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Research paper

Microbicidal activity measured by flow cytometry: Optimization and standardization for detection of primary and functional deficiencies

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ABSTRACT

Microbicidal activity is related to the production of reactive oxygen species (ROS) that can be measured by flow-cytometry using rhodamine 123 (R123). Few assays have been proposed to measure ROS production, usually on heparinized samples but none of them is standardized. Here we propose to improve the test by selecting polymorphonuclears (PMN) and monocytes, labelled and activated in one step to keep the test short, and to standardize the process even between different systems (i.e. Navios™ and FACSCanto™) using fluorescence intensity target setting ("FITS"). We applied this test on 15 patients without inflammation, 19 patients from an intensive care unit (ICU) and 11 healthy volunteers.

Results: Provided calcium restitution, we show that the test can be performed on EDTA that is a better sample preservative. The results were highly correlated between instruments ($r^2 = 0.898$). PMN CD16 (and not CD14) expression was altered under stimulation with *E. coli* (MdFI = 239.3 ± 93.5) or PMA (139.7 ± 76.8) as compared to resting sample (307.6 ± 145.1). RH123 was strongly and homogeneously induced by PMA (14.2 ± 6.6) and more heterogeneously by *E. coli* (MdFI 21.9 ± 23.4) as compared to unstimulated PNN (0.9 ± 1.3, $p < 0.0001$). The test is useful not only for genetic disorders but also for secondary deficiencies as observed in ICU (*E. coli* RH123 MFI = 10.5 ± 11.1 patients vs 30.1 ± 26.5 in healthy donors). In ICU, CD16 expression was already altered on unstimulated samples (MdFI = 197.4 ± 131.2 vs 418, 2 ± 81.3 in healthy donors; $p \leq 0.0001$). Bacterial stimulation was dependent of the complement that partly explains deficiency to *bacterial stimulus* in ICU patients.

1. Introduction

Phagocytosis of micro-organisms or cell debris is the main task of polymorphonuclear leukocytes (PMNs). It is facilitated by plasma products such as complement components, antibodies and scavengers (McDonald et al., 2012). This mechanism is named opsonisation. Several steps in opsonisation, including complement activation and adhesion, require cations such as calcium and magnesium. Phagocytes contain a large arsenal of enzymes for proteolysis and production of reactive oxidative species (ROS) (Petropoulos et al., 2015). Microorganism capture triggers NADPH oxidase that produces $NADP^+$, superoxide ion (O_2^-) and hydrogen peroxide (H_2O_2). Later myeloperoxidase transforms these species into hydroxyl radicals (•OH) and hypochlorite (ClO^-). These newly produced ROS are highly effective in killing and digesting ingested particles including bacteria and fungi (microbicidal activity) (Marchi et al., 2014; Letiembre et al., 2005). Inherited dysfunctions of NADPH oxidase or more rarely myeloperoxidase are responsible for defect in ROS production and accumulation of phagocytized products leading to chronic granulomatous disease (CGD) (Emmendorffer et al., 1994). Microbicidal activity can be measured through the capacity of the cells to produce ROS for the diagnosis of CGD.

Tissue infection induces systemic effects with fever and inflammatory metabolism that includes production of acute phase proteins including opsonins. Systemic effects are induced by bacterial components such as lipopolysaccharide (LPS) or peptidoglycan (De Marzi et al., 2015) and are mediated by the release of cytokines such as γIFN, TNFα or IL-6 (Fernando et al., 2014; Striz et al., 2014) preparing peripheral cells for phagocytosis and migration to the inflammatory site. At later stage, cell exhaustion and regulatory mechanisms induce a transient depression of phagocytosis called immunoparalysis (Volk et al., 2000). Immunoparalysis can be prolonged in sepsis, extended burning, injury or surgery and contributes in the severity of secondary infections

Abbreviations: HLA-DR, human leucocyte antigen D-related; CRP, C-reactive protein; PMN, polymorphonuclear neutrophil; ROS, reactive oxygen species; *E. coli*, *Escherichia coli*; DHR123, dihydrorhodamine 123; R123, rhodamine 123; EDTA, ethylene diamine tetra acetic acid; BSA, bovine serum albumin; PMA, phorbol 12 myristate 13-acetate; CV, coefficient of variation; NADPH, nicotin amide adenine dinucleotide phosphate; PBS, phosphate-buffered saline; MdFI, median florescence intensity; dim, diminished; FSC, forward scatter; SSC, side scatter; FITC, fluorescein isothiocyanate; PE-Cy7, phycoerythrine cyanin 7; APC, allophycocyanin; NS, non-significant; LPS, lipopolysaccharide; IL, interleukine; γIFN, gamma Interferon; CGD, chronic granulomatosis disease; ICU, intensive care unit.

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leading and consequently a high mortality rate in intensive care units (ICU) (Fitrolaki et al., 2013; Trimmel et al., 2012; Janols et al., 2014). Immunoparalysis can be analyzed in peripheral blood cells of ICU patients. As an example, the high affinity IgG receptor FcγRI (CD64) (Li et al., 2013) is up regulated while the low affinity FcγRIII receptor (CD16) on PMNs (Poehlmann et al., 2009) and HLA DR molecule expression on the surface of monocytes are down-regulated (Gouel-Cheron et al., 2012; Lambert et al., 2015). These markers have been shown to predict a poor clinical outcome of patients (Monneret and Venet, 2015; Spittler and Roth, 2003).

Microbicidal activity can be measured by flow cytometry. Several procedures have been proposed for diagnosis of CGD and can be applied for Immunoparalysis. They are usually based on measurement of production of ROS. ROS induces oxidization of probes such as dihydrorhodamine 123 (DHR123) into fluorescent rhodamine 123 (R123) that can be measured in individual cells by flow cytometry (Petropoulos et al., 2015; Chen and Junger, 2012). These methods can be easily implemented to provide more precise and reproducible output, e.g. by adding selective markers for granulocytes. Furthermore, in routine cell analysis, the use of ethylene–diamine–tetra-acetic acid (EDTA) is recommended as the best preservative anticoagulant (Davis et al., 2013; Banfi et al., 2007). But by chelating calcium, EDTA not only prevents coagulation but also cell aggregation, complement activation and phagocytosis (Elsner et al., 1994). However, standardization of the test is required in accreditation process according to ISO 15189 (Sack et al., 2013) and attempts have been recently proposed, even between different systems (instruments, antibodies.) (Kalina et al., 2012; van de Loosdrecht et al., 2009; Solly et al., 2013).

In the present report, we propose to implement and standardize a microbicidal test, independently of the reagent and instrument used, for the diagnosis of CGD and we show its interest in monitoring immunoparalysis.

2. Material and method

2.1. Blood sample

The study was performed at University Hospital of Saint-Etienne (France) on blood samples collected from 15 adults patients from cardiac unit without biological sign of acute inflammation (group B), 19 patients from ICU with recent and eventually ongoing sepsis (group C) and 11 healthy volunteers from occupational medicine (group A). Venous blood was collected on EDTA or lithium heparin tubes and tested within 12 h of sampling. Patients from ICU had inflammation status as shown by raised level of CRP (from 14 to 366 mg/L; ref. value < 5 mg/L) and ongoing bacterial infection was identified in 8 patients (gram negative bacteria, $n = 5$, gram positive bacteria, $n = 4$). For legal reasons, this technical study was performed on anonymous samples. No more medical information was available and measurement of CRP was not clinically justified in control donors and patients. All donors were older than 35 years and approximately equally distributed between males or females.

2.2. Reagents

All reagents for phagocytosis induced oxidative burst assays were taken from FagoFlowEx® Kit (Cat. No. ED7042, EXBIO, Praha, Czech Republic) containing standardized lyophilized *Escherichia coli* bacteria (*E. coli*), lyophilized DHR123, lyophilized Phorbol 12-myristate 13-acetate (PMA) as a stimulation control and ready-to-use lysing solution. Labeled monoclonal antibodies CD16-phycoerythrin–cyanine (PE-Cy7; clone 3G8) and CD14-allophycocyanin (APC; clone MEM-15) were also purchased from EXBIO.

EDTA samples were supplemented with Ca^{2+} at 10 mmol/L and calcium heparinate at 250 UI/ml final concentrations. Ca^{2+} replacement buffer was optimized on the criteria of sample coagulation and activation process. Different doses of calcium and heparin were tested around concentrations usually used. Suboptimal doses induced coagulation or lack of activation. Optimal doses of calcium and heparin were finally validated by comparing 10 individuals simultaneously analyzed on heparin and EDTA anti-coagulated blood samples, EDTA being supplemented with Ca and heparin.

The assay was also simplified. Cell stimulation and antibody labelling were performed in one step for 20 min in 37 °C water bath followed by fixation and red blood cells lysis done according to the manufacturer's instructions. In order to better preserve the sample, PBS-bovine serum albumin 10 g/L (MP Biomedical Solon OH), EDTA (0.5 G/L; MP Biomedical) was added after erythrolysis for data acquisition.

Samples were analyzed on Navios™ flow cytometer (CE-IVD; 3 lasers, 10 colors) using Navios™ software (Beckman-Coulter; Fullerton, CA) within 1 h from their final wash. The flow cytometer was daily calibrated on a routine basis using fluorescent microbeads (Flowset™, Beckman-Coulter). R123 median fluorescence intensity (MdFI) was measured on at least 5.000 PMNs selected on CD16 labelling versus side-scatter (SSC) and on monocytes selected on CD14/SSC. Data analyses were performed using Kaluza™ software (Beckman Coulter).

2.3. Standardization between instruments

Protocol standardization on time and between laboratories can be managed by keeping fluorescence detections at the same levels on time as daily checked but also between different instruments using beads with standardized fluorescence intensity. As previously described (Solly et al., 2013), settings were first optimized on Navios™; fluorescence targets were measured using calibration beads (Cyto-Cal Multifluor Calibrator™, Thermo Scientific, Carlsbad CA) and voltages adjusted on BD FACSCanto™ II cytometer (BD Biosciences, San Jose, CA) to get the same fluorescence targets. The method was named as fluorescence intensity target setting procedure (FITS). On these settings, 20 samples, same preparation, were cross-analyzed on the two instruments in delays that did not exceed 3 h.

2.4. Statistical analyses

Statistical analysis was performed using, paired student's *t*-test, linear regressions and are expressed as median ± 1 SD. Coefficient of variation (CV) was calculated as 1 SD/mean.

2.5. Legal issues

This technical study was performed on anonymous samples provided for diagnosis purpose in accordance with French Law.

3. Results

3.1. Method optimization

In order to implement the test precision and robustness, we used CD16 and CD14 to select neutrophil PMNs and monocytes respectively (Figs. 1a; 2a). The labelling was performed in one step during the activation step. The one step gave same results on preliminary tests as if samples were activated and labelled in two steps. The induced microbicidal activity could be easily measured under stimulation with PMA (Figs. 1d, 2d) or *E. coli* (Figs. 1c, 2c) and compared to unstimulated sample (Figs. 1b, 2b) on the two leukocyte populations involved in phagocytosis: PMNs (Figs. 1 and 2b) or monocytes (Fig. 2c). Calcium supplementation on EDTA samples gave similar results as heparinized samples as shown on one representative sample (Fig. 2d) with a better sample stability at least up to 24 h storing at 4 °C before testing on 5 healthy donors (Fig. 2e).