Workers’ cytokines profiling upon exposure to MWCNT aerosol in occupational settings

L M Fatkhutdinova¹, T O Khaliullin¹, R Zalyalov¹, O L Vasilyeva¹, I K Valeeva¹ and I G Mustafin³

¹Department of Hygiene, Occupational Medicine, Kazan State Medical University, 49, Butlerova str., Kazan, Russia
²Central Laboratory, Kazan State Medical University, 49, Butlerova str., Kazan, Russia
³Department of Biochemistry, Kazan State Medical University, 49, Butlerova str., Kazan, Russia

¹E-mail: liliya.fatkhutdinova@gmail.com

Abstract. Recent studies have found that upon pulmonary exposure to multi-walled carbon nanotubes (MWCNTs) animals develop primarily fibrosis and granulomas in lungs. In vitro and in vivo studies also give reason to assume that local exposure could be related to remote effects, including immune system and the endothelium. To investigate the remote effect hypothesis, we have analyzed blood, nasal lavage and induced sputum samples taken from workers in the frame of the Russian epidemiological study on Carbon Nanotubes Exposure and Risk Assessment (CNT-ERA). In serum and nasal lavage no significant differences between exposure and control groups were observed with a high variability to the cytokines content. In the samples of induced sputum from exposed workers the content of IL-1β, IL-6, IL-8, TNF-α, IL-4, IL-5, IFN-γ exceeded the control group values, but after the regression models construction and bootstrap analysis, significant differences were found only for IL-1β. This study could not provide evidences of blood cytokines changes following local cytokine production in airways in workers exposed to MWCNTs. Cytokines variability in serum and nasal lavage may indicate the absence of severe systemic inflammatory response upon the existing occupational exposure to MWCNTs. Other systemic responses (including allergy-like or autoimmune reactions) should be regarded as well.

1. Introduction
Unique properties of multi-walled carbon nanotubes are in demand by different industries – in construction, engineering, energy-producing industry, medicine [1-3]. However, recently accumulated data suggest risk to human health upon the occupational contact with carbon nanotubes [4]. Nanomaterials, depending on their characteristics and compositions, could interact with the immune system in several ways and either enhance or suppress immune system function [5]. Measuring the levels of cytokines can be useful tools in evaluating nanoparticle immunotoxicity. Moreover, screening of a single reporter is inadequate; combinations of cytokine levels should be measured, due to the cytokine network influence on the immune system [5].

Th1 (Type-1) and Th2 (Type-2) cytokines are the two groups of cytokines produced by two different subpopulations of CD4+ T-cells [5,6]. They determine whether the immune response to the...
antigen would be humoral or cell-mediated. At the same time, a plurality of cells other than CD4+ may also express these cytokines – CD8+ T-cells, phagocytic cells (including monocytes, B-cells, macrophages), NK-cells, eosinophils, mast cells, basophils and others. Th1 cells secrete large quantities of interferon IFN-g, interleukin IL-2, IL-3, IL-12, granulocyte macrophage colony-stimulating factor (GM-CSF) and small quantities of tumor necrosis factor (TNF). Th2 cells secrete large quantities of IL-3, IL-4, IL-5, IL-6, IL-10, and small quantities of GM-CSF and TNF. The cytokines produced by specific T-cells (e.g. IFN-g from Th1 cells and IL-10 from Th2 cells) usually inhibit the action of other type of T-cells and thereby potentiate a particular pattern of immune response. IFN-g and IL-4 are the main markers of Th1 and Th2 cells, respectively. Various forms of cytokine secretion correspond to different functions of effector cells. Th1-cells promote cell-mediated immune response. Th2-cells are primarily helper cells that affect B-cells and induce humoral responses, such as secretion of antibodies by B-cells, mostly IgE. Both T-cell types affect each other, including the secretion of cytokines. Thus, these subpopulations are mutually antagonistic, and predominant subpopulation may determine the outcome of inflammatory or infectious process. In case of chronic inflammatory process Th1 cytokines inhibit tissue remodeling and collagen synthesis, but may lead to tissue damage, whereas Th2 response promotes fibrotic process, promoting healing of damaged areas [5] that may be related to pro-fibrotic activity of MWCNTs [7-9].

IL-1β is the most studied member of the IL-1 family [7]. This cytokine is produced by activated macrophages as a proprotein, which is proteolytically processed to its active form. This cytokine is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. Increased production of IL-1B causes a number of different autoimmune inflammatory syndromes.

Recent studies have found that upon pulmonary exposure to MWCNTs animals develop primarily fibrosis and granulomas in the pulmonary system [8-11]. However, in vitro and in vivo studies also give reason to assume that local exposure could be related to remote effects, including immune system [12] and the endothelium [13-15].

To investigate the remote effect hypothesis, we have analyzed blood, as well as nasal lavage and induced sputum samples taken from workers in the frame of the Russian epidemiological study on carbon nanotubes exposure and risk assessment (CNT-ERA).

2. Materials and Methods

2.1. MWCNT production facility

The study was conducted at two MWCNT-producing enterprises with the same reactor type. MWCNTs are produced using the catalytic vapor deposition method [16]. The technological process includes synthesis in reactor, raw product harvesting, optional grinding/disintegration and functionalization or acid treatment. MWCNT-characteristics are shown in the Table 1.

| Parameters                              | MWCNT          |
|-----------------------------------------|----------------|
| External diameter(nm)                   | 8-15           |
| Internal diameter(nm)                   | 4-8            |
| Length(um)                              | 2 and more     |
| Total amount of impurities–catalysts leftovers(%) | Up to 5 |
| Bulk density(g/cm³)                     | 0.03-0.05      |
| Specific surface area(m²/g)             | 300-320        |
| Thermal stability in air(ºC)            | Up to 600      |
Elemental carbon (EC) was evaluated in air samples taken in occupational settings, and the CNT presence was confirmed by transmission electron microscopy [17]. TEM microphotographs have shown the presence of MWCNT-containing agglomerates at all technological process stages. Agglomerates size ranged from 1 to 10 um, and no individual MWCNTs were found. Cobalt and nickel used as catalysts were found in agglomerates using EDXA analysis.

The highest MWCNT aerosol short-term concentration was registered during the reactor cleanout process, reaching 157 ug/m³; time-weighted average (TWA) concentration was 29.6 ug/m³ for the 8-hours shift respectively. Respirable fraction TWA concentrations (obtained using the cyclone) for EC at different workstations ranged from 0.53 to 6.11 ug/m³.

2.2. Study group
25 workers of both genders aged 18-65 years with no history of diagnosed chronic respiratory diseases before they started working with CNTs were taken. 11 workers who had more than 1 year contact with MWCNT aerosol composed the exposure group. The control group (14 people) included persons who were not exposed to the MWCNT aerosol, but worked at the same enterprise - engineers, secretaries, research assistants. Average age (M±SD) of exposure group was 35.5±15.8 years, controls – 30.2±10.4. There were 4 smokers among exposed personnel and 3 in controls.

2.3. Biomaterial sampling
The blood sampling was carried out aseptically by a qualified staff using a BD Vacutainer® Safety-lok™ device («Beckton Dickenson», USA) with disposable vacuum tubes containing coagulation activator (Vacutainer®, USA). After clot retraction all tubes were centrifuged at 2500 rpm, serum was removed and frozen at -80 °C.

Inhalation is a leading a potential nanoparticles exposure route in humans, and nasal cavities are the first part of respiratory tract, where direct contact with MWCNTs happens. Similar epithelial lining properties imply similar cellular responses in nasal cavities and lower parts of the respiratory system. Thus, a method of nasal lavage was selected to evaluate pro-inflammatory properties of inhalatory exposure to MWCNT [18]. The induced sputum method allowed obtaining sputum samples from deeper parts of the respiratory tract, while being non-invasive and minimally uncomfortable for the subjects.

For the nasal lavage sampling, examinee tilted his head back at an angle of 45 degrees. 4.5 ml of physiological saline, heated to 37 °C were introduced successively into each nostril using a soft catheter and 10ml syringe; after 10 seconds examinee bent to drain solution from the nasal cavity into the funnel (inner opening closed with polyamide mesh) and further into the vial. The resulting samples were centrifuged for 10 minutes at 9000 rpm and supernatant frozen at -80 °C.

Induced sputum is gathered in the absence of its spontaneous production (in normal and healthy individuals) by inhalation of aerozolized hypertonic saline, which causes an increase in bronchial secretions [19]. This method allows obtaining sputum samples from the deeper parts of the respiratory tract, while being non-invasive and minimally uncomfortable for subjects. After centrifugation of the samples and separation of the cell pellet necessary indicators were determined in the supernatant.

2.4. Cytokine profiling
Evaluation of the cytokines content in blood, nasal lavage and induced sputum samples was conducted using a flowcytometry kit Th1/2 Multiplex (eBioscienceinc., USA) on the BD Canto flow cytometer (Beckton Dickenson, USA).

2.5. Statistical analysis of the results
For statistical analysis we used R statistical package. Initial statistical analysis of cytokine profiles obtained from the employees was carried out using t-test. Besides, generalized linear models including both main effects (exposure, age, smoking) and pairwise interactions were designed and developed.
Models fitting was performed using successive elimination and Akaike information criterion (AIC) evaluation with exhaustive search and bootstrap analysis [20]. All created models were verified by the likelihood ratio test and analysis of variance. The confidence intervals of regression coefficients derived from a small sample were refined by bootstrap analysis. Bootstrap analysis included repeated sampling with replacement from the original sample, which represents the sampling distribution of the statistic. On the basis of the analyzed indicators values deviation obtained in the simulation, confidence intervals were built. Bootstrap confidence intervals (ICA) were constructed based on which we could make conclusions about the statistical significance of the relationship.

3. Results and discussion
The results of cytokines content analysis in serum, nasal lavage and induced sputum samples are shown in figures 1, 2 and 3.

![Cytokines content in serum](image)

**Figure 1.** Cytokines content in serum, pg/ml, M±m. TNF-b levels were below the detection limit in both groups.

In serum no significant differences between exposure and control groups was observed with a high variability to the cytokines content (figure 1).

In nasal lavage samples no statistically significant differences between the exposed group and unexposed individuals were detected as well (figure 2).
In the samples of induced sputum from exposed workers a definite trend has been traced – the content of IFN-g, IL-1b, IL-6, IL-8, TNF-a, IL-5, IL-4 exceeding the control group values (Fig. 3), but after the regression models construction and bootstrap analysis, significant differences were found only for IL-1b (b = 1030.9, CI=1.64, 2060.24).

The content of IL-1b, which is involved in various processes including inflammation, cell differentiation, proliferation and apoptosis, was higher in induces sputum of exposed workers compared with controls.
In siRNA experiments on human primary macrophages Palomaki et al. [21] demonstrated that the NLRP3 inflammasome was essential for IL-1β secretion induced by long, needle-like MWCNT and asbestos. NLRP3 inflammasomes are multiprotein complexes that act as a major mediator for inflammatory responses. In addition, it was shown that double-walled carbon nanotubes enhanced the release of the pro-inflammatory cytokine IL-1β from human monocytes via NLRP3 inflammasome activation pathway [22]. Arnoldussen Y. et al [23] have recently presented results indicating that IL-1 contributes to the cellular and molecular effects of CNT exposure and that the type of CNT has an important effect on the cellular response. The author used cells from IL-1α/β wild type (IL1-WT) mice and cells with reduced inflammatory potential from IL-1α/β deficient (IL1-KO).

The IL-1β cytokine is of particular interest because its production has been shown to be associated with autoimmune disorders and fibrosis [21-23]. In report made by Ilves et al. on first results of the inhalation study of rigid MWCNT biological effects in mice, a significant increase in expression of Th2-cytokine in the lung tissues and asthma-like allergic response was shown [24].

4. Conclusion
Our preliminary study could not provide evidences of blood cytokines changes following local cytokine production in airways in workers exposed to MWCNTs. Cytokines variability in serum and nasal lavage may indicate the absence of severe systemic inflammatory response upon the existing occupational exposure to MWCNTs. At the same time, other systemic responses (including allergy-like or autoimmune reactions) should be regarded.

Application of transcriptomics methods, including the identification and determination of mRNA expression patterns of certain cytokines encoding genes, may be suggested instead of direct assessment of the cytokines content in blood, as the latter is often subjected to fluctuations related to changes in environmental parameters.

References
[1] Yakovlev G, Pervushin G, Maeva I, Keriene J, Pudov I, Shaybadullina A, Buryanov A, Korzenko K and Senkov S 2013 Procedia Eng 57 407
[2] Behabtu N, Young CC, Tsentalovich DE, Kleinerman O, Wang X, Ma AW, Bengio EA, ter Waarbeeck RF, de Jong JJ, Hoogerwerf RE et al. 2013 Science 339(6116) 182
[3] Saito N, Haniu H, Usui Yu, Aoki K, Hara K, Takanashi S, Shimizu M, Narita N, Okamoto M, Kobayashi Set al. 2014 Chem Rev 114(11) 6040
[4] Fatkhutdinova LM, Khaliullin TO and Shvedova AA 2015 Nanotechnol. Russ 10(5-6) 501
[5] Elsabahy M and Wooley KL 2013 Chem Soc Rev 42 5552
[6] Wynn TA 2004 Nat Rev Immunol 4(8) 583
[7] Dinarello Ch.A. 2009 Annual Review of Immunology 27 519-550
[8] Mercer RR, Scabilloni JF, Hubbs AF, Battelli L, McKinney W, Friend S, Wolfarth MG, Andrew M, Castranova V and Porter DW 2013 Part Fibre Toxicol 10 33
[9] Porter DW, Hubbs AF, Mercer RR, Wu N, Wolfarth MG, Sriram K, Leonard S, Battelli L, Schwiegler-Berry D, Friend S et al. 2010 Toxicology 269(2-3) 136
[10] Khaliullin TO, Shvedova AA, Kisin ER, Zalyalov RR and Fatkhutdinova LM 2015 Bull Exp Biol Med 158(5) 684
[11] Shvedova AA, Yanamala N, Kisin ER, Tkach AV, Murray AR, Hubbs A, Chirila MM, Keohavong P, Sycheva LP, Kagan VE and Castranova V 2014 Am J Physiol Lung Cell Mol Physiol 306(2) 170
[12] Mitchell L A, Lauer F T, Burchiel SW and McDonald JD 2009 Nat Nanotechnol 4(7) 451
[13] Erdely A, Hulderman T, Salmen R, Liston A, Zeidler-Erdely PC, Schwiegler-Berry D, Castranova V, Koyama S, Kim YA, Endo M and Simeonova PP 2009 Nano Lett 9(1) 36
[14] Nurkiewicz TR, Porter DW, Hubbs AF, Stone S, Moseley AM, Cumpston JL, Goodwill AG, Frisbee SJ, Perrotta PL, Brock RW et al. 2011 Res Rep Health Eff Inst 164 3
[15] Stapleton PA, Minarchick VC, Cumpston AM, McKinney W, Chen BT, Sager TM, Frazer DG, Mercer RR, Scabilloni J, Andrew ME et al. 2012 Int J Mol Sci 13(11) 13781
[16] Cassell A M, Raymakers J A, Kong J and Dai HJ 1999 J Phys Chem B 103 6484
[17] Khaliullin TO, Zalyalov RR, Shvedova AA, Birch EM and Fatkhutdinova LM, 2013 Book of abstracts, II International School-Conference Applied Nanotechnology & Nanotoxicology ed A Vedyagin 23
[18] Siegel PD, Short S, Jones WG 1995 Proc. of the Sixth FIOH-NIOSH Joint Symposium on Occupational Health and Safety (8-10 August 1995, Espoo, Finland) (People and Work - Research Reports: Finnish Institute of Occupational Health) 81
[19] Paggiaro PL, Chanez P, Holz O, Ind PW, Djukanović R, Maestrelli P and Sterk PJ 2002 Eur Respir J 20(Suppl. 37) 3
[20] Moore D, McCabe G and Craig B2010 Introduction to the Practice of Statistics 7th ed (W. H. Freeman)
[21] Palomäki J, Välimäki E, Sund, J Vippola M, Clausen PA, Jensen KA, Savolainen K, Matikainen S and Alenius H 2011 ACS Nano 5(9) 6861
[22] Meunier E, Coste A, Olagnier D, Authier H, Lefèvre L, Dardenne C, Bernad J, Béraud M, Flahaut E and Pipy B 2012 Nanomedicine 8 987
[23] Arnoldussen YJ, Skogstad A, Skaug V, Kasem M, Haugen A, Benker N, Weinbruch S, Apte RN and Zienolddiny S2015 Cytokine 73(1) 128
[24] Ilves M, Rydman E, Savolainen K and Alenius H 2013 Book of abstracts, II International School-Conference Applied Nanotechnology & Nanotoxicology ed A Vedyagin 192