.1, 3 males and 22 females) were employed in this study. All SIL subjects were medically followed at Kusaka Hospital, Bizen City, Okayama Prefecture, Japan, and were employees at the brick-yard works. The amount of free silica inhaled by the SIL subjects was supposed to be as high as 40–60% as determined from their work environment where they had been working. Bizen City is located on the eastern side of Okayama Prefecture, western Japan, approximately at the midpoint between Osaka and Hiroshima. One of the key industries of Bizen City is a firebrick factory. Many factories were founded approximately 100 years ago and continue their activities at present. Although work environments have improved, prior to the economic growth period of Japan in the 1960s and 1970s, many workers inhaled silica dust and were diagnosed with pneumoconiosis. All SIL subjects were diagnosed with pneumoconiosis according to the International Labor Office (ILO) 2011 revised guidelines [51]. They showed no clinical symptoms of autoimmune diseases such as sclerotic skin,

Raynaud's phenomenon, facial erythema or arthralgia. SSc subjects were monitored in the Department of Dermatology, Kawasaki Medical School Hospital, Kurashiki City, Okayama Prefecture, Japan. Heparinized peripheral blood was drawn from the cubital vein from all subjects. All specimens were taken only when informed consent had been obtained. This study was approved by the Ethics Committee of Kawasaki Medical School and Kusaka Hospital.

### *13.2.2 Plasma DcR3 Levels and Other Clinical Parameters*

Since PBMCs were being utilized in other research projects, plasma DcR3 concentrations were determined in lieu of serum concentrations. Plasma DcR3 was measured using the DcR3 Human ELISA Kit (ab193697) (Abcam Japan, Tokyo, Japan) according to the manufacturer's instructions.

For SIL subjects, additional clinical parameters related to autoantibodies, immunological features, as well as respiratory and exposure conditions were measured.

Anti-nuclear antibody (ANA), anti-P (myeloperoxidase)-ANCA antibody, anti-C (cytoplasmic/proteinase)-ANCA antibody, Scl-70 (anti-topoisomerase 2) antibody, CENP-B (anti-centromere antibody), Rheumatoid factor, and anti-CCP: cyclic citrullinated peptide (CCP) antibody were measured using ELISA-based MESACUP ANA or CCP test kits (MBL. Co., Ltd., Nagoya, Japan).

Immunoglobulins (Ig: G, A, M and subclasses of Ig G, G1 to 4) were measured in the Okayama Medical Laboratory (Kurashiki, Okayama, Japan) using patient plasma.

Various cytokines (IL-10, transforming growth factor (TGF)-β, IL-6, Granulocyte colony-stimulating factor (G-CSF), IL-1α, sFas, sIL-2R and soluble IL-6 receptor (sIL-6R)) were measured using an ELISA kit (Human sAPO-1/Fas, sIL-6R and sIL-2R Platinum ELISA, eBioscience, Affymetrix Inc., Santa Clara, CA., and Bio-plex multiplex system using Luminex, Bio-Rad, Hercules, CA).

Respiratory examination and history of exposure included the following items: age, profusion rate (PR; according to the ILO pneumoconiosis radiological classification [51], 2011 revised guidelines, 1–4), exposure years (ExpYear; according to occupational history), subjective dyspnea (numbered 1 (slight) to 4 (severe), according to the Hugh-Jones classification), percentage of forced expiratory volume in 1 second (FEV1), peak flow rate (PFR) at 25% forced vital capacity (FVC)/Height (v25H), and percent volume capacity (%VC). All items requiring measurement were obtained at Kusaka Hospital. The nearest clinical information was collected when blood samples were collected from Kusaka Hospital. Since all SIL patients receive medical examinations specific to pneumoconiosis every 6 months, all respiratory as well as immunological data represented the nearest actual data during a period of at most 6 months. Pulmonary function tests were performed by skilled clinical laboratory technologists belonging to Kusaka Hospital.

### *13.2.3 Statistical Analyses*

All statistical analyses were performed using SPSS v. 21 (Japan IBM Co. Ltd., Tokyo, Japan). A comparison of plasma DcR3 levels among HV, and SIL (actual and age-corrected values) and SSc (actual and age-adjusted values) subjects was analyzed using a bilateral Student's T test. Multiple regression analysis was performed to identify items which contribute to the DcR3 value among the other clinical parameters including autoantibodies, cytokines, and respiratory parameters. Additionally, factor analysis among the autoantibodies, cytokines and respiratory parameters was performed in an effort to understand the role of DcR3 and its relationship among the various other clinical items. Furthermore, SIL subjects were divided into two groups according to their serum DcR3 levels, comprising high and low groups of eight and twelve SIL subjects, respectively, since approximately 40% of SIL subjects showed higher serum DcR3 levels compared with HV. Thereafter, differences between high and low DcR3 groups in SIL were analyzed using a student t-test with all the parameters studied here.

## 13.3 Results of Serum DcR3 Levels Determined in Silicosis

### *13.3.1 Plasma DcR3 Levels*

Figure 13.2a shows the distribution of age and plasma DcR3 levels among HV, and SIL and SSc subjects. Unfortunately, age-matched HV were not collected so that a comparison of DcR3 levels could be made with SIL or SSc subjects. A certain population of SIL and SSc subjects showed higher levels of DcR3. Thereafter, comparisons between groups were performed.

As shown in Fig. 13.2b, the actual values of DcR3 in SIL subjects were significantly higher than those in HV. Additionally, those levels in SSc subjects were also higher than those in HV. However, since the average age of SIL and SSc subjects was relatively high, it was very difficult to collect samples from HV whose ages matched the SIL and SSc subjects in this study. Thus, multiple regression analysis was performed. As a result, no significant differences were found between HV and SIL subjects or HV and SSc subjects when age was the confounding factor.

Thereafter, although the data for HV indicated that age and DcR3 levels showed no correlation ($\rho = 0.201$, $p = 0.409$), the following formula was generated: DcR3 value = 0.624 + Age × 0.00353. If all SIL subjects were adapted to this formula, the age-adjusted DcR3 (aaDcR3) value was 0.888 ± 0.019 (average ± standard deviation (SD), while in the case of SSc subjects the value was 0.844 ± 0.043. A comparison of actual values and age-assumed values of DcR3 for SIL subjects was not significant ($p = 0.114$), while that for SSc subjects showed a slight tendency ($p = 0.056$) as analyzed by the bilateral student's T test. However, 8 out of 20 (40%) SIL subjects showed DcR3 values higher than the average+2SD calculated from the

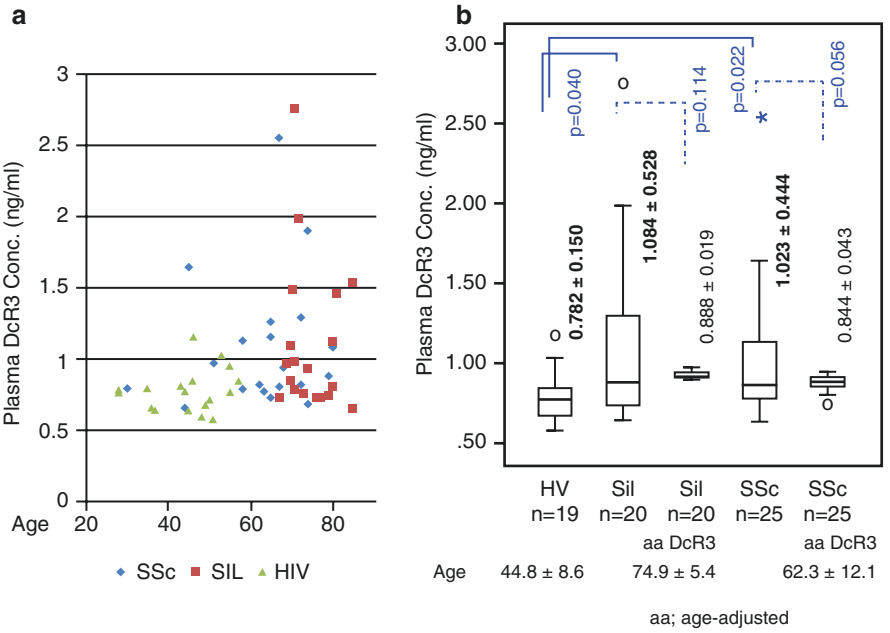


**Fig. 13.2** (**a**) Distribution of plasma DcR3 levels and age among healthy volunteers (HV: green triangle), and subjects with silicosis (SIL: red square) or systemic sclerosis (SSc: blue diamond). (**b**) Plasma DcR3 concentrations in HV, and SIL and SSc subjects. For the SIL and SSc subjects, age-adjusted (aa)-DcR3 values are indicated on the right side of the actual values. Additionally, blue-colored areas indicate SIL and SSc subjects with a higher than average + 2 times standard deviation DcR3 value calculated from the formula extracted from the data for HV

age-assumed DcR3 of SIL subjects. Similarly, 11 out of 25 (44%) SSc subjects showed DcR3 values higher than the average+2SD calculated from the age-assumed DcR3 of SSc subjects.

Additionally, differences between the higher DcR3 group (8 SIL subjects) and the lower DcR3 group (12 SIL subjects) were examined for all other clinical parameters which were measured in this study. Although DcR3 values showed significant differences (average and standard deviation: 0.760 ± 0.074 in lower group subjects and 1.321 ± 0.351 in higher group subjects, $p = 0.0025$), there were no significant differences among all clinical parameters examined.

Taken together, there was no significant difference was found in DcR3 levels among HV, and SIL and SSc subjects. However, certain SIL and SSs subjects showed higher levels of DcR3. This indicated that a consideration of the biological role of DcR3 in SIL may assist in delineating the occurrence of autoimmune disorders found in SIL and the pathophysiology associated with silica-induced disorder of autoimmunity. To be certain, plasma DcR3 levels should be examined in SIL patients for any indication of immunological alterations caused by silica exposure.

### 13.3.2 Correlation between DcR3 Values and Other Parameters in SIL

The correlation between DcR3 values and other parameters examined in this study in SIL were analyzed. As shown in Table 13.1, DcR3 values only showed significant

**Table 13.1** Correlation between plasma DcR3 values and other clinical parameters in SIL

| Correlations between titer of auto-antibodies and DcR3 | | | Correlations between immunoglobulins and DcR3 | | |
|---|---|---|---|---|---|
| ANA | $\rho$ | 0.159 | Total IgG | $\rho$ | 0.074 |
| | p | 0.557 | | p | 0.787 |
| P-ANCA | $\rho$ | 0.324 | $IgG_1$ | $\rho$ | 0.332 |
| | p | 0.222 | | p | 0.208 |
| C-ANCA | $\rho$ | 0.453 | $IgG_2$ | $\rho$ | –0.353 |
| | p | 0.078 | | p | 0.18 |
| Scl-70 | $\rho$ | –0.256 | $IgG_3$ | $\rho$ | 0.05 |
| | p | 0.339 | | p | 0.854 |
| CENP-B | $\rho$ | –0.029 | $IgG_4$ | $\rho$ | 0.203 |
| | p | 0.914 | | *p* | 0.451 |
| RF | $\rho$ | –0.079 | IgA | $\rho$ | –0.279 |
| | p | 0.77 | | p | 0.295 |
| CCP | $\rho$ | –0.206 | IgM | $\rho$ | –0.035 |
| | p | 0.444 | | p | 0.897 |
| Correlations between cytokines and DcR3 | | | Correlations between respiratory parameters and DcR3 | | |
| IL-10 | $\rho$ | 0.438 | Age | $\rho$ | –0.18 |
| | p | 0.09 | | p | 0.505 |
| **TGF-β** | $\rho$ | **0.674** | PR | $\rho$ | 0.074 |
| | **p** | **0.004** | | p | 0.786 |
| IL-6 | $\rho$ | 0.326 | ExpYear | $\rho$ | 0.126 |
| | p | 0.217 | | p | 0.641 |
| G-CSF | $\rho$ | 0.282 | Dyspnea | $\rho$ | 0.295 |
| | p | 0.289 | | p | 0.267 |
| IL-1α | $\rho$ | 0.215 | FEV1.0 | $\rho$ | 0.082 |
| | p | 0.425 | | p | 0.762 |
| sFas | $\rho$ | –0.241 | v25H | $\rho$ | –0.136 |
| | p | 0.368 | | p | 0.617 |
| **sIL-2R** | $\rho$ | 0.556 | %VC | $\rho$ | –0.385 |
| | p | 0.025 | | p | 0.141 |
| sIL-6R | $\rho$ | 0.200 | | | |
| | p | 0.458 | | | |

*SIL* silicosis patients, *DcR3* decoy receptor 3, *ABA* anti-nuclear antibody, *P-ANCA* myeloperoxidase-antineutrophil cytoplasmic antibody, *C-ANCA* cytoplasmic/proteinase-ANCA, *Scl-70* anti-topoisomerase antibody, *CENP-B* Centromere protein B, *RF* rheumatoid factor, *CCP* cyclic citrullinated peptide, *Ig* immunoglobulin, *IL* interleukin, *TGF* transforming growth factor, *G-CSF* granulocyte-colony stimulating factor, *sFas* soluble Fas, *sIL-2R* soluble IL-2 receptor, *PR* profusion rate, *ExpYear* exposure year to silica, *FEV* forced expiratory volume, *v25H* peak flow rate (PFR) at 25% forced vital capacity/Height

correlation with TGF-β (ρ = 0.674, $p = 0.004$) and sIL-2R (ρ = 0.556, $p = 0.025$) values. Both correlations were significant and strong.

From our previous reports [28], sIL-2R levels were elevated in SIL and the average values were located between those of HV (lower) and SSc (higher) subjects. Additionally, sIL-2R values were not correlated with ANA or Scl-70, but were correlated with CENP-B autoantibodies. Moreover, sIL-2R values were not correlated with any respiratory parameters.

Taken together, the DcR3 values in SIL seemed to be related to immunological alterations found in SIL, but not to respiratory changes in SIL. Furthermore, DcR3 values did not seem to be related to any of the autoantibodies examined, and seemed to be associated with alterations in cytokine production in SIL.

### *13.3.3 Multiple Regression Analysis*

In an effort to identify other clinical parameters that regulated DcR3 values in SIL, multiple regression analysis was performed. The parameters included in this assay are listed in Fig. 13.3a. Autoantibodies known to be associated with autoimmune diseases and which are often associated with SIL, such as SLE, SSc, and

**a** Parameters: DcR3, ANA, P-ANCA, C-ANCA, Scl-70, CENP-B, RF, CCP, total IgG, IL-10, TGF-β, IL-6, IL-1 α, sFas, sIL-2R, sIL-6R, Age, PR, ExpYear, dyspnea, $FEV_{1,0}$, v25/H, %VC

**b** **Formula:**
**DcR3 = 0.922 + 0.020 x [G-CSF] + 0.935 x [TGFβ] + 0.005 x [CENP-B]- 0.118 x [Scl-70] - 0.401 x [sFas]**

**c**

| | Coefficient | Significance | Importance |
|---|---|---|---|
| constant | 0.922 | 0.002 | |
| G-CSF | 0.020 | 0.001 | 0.320 |
| TGF-β | 0.935 | 0.001 | 0.296 |
| CENP-B | 0.005 | 0.002 | 0.208 |
| Scl-70 | -0.118 | 0.014 | 0.113 |
| SFas | -0.401 | 0.052 | 0.062 |

**d**

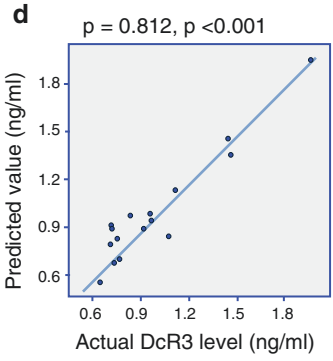


**Fig. 13.3** Multiple regression assay to determine which parameters are related to the DcR3 value. (**a**) Parameters applied to the multiple regression assay. (**b**) Formula employed to determine DcR3 levels extracted from the multiple regression assay. (**c**) Significance of individual variables closely related to DcR3 values in SIL according to the formula shown in Panel B. Panel D: Plot of individual SIL cases showing DcR3 values predicted using the formula in panel B and actual DcR3 values

ANCA-related vasculitis, cytokines and various respiratory and exposure related parameters, were included in the analysis. As a result, the following formula for determining DcR3 values was generated: DcR3 = 0.922 + 0.02 × [G-CSF] + 0.392 × [TGF-β] + 0.005 × [CENP-B]−0.118 × [Scl-70] − 0.401 × [sFas] (Fig. 13.3b). The importance of these extracted parameters is shown in Fig. 13.3c. The significance of these parameters revealed that utilization of sFas values alone are insufficient. Even though a correlation between DcR3 and G-CSF was not found using a simple assay, this formula indicated that G-CSF had some correlation with DcR3 values. Additionally, with respect to CENP-B and Scl-70, although both were detected specifically in SSc subjects, the pathophysiology differed between CENP-B-positive and Scl-70-positive SSc subjects. The former is thought to occur dominantly in localized SSc, such as Morphea, while the latter is observed in systemic SSc and in patients with lung fibrosis and other organic complications such as esophageal sclerosis. With the aforementioned formula, the correlation rates were relatively lower than other parameters, and the positive and negative coefficients found in CENP-B and Scl-70 may be important when considering the type of SSc complications found in SIL.

The values predicted using this formula and the actual DcR3 values are shown in Fig. 13.3d. A strong positive correlation was found and this formula seemed to be reasonable. In other words, the parameters comprising the formula may be related to the pathology of DcR3 in SIL.

### *13.3.4 Factor Analysis*

Factor analysis is a statistical method used to evaluate variability among observed and correlated variables in terms of a potentially lower number of unobserved variables called factors. In this study, we set out to determine whether the titer value of the DcR3 level in SIL was related to respiratory variables or immunological variables.

As shown in Table 13.2, four factors were extracted. DcR3 was extracted only in Factor 1 (the most contributing factor among the four factors shown for the contribution ratio). DcR3 was associated with IL-6 (+0.901), G-CSF (+0.839), strongly with C-ANCA (+0.9811), and slightly but significantly with ANA (+0.422) and P-ANCA (+0.502). All coefficient values were positive. This means that the elevation of DcR3 in SIL is associated with an increase in these parameters, and specifically an increase in IL-6 and G-CSF, as well as increases in ANA and ANCA autoantibodies. Among other factors, factor 2 seemed to be an immunological factor which indicated an increase in IL-10 and TGF-β with positive correlations for ANA, P-ANCA, and Scl-70. Taken together with the results of the multiple regression assay and coefficient value of 0.168 for DcR3, this factor seemed to indicate immunological dysregulation in SIL toward ANCA-related vasculitis or SSc without any correlation with elevated DcR3. Factor 3 comprised two SSc-related autoantibodies and IL-10, and was dependent on age. This might indicate the presence of

**Table 13.2** Factor analysis of clinical parameters in SIL

| Factor | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Contribution ratio | 16.906 | 11.971 | 11.534 | 11.332 |
| **DcR3** | ***0.864*** | 0.168 | 0.119 | −0.004 |
| ANA | ***0.422*** | ***0.511*** | 0.536 | 0.245 |
| P-ANCA | ***0.502*** | ***0.611*** | 0.078 | 0.117 |
| C-ANCA | ***0.811*** | 0.055 | −0.205 | −0.039 |
| Scl-70 | −0.289 | ***0.500*** | ***0.637*** | 0.033 |
| CENP-B | 0.118 | −0.110 | ***0.741*** | −0.106 |
| RF | 0.103 | 0.322 | 0.338 | ***0.730*** |
| CCP | −0.184 | −0.130 | −0.109 | −0.019 |
| Total IgG | 0.283 | −0.016 | ***0.424*** | −0.252 |
| IL-10 | −0.024 | ***0.496*** | ***0.497*** | 0.198 |
| TGF-β | 0.267 | ***0.884*** | −0.022 | −0.069 |
| IL-6 | ***0.901*** | 0.115 | −0.082 | 0.225 |
| G-CSF | ***0.839*** | −0.02 | 0.039 | 0.136 |
| IL1α | 0.168 | 0.113 | 0.206 | −0.186 |
| sFas | −0.336 | 0.214 | −0.011 | 0.062 |
| sIL2R | 0.164 | 0.195 | 0.104 | 0.07 |
| sIL6R | −0.064 | ***0.748*** | 0.049 | −0.247 |
| Age | −0.218 | 0.071 | ***0.769*** | 0.034 |
| PR | −0.106 | −0.04 | −0.012 | 0.343 |
| ExpYear | 0.227 | −0.168 | 0.126 | 0.007 |
| Dyspnea | 0.282 | −0.119 | −0.317 | ***0.603*** |
| $FEV_{1.0}$ | −0.085 | 0.212 | 0.196 | ***−0.793*** |
| v25H | −0.098 | 0.183 | −0.040 | ***−0.817*** |
| %VC | −0.184 | 0.004 | −0.315 | 0.197 |

*SIL* silicosis patients, *DcR3* decoy receptor 3, *ABA* anti-nuclear antibody, *P-ANCA* myeloperoxidase-antineutrophil cytoplasmic antibody, *C-ANCA* cytoplasmic/proteinase-ANCA, *Scl-70* anti-topoisomerase antibody, *CENP-B* Centromere protein B, *RF* rheumatoid factor, *CCP* cyclic citrullinated peptide, *Ig* immunoglobulin, *IL* interleukin, *TGF* transforming growth factor, *G-CSF* granulocyte-colony stimulating factor, *sFas* soluble Fas, *sIL-2R* soluble IL-2 receptor, *PR* profusion rate, *ExpYear* exposure year to silica, *FEV* forced expiratory volume, *v25H* peak flow rate (PFR) at 25% forced vital capacity/Height

age-related abnormalities in the autoimmune conditions present. Finally, factor 4 was a respiratory factor with positive correlations with RF.

## 13.4 Discussion Regarding Serum Levels of DcR3 in Silicosis

DcR3 levels were reported to be elevated in various immune diseases as well as in certain cancers. This may be related to T cell activation or other immune activations. Although our previous study showed higher DcR3 gene expression in PBMCs

derived from SIL subjects compared with HV [31], we considered that elevated DcR3 expression may contribute to the protection of responder T cells from apoptosis, thus resulting in longer survival of these cells, and subsequently some T cell clones reacting with self-antigens would survive longer to induce clinical manifestations of autoimmune diseases [25, 26]. This theory may partly explain the pathogenesis which occurs with autoimmune diseases in SIL.

In this study, plasma DcR3 values were measured and the elevated DcR3 values found were considered in terms of the pathophysiology of SIL. Some SIL subjects showed higher levels of DcR3 similar to SSc subjects. An examination of DcR3 levels alone does not seem to be particularly useful in terms of the early detection of immunological abnormalities in SIL. Additionally, the limitation of this study concerns the unmatched age distribution between HV and SIL subjects, although aaDcR3 levels were used to compare serum DcR3 levels among HV, and SIL and SSc subjects. However, from the correlation studies, and multiple regression and factor analyses, DcR3 levels were found to be correlated with immunological, but not respiratory, variables. These results suggested that DcR3 may play some role in silica-induced disorders of autoimmunity.

From the simple correction analysis, DcR3 values were only correlated with TGF-β and sIL-2R. As we reported previously, an examination of sIL-2R in SIL seemed to indicate chronic T cell activation in SIL [28]. However, since sIL-2R in SIL was not related to sFas, sIL-2R was not related to cellular mechanisms involved in the long-term survival of responder T cells activated with silica particles or various self-antigens [28]. However, sIL-2R was correlated with CENP-B, but not Scl-70, and ANA titers [28]. These data indicated that the pathophysiology hidden with respect to the elevation of sIL-2R is related to localized SSc [52–55]. Additionally, factor analysis with sIL-2R in our previous reports showed that the sIL-2R level was extracted together with other immunological parameters such as Ig G, CENP-B and sFas. These results can be accounted for by considering the possibility that sIL-2R in SIL may indicate T cell activation with CENP-B related to SSc.

In this study, although the clinical parameters differed from our previous assay analyzing sIL-2R in SIL, certain findings were obtained. DcR3 values were related to TGF-β, G-CSF, and IL-6 among the cytokines, and with ANA as well as P- or C- ANCA among the various autoantibodies. These results may help to further our understanding of the mechanisms involved in the cellular alterations observed in SIL complicated with ANCA-related vasculitis. Many reports have recently highlighted this complication in SIL [20–22]. Considering the cellular role of DcR3, elevated DcR3 levels may protect responder T cells from apoptosis caused by Trail. Although the role of DcR3 resembles that of sFas, and we previously reported increased expression of DcR3 in PBMCs derived from SIL subjects, we considered the similarity between sFas and DcR3 and found that these two molecules showed no correlation in this study. Thus, it is necessary to examine the role of elevated DcR3 levels in the immune system of SIL patients which precedes the occurrence of autoimmune diseases. In this regard, the correlation with ANCA-autoantibodies found in the factor analysis may be revealing (Table 13.2). ANCA-related vasculitis is known to be strongly correlated with B cells given the efficacy of anti-CD20

monoclonal antibody-rituximab therapy [56–58]. Thus, T cells protected against apoptosis by DcR3 may possess functionality that strongly influences B cell function. This speculation may be supported by the combined extraction of IL-6 with DcR3, and ANCA antibodies in the factor analysis.

Additionally, the role of TGF-β in the occurrence of dysregulation of autoimmunity or chronic exposure and retention of silica particles in the pulmonary region and related lymph nodes has to be considered [59–63]. The simple correlation and multiple regression assays indicated a close relationship between DcR3 and TGF-β. TGF-β is correlated much more strongly with lung fibrosis in SIL rather than with the occurrence of autoimmune disease. Thus, DcR3 may also be related to pulmonary fibrogenesis in SIL, although factor analysis did not indicate any correlation of DcR3 with respiratory parameters.

## 13.5 Conclusions

Plasma DcR3 values in SIL may be representative of dysregulation of autoimmunity found in SIL, and may be utilized as a predictive indicator in SIL cases with respect to the onset of autoimmune diseases such as RA, SLE, SSc, and ANCA-related vasculitis [15–22]. Measurement of DcR3 levels in SIL may be useful in the early diagnosis of complicated autoimmune diseases, where some SIL patients tend to develop worse immune conditions with better respiratory conditions. Moreover, it would be important to consider the role of DcR3 at the cellular and molecular levels in relation to the onset of autoimmune diseases. Further studies are required to address these all-important issues.

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**Conflicts of Interest** All authors declare no competing interests regarding this study.

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# Chapter 14
# Reduction of Antitumor Immunity Caused by Asbestos Exposure

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**Abstract** Asbestos fibers are known to cause not only benign pulmonary and pleural diseases such as asbestosis and pleural plaque, but also malignant tumors such as lung cancer and malignant mesothelioma. In addition to the carcinogenic activities possessed by the fibers themselves, it has been considered that asbestos fibers may affect the human immune system. In this review, a cell culture model using a human T cell line exposed to asbestos fibers continuously and at relatively low doses to mimic exposure of environmentally and occupationally exposed people to these fibers is introduced. Although transient and high-dose exposure caused cell apopto-

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sis, the cell line employed acquired resistance to asbestos-induced apoptosis with continuous exposure as a result of various cellular and molecular changes such as changes in cytokine production and cytoskeletal molecules. On the other hand, changes in various immune cells such as cytotoxic T lymphocytes, natural killer cells, T helper cells, and regulatory T cells by in vitro exposure using certain cell lines as well as freshly isolated peripheral blood immune cells derived from healthy volunteers revealed impairment of antitumor immunity. Thereafter, the findings obtained were confirmed using peripheral blood immune cells derived from asbestos-exposed patients with pleural plaque or mesothelioma. The findings are also shown in this chapter. Further research should explore the effects of asbestos fibers on other immune cells such as Th17, investigate the development of diagnostic markers using altered immune cells, and pursue the identification of physiological substances from plants and other sources that can halt or recover the antitumor immunity caused by asbestos exposure.

**Keywords** Asbestos · Antitumor immunity · T helper (Th) cell · Cytotoxic T lymphocyte (CTL) · Natural killer (NK) cell · Regulatory T (Treg) cell

## 14.1 Introduction

Asbestos fibers can cause not only lung fibrosis known as asbestosis, but also malignant tumors such as lung cancer and malignant mesothelioma (MM) [1–4]. Additionally, benign diseases can also occur following asbestos exposure, such as pleural plaque (PP), diffuse pleural thickening, benign pleural effusion, and rounded atelectasis. Among these asbestos-related diseases, malignant tumors are the most important in terms of prognosis and the long latency period that usually ensues following exposure to asbestos. For example, the appearance of MM occurs 30–50 years after initial exposure [1–4]. Once MM occurs, most of the MM tumors progress rapidly, thereby resulting in poorer prognosis of the disease, notwithstanding the development of various novel approaches such as molecular targeting therapies [5, 6] and surgical treatments such as pleurectomy/decortication [7–9].

With regard to the carcinogenicity of asbestos fibers, the most important factor appears to be the iron which is included in amphibole asbestos materials, e.g., crocidolite ($Na_2Fe_2$+3$Fe_3$+2$Si_8O_{22}(OH)_2$) and amosite ($Fe_7Si_8O_{22}(OH)_2$) [10]. Although other amphibole materials such as actinolite ($Ca_2(Mg, Fe)_5(Si_8O_{22})(OH)_2$) and anthophyllite ($(Mg, Fe)_7Si_8O_{22}(OH)_2$) contain iron, these fibers have not been used in industry. Other amphibole materials such as tremolite ($Ca_2Mg_5Si_8O_{22}(OH)_2$) do not possess iron. Thus, crocidolite and amosite are considered stronger and potentially more dangerous in terms of asbestos-induced cancers. On the other hand, serpentine fibers such as chrysotile ($Mg_3(Si_2O_5)(OH)_4$) do not contain iron. Thus, although most industrial uses employed chrysotile, its carcinogenicity was considered to be the lowest. However, recent studies have demonstrated the carcinogenicity of chrysotile fibers [10–14]. Animal models showed higher frequencies of

chrysotile-induced mesothelioma compared with other asbestos fibers [15, 16]. Additionally, chrysotile showed a capacity to adhere to red blood cells and cause increased levels of iron in the body by hemolysis [17]. The International Labor Organization (ILO) then declared that "all forms of asbestos, including chrysotile, are considered as known human carcinogens" [12].

The iron yields oxygen stress and produces reactive oxygen species (ROS) that result in genotoxicity to nearby cells. When alveolar macrophages come into contact with asbestos fibers, they are unable to effectively treat the fibers as foreign entities due to the fibers being rigid and long [18–20]. As a result, these macrophages produce ROS and are referred to as "frustrated macrophages." In addition to the aforementioned theories, asbestos fibers can directly attack surrounding cells. Due to their rigid characteristics, fibers can damage chromosomes. Moreover, fibers can adsorb various carcinogenic substances which are inhaled into the lungs such as tobacco smoke and air pollutants [21, 22].

On the other hand, the effects of asbestos fibers on the human immune system have not been well documented, except with the investigation of cellular and molecular mechanisms related to the signaling of these fibers as foreign and a danger by inflammasomes included in alveolar macrophages as antigen presenting cells [23, 24]. In the case of asbestos fibers, the pattern-recognition receptor NALP3 (NACHT, LRR, and PYD domains-containing protein 3) plays a role with ASC (apoptosis-associated speck-like protein containing a CARD) and caspase-1 inflammasome which results in the activation of caspase-1. Thereafter, pro-inflammatory cytokines such as interleukin (IL)-1β and IL-18 are secreted to promote fibrogenic changes in lung fields [25, 26]. However, these recognition processes represent just the initial events following the entry of asbestos fibers into the human body. Given their physical characteristics, fibers are retained in the lung fields as well as related lymph nodes. Then, various circulating lymphocytes may have repeat encountering with fibers. These phenomena may cause cellular and molecular alterations in these lymphocytes.

A number of reports have detailed the use of in vitro experiments to investigate the effects of asbestos on lung alveolar epithelial cells and pleural mesothelial cells in terms of the progression of cells toward a cancerous state [27–30]. Most of these trials demonstrated the importance of ROS production and activation of mitochondrial apoptotic pathways by transient and relatively high-dose exposure. It can be speculated that an accumulation of damage to the genome by ROS might prevail, and that certain cellular mechanisms may be enacted that place cells on a non-apoptotic pathway. Thereafter, these cells may then possess the cellular and molecular characteristics of cancer cells.

These experimental procedures could be utilized to clarify the immunological effects of asbestos fibers, and to establish continuous and relatively low-dose exposure conditions in an effort to delineate the cellular and molecular changes that occur in environmentally and occupationally exposed people to asbestos. Consequently, our strategies to explore the immunological effects of asbestos fibers on various lymphocytes such as T helper (Th) cells, regulatory T cells (Tregs), cytotoxic T lymphocytes (CTLs), and natural killer (NK) cells included the following approaches:

1. Applying cell lines and established cell culture models to continuous and low-dose exposure.
2. Generating an ex vivo model using freshly isolated peripheral blood mononuclear cells (PBMCs) or sorted specific lymphocyte fractions for activation in a cell culture system in the absence or presence of asbestos fibers.
3. If certain changes were observed from the aforementioned approaches, confirming such findings using peripheral blood lymphocytes derived from PP and MM patients, as both are considered to be caused by exposure to asbestos.

These strategies to explore the immunological effects of asbestos fibers have previously been reported [31–33]. In this review, one example will be given of the cellular and molecular alterations found resulting from continuous exposure of the human T cell leukemia virus (HTLV)-1 immortalized polyclonal cell line MT-2 to chrysotile and crocidolite asbestos fibers. Additionally, the effects of asbestos exposure on CTLs, NK cells, Th cells, and a Treg cell line model will also be summarized in terms of antitumor immunity.

## 14.2 Cellular and Molecular Alterations of the MT-2 T Cell Line Exposed Continuously to Asbestos Fibers

Using the MT-2 cell line, cellular events caused by transient and relatively high-dose exposure to chrysotile or crocidolite were examined [34, 35]. Dependent on iron content, ROS production was higher in cultures with crocidolite compared to chrysotile cultures, whereas growth inhibition and the appearance of apoptosis were slightly greater in chrysotile compared to crocidolite cultures. In both crocidolite and chrysotile cultures, MT-2 cells proceeded toward apoptosis via increased phosphorylation of proapoptotic mitogen-activated protein kinase (MAPK) signaling molecules such as p38 and JUN, increased release of cytochrome c from mitochondria to the cytoplasm, decreased expression ratio of Bcl-2/BAX, and activation of caspase-9 and caspase-3 [34, 35].

After more than 8 months of continuous and low-dose exposure (causes less than half of the cells to proceed toward apoptosis by transient exposure) to chrysotile or crocidolite fibers, MT-2 cells acquired resistance to asbestos-induced apoptosis. The monitoring of apoptosis continued monthly by transient high-dose exposure to fibers after removal of fibers employed for continuous exposure. A number of interesting findings have been found with respect to the cellular and molecular characteristics in all of these sublines (continuously exposed MT-2 cells; there are independently established sublines, three were exposed to chrysotile A, three to chrysotile B, and four to crocidolite). For example, IL-10 was overproduced in the sublines IL-10 was overproduced and regulated by Src kinase. This overproduction of IL-10 activated signal transducer and activator of transcription 3 (STAT3) via phosphorylation and upregulated Bcl-2 located down stream of STAT3 by autocrine usage of IL-10 [36]. This represents one route to apoptosis resistance or enhanced survival. Additionally, sublines showed overproduction of transforming growth factor (TGF)-β. The

autocrine mechanism involving use of TGF-β caused phosphorylation of p38, thereby resulting in increased levels of phosphorylated SMAD3 and decreased levels of phosphorylated SMAD2 [37]. Therefore, these sublines showed resistance to TGF-β-induced growth inhibition found in MT-2 original cells [37].

The other interesting feature of the sublines was reduced expression of forkhead box protein O1 (FoxO1), which regulates various apoptosis-related molecules. Examination of proapoptotic molecules such as p53 upregulated modulator of apoptosis (Puma), Bcl-2 interacting mediator (Bim), and Fas ligand in sublines revealed decreased expression [38]. This represents another route to acquire apoptosis resistance by decreasing proapoptotic signals.

Moreover, various cytoskeletal molecules were altered following long-term continuous exposure to asbestos in MT-2 cells. Additional phosphorylation and increased expression of β-actin were detected in sublines. Furthermore, myosin9, vimentin, and tubulin β2 extracted from sublines showed increased binding capacity to chrysotile fibers [39]. These findings were considered to be reasonable since MT-2 cells are incapable of digesting fibers and repeatedly encounter fibers on their cell surface. Therefore, changes in cytoskeletal molecules occurred as a result of continuous exposure. Although the precise impact on cellular function caused by these changes have yet to be delineated, an examination of the alteration of other molecules on the cell surface is important in determining the effects of fibers on immune cells.

All of these findings are schematically represented in Fig. 14.1.

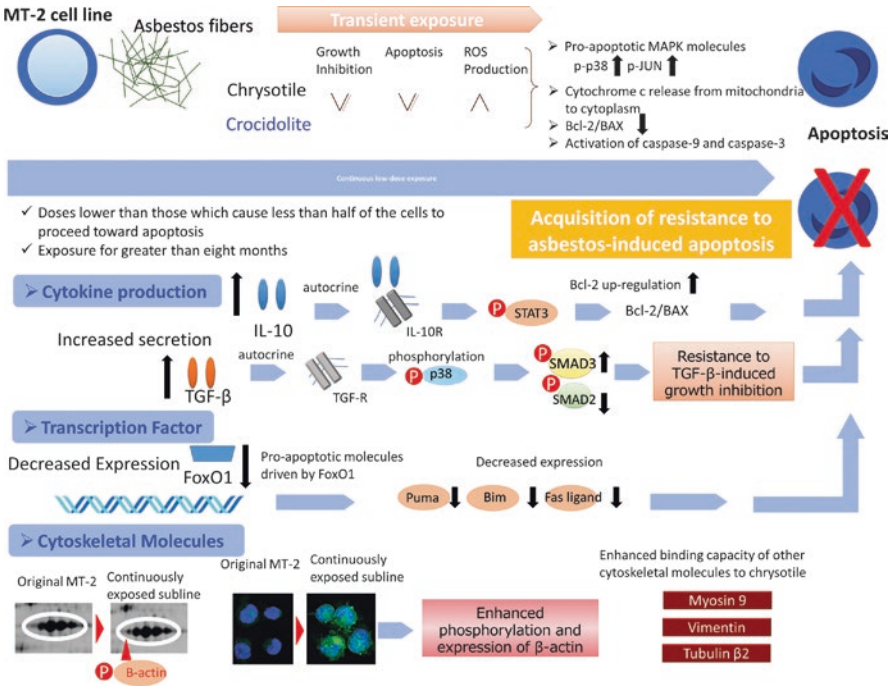


**Fig. 14.1** Schematic presentation of cellular and molecular alterations of the MT-2 T cell line continuously exposed to asbestos. Detailed explanations are described in the text

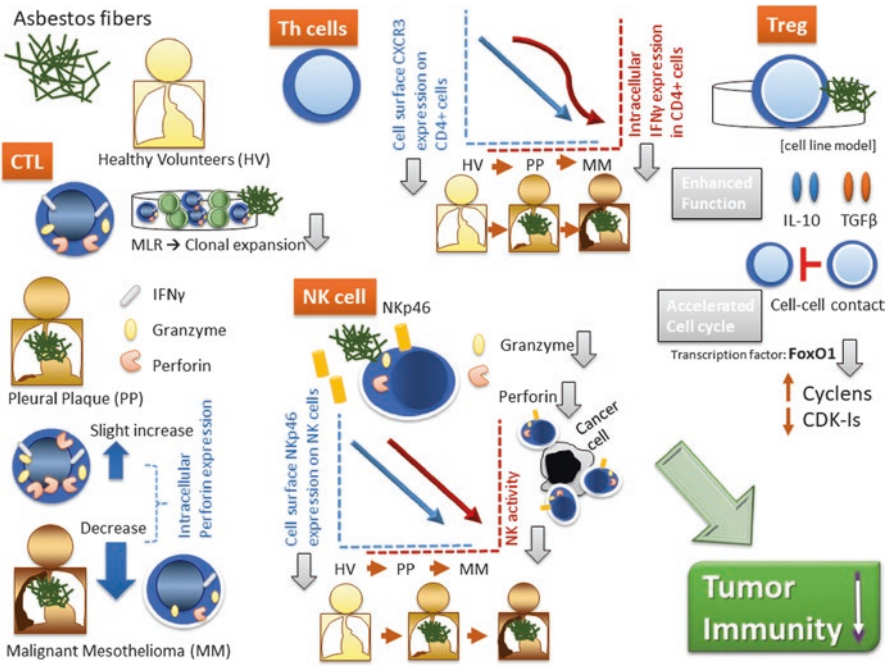


**Fig. 14.2** Schematic presentation of the decrease of antitumor immunity caused by exposure to asbestos fibers in CTL, NK, Th, and Treg cells

## 14.3 Reduced Antitumor Immunity Caused by Exposure to Asbestos Fibers

The experimental results as well as analyses using clinical specimens (peripheral blood immune cells) derived from HV as well as PP and MM patients are schematically summarized in Fig. 14.2.

## 14.4 CTLs

Since CTLs are important when considering antitumor immunity, the effects of chrysotile on a mixed lymphocyte reaction (MLR) assay to examine the transition of CD8+ naïve T lymphocytes toward CTLs were analyzed in the absence or presence of fibers. As a result, supplemented chrysotile caused a decrease in CD8+ cell proliferation and inhibited cellular transition from naïve to effector/memory type CD8+ cells as determined by examining cell surface molecules such as CD45RA, CD45RO, and CD25. Additionally, intracellular granzyme B and IFN-γ levels were lower compared to the MLR in the absence of supplemented chrysotile. The

supernatant of the MLR showed decreased levels of IFN-γ and tumor necrosis factor (TNF)-α when chrysotile was supplemented in the MLR. All of these findings indicated that asbestos fibers suppress the clonal expansion of CTLs [40, 41].

Thereafter, the intracellular cell-attacking granules, containing cytotoxic molecules such as granzyme and perforin, were examined using PBMCs derived from HV as well as PP and MM patients after overnight stimulation with phorbol myristate acetate (PMA) and ionomycin. Interestingly, the percentage of intracellular granzyme B+ and perforin+ cells in PMA/ionomycin-stimulated CD8+ lymphocytes was higher in PP patients compared to HV. Additionally, intracellular levels of perforin+ cells in stimulated CD8+ cells derived from MM patients were lower compared to those derived from PP patients. These results indicated that MM patients possess impairment of stimulation-induced cytotoxicity of peripheral blood CD8+ lymphocytes, while PP and MM patients possess a common alteration of those lymphocytes, namely, an increase in memory cells (percentage of perforin+ cells and CD45RA− cells in fresh CD8+ lymphocytes of PP and MM groups were higher compared to HV), possibly related to asbestos exposure. It is noteworthy that intracellular perforin levels differed between PP (noncancerous) and MM (cancerous) patients. Thus, the effects of asbestos on CTLs may be altered due to complicated physiopathological conditions [40, 42].

## 14.5 NK Cells

The effects of asbestos fibers on human NK cells were reported in our previous review "Dysregulation of the immune system caused by silica and asbestos" [43].

Briefly, the human NK cell line YT-CB was employed for studies. Then, ex vivo exposure of freshly isolated and activated NK cells from HV to asbestos fibers was investigated. These experiments showed decreased levels of certain NK cell-activating receptors such as 2B4, NKG2D, and NKp46. Thereafter, the correlation between these activating receptors and the cytotoxic activity of NK cells derived from HV as well as PP and MM patients were examined [44, 45]. As shown in Fig. 14.2, expression of NKp46 and cytotoxicity showed a significant positive correlation, where decreased NKp46 expression resulted in weaker cytotoxic potential of the analyzed NK cells. Moreover, the extent of NKp46 expression on NK cells gradually decreased in the order HV to PP to MM. NK cells from MM patients displayed the lowest expression. Additionally, it was supposed that if the NKp46 expression were examined in PP patients, that higher expression may be present only in PP patients even if they were exposed to asbestos, whereas lower expression may require careful medical evaluations for risk of mesothelioma onset. Additionally, the reduction in cytotoxicity was accompanied with reduced intracellular signaling via receptors, phosphorylation of the extracellular signal-regulated kinase (ERK), and decreased degranulation of cell-killing granules containing perforin [44, 45].

These findings indicated that asbestos exposure results in decreased NK cell cytotoxicity with lower expression of NKp46.

## 14.6 Th Cells

As described above, the HTLV-1 immortalized polyclonal T cell line MT-2 was exposed to asbestos fibers and continuously exposed sublines were established [35, 36]. From the cDNA microarray data and pathway analysis using those data of the comparison between MT-2 original cells and sublines, there were some interesting findings regarding antitumor immunity [46, 47]. One finding involved the molecular pathway leading to IFN-γ. The expression of many molecules involved in this pathway decreased in sublines. Additionally, and related to this pathway, the expression of CXC chemokine receptor (CXCR) 3 was reduced in sublines.

After confirming the reduced expression of CXCR3 in sublines by real-time RT-PCR as well as using flow cytometry and immunohistochemical assays for a comparison of protein expression between sublines and MT-2 original cells, freshly isolated CD4+ cells from peripheral blood of HV were examined for surface expression of CXCR3 after ex vivo stimulation with anti-CD3 and anti-CD28 antibodies supplemented with IL-2 in the absence or presence of chrysotile fibers. After 4 weeks, surface CXCR3 was significantly reduced when fibers were present; however, other chemokine receptors such as CCR5, which was not detected by cDNA microarray, remained unchanged under these ex vivo conditions. Moreover, intracellular protein and mRNA expression of IFN-γ were also reduced under these ex vivo conditions [46, 47].

Surface expression of CXCR3 on freshly isolated CD4+ cells from HV as well as PP and MM patients were then compared. From the results shown in Fig. 14.2, it can be seen that the extent of CXCR3 expression decreased in the order HV to PP, and then markedly decreased in CD4+ cells derived from MM patients. Additionally, these cells were stimulated with anti-CD3 and anti-CD28 antibodies and IL-2 for 5 days. Then, intracellular IFN-γ positive cells were analyzed. As a result, although there was no difference between CD4+ cells derived from HV and PP patients, the number of intracellular IFN-γ-positive CD4+ cells from MM patients was significantly lower compared to that of HV and PP patients [46, 47].

CXCR3 is considered to be important in facilitating the introduction or movement of cancer-attacking T cells near tumor cells. Additionally, IFN-γ plays an important role in tumor cell damage. Thus, these findings also indicated that asbestos exposure results in reduced antitumor immunity.

## 14.7 Cell Line Model of Tregs

The MT-2 cell line is considered to possess Treg function ([48]: [49]). Hence, the Treg function associated with suppressing the proliferation of responder T cells was compared between MT-2 original cells and the aforementioned sublines. Freshly isolated peripheral blood CD4+ cells were activated by anti-CD3 and anti-CD8 antibodies with in vitro differentiated auto-dendritic cells. Into this activated culture

were added irradiated MT-2 and one subline (designated as CB1 cells) to represent the role of Tregs. The antiproliferative effects against responder T cells were found to be significantly enhanced in CB1 cells compared to original MT-2 cells. Additionally, as mentioned above, sublines showed increased production of IL-10 and TGF-β [50]. Since these two cytokines are known as typical soluble factors secreted from Tregs and which contribute to the inhibitory function of Tregs, IL-10, or TGF-β was silenced using a siRNA method in the CB1 subline, and the suppressive effects against freshly isolated and activated peripheral CD4+ cells derived from HV were examined using a Transwell culture assay. As a result, it was found that approximately half of the suppressive effects were canceled by silencing IL-10 or TGF-β [50].

Moreover, it is known that FoxO1 regulates several molecules involved in cell cycle progression such as cyclin D1 and cyclin-dependent inhibitors (CDK-I) including INK4 family members such as p16INK4a and p15INK4b and Cip/Kip family CDK-I members including p21Cip1 and p27Kip1. FoxO1 inhibition regulates accelerating molecules, but stimulation regulates braking molecules. As mentioned above, since FoxO1 expression is specifically reduced in continuously exposed sublines of MT-2 [38], the expression of these cell cycle regulators in original MT-2 cells and sublines was determined and compared. As a result, it was found that accelerating molecules were upregulated while CDK-I was downregulated. Thereafter, the S/G1 ratio in cell cycle phases analyzed by flow cytometry was higher in sublines compared to original MT-2 cells [51]. These results indicated that asbestos exposure causes rapid progression of the cell cycle in Tregs and results in increased volume in Tregs.

These results indicated that Tregs exposed to asbestos fibers possess enhanced function via cell–cell contact (including the effects of membrane-bound TGF-β in addition to overproduction of functional cytokines IL-10 and TGF-β. Furthermore, Tregs exposed to asbestos undergo rapid proliferation by altering the regulation of FoxO1. Unfortunately, although the function of peripheral blood or tumor-surrounding Tregs derived from PP and MM patients have yet to be examined, these cell line models also suggest that asbestos exposure causes a reduction of antitumor immunity.

## 14.8 Conclusion

This review presented experimental approaches examining the immunological effects of asbestos fibers using human cell lines and freshly isolated lymphoid cells derived from HV, and provided detailed confirmation of certain findings using peripheral blood immune cells derived from PP and MM patients exposed to asbestos. Exposure to asbestos altered the cellular and molecular characteristics of various immune cells such as Th, Treg, CTL, and NK cells. Additionally, most of the changes suggested a reduction of antitumor immunity in asbestos-exposed populations. However, detailed examinations regarding the effects of asbestos fibers on

Th17, dendritic cells, small populations of T cells such as γδT and other cells have yet to be undertaken.

One potential clinical use of these findings is to provide a comprehensive assessment of the immune status of high-risk groups such as past (and present) workers involved in asbestos manufacturing, asbestos handling, building demolition, rubble processing, and other asbestos-related activities, rather than just making a diagnosis of PP or other asbestos-related pathological changes by chest X-ray or CT imaging. The immunological findings may be examined by drawing peripheral blood from subjects.

The other possibility is to identify physiological substances from plants and other sources that can halt the reduction of or reduce the antitumor immunity caused by asbestos exposure. If certain substances can be administered orally on a daily basis to the aforementioned high-risk groups, this may assist in the chemoprevention of asbestos-related diseases and the development of malignant tumors.

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