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Quantitative Measurement of Monocyte HLA-DR Expression in the Identification of Early-Onset Neonatal Infection

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Key Words
Infection, early-onset · HLA-DR · Pneumonia

Abstract

Background: This study aimed to evaluate the diagnostic utilities of monocyte HLA-DR as an infection marker in the identification of early-onset clinical infection and pneumonia in newborn infants. ***Methods:*** Term newborns in whom infection was suspected when they were <72 h of age were eligible for enrollment in the study. C-reactive protein (CRP), monocyte HLA-DR and neutrophil CD64 expressions were quantitatively measured at the time of sepsis evaluation (0 h) and 24 h afterwards by flow cytometry and standard laboratory method. ***Results:*** A total of 288 infants with suspected sepsis were investigated, and 93 were found to be clinically infected. There were no significant differences in monocyte HLA-DR expression between the infected, non-infected and control groups at 0 h (median (interquartile range): 13,986 (10,994–18,544), 14,234 (12,045–17,474) and 18,441 (14,250–21,537) antibody phycoerythrin (PE) molecules bound/cell), and between infected and non-infected infants at 24 h (median (interquartile range): 17,772 (12,933–25,167) and 19,406 (14,885–24,225) antibody PE molecules bound/cell). The areas under the receiver operating characteristics (ROC) curves for HLA-DR, CD64 and CRP were 0.52–0.54, 0.88–0.94 and 0.75–0.77, respectively. We were unable to determine an optimal cutoff value for HLA-DR, as the diagnostic utilities of any cutoff point on the ROC curves were unable to satisfy the criteria (i.e. sensitivity and specificity ≥80%) for consideration as an useful diagnostic marker of infection. ***Conclusions:*** Our findings did not support the use of monocyte HLA-DR alone or in combination with other infection markers in the diagnosis of early-onset clinical infection and pneumonia in term newborns.

Introduction

Early-onset neonatal infection remains an important cause of morbidity and mortality in the immediate postnatal period [1]. The diagnosis of infection in newborn infants is difficult because early clinical signs and symptoms are often subtle and inconspicuous, and can easily be confused with non-infective causes such as transient tachypnea of the newborn, cyanotic congenital heart diseases, meconium aspiration syndrome and hypoxic-ischemic encephalopathy [2]. Commonly used hematologic indexes, including differential white cell counts and platelet counts are relatively insensitive and unreliable [3]. As

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a consequence, neonatal clinicians tend to have a very low threshold for performing sepsis screening and prescribing broad-spectrum antibiotics for newborns who present with non-specific clinical manifestations or in association with maternal risk factors of perinatal infection [2, 4–6]. Thus, understanding the expression of cell surface proteins and their functions on different inflammatory cells in newborn infants can potentially assist in the search for suitable infection markers to better differentiate between infected and non-infected infants. The monocyte/macrophage system is known to play a crucial role in host body defense. These inflammatory cells participate in phagocytosis, antigen presentation and production of cytokines and/or chemotactic factors mediated through the expression of cell surface molecules [7]. HLA-DR is one of the key cell surface molecules expressed on monocytes, and is responsible for antigen presentation to T cells and initiation of the inflammatory cascade during infection [8]. Paradoxically, the expression of HLA-DR on monocytes has been reported to be decreased in adult patients with sepsis after severe trauma and major surgery [9–12], and this cell surface molecule has been advocated as an early indicator of immunological deviation associated with the development of severe infection in these patients.

Monocyte HLA-DR was specifically chosen in this study for evaluation as an infection marker in the diagnosis of early-onset neonatal sepsis. Reports on adult patients suggested that the decrease in expression of HLA-DR could differentiate patients with sepsis from those who encountered only physical trauma or underwent surgery [9–12]. This characteristic may theoretically enable infants who acquired perinatal infection to be identified and separated from those who experienced only traumatic delivery. Further, the measurement of monocyte HLA-DR by flow cytometry and QuantiBRITE beads has the advantage of (1) using a very small volume of blood (whole blood 50 μl) for laboratory analysis; (2) assessing the diagnostic marker quantitatively, and (3) obtaining the results within 4 h after the specimen reaches the laboratory. This study aimed (1) to determine the diagnostic utilities (sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV)) of monocyte HLA-DR as an infection marker for identifying early-onset clinical infection and pneumonia in term newborns; (2) to define its optimal cutoff value using the receiver operating characteristics (ROC) curve so that this value may be used as a reference with which future studies can be compared, and (3) to compare the usefulness of monocyte HLA-DR with neutrophil CD64 and C-reactive protein (CRP) for diagnosing early-onset neonatal infection.

Patients and Methods

Term (≥ 37 weeks gestation) newborn infants with (1) postnatal age <72 h; (2) signs and symptoms suggestive of early-onset clinical infection or pneumonia, and requiring full sepsis evaluation and systemic antibiotic treatment, and (3) parental consent, were eligible for enrollment in the study. Infants with major congenital anomalies or chromosomal abnormalities and those with positive family histories of immunodeficiencies were excluded. The use of intrapartum antibiotics, however, did not preclude the infants from participating in the study, as we wished to strictly follow routine clinical practice. The infants were recruited over a 24-month period.

The criteria of performing infection screening and the classification of infection episodes have been described in detail in our previous study [6]. A brief summary is provided below.

Infection Screening

Term newborns presenting with clinical signs and symptoms suggestive of early-onset clinical infection or pneumonia received a full sepsis workup. In brief, these clinical features included: unstable temperature; poor peripheral circulation with prolonged capillary refilling time >3 s; signs of respiratory distress or requirement of oxygen supplementation to maintain a satisfactory saturation; abnormal fontanelle tension or convulsion, and abnormal gastrointestinal manifestations such as persistent vomiting, abdominal distension and stool mixed with blood and mucus [6]. All infants were recruited at the time of sepsis evaluation (0 h). A full sepsis screen was performed in each suspected episode, and included surface, urine, stool (infants suspected of enterocolitis), blood, cerebrospinal fluid (CSF) and endotracheal aspirate (infants on respirator) specimens for microscopy and bacterial cultures. Chest radiographs were routinely performed but abdominal radiographs were only requested if the patients were suspected of having intra-abdominal sepsis. In addition to the routine hematological and biochemical investigations such as complete blood counts and blood gases, serial CRP measurements and EDTA, blood samples (0.2 ml) were obtained for monocyte HLA-DR and neutrophil CD64 analysis by flow cytometry. The first CRP and EDTA sample was taken at the time of the initial sepsis evaluation (0 h) and the second sample was obtained 24 h after the onset of clinical presentation [6].

Classification of Infection Episodes

Three categories of 'infection' episodes were prospectively defined. The definition of each category has also been described in detail in our previous study [6]. The 3 groups are summarized as follows.

Infected Group. The infected group consisted of infants who were microbiologically confirmed to have septicemia, meningitis, peritonitis, or radiologically or laparotomy-confirmed necrotizing enterocolitis (Bell's classification ≥ stage II, with or without positive peritoneal culture and/or blood culture) [13]. Clinical pneumonia was diagnosed on the basis of respiratory signs in conjunction with abnormal chest radiological findings. These images were digitally stored and evaluated by 2 independent investigators, a pediatric radiologist (W.C.W.C.) and a neonatologist (P.C.N.), who were both blinded to the patients' identities and results of the infection markers. The assessors specifically looked for air space consolidation and peribronchial or perivascular interstitial infiltration [6].

Non-Infected Group. The non-infected group consisted of infants who met the initial infection screening criteria but were subsequently found to be non-infected with sterile blood and CSF cul-

tures, normal chest and abdominal radiographs, and the patients continued to improve after antibiotic treatment had been discontinued. Positive surface culture alone was considered as bacterial colonization and was not classified as genuine neonatal sepsis.

Control Group. Ten well infants also had their blood taken once within the first 72 h of life for monocyte HLA-DR, neutrophil CD64 and CRP measurements. The collection of blood samples coincided with other standard blood-taking procedures such as serum bilirubin and plasma hematocrit measurements.

Measurement of CRP and Cell Surface Markers

Blood samples were collected by venipuncture, kept at 4°C and immediately transported to the laboratory. Levels of CRP were quantified by a turbidity assay kit according to the manufacturer's instruction (Behring Diagnostics Inc., Westwood, Mass., USA). Expressions of HLA-DR and CD64 on cell surfaces of monocytes and neutrophils were measured by a quantitative method using flow cytometry as described previously [6]. In essence, the QuantiBRITE PE beads (Becton Dickinson Immunocytometry Systems (BDIS), San Jose, Calif., USA) conjugated with four predefined levels of phycoerythrin (PE) molecules were used to construct a standard linear regression curve (QuantiQUEST software, BDIS) for each analysis. The antibodies for HLA-DR and CD64 were of QuantiBRITE grade (≥95% 1:1 antibody:PE ratio) with the former antibody being non-reactive with HLA-DQ or HLA-DP molecules. For the enumeration of HLA-DR antigens on monocytes, 50 μl of EDTA blood was stained with 20 μl of QuantiBRITE anti-HLA-DR PE/anti-monocyte peridinin chlorophyll protein (PerCP)-Cy5.5 antibodies for 30 min in the dark at room temperature. Red blood cells were then lysed by adding 0.45 ml 1 × FACS lysing solution for 30 min before analysis using the FACSCalibur Cytometer and CellQuest software. Five thousand events of monocytes, as recognized by the CD14 antibody and cyanine dyes, were acquired by gating on the fluorescence (FL)-3 (anti-monocyte PerCP-Cy5.5) versus side scatter plot. Expressions (geometric mean) of HLA-DR were measured on the FL-2 histograms. HLA-DR-PE binding sites per cell were computed from the standard curve of QuantiBRITE beads. Similarly, we measured the expression of CD64 on neutrophils by staining 50 μl of whole blood with 20 μl of CD64-PE/CD45-PerCP (BDIS) antibodies for 60 min and a further lysing for 60 min before cytometric analysis. Twenty thousand events were acquired for each sample. The neutrophil population was gated by their CD45/side scatter profile and the expression of CD64 (geometric mean) quantified on the FL-2 plots.

Statistical Analysis

The demographic data and the levels of infection markers among the infected (0 h), non-infected (0 h), and control groups were compared using the Kruskal-Wallis test and χ^2 test, where appropriate. The Mann-Whitney U test was used to assess the levels of infection markers at 24 h between infected and non-infected infants. Since there was no recommended diagnostic cutoff value for monocyte HLA-DR in term newborns, a ROC curve was constructed for each sampling time point. The optimal cutoff value for HLA-DR was determined on the curve by minimizing the number of misclassified episodes. As the diagnostic marker should ideally identify all genuinely infected infants (i.e. 100% sensitivity) and at the same time would not misclassify too many non-infected cases (i.e. high specificity), the optimal cutoff value was, therefore, chosen with the sensitivity approaching 100% and specificity >85% [3, 6, 14, 15]. However, if the diagnostic marker was unable to satisfy the above criteria, the optimal cutoff value would be chosen so that both the sensitivity and specificity approached 80% [6, 14, 15]. The calculated optimal cutoff value enables us to work out the sensitivity, specificity, PPV, and NPV of HLA-DR at the most appropriate sampling time for diagnosing early-onset clinical infection and pneumonia in term infants. A combination of tests was considered positive when any one of the selected markers exceeded its respective cutoff value. All statistical tests were performed by SPSS for Windows (Release 11.5; SPSS Inc., Chicago, Ill., USA). The level of significance was set at 5% in all comparisons.

Ethical Approval

The study was approved by the Research Ethics Committee of the Chinese University of Hong Kong. Written informed consent was obtained from the parents for all patients studied.

Table 1. The clinical characteristics of the patients studied

	Infected group	Non-infected group	Control group
Number of infants	93	195	10
Gestational age, weeks	39.7 (38.9–40.4)	40.0 (38.6–40.7)	39.1 (37.9–40.5)
Birth weight, g	3,340 (3,143–3,603)	3,260 (2,920–3,585)	3,223 (2,829–4,121)
Male:female	63 (68%):30 (32%)	111 (57%):84 (43%)	6 (60%):4 (40%)
Apgar scores			
1 min	9 (8–9)	9 (8–9)	9 (9–9)
5 min	10 (9–10)	10 (9–10)	10 (9–10)
Umbilical arterial cord blood			
pH	7.26 (7.19–7.30)	7.26 (7.19–7.30)	7.23 (7.21–7.27)
Base excess, mmol/l	–4.8 (–7.2 to –2.7)	–5.0 (–6.9 to –2.9)	–4.3 (–6.5 to –2.7)
Singleton:twins	91 (98%):2 (2%)	189 (97%):6 (3%)	10 (100%):0 (0%)
Inborn:outborn	91 (98%):2 (2%)	192 (99%):3 (1%)	10 (100%):0 (0%)

Results are medians (interquartile ranges) or numbers of patients (%).

Results

The clinical characteristics of the infants studied are summarized in table 1. A total of 288 term infants with suspected clinical sepsis were recruited. Approximately 26% (n = 78) of the patients participated both in this and our previous studies [6]. Of the recruited patients, 93 and 195 infants were classified in the infected and non-infected groups, respectively. In addition, 10 well term infants were enrolled in the control group.

There were no significant differences in demographic characteristics between the groups (table 1). None of the infants died because of neonatal sepsis. The clinical diagnoses of infected and non-infected infants are summarized in table 2. Table 3 compares the serum CRP concentrations, neutrophil CD64 and monocyte HLA-DR expressions at 0 and 24 h. As expected, there was no significant difference in all 3 infection markers between non-infected patients and control subjects. CD64 and CRP in infected infants were significantly elevated at both 0 and 24 h compared with non-infected patients and control subjects, whereas HLA-DR expression did not differ significantly between the 2 groups (table 3).

Table 2. The clinical diagnosis of infected and non-infected infants

Clinical diagnosis	Number of cases
Infected infants	93
Septicemia	
Streptococcus agalactiae[a]	1
Necrotizing enterocolitis	1
Clinical pneumonia (with organisms isolated from surface culture)[b]	
Streptococcus spp.	17
Escherichia coli	4
Clinical pneumonia (with no organism isolated from surface culture)	70
Non-infected infants	195
Non-infected pulmonary causes	
Transient tachypnea of the newborns	79
Aspiration syndrome (meconium or blood)	14
Pneumothorax	6
Idiopathic persistent pulmonary hypertension of the newborn	3
Pethedine-induced apnea	11
Other conditions presenting with respiratory signs and symptoms	
Hypoxic-ischemic encephalopathy	3
Polycythemia	4
Subgaleal hemorrhage	4
Laryngomalacia	1
Cardiovascular causes	
Ventricular septal defect	1
Patent ductus arteriosus	1
Gastrointestinal causes	
Gastroesophageal reflux	3
Sucking incoordination	3
Maternal or neonatal fever (for infection screening)[c]	57
Suspicious infected cases[d]	5

[a] This patient also had meningitis and pneumonic changes on chest radiographs.

[b] A total of 71 infants had positive surface cultures and 21 were associated with abnormal chest radiographic findings.

[c] A total of 77 infants received infection screening for maternal or neonatal fever and 57 were classified as being non-infected.

[d] These patients ran a severe clinical course, and had negative blood culture and radiographic findings. They were suspected of having genuine neonatal sepsis and received a full course of antibiotics.

The areas under the ROC curves for HLA-DR, CD64 and CRP were 0.52–0.54, 0.88–0.94 and 0.75–0.77, respectively. However, we were unable to determine an optimal cutoff value for HLA-DR, as the diagnostic utilities of any cutoff point on the ROC curve were unable to satisfy the predetermined criteria [3] for consideration as a useful marker of infection. For example, setting the cutoff value of monocyte HLA-DR expression at ≤11,718 antibody-PE molecules bound/cell would give the sensitivity and specificity of 31 and 80%, whereas these parameters at HLA-DR expression ≤20,606 were 80 and 12% at 0 h, respectively (table 4). The optimal cutoff values for CD64 and CRP in term infants had already been defined to be 6,136 antibody-PE molecules bound/cell and 10 mg/l in our previous studies [6, 15]. Using these cutoff values, the diagnostic utilities of CD64 and CRP at 0 and 24 h are summarized in table 4. Of the studied markers, CD64 has the highest sensitivity and NPV at both 0 and

Table 3. Levels of monocyte HLA-DR and neutrophil CD64 expressions, and serum CRP concentrations at the initial sepsis evaluation (0 h) and at 24 h

	Infected group (n = 93)	Non-infected group (n = 195)	Control group (n = 10)
At 0 h			
HLA-DR, antibody-PE molecules bound/cell	13,986 (10,994 – 18,544)	14,234 (12,045 – 17,474)	18,441 (14,250–21,537)
CD64, antibody-PE molecules bound/cell	8,299 (6,542–11,942)[a]	3,938 (2,914–5,281)	3,002 (2,121–4,712)
CRP, mg/l	7.2 (3.0–16.4)[a]	3.0 (3.0–5.4)	3.0 (1.3–3.0)
At 24 h			
HLA-DR, antibody-PE molecules bound/cell	17,772 (12,933–25,167)	19,406 (14,885–24,225)	–
CD64, antibody-PE molecules bound/cell	9,754 (8,040–14,212)[a]	4,545 (3,459–5,668)	–
CRP, mg/l	11.6 (4.6–23.4)[a]	3.9 (3.0–7.0)	–

Results are medians (interquartile ranges).

[a] Levels of neutrophil CD64 expression and serum CRP concentrations in infected patients are significantly increased compared with the corresponding levels of non-infected infants and control subjects at 0 h and 24 h ($p < 0.001$).

Table 4. Comparison of sensitivity, specificity, and positive and negative predictive values of the markers at 0 and 24 h after the onset of infection

Infection markers	Cutoff values	0 h				24 h			
		sensitivity	specificity	PPV	NPV	sensitivity	specificity	PPV	NPV
Single marker									
HLA-DR, antibody-PE molecules bound/cell	≤11,718	0.31	0.80	0.42	0.71	0.19	0.92	0.55	0.76
	≤20,606	0.80	0.12	0.30	0.54	0.58	0.45	0.33	0.69
CD64, antibody-PE molecules bound/cell	≤6,136	0.78	0.90	0.78	0.90	0.94	0.81	0.70	0.96
CRP, mg/l	≤10	0.42	0.91	0.70	0.77	0.53	0.83	0.60	0.79
Combination of markers									
HLA-DR or CD64, antibody-PE molecules bound/cell	≤11,718 or ≤6,136	0.84	0.70	0.57	0.90	0.94	0.76	0.65	0.96
	≤20,606 or ≥6,136	0.97	0.07	0.33	0.81	0.96	0.37	0.42	0.95

PPV = Positive predictive value; NPV = negative predictive value.

24 h. In addition, a comparison of the diagnostic utilities of monocyte HLA-DR in combination with neutrophil CD64 versus individual markers suggested that the use of multiple markers could only marginally improve the sensitivity at 0 h but adversely affect the specificity of the tests (table 4).

Discussion

The impact of major surgery and trauma on adult patients resulted in a significant decrease in the expression of HLA-DR on monocytes [9–12]. This effect was further augmented in patients with severe sepsis [9–12]. A recent study in the neonatal age group indicated that the expression of monocyte HLA-DR was not affected by gestational age or hypoxic-ischemic injury [16]. However, the level was significantly lower in healthy neonates than in adults and was further decreased in neonates who developed septicemia or respiratory distress syndrome [16]. Although the study illustrated many relevant and practical issues concerning the potential use of HLA-DR as an indicator of systemic neonatal sepsis, the antigen expression was not quantitatively measured such that it could be compared among different laboratories. The diagnostic utilities of monocyte HLA-DR were also not defined. Further, our previous study on neutrophil CD64 and CRP in term neonates suggested that CD64 was a sensitive (sensitivity 96%) but only moderately specific (specificity 81%) infection marker for identifying early-onset clinical sepsis and pneumonia [6]. The serum CRP level was shown to be influenced by surgery or trauma to body tissue. Hence, monocyte HLA-DR was chosen as an additional marker to neutrophil CD64 and CRP because we anticipated that a decrease in the expression of this surface antigen could further differentiate newborns with sepsis from those who experienced only traumatic delivery. Thus, the inclusion of HLA-DR might assist in improving the diagnostic utilities of the existing infection markers.

Our findings suggested that quantitative measurement of monocyte HLA-DR did not contribute to differentiating newborns with clinical infection or pneumonia from those without infection. The areas under the ROC curves at 0 and 24 h were only 0.52 and 0.54, and a clinically useful cutoff value could not be determined using the criteria of an 'ideal' diagnostic marker [3]. The infants with group B streptococcus septicemia and necrotizing enterocolitis had relatively low monocyte HLA-DR expressions of 8,614 and 10,658 antibody-PE molecules bound/cell. Nonetheless, 9 and 23 non-infected infants also had HLA-DR expression below these levels, respectively. Our results suggested that newborns with clinical pneumonia could not be identified by monocyte HLA-DR alone, as there was much overlap in the levels of antigen expression between the infected and non-infected patients. In contrast, the results of neutrophil CD64 and CRP were similar to those of our previous study [6] and their diagnostic utilities were superior to monocyte HLA-DR. The use of multiple markers, including monocyte HLA-DR and neutrophil CD64 could only marginally improve the sensitivity of the tests, but would seriously affect the specificity of the results (table 4).

The stringent criteria in recruitment of patients and standardization of methodology in evaluating infection markers [17] should ensure objective and correct classification of cases. In this study, we prospectively standardized the definitions of clinical infection and pneumonia. The same definitions were applied to our previous study [6] and were proven to be highly discriminatory for infection markers such as neutrophil CD64. The inclusion of only term infants also eliminated the enrollment of newborns with respiratory distress syndrome which might affect the level of HLA-DR expression. Further, the independent assessors were effectively blinded from the results of infection markers and the identities of the patients during the classification process, and the relatively large sample size of this study could also minimize the magnitude of misclassification of cases.

Several plausible explanations could account for the disparity between the findings in adults [9–12, 16] and the results of the current study. Firstly, the 'normal' range of HLA-DR in well and completely asymptomatic term infants was wide (10,938–25,312 antibody-PE molecules bound/cell). Control infants could have relatively low monocyte HLA-DR expressions. Secondly, and more importantly, the types of infection described in the cohort of Kanakoudi-Tsakalidou [16] and in the adult studies [9–12] were mainly systemic infections secondary to septicemia, whereas early-onset neonatal sepsis in our cohort was primarily due to localized pneumonia (i.e. single organ infection). Thus, we speculate that the relatively less severe pulmonary infection might not be able to initiate the same degree of systemic inflammatory response. Further, a recent study in neonates and small children indicated that increases in the proportion of CD14+ CD16+ monocytes correlated with clinical symptoms of sepsis much better than the reduction of HLA-

DR expression on monocytes [18]. Thirdly, the methods of measurement of HLA-DR were different between the various studies [9–12, 16]. We chose a quantitative assessment by flow cytometry which should theoretically give a more accurate measurement compared with the semiquantitative methods used by other investigators [9–12, 16].

In conclusion, our findings do not support the use of monocyte HLA-DR alone or in combination with other infection markers for the diagnosis of early-onset clinical infection and pneumonia in term newborns. None of the cutoff values from the ROC curve could satisfy the predetermined criteria for monocyte HLA-DR to be classified as a useful marker of infection.

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