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The effect of assay type and sample matrix on detected cytokine concentrations in human blood serum and nasal lavage fluid

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ABSTRACT

Cytokine concentrations in biological fluids are widely used markers for activation of immunological processes. Confirming the reproducibility of measurements is important, especially in longitudinal or multicenter studies where time between analyses or different analyzing laboratories increases the intra-assay variability. In this study, the reproducibility of the cytokine analysis conducted with different assay platforms was studied by comparing the results of two cytokines [interleukin (IL)-6 in serum and nasal lavage fluid (NAL) and IL-8 in NAL] analyzed with Meso Scale Discovery (MSD) ultra-sensitive single and multiplex assay kits ($n = 76$). In addition, the difference in cytokine levels between two biological sample matrices was studied by comparing the results of altogether 9 cytokines [IL-6, IL-2, IL-8, IL12p70, IL-1β, granulocyte–macrophage colony-stimulating factor (GM-CSF), interferon (IFN)γ, IL-10 and tumor necrosis factor (TNF)α] measured from serum and NAL of the same study subjects ($n = 460$).

The results show that the cytokine concentrations analyzed with single and multiplex assays are concordant but not equal. Comparison of the different matrices revealed that cytokine concentrations in serum do not correspond with concentrations detected in nasal lavage fluid.

It can be concluded that comparability of the results from single and multiplex analysis of cytokines is high, but the concentrations should not be compared directly with each other. The differences between concentrations analyzed from serum and nasal lavage fluid indicate that the levels are specific for each matrix and represent distinct immunological conditions.

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1. Introduction

Cytokines are recognized as important molecules in cellular signaling in both healthy and diseased individuals [1–3]. However, the complexity and diverse nature of these proteins calls for techniques able to analyze simultaneously multiple cytokines or composition of cytokine networks rather than concentrations of single analytes. During the last decade, multiplex technology has enabled the analysis of large panels from even smaller sample volumes, widening significantly the scope of cytokine research. Before this technology is fit for use in study settings involving multiple centers or longitudinal design, the reproducibility of the method has to be proven. There are several reports on comparison of multiplex methods against the enzyme-linked immunosorbent assay (ELISA) showing good agreement in culture supernatants [4,5] but deteriorating quantitative agreement in more complex sample

Abbreviations: ELISA, enzyme linked immunosorbent assay; IA, immunoassay; RT-PCR, reverse transcription polymerase chain reaction; MSD, Meso Scale Discoveries; PBS, Phosphate-Buffered Saline; IL, interleukin; IFN, interferon; GM-CSF, granulocyte–macrophage colony-stimulating factor; TNF, tumor necrosis factor; NAL, nasal lavage.

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matrices [6,7]. Typically, the absolute levels of cytokines can be affected, e.g., by choice of supplier for the reagents [8], which complicates the comparison between different platforms even further.

Techniques for measuring cytokines from biological liquids include several methods utilizing antibody–antigen reactions, e.g., ELISA, other immunoassays (IA), multiplex bead assay and multiplex suspension array, and also methods detecting the gene expression level, e.g., RT-PCR [9]. ELISA and IA require a large amount of sample whereas multiplex bead assay or multiplex suspension array detects numerous proteins simultaneously from low sample volume [9–11]. The MSD kit assays used in this study are based on sandwich immunoassay technique, where cytokines in liquid sample bind to capture antibodies immobilized on the working electrode surface and the labeled detection antibodies in turn bind the captured cytokine. MSD SULFO-TAG™ label is electrochemiluminescent compound and it emits light when voltage is applied to the plate electrode, providing high sensitivity for the detection of low level of cytokines [12].

There are relatively few data available on variation of cytokine concentrations in different biological matrices, but studies with healthy volunteers show that concentrations vary according to sample material [9,11,13]. Cytokines are typically produced locally, and the capability to produce certain cytokines is cell-specific, thus the concentrations of cytokines are likely to differ according to the sampling material and site. Cytokines have been measured, e.g., from blood, serum, plasma, urine, saliva, conjunctival sac fluid, gingival crevicular fluid, cultured and collected blood cells, nasal lavage fluid, induced sputum, bronchoalveolar fluid and exhaled breath condensate [9,14–16]. In addition to the differences in the production of cytokines at different sites, also the sample matrix itself may affect the concentrations by hindering the analysis. This is evident especially when analyzing complex matrices such as serum or highly diluted samples such as exhaled breath condensate or nasal lavage fluid [14,17]. The improved sensitivity of the assays has made it possible to detect significant differences in cytokine profiles also at subclinical level, although the biological relevance of differences in very low concentrations is questionable.

The aim of this study was to determine the reproducibility of the cytokine analysis in multiplex and singleplex assays from the same manufacturer and to compare the cytokine levels in corresponding serum and nasal lavage fluid samples. The collected data provides crucial information for assessing the reliability of the data obtained with multiplex platform and usability of the methodology, e.g., in multicenter research projects.

2. Materials and methods

2.1. Sample collection

The analyzed serum and nasal lavage samples were collected for the multinational research project "Health Effects of Indoor Pollutants: Integrating microbial, toxicological and epidemiological approaches (HITEA)". As a part of the project, a cross-sectional and longitudinal study of teachers' health in moisture damaged and reference school buildings in three European countries (Spain, The Netherlands and Finland) was conducted. Samples of blood and nasal lavage fluid were collected in three sampling campaigns within a year; April–June 2009 (before the summer holidays), August–September 2009 (after summer holidays) and January–March 2010.

The blood and nasal lavage samples were taken from the same teachers by a centrally trained healthcare professional during the school visits. All samples were transported within 8 h at +4 °C to the processing laboratory. For each study subject, one 8.5 ml serum tube with separating gel was filled and allowed to stand for 30 min. The serum was separated by centrifugation (1780 × *g*, 15 min, room temperature), after which it was aliquotted and frozen (−70 °C). The nasal lavage samples were taken by lavaging the nasal cavities with altogether 9 ml of warm physiological solution [Phosphate-Buffered Saline (PBS), Gibco®, Life Technologies, Paisley, UK] [18]. The samples were centrifuged (425 × *g*, 10 min, room temperature) and the separated supernatant was aliquotted and frozen (−70 °C).

2.2. Cytokine assay

The cytokine analyses were completed in Inhalation Toxicology Laboratory at University of Eastern Finland using Meso Scale Discovery (MSD) Sector Imager™ 2400A with Discovery Workbench® 3.0 software. The samples were analyzed both with MSD® Human IL-6 Ultra-Sensitive Kit and MSD® Human TH1/TH2 10-Plex Ultra-Sensitive Kit (for IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p70, IL-13 and TNFα), both purchased from MSD (Rockville, MD, USA). First, a subset ($n = 76$) of serum and nasal lavage samples were analyzed according to manufacturer's instructions and using reagents provided with the kit. Assay diluent was added to all wells of a pre-coated 96-well plate and samples, standards and controls were incubated for 2 h in room temperature on an orbital shaker. After washing three times with wash buffer (PBS + 0.05% Tween 20), each analyte bound to a specific spot was further conjugated by adding detection antibody and incubating for 1 h in room temperature. After washing, the read buffer was added to each well and the amount of analyte was assessed by detecting the electrochemiluminescence signal with a sensitive camera. The concentration of the analyte was calculated by comparing the results to the standard curve reconstituted in the provided assay diluent.

Based on the results of the subset, nine cytokines (including one not tested in the subset, GM-CSF) were selected to be measured from the whole set of serum ($n = 470$) and nasal lavage ($n = 489$) samples and the method was modified to improve sensitivity and repeatability of the analysis. Briefly, a blocking step was added to minimize the nonspecific binding of antibodies, and the incubation time with samples was increased to overnight (+4 °C, shaker) to improve the sensitivity of the assay. In serum samples, 9 cytokines (IL-2, IL-8, IL12p70, IL-1β, GM-CSF, IFNγ, IL-6 IL-10 and TNFα) were analyzed using MSD® Human ProInflammatory 9-Plex Ultra-Sensitive Kit, whereas in nasal lavage samples, 8 cytokines (IL-2, IL12p70, IL-1β, GM-CSF, IFNγ, IL-6, IL-10, TNFα) were analyzed with MSD® Human ProInflammatory Custom 8-Plex Ultra-Sensitive Kit and IL-8 was measured using MSD® Human IL-8 Ultra-Sensitive Kit. A separate analysis of IL-8 from the nasal lavage samples was done because in some cases the high concentrations of IL-8 appeared to disturb the analysis of other cytokines in the multiplex-system. The detection limit was defined for each cytokine and for each plate separately by using the Meso Scale Discovery Sector Imager™ 2400A with Discovery Workbench® 3.0 software. The lower limit of detection was the calculated concentration of the signal that is 2.5 standard deviations over the zero calibrator of the kit (Table 1). The samples were measured in duplicates, thus the resulting concentration is the mean of the two replicate samples.

2.3. Statistical analysis

The data description values and statistical tests were computed by IBM SPSS Statistics Version 19. The data was not normally distributed, thus non-parametric Wilcoxon matched-pairs signed-ranks test and Spearman rank correlation were used to analyze the differences and strength of association between the groups. The values below detection range were included in the statistical analysis, but the sensitivity analysis was done also by analysing the data with only values above the detection limit.

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