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Effect of the anticoagulant, storage time and temperature of blood samples on the concentrations of 27 multiplex assayed cytokines – Consequences for defining reference values in healthy humans

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ABSTRACT

Cytokines are potentially useful biomarkers of sepsis and other inflammatory conditions. Many cytokines can be released by leukocytes and platelets after sampling. The sampling and processing techniques are consequently critically important to measure the *in vivo* levels. We therefore examined the effects of four different anticoagulants, EDTA, citrate, lepirudin, heparin compared to serum, on the levels of 27 different cytokines. The effects of storage temperature, freezing and thawing on the plasma cytokines were examined. Cytokines were analysed using a multiplex immunoassay. The cytokine levels in serum were significantly higher compared with plasma, consistent with release of cytokines *in vitro* during coagulation. In general, the lowest values for all cytokines were found in EDTA samples, stored on crushed ice, centrifuged within 4 h and thereafter stored at −80 °C. MCP-1 and MIP-1β levels were highest in heparin plasma and storage of blood for up to 4 h at room temperature significantly increased the interleukin (IL)-2, IL-6, IL-8, IFN-γ and GM-CSF levels in EDTA plasma, indicating post-sampling release. In contrast, the IP-10 levels were unaffected by sample storage at both temperatures. Our results indicate that the cytokines were more stable in plasma than in whole blood after sampling. Thus, cytokines should be analysed in EDTA plasma samples stored on ice and centrifuged within 4 h. Based on these data, the reference ranges of 27 cytokines in EDTA plasma in 162 healthy human donors were calculated.

1. Introduction

Cytokines, chemokines and growth factors are potential biomarkers of sepsis and other inflammatory diseases [1–3]. These compounds are extensively used as biomarkers of inflammation in a number of human diseases and inflammatory conditions [4–10]. In the case of many analytes in general, much attention has been focussed on the pre-analytical sampling conditions [11,11–13]. Although poorly studied, the sampling and processing techniques used for analysis of the cytokine levels in plasma and/or serum samples might also be important. To obtain cytokine values representative of the *in vivo* conditions, the sampling techniques must prevent cytokines from being released by blood leukocytes after sampling. The time and the storage temperature between the sampling and centrifugation steps might therefore be important.

Human blood leukocytes, including monocyte-macrophages and neutrophils, synthesize a number of cytokines after stimulation with an endotoxin or bacteria through Toll-like receptor activation [14]. A number of other stimuli, including bacterial products [15], complement fragment C5a [16], immune complexes, complement activation, C5a [17] and calcium ionophores also stimulate cytokine synthesis [18]. Coagulation activation itself and thrombin can also enhance the release

Abbreviations: EDTA, ethylenediamine tetraacetic acid; IL, interleukin; RT, room temperature

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of IL-1β, IL-6 and TNF by monocytes through activation of protease-activated receptors [19]. Thrombin can activate protease-activated receptor (PAR)-1 and PAR-4, leading to leukocyte activation and cytokine release [19].

When blood is drawn into plastic tubes, complement and coagulation activation by the plastic surface leads to the activation of immune-competent cells and platelets, in part due to their recognition of this foreign surface [20]. This phenomenon of activation by foreign surfaces is termed bioincompatibility and opposite biocompatibility [20]. Complement is rapidly activated by plastic and other foreign surfaces and participates in the activation of platelets and leukocytes [21]. Coating plastic tubing with heparin effectively reduces complement activation and cytokine release [22]. The release of the anaphylatoxin C5a is involved in cytokine release triggered by several different stimuli [17,23]. In addition, coagulation activation and thrombin can also stimulate immune-competent cells, including leukocytes, leading to cytokine release [24].

Previously, most studies used serum for cytokine measurements [25]. The manufacturer of many ELISA and immunoassay kits for cytokine analyses also recommends using serum samples for the analyses. However, several studies indicated that coagulation activation is linked to inflammation in a process called immunothrombosis [26]. The potential role of thrombin in coagulation activation leading to cytokine release in human whole blood samples is yet to be examined. Several anticoagulants, such as EDTA and citrate, bind calcium and thereby inhibit both coagulation and the activation of the complement system. However, newer anticoagulants, such as hirudin and its recombinant analogue lepirudin, which specifically inhibit thrombin, have not been evaluated as anticoagulants for use in cytokine analyses. In contrast to heparin and calcium-binding anticoagulants, such as EDTA and citrate, lepirudin has no adverse effects on complement activation [27]. Lepirudin has therefore been considered the preferred anticoagulant for whole blood when the role of complement activation *in vitro* is examined [27]. The levels of 27 different cytokines, interleukins and growth factors in EDTA, citrate, lepirudin and heparin plasma and serum obtained from ten healthy blood donors were therefore examined. The influence of storage time and temperature before and after centrifugation on cytokine levels were also studied.

2. Materials and methods

2.1. Study population

To examine the effects of temperature and storage time on cytokine levels, venous blood was collected from ten healthy blood donors (five females and five males). The donors were without any medication and did not experience fever or other symptoms of infectious or inflammatory illness during the two weeks prior to blood sampling. The blood donors provided written informed consent. The study was approved by the regional ethics committee of the northern Health Region of Norway. To determine the reference ranges, EDTA plasma samples from the following groups of healthy donors were included: (1) 49 healthy blood donors from the Blood Bank of Nordlandssykehuset Bodø, (2) 42 healthy controls in a clinical epidemiological study performed in Nordland County, Norway, and (3) 71 healthy persons from a local study on health care workers; giving a total of 162 healthy Norwegian persons (107 females and 55 males, aged range 18–85). The samples were obtained by venipuncture after an overnight fast, between 8 and 9 a.m. and were immediately placed on ice, centrifuged within 15 min at +4 °C and stored at −80 °C until analysis.

2.2. Blood sampling and sample tubes

To prevent contamination by tissue thromboplastin during sampling, blood was first drawn into one dry 5-mL Vacutainer® tube (Becton Dickinson (BD), Plymouth, UK), which was discarded. Winged blood collection sets (BD Vactuainer® Safety-Lok™, needle gauge 21, needle length 0.75 inches, tubing length 7 inches, with luer locks (Franklin Lakes, NJ, U.S.A.) were used to collect the blood. Tubes were filled in the following order: blood collected in lepirudin-containing tubes (Refludan®, 50 μg/mL) was added to 4.5-mL Nunc™ polypropylene CryoTubes™ (NUNC A/S, Roskilde, Denmark) using lids from dry 4.5-mL BD Vacutainer® tubes, blood for serum samples was collected in tubes without an additive (Vacuette®, 2 mL), in 3.2% sodium citrate Vacuette® tubes (2 mL), in lithium heparin tubes (Vacuette®, 4 mL), in K3EDTA tubes (Vacuette®, 2 mL), and in serum sep clot activator tubes (Vacuette®, 4 mL). All Vacuette® tubes were purchased from Greiner Bio-One GmbH (Kremsmünster, Austria). All of the tubes were held vertically during sampling to prevent contamination by anticoagulants between using the different tubes. All tubes were gently rotated eight times by hand after sampling.

2.3. Whole blood storage conditions

After the blood samples were collected, one tube (T0) was immediately centrifuged at 3220*g* for 15 min at 4 °C and stored at −80 °C. Two tubes were stored at +4 °C and two tubes were stored at room temperature (RT), one for one hour (T1 h) and one for four h (T4 h). In the case of the two serum sampling tubes, the same procedure was initiated after one h of coagulation at room temperature.

2.4. Treatment of plasma samples

Four healthy blood donors were selected and using the same blood sampling technique. We chose four different plasma collection tubes, as follows: (1) lepirudin-containing tubes, to which EDTA (10 mM final concentration) was added immediately before centrifugation to prevent further complement activation, (2) citrate-containing tubes (3.2% sodium citrate), (3) EDTA-containing tubes (4.43 mM final concentration)

To obtain higher levels of cytokines for testing cytokine stability, some of the lepirudin anticoagulated whole blood samples were incubated with *E. coli* (1×10^7/mL) in Nunc™ cryotubes. The Nunc tubes were incubated for 2 h at 37 °C before centrifugation. Control samples were obtained by immediate centrifugation (T0). To examine the stability of cytokines in the plasma samples, the samples were stored in Nunc tubes in the dark at either RT or at +4 °C for two, four and 24 h. The *E. coli* strain LE392 (ATCC 33572) was obtained from American Type Culture Collection (Manassas, VA, U.S.A.). *E. coli* was grown overnight on a lactose dish, after which 5–10 colonies were transferred to LB-medium (1% tryptone, 0.5% (w/v) yeast extract, 1% (v/v) NaCl) purchased from Becton Dickinson (Sparks, MD) and grown overnight. The bacteria were harvested and washed once with Dulbecco's PBS without Ca^{2+} and Mg^{2+} by centrifugation at 3220*g* for 10 min at 4 °C. The bacteria were aliquoted, heat inactivated for 1 h at 60 °C and stored at −80 °C. A frozen ampoule was thawed at ambient temperature and the cells were washed six times with PBS by centrifugation at 3220*g* for 10 min at 4 °C.

2.5. Freezing and thawing of plasma

The effect of freezing and thawing on the cytokines was examined using the T0 plasma samples (sodium citrate, 4.43 mM EDTA samples). The samples were frozen (−80 °C) and then thawed on ice (0 °C) for 20 min, followed by one h of storage on ice to simulate standard sample handling. The samples were then maintained in a −80 °C freezer for a minimum of 2 h to ensure freezing. The samples were then frozen and thawed 1, 3 and 6 times.

2.6. Cytokine analyses

The cytokines in the plasma and serum samples were analysed using a Bio-Plex Human Cytokine 27-Plex Panel kit purchased from Bio-Rad