Immunological Assays as an Opportunity of Assessment of Health Risks of Airborne Particle Mixture Including Nanoparticles

Táňa Brzicová1, Ivo Lochman2, Pavel Danihelka1, Alexandra Lochmanová3, Karel Lach2, Vladimír Mička1,2

1 VŠB – Technical University of Ostrava, Faculty of Safety Engineering, Lumírova 13, 700 30 Ostrava-Výškovice, Czech Republic
2 Institute of Public Health Ostrava, Partyzánské náměstí 7, 702 00 Ostrava, Czech Republic
3 University of Ostrava, Faculty of Medicine, Syllabova 19, 703 00 Ostrava-Zábřeh, Czech Republic

E-mail: tana.brzicova@vsb.cz

Abstract. The aim of this pilot study was to evaluate perspectives of the assessment of non-specific biological effects of airborne particulate matter including nanoparticles using appropriate immunological assays. We have selected various *in vitro* immunological assays to establish an array allowing us to monitor activation of the cell-mediated and humoral response of both the innate and adaptive immunity. To assess comprehensive interactions and effects, the assays were performed in whole blood cultures from healthy volunteers and we used an original airborne particle mixture from high pollution period in Ostrava region representing areas with one of the most polluted air in Europe. Even if certain effects were observed, the results of the immunological assays did not prove significant effects of airborne particles on immune cells’ functions of healthy persons. However, obtained data do not exclude health risks of long-term exposure to airborne particles, especially in case of individuals with genetic predisposition to certain diseases or already existing disease. This study emphasizes the *in vitro* assessment of complex effects of airborne particles in conditions similar to actual ones in an organism exposed to particle mixture present in the polluted air.

1. Introduction

Nanoparticles have always been present in airborne solid matter, but contemporary development of engineered nanoparticles, together with an increasing number of unintentionally produced ultratine particles and nanoparticles, draw attention to corresponding adverse health effects. The same properties that make nanomaterials unique and useful may be on the other side associated with unforeseen adverse health effects [1]. Since the nanomaterials are widely used and actually ubiquitous in the environment, research on the suitable methods of their potential toxicity evaluation represents a global challenge. The experience with studies on adverse health effects of airborne particles implies the importance of the influence of immune system functions, especially through the reactive oxygen species generation and pro-inflammatory action [2].

Published under licence by IOP Publishing Ltd
The essential role of the immune system is the recognition of foreign potential dangerous agents and their subsequent elimination from the organism. However, these processes can be accompanied by various harmful actions on the cells and tissues and can be the cause of the adverse health effects relating to the nanoparticle exposure both in workplaces and the environment. The health risk assessment is complicated by the high number of nanomaterial characteristics which can influence their effects on the immune system and the human health. The recognition versus non-recognition of nanomaterials by immune-competent cells can determine their distribution as well as their toxicological potential [3]. Particles can either stimulate or suppress immune responses or act indirectly on components of the immune system, for instance through modulation of secretion of cytokine and other immune system regulatory molecules [3].

With regard to the marginal role of the immune system on the homeostasis in an organism, there is a presumption that an array of appropriate immunological assays which monitor activations of various immune system components and functions should represent a suitable screening tool for the assessment of harmful nanomaterial action on the human health.

Nanoparticle compatibility with the immune system and thus the ability of the immune system to react on the presence of nanoparticles in an organism is largely determined by the particle surface chemistry. Small size of nanoparticles provides large surface area, where more other pollutants can be adsorbed and in this form transported into the human body. Moreover, the smaller the particles are, the deeper they can penetrate into the respiratory tract and there are evidence that some nanoscale particles cross the biological membranes [4].

2. Methods and Materials

2.1. Particulate matter sample preparation

Owing to the restricted amount of available nanoparticles, an integral sample containing fine airborne particles less than 2.5 micrometers in diameter (PM$_{2.5}$) was used in this pilot study. In case of positive assay results, the ultrafine fraction of airborne particles (PM$_{0.1}$) would be collected and tested. Particulate matter was obtained from industrial measuring station located in Ostrava-Radvanice (the district of the Ostrava City in the Moravian-Silesian region, the Czech Republic), representing area with one of the most polluted air in Europe. Recent measurements have shown significant number of nanoparticles present in the polluted air in Ostrava region [5]. Data from epidemiologic studies suggest adverse health impact on local population associated with heavy airborne particle pollution [6,7]. The average PM$_{2.5}$ concentration during the sampling period was 175 $\mu$g/m$^3$. The main sources of the air pollution in this region are industry (especially metallurgy, fuel processing and chemical industry), local heating, traffic and emission transport from industrial areas in Poland. The situation is even worsened by geomorphological condition supporting concentration of the air pollution [8]. With regard to the presence of heavy industry in this region, the metals bound on the airborne particle can be presumed. This presumption was confirmed by chemical analysis performed on the particle sample suspension. Significant amount of lead, zinc, cadmium, arsenic and antimony was detected. These elements are a common part of the fly ash from combustion processes [9].

PM$_{2.5}$ fraction was collected on mixed cellulose ester (MCE) membrane filters (Millipore Co., Ltd., USA, 47 mm diameter, 0.8 $\mu$m pore size). The sampling flow rate was 16.7 l/min. Particulate matter was collected on November 13–14, 2011 (19.11 hours, total air volume 19.185 m$^3$).

The filter exempt from sampler head was kept in Petri dish covered with paper box to restrain light exposition. After stabilizing at constant temperature (22–24 °C) and humidity (40–50 %), the filter was weighed on an electronic balance (Sartorius BP211D).

To obtain particulate matter suspension, the filter was sonicated in beaker filled with 250 ml of distilled water using Ultrasonic compact cleaner 4L PS04000A. Sonication time was one and one minute consequently, with the pause to prevent overwarming of the water suspension. Obtained suspension was retained in the refrigerator. Just before the use, the suspension was resonicated and diluted. A part of the suspension was chemically analyzed by Inductively Coupled Plasma –
Mass Spectrometry (ICP-MS) using model THERMO X-Series2. Chemical analysis was aimed to detection of selected metals.

2.2. Biological material withdrawing and preparation

The immunological assays were performed on the whole blood culture of healthy volunteers. Such an approach is suitable in case of screening studies due to comprehensive evaluation of interactions and effects of the particle sample, as well as easy taking of the biological material. When positive assay results occur, it is possible to continue in testing using isolated blood cell populations.

Blood samples were collected from three healthy volunteers (two men, one woman, aged from 25 to 55 years) into Vacutainer Tubes containing heparin as an anticoagulant. Immunological assays were performed within three hours after blood taking. Whole blood analysis (total red blood cell count, total and differential white blood cell counts) was determined using Sysmex XS-800i analyser.

2.3. Chemiluminescence assay

The assay was performed according to SOP 25.47 CKL of Institute of Public Health Ostrava [10]. Briefly, the sample was diluted to obtain four tested concentrations (30 pg/ml, 300 pg/ml, 3,000 pg/ml, 134 µg/ml) of original dust in Hank’s Buffered Salt Solution (HBSS). 2 ml of HBSS and 10 µl of luminol (used to enhance detection of the light) were pipetted into all scintillation viols. The viol representing negative control was left without application of any stimulant. Into the viol representing positive control, 50 µl of opsonized zymosan were added. The tested particle sample in four various concentrations was added into the other viols. 20 µl of blood were pipetted into the viols in 40-second intervals. After vortexing, all viols were placed into Tri-Carb Scintillation Counter and left to temperate for 15 minutes at 37 °C.

The measured results, expressed in the terms of cpm (counts per minute) were read 30, 60 and 90 minutes after the adding of the blood. The test results were statistically analyzed using pared Student’s t-test and one-way ANOVA.

2.4. Basophil degranulation assay

Commerce kit BasoFlowEx® was used to evaluate basophil degranulation. The assay was performed according to the kit instructions [11]. Briefly, the sample was diluted to obtain four tested concentrations (30 pg/ml, 300 pg/ml, 3,000 pg/ml, 134 µg/ml) of original dust in Physiological Buffered Solution (PBS).

The tube representing negative control was left without application of any allergen. Into positive control tube, 10 µl of Stimulation Control were added. The tested airborne particle sample in four varying concentrations was added into the others tubes. Into all tubes, 100 µl of whole blood were added and stimulated by adding of 100 µl of stimulation buffer for 15 minutes at 37 °C. Then, 300 µl of Staining Reagent were added and incubated for 20 minutes at 6 °C. Thereafter 300 µl of Lyzing Solution were added, mixed and incubated for 5 minutes at laboratory temperature. To red blood cell lysis, 3 ml of demineralized water were added into each tube and incubated for 5 minutes at laboratory temperature. Then the tubes were centrifuged for 5 minutes at 300 g, the supernatant was removed and pellet was resuspended in 0.2 ml of PBS. Cell surface expression of CD63 was measured by flow cytometry within 2 hours after staining. Obtained data were visualized on the side-scatter (SSC) versus fluorescence intensity in PE channel (FL2) dot-plot. The gate for basophil population (CD203c positive, SSC low) was set and the gated basophils were brought to histogram.

2.5. Lymphocyte proliferation assay

The assay was performed according to SOP 25.47 CKL of Institute of Public Health Ostrava [12]. Briefly, the sample was diluted to obtain four tested concentrations (30 pg/ml, 300 pg/ml, 3,000 pg/ml, 134 µg/ml) of original dust in Minimum Essential Medium (MEM). The assay was performed in microplate wells in triplicates. Into three wells representing negative controls, only 200 µl of MEM were added. Into positive control wells, 200 µl of cultivated media with phytohemaglutinin (PHA)
as a standard mitogen were added. 200 µl of the tested sample in four varying concentrations were pipetted into the other wells. Then 10 µl of blood were added into each well, vortexed and incubated in atmosphere with 5% CO₂ for 72 hours. After incubation, 10 µl of [³H]thymidine were added into each well and the microplate was returned into the CO₂ incubator for 24 hours. Finally, the content of each well was recovered with a cell harvester. A stripe of filter paper was put onto the sturdy paper and let drying. Drought rounds of the filter paper were taken out with tweezers and placed into particular flasks fulfilled with 1.5 ml of scintillation liquid. Then the flasks were transported into Tri-Carb scintillation counter to measure the incorporated radioactivity. The assay results were counted as an average of three values for a individual well, and expressed in the terms of cpm (counts per minute). The test result was statistically analyzed using pared Student’s t-test and one-way ANOVA.

2.6. Cytokine release evaluation

Commerce kit Human Cytokine 25-Plex Panel was used to evaluate the release of 25 selected cytokines. The assay was performed according to the assay kit’s instructions [13]. Briefly, the airborne particle sample was diluted to obtain the tested concentration of 3,000 pg/ml in Roswell Park Memorial Institute Medium (RPMI). The filter plate was pre-wetted with 200 µl of working Washing Solution. The Washing Solution was aspirated using a vacuum manifold. 25 µl of the beads with defined spectral properties conjugated to protein-specific capture antibodies were pipetted into each well and incubated for 2 hours. Thereafter, protein-specific biotinylated detector antibodies were added and after 1-hour incubation the exceeding antibodies were washed and the wells drought again. Streptavidin conjugated to the fluorescent protein, R-Phycocerythrin (Streptavidin-RPE), were added and washed after 30-minute incubation. Finally, the beads were analyzed using Luminex detection system which enables monitoring of the spectral properties of the beads and the amount of associated R-Phycocerythrin (RPE) fluorescence, therefore the concentration of proteins can be determined.

Polynomial regression was used for the calibration curves. Positivity index (values of PM₂·₅ stimulated cultures in relation to negative control values) was used to reciprocal comparison of individual cytokine release.

3. Results

3.1. White blood cell counts

Total red blood cell and total white blood cell counts and differential white blood cell counts of all three blood donors correspond with reference values [14]. Therefore, the results of immunological assays should not be influenced by abnormalities of haematological parameters of the donors.

3.2. Chemiluminescence assay

Airborne particle sample was cultivated in four concentrations in the range from 30 pg/ml to 134 µg/ml. Stimulation effect was compared with the negative control (sample without any stimulant). Positive control with application of standard stimulant opsonized zymosan was used to confirm the assay functionality. The assay results are expressed in terms of cpm (counts per minute). The values were read 30, 60 and 90 minutes after the incubation had begun.

As seen on Figure 1, the presence of airborne particle sample did not significantly enhance cpm values compared with the values of negative controls. Despite the increased values observed in some measurements, the no effect hypothesis was statistically confirmed. Pared Student’s t-test was used to compare results measured for single concentrations with negative control values. Only in one case (concentration of 3,000 pg/ml, incubation time 30 minutes) significant difference between negative control and the particle sample was recorded. With regard to low number of measurements and the other negative results the response cannot be attributed to the action of airborne particles. The results of one-way ANOVA confirmed that the sample did not have stimulant effect.
3.3. Basophil degranulation assay

Airborne particle sample was cultivated in four concentrations in the range from 30 pg/ml to 134 µg/ml. Stimulation effect was compared with the negative control (sample without any stimulant). Positive control with application of standard stimulant was used to confirm the assay functionality. The assay results are expressed in percentage of activated basophils.

For a right interpretation of the basophil degranulation assay results, it is necessary to achieve convenient values of both negative and positive control. According to BasoFlowEx® Kit user manual, negative control result should be lower than 10 % of activated basophils and contrarily positive control should be higher than 10 % of activated basophils. These conditions were fulfilled.

The cut-off limit between negative and positive response against inhalation allergens when allergies are assessed was established on 15 % of activated basophils. As seen on Figure 2, percentage values of activated basophils did not exceed this limit in any of tested airborne particle concentrations. Therefore, the tested sample did not stimulate significant basophil degranulation.

3.4. Lymphocyte proliferation assay

Airborne particle sample was cultivated in four concentrations in the range from 30 pg/ml to 134 µg/ml. Stimulation effect was compared with the negative control (sample without any stimulant). Positive control with application of standard stimulant PHA was used to confirm the assay functionality. The assay results are expressed in terms of cpm (counts per minute) as an average from triplicate values.

Slight growth in cpm compared with negative control was recorded with particle concentrations of 300 pg/ml and 134 µg/ml, as seen on Figure 3. Pared Student’s t-test as well as one-way ANOVA confirmed significant stimulant effects on p-value < 0.05 of airborne particle sample in concentrations of 300 pg/ml and 134 µg/ml on lymphocyte activation.
3.5. Cytokine release evaluation
We used commerce kit Human Cytokine 25-Plex Panel enabling to evaluate production of 25 cytokines divided according to their biological function in four panels:

- Inflammatory Panel: GM-SCF, IL-1β, IL-1RA, IL-6, IL-8, TNF-α
- Th1/Th2 Panel: INF-γ, IL-2, IL-2R, IL-4, IL-5, IL-10
- Cytokine II Panel: INF-α, IL-7, IL-12, IL-13, IL-15, IL-17
- Chemokine Panel: Eotaxin, IP-10, MIG, MCP-1, MIP-1α, MIP-1β, RANTES

Airborne particle sample was cultivated in a concentration of 3,000 pg/ml. Stimulation effects were compared with the negative control (sample without any stimulant). Positive control with application of standard stimulant PHA was used to confirm the assay functionality. The assay results were expressed as positivity index, which allow us the comparison of even very different concentrations of individual cytokines. The assay was performed with three incubation periods: 6, 24 and 72 hours. The assay results are shown on Figure 4.

![In vitro cytokine release evaluation in cultures of whole blood after stimulation with PM$_{2.5}$ sample. The results are expresses as a positivity index (with and without PM$_{2.5}$ stimulation concentration ratio). Plots are constructed for particular stimulation times from values of three healthy volunteers.](image)

4. Discussion

Nanoparticle toxicity assessment arises from studies concerning the toxicity effect of airborne particulate matter. Epidemiological and toxicological studies indicate that airborne particulate matter can considerably contribute to morbidity and mortality, especially in regions heavy laden by industry and traffic [6, 15, 16]. Suspended airborne particles form a multicomponent mixture with particles varying in their size and chemical composition. Particle diversity together with their possible synergy effects complicates the health risk assessment. This is why adverse health effects of airborne particulate matter cannot be derived only from mass concentrations in the air. Therefore, there is a need to search for suitable methods able to assess negative particulate matter action on human health.

Presented study deals with the short-term effects of real airborne particulate matter on the human immune cells’ functions. With regard to the integrating and protective role of the immune system, there is a presumption, that influence of the immune system functions can be the cause of adverse health effects relating to the airborne particle exposure.
To assess immunomodulatory effects, four in vitro immunological assays allowing us to monitor both the humoral and cell-mediated components of the innate and acquired immune responses were used.

The basic idea was that if significant immunological response was observed for integral fine and ultrafine particle sample, the next step would be the separation of nanoparticles from PM in sufficient amount for the performance of the immunological assays.

4.1. Chemiluminescence assay

Stimulation of phagocytes involves generation of reactive oxygen species (ROS) called respiratory burst. The return of the excited chemical groups to the basic state is accompanied by foton emission - chemiluminescence. The chemiluminescence assay was used to measure light production by phagocyte cells in whole blood cultures representing the release of reactive oxidant species [10].

This assay enables us to detect the impact of the tested particle sample on the oxidative burst of phagocytic cells in whole blood represented mainly by polymorphonuclears. Donaldson et al. [17] suppose that the increase of phagocyte activity induced by the presence of airborne particles in the respiratory tract can support particle clearance. However, nonspecific action of ROS can cause inflammation, cell and tissue damage. Epidemiological as well as experimental data show that reactive molecules play an important role in progress of some respiratory disease [15, 17]. Therefore, there is a need for a method able to measure the rate of oxidative molecule generation, as a screening tool for possible airborne particle associated oxidative damage evaluation.

In our study, the particle sample only in concentration of 3,000 pg/ml after 30-minute incubation significantly increased phagocytosis associated metabolic activity of leukocytes in whole blood cultures compared to the values of negative control. However, one way ANOVA did not confirm the stimulation effect of the particle sample. With regard to the low number of measurements and the negative results with the other particle sample concentrations and incubation times, the difference cannot be contributed to the action of airborne particles.

The negative assay results could be caused by absence of ligands on the particle surface able to bind on the phagocyte receptors. Both in vivo and in vitro studies have shown that particularly the presence of a biological component on the airborne particles is associated with phagocyte activation [18]. However the presence of the biological components was not investigated in our study.

Antonini et al. [19] consider luminol-dependent chemiluminescence to be a suitable method to measure the earliest events in the inflammatory process, based on their experiments with inflammatory effects of silica nanoparticles. Nevertheless in other study [20], pathogenic respirable mineral dusts did not induce higher production of ROS than non-pathogenic ones. Donaldson et al. [21] relate the pathogenicity of various nanomaterials rather to their biopersistence, than in vitro ROS production.

Negative chemiluminescence assay results obtained in presented study indeed do not exclude possible adverse effect of the airborne particle matter on the ROS generation. The situation in a real organism is more complex, the influence of other important factors, such as retention, lysosomal enzyme release, opsonisation, etc., needs to be taken into account, particularly in case of people with already existing health problems accompanied by the increase of ROS levels in the organism (COPD, asthma). Epidemiological studies confirm that those people are generally more threatened with the exposure of particle polluted air [16, 22].

4.2. Basotest

Recent increase in incidence and prevalence of allergic disease is probably caused by environmental factors, particularly by polluted air [23]. Particulate matter can serve as a vector carrying allergens (polen grains, mites, moulds) into the respiratory tract, where they can be deposited [24]. Furthermore, studies have shown that particulate matter enhance the susceptibility to common environmental allergens and trigger sensibilization to neoallergens [25, 26].

Basotest, used in this study to evaluate the allergenic properties of airborne particle sample, allows us to monitor the basophil activation after the allergen binding to IgE present on the surface of the basophils. Basophil activation led to release of inflammatory mediators and consequent manifestation
of allergic inflammation [27]. Basophil degranulation assay is based on the measurement of exposition of CD63 transmembrane protein on the surface of activated basophils [11].

The cut-off limit between negative and positive response against inhalation allergens was established on 15% of activated basophils [11]. Our results show that the airborne particle sample did not stimulate significant basophil degranulation since none of the tested airborne particle concentrations exceeded this limit.

Schober et al. [28] investigated possible synergy between organic extracts of urban aerosol and major allergen of birch pollen grains (rBet v 1) on basophil activation in birch pollen-allergic donors and healthy controls. Synergy was only observed in blood samples from allergic individuals. Samples from healthy controls did not showed upregulation of CD63 by rBet v 1. Affirmatively other studies indicates that airborne particulate matter, or its organic extract, enhances allergic inflammatory mediator realize from basophils in the presence of antigen [27]. The negative results of our study can be caused by the absence of antigens in the tested particle sample. Chemical composition, especially content of organic matter and heavy metals, plays an important role in the adjuvant effect of airborne particulate matter on the allergic response. On the other side, there are experimental works proving that even inert particles (e.g. polystyrene nanoparticles) influenced the allergy reaction [29].

Despite many studies, the accurate mechanism by which airborne particles worsen the allergic reaction is still unknown. Moreover, gas pollutants present in the polluted air can enhanced the effect of the airborne particles [30]. Recent research has revealed new mechanism by which TiO$_2$ nanoparticles cause mast cell degranulation of histamine even without previous IgE antibody-based sensitization through the activation of Ca$^{2+}$ channels [31].

4.3. Lymphocyte proliferation assay

Lymphocyte proliferation is caused by sensible lymphocyte exposition to antigens or allergens. To detect lymphocyte proliferation, the method of $[^{3}H]$thymidine incorporation into newly synthetized lymphocyte DNA was used [12]. Enhanced lymphocyte proliferation reflects more effective immune response and thus possible more intensive removing of particles from the organism. On the other side, increased lymphocyte proliferation can be related to inflammatory and autoimmune disease.

Slight increase in cpm compared with negative control values was recorded using the particle concentrations of 300 pg/ml and 134 µg/ml. However, regarding to the negative results in concentration of 3,000 pg/ml, another evaluation is needed to make a decision on the possible stimulation properties of the airborne particles on the lymphocyte proliferation.

Longer incubation time can enhance assay efficiency in case of less potent stimulants. Incubation period influenced the assay results in study of Miller et Zarkower [32]. T-lymphocytes in the spleen were found to be depressed after 7 days of pre-exposure to carbon dust. However, after 14, 21 and 28 days of pre-exposure to carbon dust 30 to 40 % of enhancement over control group responses were recorded. Worsened T-lymphocyte activity can be caused by oxidative stress related to enhanced apoptosis of T-lymphocytes.

4.4. Cytokine release evaluation

Multiplex methods, especially ALBIA, enable to analyze the cell functionality and reactivity by evaluation of the release of biologically active molecules into the cultivation media. For this pilot study we used commerce kit Human Cytokine 25-Plex Panel enabling to evaluate production of 25 cytokines divided according to their biological function in four panels: Inflammatory Panel, Th1/Th2 Panel, Cytokine II Panel and Chemokine Panel. In case of positive assay results, the set of suitable cytokines would be established to monitor the effects of particulate matter on the immune cells. Effects of the PM$_{2.5}$ sample on the release of 25 various cytokines were assessed in the particle concentration of 3,000 pg/ml. Particular immune mechanisms are involved gradually, based on the published data, we evaluated the cytokine release after 6, 24 and 72 hours after incubation [33].

With regard to the uncertainty of the assay expected on 20 %, the slight stimulation effect was found at some inflammatory cytokines (IL-6, IL-8, TNF-$\alpha$) and chemokines (eotaxin, RANTES, ...
MIP-1α). These effects were recorded only after certain incubation periods. However, the ultimate values were very low compared to the stimulation effect of PHA used as a positive control and in real organism probably would not have any impact.

5. Conclusion
Even if certain effects of the particle sample were observed compared to the results of negative controls, changes in evaluated immune response parameters were not high enough to take effect in a real organism. Thus, the results of the immunological assays did not prove significant effects of airborne particles on immune cells’ functions of healthy persons.

Results of our experiments are related to short-term action of the real airborne particle mixture on the whole blood cultures of healthy people. Obtained data indeed do not exclude health risks of long-term exposure to airborne particles, especially for individuals with genetic predisposition to certain diseases or already existing disease. Age, co- pollutants and existing disease of respiratory tract can modify the immune response to particulate matter exposure.

Suitability of selected immunological assays should be also taken into account. The main challenge of immunological studies of nanomaterials as well as airborne particles is to select an experimental approach that is free of false positive or false negative results. Modification of available assays to be applicable for nanomaterial immunomodulatory effect assessing can be an objective of our future research.

Acknowledgements
Authors thank to project INEF CZ.1.05/2.1.00/01.0036 for the support.

References
[1] Fadeel B 2012 Clear and present danger? Engineered nanoparticles and the immune system Swiss. Med. Wkly. 26 142–w13609
[2] Gilmour I M, Stevens T and Saxena R K 2007 Effect of particles on the immune system In Donaldson K and Borm P (Eds.) 2007 Particle Toxicology CRC Press, NY 245–58
[3] Krug H P 2011 Quality handbook standard procedures for nanoparticle testing [S.l.], Nanommune
[4] Geiser M, Rothen-Rutishauser B, Kapp N, Schurch S, Kreyling W, Schulz H, Semmler M, Im Hof V, Heyder J and Gehr P 2005 Ultrafine particles cross cellular membranes by nonphagocytic mechanisms in lungs and in cultured cells Environ. Health Perspect. 113 1555–60
[5] Kaličáková Z 2012 Air contamination of Moravia-Silesia region by nanoparticles Master’s thesis VSB – Technical University of Ostrava, Faculty of Mining and Geology
[6] Tomášková H, Tomášek I, Šlachtová H and Šebáková H 2011 Impact assessment of PM10 concentrations on mortality and morbidity in Ostrava during smog episodes Hygiena 1 5–10
[7] Dostál M and Šrám R J 2010 The study on the health status of children from Ostrava-Radvanice and Ostrava-Bartovice Ochrana Ovzduší 5–6 35–8
[8] Jančík P, Pavlíková I and Bitta J 2009 Analýza kvality ovzduší na území města Ostravy Ochrana ovzduší 4 24–7
[9] Meij R, Janssen L H J M and van der Kooij J 1986 Air pollutant emissions from coal fired power stations KEMA Sci. Tech. Rep. 6 651–69
[10] Lochmanová A 2005 Polymorphonuclear Metabolic Activity Evaluation Using Chemiluminescence Assay SOP 25.47 CKL ZÚ Ostrava
[11] EXBIO BasoFlowEx® Kit Cat. No: ED7036
[12] Lochmanová A 2005 Lymphocyte proliferation Evaluation using Lymphocyte proliferation Assay with 3H thymidin incorporation SOP 25.46 CKL ZÚ Ostrava
[13] Invitrogen Corporation 2010 Human Cytokine 25-Plex Pan. Cat. No: lhc0009 User manual
[14] Kleener P (Ed.) 2006 Vnitřní lékařství Praha Galén
[15] Pope III C A and Dockery D W 2006 Health effects of fine particulate air pollution: lines that connect J. Air Waste Manag. Assoc. 6 709–42

[16] Katsouyanni K, Touloumi G, Samoli E, Gryparis A, Le Tertre A, et al. 2001 Confounding and effect modification in the short–term effects of ambient particles on total mortality: results from 29 European cities within the APHEA2 project Epidemiology 5 521–31

[17] Donaldson K, Slight J and Bolton J E 1998 The effect of products from bronchoalveolar–derived neutrophils on oxidant production and phagocytic activity of alveolar macrophages Clin. Exp. Immunol. 74 477–82

[18] Antonini J M, Dyke K V, Ye Z, Dimatteo M and Reasor M J 1994 Introduction of luminol–dependent chemiluminescence as a method to study silica inflammation in the issue and phagocytic cells of rat lung Environ. Health. Perspect. Suppl. 10 37–42

[19] Antonini J M, Roberts, J R, Yang H M, Barger M W, Ramsey D, Castranova V and Ma J Y 2000 Effect of silica inhalation on the pulmonary clearance of a bacterial pathogen in Fischer 344 rats Lung. 6 341–50

[20] Gormley P, Kowolik M J and Cullen R T 1985 The chemiluminescent response of human phagocytic cells to mineral dusts Br. J. Exp. Pathol. 66 409–16

[21] Donaldson K, Borm P J A, Castranova V and V Gulumian M 2009 The limits of testing particle–mediated oxidative stress in vitro in predicting diverse pathologies, relevance for nanoparticles Part. Fibre Toxicol. 27 6–13

[22] Kim C S and Kang C W 1997 Comparative measurement of lung deposition of inhaled fine particles in normals and patients with obstructive airway disease Am. J. Respir. Crit. Care Med. 155 899–905

[23] Gutová V 2007 Alergie – systémové onemocnění s lokálními projevy Dermatologie pro praxi 1 27–30

[24] Jin C, Shelburne C P, Li G, Potts E N, Riebe K J, Sempowski G D and Foster M A 2011 Particulate allergens potentiate allergic asthma in mice through sustained IgE–mediated mast cell activation J. Clin. Invest. 3 941–55

[25] Ishizaki T, Koizumi K, Ikemori R, Ishiyama Y and Kushibiki E 1987 Studies of prevalence of Japanese cedar pollinosis among the residents in a densely cultivated area Ann. Allergy. 58 265–70

[26] Diaz–Sanchez D, Tsien A, Fleming J and Saxon A 1997 Combined diesel exhaust particulate and ragweed allergen challenge markedly enhances human in vivo nasal ragweed–specific IgE and skews cytokine production to a T helper cell 2–type pattern J. Immunol. 5 2406–13

[27] Kepley C L, Lauer F T, Oliver J M and Burchiel S W 2003 Environmental polycyclic aromatic hydrocarbons, benzo(a)pyrene (BaP) and BaP-quinones, enhance IgE-mediated histamine release and IL-4 production in human basophils Clin. Immunol. 107 10–9

[28] Schober W, Lubitz S, Belloni B, Gebauer G, Lintelmann J, Matuschek G, Weichenmeier I, Eberlein–Konig B, Buters J and Behrendt H 2007 Environmental polycyclic aromatic hydrocarbons (PAHs) enhance allergic inflammation by acting on human basophils Inhal. Toxicol. Suppl. 1 151–6

[29] Granum B and Løvik M 2002 The effect of particles on allergic immune responses Toxicol. Sci. 65 7–17

[30] Diaz–Sanchez D and Riedl M 2005 Diesel effects on human health: a question of stress? Am. J. Physiol. Lung Cell Mol. Physiol. 289 L722–3

[31] Chen J–C and Schwartz J 2008 Metabolic Syndrome and Inflammatory Responses to Long–Term Particulate Air Pollutants Environ. Health. Perspect. 5 612–7

[32] Miller S D and Zarkower A 1974 Effects of Carbon Dust Inhalation on the Cell–Mediated Immune Response in Mice Infect. Immun. 3 534–9

[33] Largelius M, Jones P, Franck K and Gaines H 2006 Cytokine detection by multiplex technology useful for assessing antigen specific cytokine profiles and kinetics in whole blood cultured up to seven days Cytokine 33 156–65