

Innate Immunity Tests and CD69 in Septicemia of Infants

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Abstract Introduction: Sepsis is systemic inflammatory syndromes triggered by viral or bacterial infections. Clinicians have long sought reliable markers to detect sepsis early in its course and to exclude diseases of non infectious origin. The role of the phagocytic function of monocyte, neutrophils, and T-lymphocyte cells in sepsis has been poorly investigated. **Materials and Methods:** The present study has been evaluate the activity of neutrophils, monocytes by phago and phagoburst tests and the activity of lymphocyte cells by CD69 on CD4, CD8 cells of patients with septicemia by flow-cytometry. Thirty five patients and twenty healthy individuals were enrolled in the study. **Results:** Our results were showing high significant difference of Phago test, PhagoBurst test of neutrophils, also CD69-CD4 and CD69-CD8 tests on T-cells. **Conclusion:** Our results need further researches to use these markers as early diagnostic and prognostic markers.

Keywords CD69, Phago, Burst, Septicemia

1. Introduction

Sepsis remains one of the leading causes of morbidity and mortality in children despite improved understanding of the path physiology leading to better clinical management and survival [1]. Diagnosis of neonatal sepsis remains a major challenge as early signs of sepsis are often non-specific and the laboratory criteria are also not fully reliable [2]. The incidence of early-onset sepsis full term neonates is 0.1% while in premature ones is as high as 0.4 % [3]. The increased susceptibility of neonates to bacterial infections has been attributed to immaturity of innate immunity.

The phagocyte system is an essential component of innate immunity [4]. Macrophages, monocytes and neutrophils have the ability to phagocyte bacteria and insert it in a cellular compartment (phagosome) which role as a cytotoxic agent [5]. These cells use bactericidal pathways that depend or in depend on oxygen as a weapon to eliminate infectious agents.

The mechanism oxygen-independent involves chemotaxis, phagocytosis, degranulations and the release of lysosomal enzyme and bactericidal peptides [6], through the degranulation process occurred sections of several chemical compounds, especially MPO (myeloperoxidase) and LTF (lactoferrin), azurophilic granules and specific granules are released [7].

Recently, flow-cytometric analysis of phago, phagoburst and antigens (CD11b, CD64, CD32, CD16, CD69, CD25

and CD45) has been performed to detect and follow up neonatal sepsis [8]. CD69, a protein expressed early on the surface of stimulated T cells, is used as a marker of activation and correlates with antigen specific proliferative response of lymphocytes [9, 10].

The purpose of this study was to investigate the phago, phagoburst and CD69 T-cell activity as markers of neonatal septicemia.

2. Materials and Methods

This study was conducted at Immunology department in Riyadh Regional lab in collaboration with neonatal intensive care unit at King Saud medical city.

Subjects were classified into two groups:

Group 1: included 35 patients (17 females and 18 male) in the first year of life admitted to hospital screened for septicemia in the first week of admission by sepsis score [11] and hematological sepsis scoring system and interpreted as suspected cases of sepsis (total score 7, score ≥ 3 is suggestive of sepsis) [12]. With exclusion criteria: infants with severe congenital abnormality, severe asphyxia and baby with severe low birth weight.

Group 2: included 20 apparently healthy controls, matched by age and sex with the patients.

Full clinical examination for symptoms and signs of sepsis. Laboratory investigations included complete blood picture, C-reactive protein and blood culture.

Analysis was done for phago test, phagoburst test and CD69/CD4, CD69/CD8 fast immune test by FACSCanto™II flow-cytometry. Whole blood, collected in a vacutainer blood collection tube containing sodium

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heparin.

Phagotest™ allows the quantitative determination of leukocyte phagocytosis (ingestion of bacteria). It measures the percentage of phagocytes which have ingested bacteria and their activity (number of bacteria per cell). The phagocytosis test kit contains fluorescein-labelled opsonized *Escherichia coli* bacteria and other necessary reagents. BD Biosciences (Cat.NO.341060). Heparinized whole blood is incubated with reagent B (FITC-labeled *E.coli* bacteria) at 37°C, a negative control sample remains on ice. The phagocytosis is stopped by placing the samples on ice and adding reagent C (quenching solution). This solution allows the discrimination between attachment and internalization of bacteria by quenching the FITC fluorescence of surface bound bacteria leaving the fluorescence of internalized particles unaltered. After two washing steps with reagent A (wash solution) erythrocytes are then removed by addition of reagent D (lysing solution). The DNA staining solution (Reagent E), which is added just prior flow cytometric analysis, excludes aggregation artifacts of bacteria or cells.

PhagoBurst test allows the quantitative determination of leukocyte oxidative burst. The Burst test kit contain unlabeled opsonized *E. coli* bacteria as particulate stimulus, the protein kinase C ligand phorbol 12-myristate 13-acetate (PMA) as high stimulus and the chemotactic peptide N-formyl-MetLeuPhe (fMLP) as low physiological stimulus,

dihydrorhodamine(DHR) 123 as a fluorogenic substrate and necessary reagents. BD Biosciences (Cat.NO.341058) heparinized whole blood is incubated with the various stimuli at 37°C, a sample without stimulus serves as negative background control. Upon stimulation, granulocytes and monocytes produce reactive oxygen metabolites (superoxide anion, hydrogen peroxide, hypochlorous acid) which destroy bacteria inside the phagosome. Formation of the reactive oxidants during the oxidative burst can be monitored by the addition and oxidation of DHR 123. The reaction is stopped by addition of lysing solution, which removes erythrocytes and results in a partial fixation of leukocyte. After one washing step with washing solution, DNA staining solution is added to exclude aggregation artifacts of bacteria or cells. The percentage of cells having produced reactive oxygen radicals are then analyzed as well as their mean fluorescence intensity (enzymatic activity).

CD69 fast immune assay: Lymphocyte response is measured after only 4 hour incubation with the stimulus in a three-color lyses/no wash flow-cytometric assay. BD Biosciences [Cat. NO.340365, for CD4FITC/CD69PE/CD3PerCP, Cat.NO.340367 for CD8FITC/CD69PE/CD3PerCP].

3. Results and Discussion

Table 1. Phago Tes

Student t test:

Test	Groups	N	Mean	SD	t	p	Sig.
Phg_Mono	Control	20	80.6	7.6185	0.73	0.469	NS
	Cases	35	78.96	8.6739			
Phg_neutro	Control	20	81.3	7.392	-2.571	0.015	S
	Cases	35	86.329	6.1902			

Student t Test: For Phago-Monocyte cells and Phago-neutrophil cells, 20 normal healthy control groups and 35 Patients group.

Table 2. PhagoBurst Test

Test	Groups	N	Mean	SD	t	p	Sig.
Burst_Mono	Control	20	83.995	8.438	0.776	0.441	NS
	Cases	35	81.466	15.7367			
Burst_neutro	Control	20	79.435	9.0829	-4.245	0	HS
	Cases	35	90.169	8.9117			

Student t Test: For Burst-Monocyte cells and Burst-neutrophil cells, 20 normal healthy control group and 35 patients group.

Table 3. CD69 Fastimmune Test

Test	Groups	n	Mean	SD	t	p	Sig.
CD69/CD4	Control	20	74.3	5.1412	-3.378	0.001	HS
	Cases	35	80.477	8.4151			
CD69/CD8	Control	20	75.2	8.0302	-2.459	0.019	S
	Cases	35	80.554	7.2861			

Student t Test: For CD69/CD4 and CD69/CD8, 20 normal healthy Control group and 35 Patients group.

Table 4. CD69/CD4 Test correlation**Pearson Correlation Test:**

Test		Phg_Mono	Phg_neut	Burst_Mono	Burst_neut
CD69/CD4	r	0.176	-0.076	-0.081	-0.071
	p	0.311	0.665	0.645	0.685

Pearson Correlation Test between: CD69/CD4 and Phago-monocyte cells, Phago-neutrophil cells, Burst-monocyte cells, Burst-neutrophil cells in patients group.

Table 5. CD69/CD8 Test correlation

Test		Phg_Mono	Phg_neut	Burst_Mono	Burst_neut
CD69/CD8	r	-0.057	-0.069	0.187	-0.109
	p	0.747	0.695	0.283	0.534

Pearson Correlation Test between: CD69/CD8 and Phago-monocyte cells, Phago-neutrophil cells, Burst-monocyte cells, Burst-neutrophil cells in patients group.

The term sepsis implies the presence of infection with sign of systemic body response (tachycardia, tachypnea, fever, etc.) and condition of severe sepsis and septic shock, further disturbance in organ perfusion (impaired consciousness, hypoxia, oliguria) require fluid administration and inotropic and / or vasopressor drug, respectively [13].

Sepsis is an enormously complex clinical syndrome that arises from the activation of an innate host response to danger. Sepsis is associated with infections of bacteria, viruses, fungi and endotoxins, whether or not being evidence by culture. The above systematic inflammatory response in humans occurs not only in sepsis but also in other non-infectious conditions such as pancreatitis, ischemia, server trauma, etc.).

Early diagnosis increases the possibility of starting a specific therapy in time [14, 15].

Infections by a variety of pathogens are a significant cause of morbidity and mortality during neonated and childhood. The susceptibility of neonates to bacterial infections has been attributed to immaturity of innate immunity. It is considered that one of the impaired mechanisms is the phagocytic function of neutrophils and monocytes [16]. Phagocytosis is the important factor that plays a role in acute inflammatory response due to its ability to destroy various pathogens efficiently [17]. A number of factors contribute to the efficient function of phagocytic system. These factors include the presence of adequate numbers of monocytes and neutrophils in peripheral blood, the ability to respond to signals from sites of inflammation, the migration to these sites and the capacity to ingest and kill the invaded micro-organisms [4, 5]. Neutrophils are considered to participate in the acute response against pathogens in many tissues [18].

Neonatal sepsis is showing a very significant increase in phagocytosis activity due to qualitative and quantitative neutrophils changes as told by Ari Yunanto 2013, and This result is agree with our result, 35 patients group show neutrophil phagocytosis mean \pm SD (86.329% \pm 6.1902) and 20 health control group mean \pm SD (81.3% \pm 7.392) p-value 0.015. But there is no significant change in monocyte

phagocytosis (Table 1).

In response to bacterial pathogens entrance, neutrophils will move into the infected tissue, then activate to form a reactive oxygen compounds. This event called respiratory burst involving the NADPH oxidizeactivation [19]. Respiratory burst that is a rapid uptake of molecular oxygen and transformation into reactive oxygen compounds. Reactive oxygen compounds can trigger oxidative damage to macromolecule, leading to lipid peroxidation, amino acid chains oxidation, cross links protein formation, polypeptide chain oxidation forming protein fragmentation, DNA strands ruptured [7].

Ari Yunato 2012 told that after going through the process of phagocytosis, degranulation process will occur, with secretion of several chemical compound, as myeloperoxidase (MPO) and lactoferrin (LTF). MPO is usually used as a marker of accumulation of neutrophils in tissue and it is a marker of neutrophil activity measured in plasma [18]. In our study the result of activation percentage of neutrophil burst test show high significant difference between patients and controls groups mean \pm SD (90.169 \pm 8.9117), (79.435 \pm 9.0829) respectively.

There is no significant difference between monocyte burst test in patients group and control group (Table 2).

Early diagnosis of the severity in sepsis is very important, increasing the possibility of starting timely and specific treatment [15, 20]. Biomarkers can have an important place in this process because they can indicate the presence or absence or severity of sepsis [21, 22].

CD10, CD11, CD14, CD18, CD25, CD28, CD40, CD48, CD54, CD60, CD80 and CD163 are the cell marker proteins which levels have been correlated to sepsis and septic shock prognosis. From those CD10 and CD11c are found in decrease levels in septic patients. Neutrophil CD11b and CD64 appear to be promising markers for diagnosis of early and late onset infections. CD11b is normally expressed in low concentration on the surface of neutrophils and its expression is increased 2-4 times more in infants and adults with positive blood culture for sepsis. CD11b seems to be very useful in early diagnosis of neonatal sepsis. CD14,

CD25, CD28, CD40 and CD163 are significant different between septic patients with good prognosis and those with very bad prognosis in the 28th day from sepsis diagnosis. CD69 is increased in septic patients [23-26].

The present study revealed high significant difference between patients group (80.477 ± 8.4151), (80.554 ± 7.2861) and control group (74.3 ± 5.1412), (75.2 ± 8.0302) regarding mean percentage \pm SD expressions of CD69/CD4, CD69 / CD8, respectively (Table 3). These results are correlated with the results was done by Azza et al, 2013 that show high significant difference between patients group and control group expression of CD69 on T-lymphocytes cells [27].

Our results were showing no significant correlation in patients group between CD69/CD4 and phago, phagoburst tests (table 4), also no significant correlation between CD69/CD8 and phago, phagoburst tests (table 5).

Sepsis is a very complex chain of events including inflammatory and anti-inflammatory processes, circulatory abnormalities and humeral and cellular reactions [28, 29].

4. Conclusions and Recommendations

Our study has demonstrated high significance of phagocyte and phago-burst tests activity of neutrophil cells, as innate immunity, and CD69 as T-cell marker activity in the first week of neonatal septicemia. And they may help in diagnosis and follow up of patients. Further research is needed to use these markers as early diagnostic onset markers of neonatal sepsis and as prognostic markers.

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