

Immunophenotyping of Lymphocyte Subpopulations and pre-inflammatory mediators in neonatal sepsis**¹Mostafa Zakaria, ²Mona Rafaat, M.D.**¹*Department of Pediatrics, Faculty of medicine, Cairo University,*²*Clinical Pathology Department, National research Center*

Mostafa Zakaria, Mona Rafaat, M.D.; Immunophenotyping of Lymphocyte Subpopulations and pre-inflammatory mediators in neonatal sepsis

ABSTRACT

Objective: Sepsis is one of the major causes of neonatal morbidity and mortality. The present study was designed to elucidate the changes in the lymphocyte subsets and in the pre-inflammatory mediators; IL-6 and IL-1b in full-term neonates with sepsis. **Patients and methods:** A prospective study was carried out at the neonatology intensive care unit of Cairo University in the period from June 2007 to June 2009 and included 96 full term neonates, classified into 2 groups. A Septic group (n = 50) and a control group (n=46). The parameters studied serially were: complete blood count, differential WBCs, blood culture, C- reactive protein. The lymphocyte subsets including; CD3+, CD4+, CD8+, NK cells, and B cells. Also the interleukins including; IL-1b and IL-6, and the immunoglobulins (Igs) including IgA, IgG, and IgM. **Results:** CRP, IL1-b, and IL6 were significantly higher in the sepsis group than in the control group. IL-6 > 90 pg/ml was an excellent marker with high sensitivity and specificity. A significantly elevated absolute and percentage counts of CD19+, CD 4+ and NK cells in the septic group, while total lymphocytic count, CD3+ and CD8 + were not significantly different. **Conclusion:** this study has shown that the combination of IL-6 with CRP can offer a good diagnostic accuracy in the detection of sepsis in neonates. NK cells are elevated in documented sepsis and it may be considered as an early diagnostic marker.

Key words: neonates -sepsis- lymphocytes –pre-inflammatory mediators- Immunophenotyping**Introduction**

Neonates are vulnerable to bacterial infections, and sepsis is one of the major causes of neonatal morbidity and mortality. It is important to identify neonatal infection as early as possible, but clinical signs are usually unreliable in neonates, while the routine diagnostic tests lack precision [1]. C-reactive protein (CRP) and white blood count (WBC) have been used as early markers of infection in neonates. CRP is a specific but not a sensitive marker in the early stages of neonatal sepsis, while the WBC appears to be unreliable [2,3].

Recent evidence showed that neonates react to bacterial sepsis with an exaggerated inflammatory response, which may contribute to the high mortality observed in neonatal sepsis [4,5]. The neonatal immune response, however, includes increased production of other inflammatory mediators such as Interleukin-1 (IL-1), IL-6 and TNF- α , the assessment of which may improve diagnostic accuracy in suspected sepsis [5,6].

Studies in adults with sepsis have shown considerable changes in the subsets of lymphocytes, especially in the T-helper cells, B cells, and natural killer (NK) cells [7-9]. There are indications that NK

cells have a special role as a component of the innate immune system [7]. Although lymphocyte subsets have been extensively studied in adult patients with sepsis, little is known about their profile in infected neonates [10].

This study was designed to investigate the changes in the lymphocyte subsets and in the pre-inflammatory mediators; IL-6 and IL-1b in full-term neonates with sepsis to document possible changes in their levels during the course of infection, and to evaluate their efficacy as early predictors of neonatal sepsis

Patients and methods:

This prospective study was carried out at the neonatology intensive care unit of Cairo University in the period from June 2007 to June 2009 and included 96 full term neonates, classified into 2 groups. A Septic group (n = 50) where sepsis was confirmed by a positive blood culture accompanied by compatible signs and symptoms, and a control group, which included infection-free full term neonates (n=46), without clinical findings or maternal risk factors for infection, admitted for minor problems or nursed in the neonatal ward at the

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same period. The study was approved by the ethical committee of the University Pediatric hospital.

All newborns were subjected to detailed history with special emphasis on perinatal history regarding maternal medical diseases especially diabetes. Maternal infections during pregnancy as well as the intake of any medications were also recorded. Obstetric history in previous pregnancies (abortion, stillbirth ...etc) as well as in the current pregnancy was taken. In addition, Natal history especially mode of delivery, premature rupture of membranes and maternal fever was recorded.

All cases were then subjected to full clinical examination, especially for the clinical signs of infections, namely poor peripheral perfusion, capillary refilling time >3 seconds, hypotension, hypothermia or hyperthermia, hypoglycemia, poor feeding, hypotonia, vomiting, abdominal distension, increased needs for ventilation or respiratory instability, tachypnea, increased or decreased heart rate, apneic episodes, seizures in the absence of perinatal asphyxia.

With the first signs of infection, sepsis screening tests were carried out. The parameters studied serially in all infants were: complete blood count, differential WBCs, blood culture, C reactive protein, blood glucose and calcium. The lymphocyte subsets including: CD3+, CD4+, CD8+, NK cells, and B cells. Also the interleukins including: IL-1b and IL-6, and the immunoglobulins (Igs) including IgA, IgG, and IgM,

Methods:

- Immunophenotyping of lymphocyte subsets:

Samples were collected from all cases into EDTA containing tubes and were processed within 24 hours on a FACSORT (B-D) flow cytometer. Cells were tested for CD3 (pan T), CD4 (T helper), CD8 (T cytotoxic), CD19 (B cells) and CD16+CD56 (NK cells) using IMK lymphocyte Kit (BD cat no 349525).

- Measurement of interleukins IL-1b and IL-6:

Serum level IL-6 and IL-1 β are measured by quantitative sandwich enzyme immunoassay technique (Quantakine ELISA catalog number D6050 and DLB50 respectively).

Statistical analysis:

Data were statistically described in terms of mean \pm standard deviation (SD), median and range, or frequencies (number of cases) and percentages when appropriate. Comparison of numerical variables between the study groups was done using Student *t* test for independent samples in comparing 2 groups, and one way analysis of variance

(ANOVA) test with multiple 2-group comparisons in comparing more than 2 groups. For comparing categorical data, Chi square (χ^2) test was performed. Exact test was used instead when the expected frequency is less than 5. Correlation between various variables was done using Spearman rank correlation equation. *p* values less than 0.05 was considered statistically significant. All statistical calculations were done using computer programs SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows.

Results:

Of the 50 babies recruited, 10 cases (20%) developed early onset sepsis (age : 2 ± 1), While 40 cases (80%) developed late onset sepsis (age 15 ± 5). The weight was significantly lower in the study group than in the control group (P value < 0.001), while no significant differences were observed in the length and head circumference. Both the heart rate and respiratory rate were higher in the study group than in the control group (P value < 0.001).

Lethargy and poor feeding were the main presenting features (100% of septic cases), followed by abdominal distention and hypothermia (90% of septic cases).

Total leucocytic count, absolute neutrophilic count, hemoglobin percentage and platelet count were significantly lower in septic neonates in comparison to the non septic group. Similarly Glucose and calcium levels were significantly lower in the septic group.

Regarding the results of blood culture, the following organisms were isolated; Klebsiella (15 cases: 30%). E coli (12 cases: 24%), streptococcus group B (10 cases: 20%) Staphylococcus coagulase negative (6 cases: 12%) listeria (4cases: 8%) and Pseudomonas (3cases: 6%).

In the current study, CRP, IL-1b, and IL6 were significantly higher in the sepsis group than in the control group. CRP > 15 mg /l, was shown to have relatively good specificity (0.76), but its sensitivity was low (0.60). Meanwhile, IL-6 > 90 pg/ml was an excellent marker with high sensitivity and specificity, and IL-1b > 1.9 pg / ml was a specific but not a sensitive index of infection.

Regarding Immunoglobulins, IgM was higher in the sepsis group, while IgG was lower in the sepsis group than in the control group, the difference was significant. Similarly IgA was also higher in the sepsis group than in the control, but the difference was not significant.

Immunophenotyping of lymphocyte subsets showed a significantly elevated absolute and percentage counts of CD19+, CD 4+ and NK cells in the septic group in comparison to the other non septic, while total lymphocytic count, CD3+ and CD8 + were not significantly different.

Table 1: Shows the clinical presentations of septic cases.

Clinical presentations	Septic neonates (n : 50)	
	Number	Percent
Lethargy	50	100%
Poor feeding	50	100%
Abdominal distention	45	90%
Hypothermia	45	90%
Vomiting	45	80%
Respiratory distress	40	80%
Jaundice	15	30%
Umbilical sepsis	20	40%

Table 2: Shows hematological findings in the study and control groups.

	Control group (n : 46)	Septic group (n : 50)	P value
• Hemoglobin g/dl	15.40±1.43	11.44±3.27	<0.001
• White Blood count/cmm	14530±2809	7650±4132	<0.001
• Neutrophils ×10 ⁶ /l	9054±2201	4655±2210	<0.001
• Band/Total ratio×10 ⁶ /l	0.06±0.02	0.30±0.18	<0.001
• Eosinophils×10 ⁶ /l	142±14	128±12	Ns
• Monocytes×10 ⁶ /l	435±126	422.128	Ns
• Platelets/cmm	237.7±50.81	110.80±80.2	<0.001

Table 3: Shows laboratory data in the study and control group.

	Control group n:46	Septic group n:50	P value
CRP: mg/l	1 ± 1.4	27 ± 35	0.001
IL-6 : pg/ml	3.1 ± 3.2	140 ± 80	0.022
IL-1b:pg/ml	1.1 ± 1.3	3.9 ± 1.2	0.035
IgG: mg/dl	930 ± 310	650 ± 290	0.045
IgM: mg/dl	12.2 ± 7.8	23 ± 12	0.001
IgA: mg/dl	2.9 ± 1.3	3.6 ± 7	Ns

Table 4: Shows Immunophenotyping of lymphocyte subpopulations in study and control groups.

	Control group (n : 46)	Septic group (n :50)	P.value
Lymphocytes			
Absolute	2236±1390	2302±1180	ns
%	24.2±8.5	25.3±6.4	ns
CD ₃ (Total T)			
Absolute	1820±665	1724±1709	ns
%	65.7±12.7	64.0±14.6	ns
CD ₄ (Helper)			
Absolute	973±602	1203±520	<0.001
%	33.5±8.2	37.7±12.3	<0.001
CD ₈ (cytotoxic)			
Absolute	890±543	706±478	ns
%	28.8±10.3	29.7±9.5	ns
CD ₄ /CD ₈	1.25±0.43	1.5±0.70	
CD ₁₉ (B cells)			
Absolute	250±219	789±541	<0.001
%	10.2±5.3	18.5±5.1	<0.001
NK cells			
Absolute	190±124	280±210	<0.001
%	9.1±5.3	12.8±12.4	<0.001

Discussion:

Sepsis is one of the major causes of neonatal morbidity and mortality. Early diagnosis poses a challenge because of the non-specific nature of the signs and symptoms and the non-availability of microbial culture results in the first few days, while the routine diagnostic tests lack precision [1]. The present study was designed to elucidate the changes in the lymphocyte subsets and the pre-inflammatory mediators; IL-6 and IL-1b in full-term neonates with sepsis to document possible changes in their levels during the course of infection, and to evaluate their efficacy as early predictors of neonatal sepsis.

In the current study we could demonstrate an increase in CRP and in the two studied cytokines, IL-1b, IL-6, in the group of neonates with sepsis, to values higher than healthy control subjects. This is in agreement with the results of Ng *et al.*, [3] and Hotoura *et al.* [11]. Regarding their diagnostic accuracy to detect neonatal sepsis, CRP >15 mg/L, was a specific but not a sensitive index. This may be because of the fact that the sepsis workup in our study was performed early, with the first suspicion of infection, at which time CRP has not reached its highest levels yet. On the other hand, Philip *et al.* [12] and Pourcyrus *et al.* [13] have shown that repeated CRP values may be a better diagnostic tool

for neonatal infection, but introduction of antibiotics cannot always be postponed awaiting the results of serial CRP evaluation.

Hotoura *et al.*, [11] suggested a cut-off value of IL-6 > 60 pg/ml proved to be a more sensitive and specific index for early diagnosis of sepsis. Our study revealed a cut-off value of IL-6 > 90 pg/ml. Our findings and the findings of Hotoura *et al.*, although different in figures, confirm those of earlier researchers who considered IL-6 to be a very precise early marker of neonatal infection [3,14,15]. Some investigators have proposed the use of a combination of markers, such as IL-6, which is an acute reactor, and CRP, which increases later in the course of sepsis [3,15,16].

Few clinical studies were reported on lymphocyte subsets in neonates with infection, and those published provided inconsistent results [17-21]. Aygun *et al.* found levels of CD3+, CD4+, and CD8+ in 12 neonates with proven sepsis similar to those of control subjects in absolute numbers, but with a lower percentage of total lymphocytes and CD4+ [20]. Hotoura *et al.* found higher CD3+, CD4+, and CD8+ in full-term neonates with sepsis, but their ratios did not differ from those of the controls [21]. Sofatzis *et al.* [17] found lower mean CD3+, CD4+, CD18 and CD11a and CD4+/CD8+ ratio in 20 preterm and term neonates with sepsis, compared with 23 healthy control subjects. Similarly, Juretic *et al.* [18] showed that preterm neonates with sepsis have lower CD3+ and CD4+ than uninfected premature neonates. In the present study, significantly elevated absolute and percentage counts of CD19+, CD 4+ in comparison to other non septic cases were found, while total lymphocytic count, CD3+ and CD8 + showed no significant differences. The increased level of CD19+ and CD 4+ , in the current study, support the previous study of Schultz *et al* [4] who reported increased IL- 6 early in the course of infection. IL- 6 is a cytokine produced mainly by CD4+ cells and it is responsible for B cell replication and immunoglobulin production and it is the major inducer of hepatic protein synthesis including CRP and fibrinogen during early acute phase response [16].

NK cells have recently been implicated in processes of microbial immunity, including the onset of sepsis [22]. The neonatal defense is initially dependent on the innate immune system, as antigen-specific immunity develops later in life, and the NK cells, which are a part of the innate immune system are found in higher numbers in neonates than in older children and adults [23,24]. The neonates with sepsis in the present study had elevated percentages of NK and B cells. Preterm neonates with sepsis or suspected infection were found to have an increase in NK cells and also a significant increase in B cells [21]. It has been speculated that there may be a protective effect of increased NK cells for the infected host [7].

Upregulation of many surface activation markers on peripheral blood derived T cells, monocytes, and NK cells was recently found in neonates with sepsis [25]. Adults with severe sepsis showed an increase in NK cells, which provided a survival benefit [7]. NK cells are both targets of interferon-gamma (IFN- γ) action and producers of IFN- γ , and it has been shown that NK cells constitute early sources of IFN- γ during infection [26]. IFN- γ plays an important role in both innate and adaptive immune responses, inducing production of TNF- α and IL-12 from mononuclear phagocytes and then augmenting their bacteriostatic action [26]. The role of IFN- γ as an early marker of neonatal infection is yet to be studied. Studies in adults have shown either decreased or increased B cell numbers in patients with sepsis; the former may be a phenomenon occurring later in the course of the sepsis [7,8].

IgM, unlike IgG, does not cross the placental barrier, and its elevation implies the neonate's own postnatal production as a reaction to infective agents. IgM was elevated in the neonates with sepsis. Other researchers also have found an elevation of IgM in neonates with sepsis and have proposed that it may be used, coupled with IL-6, as an early detector of neonatal sepsis [25]. In that study, IgM levels were higher in sepsis and suspected infection groups, compared to healthy neonates.

The IgG levels were lower in neonates with sepsis, compared to the control subjects. A causative could be speculated between low IgG levels and sepsis, with the reservation that biochemical IgG values were measured rather than functional parameters that could establish a functional deficit.

Conclusion:

This study has shown that the combination of IL-6 with CRP can offer a good diagnostic accuracy in the detection of sepsis in neonates. NK cells are elevated in documented sepsis and it may be considered as an early diagnostic marker. Serial measurement of infection markers will certainly improve the diagnostic sensitivity of these tests, because in most circumstances it is uncertain at which stage of the infection the specimen should be taken for analysis. In addition, the use of multiple markers, will further enhance the diagnostic accuracy of these mediators in identifying infected cases

Conflict of interest:

The authors declare that they have no conflict of interest

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