Flow Cytometric Assays Assessing Phagocytic Function for Patient Evaluation and Clinical Trial Testing

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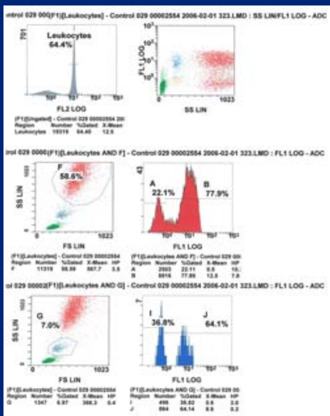
Abstract

Phagocytosis is an essential arm of host defense against bacterial or fungal infections. Two flow cytometric tests were evaluated to measure granulocyte and monocyte phagocytic activity in heparinized whole blood. In ten healthy volunteers, the percentage of phagocytizing cells (%PC) after in vitro incubation with *E. coli* (Phagotest®), phagocytic activity (Mean Fluorescence Intensity, MFI), percentage of phagocytic oxidative burst (%OC, Phagoburst®), and level of free oxygen radical production (MFI of Rhodamine 123) were analyzed by flow cytometry. Normal granulocyte ranges under optimal Phagotest conditions were 96- 99 %PC and 360-590 MFI. Using an optimized Phagoburst assay, normal granulocyte ranges were 89-97 %OC and 124-320 MFI. For pharmaceutical testing purposes, a modified E. coli incubation time and a reduced stimulation suspension level consistently obtained lowered %PC, %OC and MFI ranges useful for testing investigational medications. Inter- and intra-assay precision was measured on triplicate samples and stability was assessed in fresh, 24 and 48 hour post-venipuncture blood. Cell viability remained above 95.8% over 48 hours. Phagocytic and oxidative burst activity may be reproducibly evaluated in patients or clinical trial volunteers with flow cytometric assays.

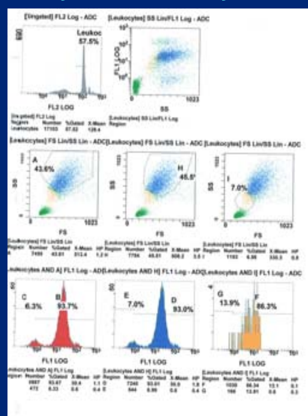
Methods and Materials

The Phagotest® and Phagoburst® kits (ORPEGEN Pharma, Heidelburg, Germany) quantitatived leukocyte phagocytosis and oxidative burst, respectively, in heparinized whole blood (WB). Phagotest involved pre-cooled WB incubated with fluorescein-labeled opsonitzed E.coli bacteria for 10 min. at 37°C, quenching, washing, lysing red blood cells, washing, staining DNA, and flow cytometric analysis. Similarly, in Phagoburst pre-cooled WB was incubated with 2×10^8 opsonized, non-labelled E. coli for 10 min. at 37°C and dihydrorhodamine 123, which fluoresces on reaction with oxygen species produced during oxidative burst, for 10 min. at 37°C. Lysing buffer stopped the reactions. Cells were washed, DNA stained, and oxidative burst measured by flow cytometric analysis. A secondary set assay conditions were experimentally derived at a level where both increased and decreased phagocytic or oxidative burst activity could be monitored after exposure to investigational medications or nutraceuticals. In the pharmaceutical testing format the fluorescein-labeled opsonitzed E.coli bacteria was incubated for 2.5 minutes to yield of approximately 70% of granulocytes and 50% of monocytes engaged in phagocytosis. Similarly, the secondary format for Phagoburst used 0.2×10^8 E. coli and shifted the average % oxidizing granulocytes and monocytes to about 50% and 25% respectively.

Phagotest Flow Cytometric Analysis



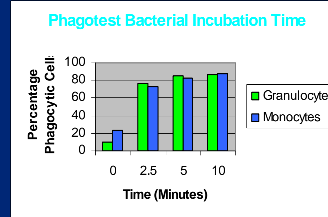
Phagoburst Flow Cytometric Analysis

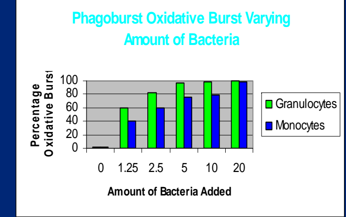


Leukocytes were identified based on the fluorescence in the FL2 channel. Monocytes and granulocytes were identified based on the side and forward scatter profile of the leukocytes. Oxidative burst or phagocytosis was monitored by observing the FL1 channel fluorescence (MFI and %+) of monocytes and granulocytes separately.

Results

For both tests the amount of bacteria and time were varied to optimize for pharmaceutical testing. Time proved most critical for Phagotest and amount of bacteria for Phagoburst.





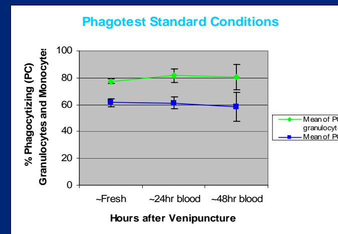
Phagotest Assay Summary

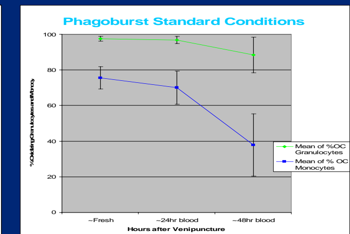
	Granulocyte % Phagocytosis	Granulocyte Mean Fluorescence Intensity	Monocyte % Phagocytosis	Monocyte Mean Fluorescence Intensity
Standard Conditions				
Normal Range	69.4-89.9	15.5-19.7	52.1- 71.4	10.6-17.4
Pharmaceutical Testing Format				
Normal Range	71.6 - 83.9	11.3 -15.0	42.7 - 65.9	6.9 - 12.1

Phagoburst Assay Summary

	Granulocyte % Oxidizing Cells	Granulocyte Mean Fluorescence Intensity	Monocyte % Oxidizing Cells	Monocyte Mean Fluorescence Intensity
Standard Conditions				
Normal Range	89.0 - 97.2	12.4 - 32.0	50.1 - 76.6	8.0 - 12.2
Pharmaceutical Testing Format				
Normal Range	23.9 - 72.3	4.2 - 8.3	12.8 - 39.9	4.3 - 6.0

Intra-assay and inter-assay percent coefficient of variations (CVs) ranged from 1.5 - 11.0% and 1.5 -15.5% for the standard assays and from 2.8 – 16.0% and 3- 20% for the pharmaceutical testing assay, respectively.





Conclusions

- Phagotest and Phagoburst may be adapted to test medicinal changes in phagocytosis and oxidative burst.
- The standard and pharmaceutical testing conditions can be standardized to maintain coefficient of variations CVs below 25%.
- Fresh samples are optimal for these assays

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