

Expression of CD64 as a potential marker of neonatal sepsis

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The aim of this study was to identify a novel immunological indicator useful for the early diagnosis (through a rapid and single determination) of neonatal sepsis (NS). Peripheral blood samples were taken from 63 neonates, who were classified into four groups: proven NS (n=17); clinical NS (n=14); disease without infection (n=17); and healthy newborns (n=15). Neutrophil expression of CD64, CD43, CD44, CD50, CD62L and Mac-1, and plasma levels of interleukin (IL)-1 β , IL-6, tumor necrosis factor- α (TNF- α) and soluble L-selectin (sCD62L), were determined. Expression of CD64 was significantly enhanced in the group with proven sepsis and clinical NS compared to newborns without infection (p < 0.05). Eight newborns with proven or clinical sepsis, but only one with disease without infection, showed an increased percentage of CD64⁺ cells (diagnostic specificity = 96.8%). No significant differences were found in the expression of the other leucocyte differentiation antigens studied. As previously described, TNF- α and IL-6 levels were significantly elevated in newborns with proven or clinical sepsis compared to neonates without infection (p < 0.05). Our results suggest that, through a single determination, the enhanced expression of CD64 is a highly specific indicator of NS, although its diagnostic sensitivity is low (25.8%). In contrast, we found that plasma levels of IL-1 β and sCD62L, as well as the expression of Mac-1, CD43, CD44, CD50, and CD62L, do not appear to be useful for the diagnosis of NS.

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Neonatal sepsis (NS) remains as a major cause of morbidity and mortality in the newborn, mainly among preterm and low-birth-weight infants (1). Early diagnosis of sepsis in the neonate is difficult because symptoms and signs are subtle and non-specific. The current gold standard for confirming the diagnosis of NS is isolation of the causal micro-organism by blood culture. However, blood culture results are not available until 24–48 h after starting the culture, and they are often negative in cases of pneumonia and meningitis, or even in fatal generalized bacterial infection (2). Many laboratory tests, including different leucocyte indexes and acute-phase proteins, have been evaluated for use in the diagnosis of NS. However, the inability of such screening tests to provide definitive guidelines has compelled neonatologists to search for other indicators (3).

Sepsis is a syndrome characterized by a systemic inflammatory response in which the

cytokines produced by immune cells in response to bacterial products are important mediators. The cardio-respiratory abnormalities and other organ dysfunctions associated with sepsis seem to be a result of the action of monocyte-derived cytokines, such as tumor necrosis factor- α (TNF- α), interleukin (IL)-1 and IL-6 (4). Increased plasma levels of these cytokines have been found in sepsis, and a significant correlation between high plasma levels of these cytokines and poor clinical outcome has been reported (5–9).

An important early event in the generation of the inflammatory response is the cytokine-induced activation of neutrophils, the primary function of which is to eliminate invading micro-organisms. In order to achieve this, neutrophils must extravasate, a phenomenon that is mediated by cell-surface adhesion molecules, including L-selectin (CD62L) (10). This adhesion receptor is proteolytically cleaved from the leucocyte surface

after cell activation, generating a soluble form that can be detected in serum (11,12). Other adhesion molecules, such as CD43, CD44 and CD50 [intracellular adhesion molecule-3 (ICAM-3)], that are expressed by neutrophils, are also cleaved from the cell surface during cell activation, down-regulating their expression on activated neutrophils (11,13). In contrast, the leucocyte integrin Mac-1 (CD11b/CD18), and the Fc γ receptor I (CD64), behave as activation antigens in neutrophils, increasing their expression on the cell surface after leucocyte activation (14,15). Therefore, it is expected that during NS, bacterial products stimulate the release of IL-1 β , IL-6 and TNF- α , inducing the activation of neutrophils and resulting in changes in the expression of leucocyte-differentiation antigens, with an increase in Mac-1 and CD64 levels and a decrease in those of CD43, CD44, CD50 and CD62L. Thus, detection of leucocyte-differentiation antigen by flow cytometry may prove to be a very fast and simple method for the diagnosis of NS. The aim of this work was to identify, using a rapid and single determination, a novel immunological indicator useful for the early diagnosis of NS. We found that the enhanced expression of CD64, through a single determination, has a high specificity for the diagnosis of NS (96.8%), whereas CD43, CD44, CD50 and CD62L expression levels do not seem to be useful for the diagnosis of this condition.

Patients and methods

Subjects

Neonates in the first 28 days of life, who were admitted to the neonatal intensive care unit at the Hospital Central 'Dr Ignacio Morones Prieto', were included in the study. Neonates were classified into one of the following four groups:

1 Proven sepsis (n=17): clinical sepsis plus a positive bacterial blood culture.

2 Clinical sepsis (n=14): negative bacterial culture but the presence of three or more clinical signs of infection such as fever, temperature instability, tachycardia, tachypnea, abdominal distension, respiratory distress, seizures, compromised microcirculation, apnea, cyanosis, oliguria, gastrointestinal bleeding, or petechiae. All newborns were evaluated by two clinicians.

3 Disease without infection (n=17): neonates with the final diagnosis of idiopathic respiratory distress syndrome (n=4), perinatal asphyxia (n=6), meconium aspiration syndrome (n=2), transient tachypnea of the newborn (n=2), and seizures (n=3).

4 Healthy newborns (n=15).

In the last group were included neonates from whom a blood sample had been taken because of suspicion of different diseases, but a normal result observed and no illness subsequently detected.

Informed consent was obtained from the parents in all cases, and the Ethics Committee of the Hospital Central 'Dr Ignacio Morones Prieto' approved this study. Gestational age, birth weight, and postnatal age were recorded and are shown in Table 1.

Blood samples

Most samples of peripheral blood (83%) were obtained when infection was suspected, and only 17% were taken later, in all cases during the first 24 h of clinical suspicion of NS. In accordance with the aim of the study, only one blood sample was obtained, simultaneously with that taken for bacterial culture and leucocyte count. Samples from groups 3 and 4 were taken at the time of blood drawn for the laboratory tests and within the first 48 h of their admittance. Four neonates with proven or clinical sepsis had received antibiotics before the blood sample was obtained. Blood samples were immediately transported on ice to the laboratory, and they were processed upon arrival. Plasma was obtained from the heparinized blood (20–30 U/ml) by centrifugation (200 $\times g$ for 10 min) and stored at -20°C for the subsequent quantification of cytokines (IL-1 β , IL-6, and TNF- α) and soluble L-selectin (sCD62L); the rest of the blood sample was used in flow cytometry to detect the expression of leucocyte-differentiation antigens.

Monoclonal antibodies

Monoclonal antibodies (mAbs) against CD43, CD44 and CD50, as well as the isotype-control immunoglobulin P3x63, were kindly provided by Dr F. Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain). The anti-Mac-1 (CD11b) mAb was purchased from Serotec (Oxford, UK) and the fluorescein isothiocyanate (FITC)-conjugated antibodies against CD62L and CD64 were obtained from Becton-Dickinson (San Jose, CA, USA) and Pharmingen (San Diego, CA, USA), respectively.

Determination of plasma levels of IL-1 β , IL-6, TNF- α and sCD62L

Quantification of IL-1 β , IL-6, TNF- α , and sCD62L was performed using commercially available, high-sensitivity enzyme-linked immu-

Table 1. Main characteristics of patients included in the study

Definition	Group 1 Proven sepsis	Group 2 Clinical sepsis	Group 3 Disease without Infection	Group 4 Healthy newborns
No. of newborns	17	14	17	15
Gestational age (weeks)	34.6±0.8	36.5±0.7	36.0±1.1	36.2±0.6
Birth weight (kg)	1.9±0.3	2.4±0.2	2.2±0.2	2.2±0.2
Postnatal age (days)	11.4±2.0	8.8±1.6	2.8±0.6	3.8±1.7

Values correspond to the arithmetic mean±SE. The postnatal age of groups 3 and 4 was significantly lower than in the groups of patients with sepsis (groups 1 and 2) ($p < 0.05$). There were no significant differences among the groups regarding gestational age and birth weight ($p > 0.05$ in both cases).

nosorbent assay (ELISA)_kits (R & D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The lower detection limits of the assays were 0.1 pg/ml for IL-1 β , 0.09 pg/ml for IL-6, 0.18 pg/ml for TNF- α , and 0.1 ng/ml for sCD62L.

Expression of leucocyte-differentiation antigens by neutrophils

The expression of leucocyte-differentiation antigens by neutrophils was analyzed in whole blood using flow cytometry. Samples were maintained in silicon-coated tubes at 4°C before and during this process, to avoid non-specific *in vitro* activation of leucocytes. Whole blood (100 μ l) was incubated for 30 min with the specific mAb or the isotype-matched control P3x63 mAb. Then, an FITC-labeled goat anti-mouse IgG (Becton-Dickinson) was added and the tubes incubated for an additional 30 min on ice. For the detection of CD62L and CD64, 50 μ l of whole blood was incubated with the corresponding FITC-labeled mAb for 15 min. Finally, erythrocytes were lysed and leucocytes fixed using Q-Prep (Coulter, Hialeah, FL, USA), following which samples were analyzed in an EPICS PROFILE II flow cytometer (Coulter). Polymorphonuclear leucocytes (PMNs) were electronically selected on the basis of their side- and forward-scatter characteristics and 10 000 cells were analyzed in each sample. Results were expressed as the percentage of positive cells and/or the mean fluorescence intensity (MFI).

Statistical analysis

Data were expressed as the arithmetic mean $\pm$ standard error. Differences among the groups were tested using one-way Kruskal-Wallis analysis and the non-parametric Mann-Whitney *U*-test, Fisher's exact test, and chi-square test. The diagnostic specificity and sensitivity values were calculated using the ROC curve analysis.

Results

Characteristics of patients and controls

As stated above, the 63 neonates studied were classified into four groups (Table 1) as follows:

- 1 Proven sepsis (n=17),
- 2 Clinical sepsis (n=14),
- 3 Disease without infection (n=17), and
- 4 Healthy newborns (n=15).

There were no significant differences among the groups regarding gestational age and birth weight ($p > 0.05$). Healthy newborns were significantly younger than patients with sepsis ($p < 0.05$, Table 1) because they usually required fewer hospitalization days after delivery. In the group of proven sepsis, most of the blood cultures demonstrated the presence of the Gram-negative bacillus *Klebsiella pneumoniae*, which at the time of the study was the dominant pathogen in our hospital.

Cytokine and sCD62L plasma levels

IL-1 β plasma levels tended to be higher in patients with proven sepsis compared with the other newborns, but these differences did not reach statistical significance (Fig. 1a). In contrast, plasma levels of IL-6 were significantly higher in newborns with proven sepsis and clinical sepsis compared to patients without infection or healthy newborns ($p < 0.05$ in all cases, Fig. 1b). These results showed that IL-6 plasma levels have a high diagnostic specificity for NS (92%, ROC curve analysis). The diagnostic sensitivity, and the positive and negative predictive values for this cytokine, were 47%, 78%, and 75%, respectively. In addition, when normal plasma levels of IL-6 were defined as the arithmetic mean+3 SD (75.9 pg/ml) of the values observed in healthy newborns, we found that 13 patients with proven or clinical sepsis (41.9%), but none of those without infection, had raised levels of IL-6 ($p < 0.01$, Fig. 1b). TNF- α plasma levels were significantly increased in the group with proven sepsis compared to patients without infection or healthy newborns ($p < 0.05$ in both cases, Fig. 1c). The sensitivity and specificity of

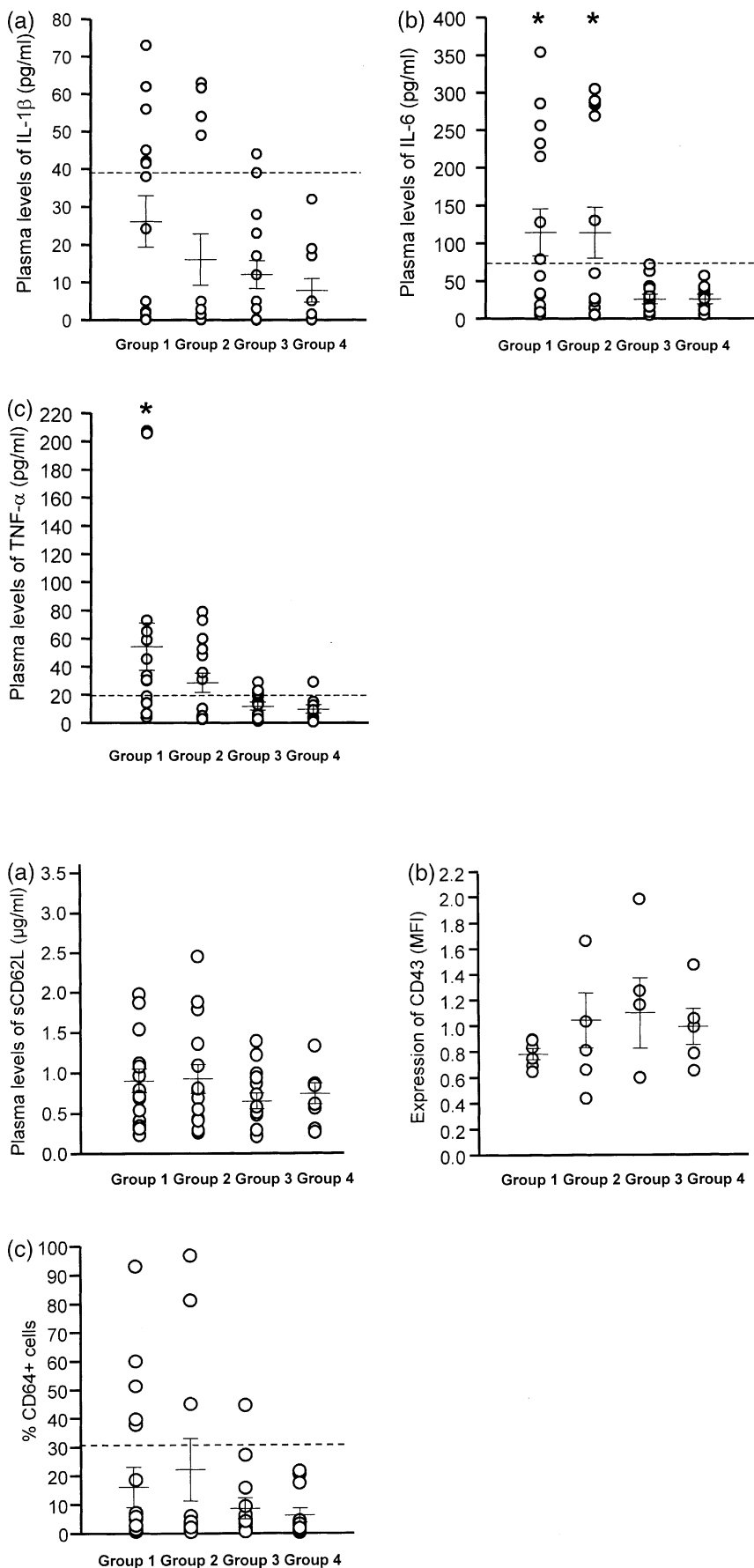


Fig. 1. Plasma levels of interleukin (IL)-1 β , IL-6 and tumor necrosis factor- α (TNF- α) in neonates with proven and clinical sepsis (groups 1 and 2, respectively), disease without infection (group 3), and healthy newborns (group 4). A blood sample was obtained from each newborn and cytokine concentrations in this blood sample were measured by using enzyme-linked immunosorbent assay (ELISA). The arithmetic mean $\pm$ SE of cytokine levels are indicated by the horizontal bars. Horizontal dashed lines represent the cut-off values (arithmetic mean+3 SD of plasma levels found in normal neonates), which were arbitrary and considered as the top normal levels of the indicated cytokines. * $p < 0.01$ compared with groups 3 and 4 (Mann-Whitney U-test).

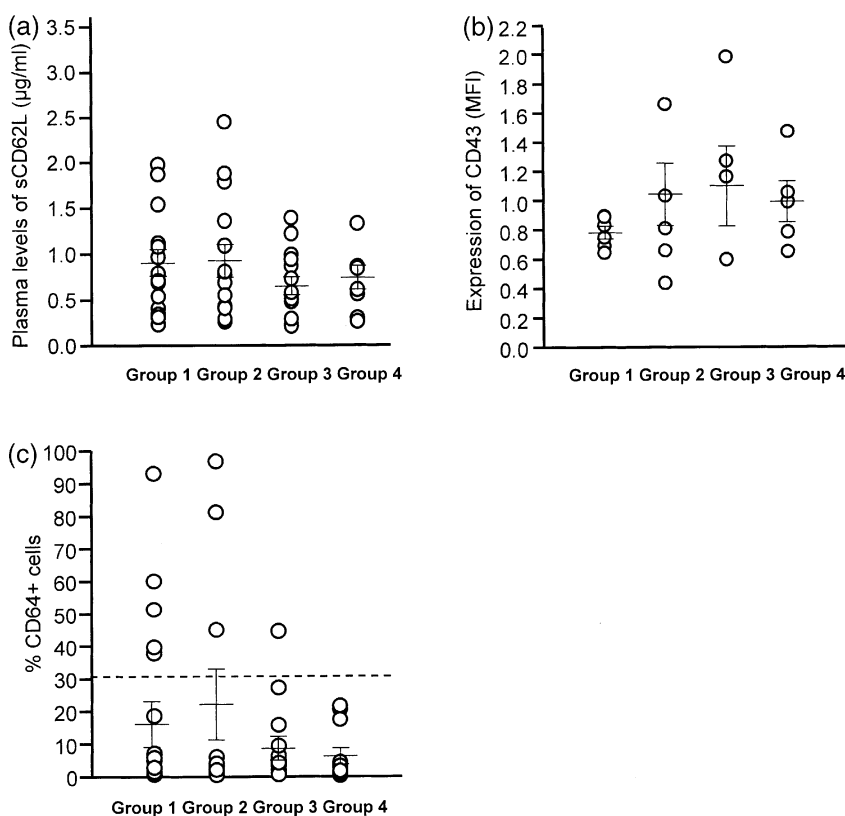


Fig. 2. Expression of cell-surface antigens by neutrophils and plasma levels of soluble L-selectin in neonates with proven and clinical sepsis (groups 1 and 2, respectively), disease without infection (group 3), and healthy newborns (group 4). A blood sample was obtained from each newborn and the concentration of soluble (s)L-selectin was measured by using enzyme-linked immunosorbent assay (ELISA) (a), whereas the membrane expression of CD43 (b) and CD64 (c) was analyzed by flow cytometry. Data correspond to the arithmetic mean $\pm$ SE of plasma levels of sL-selectin (a), the mean fluorescence intensity (MFI) of CD43 expression (b), or the percentage of CD64 $^{+}$ cells (c). The dashed line corresponds to the cut-off value (arithmetic mean + 3 SD) of the percentage of CD64 $^{+}$ cells found in normal neonates, which was arbitrary and considered as the top normal level.

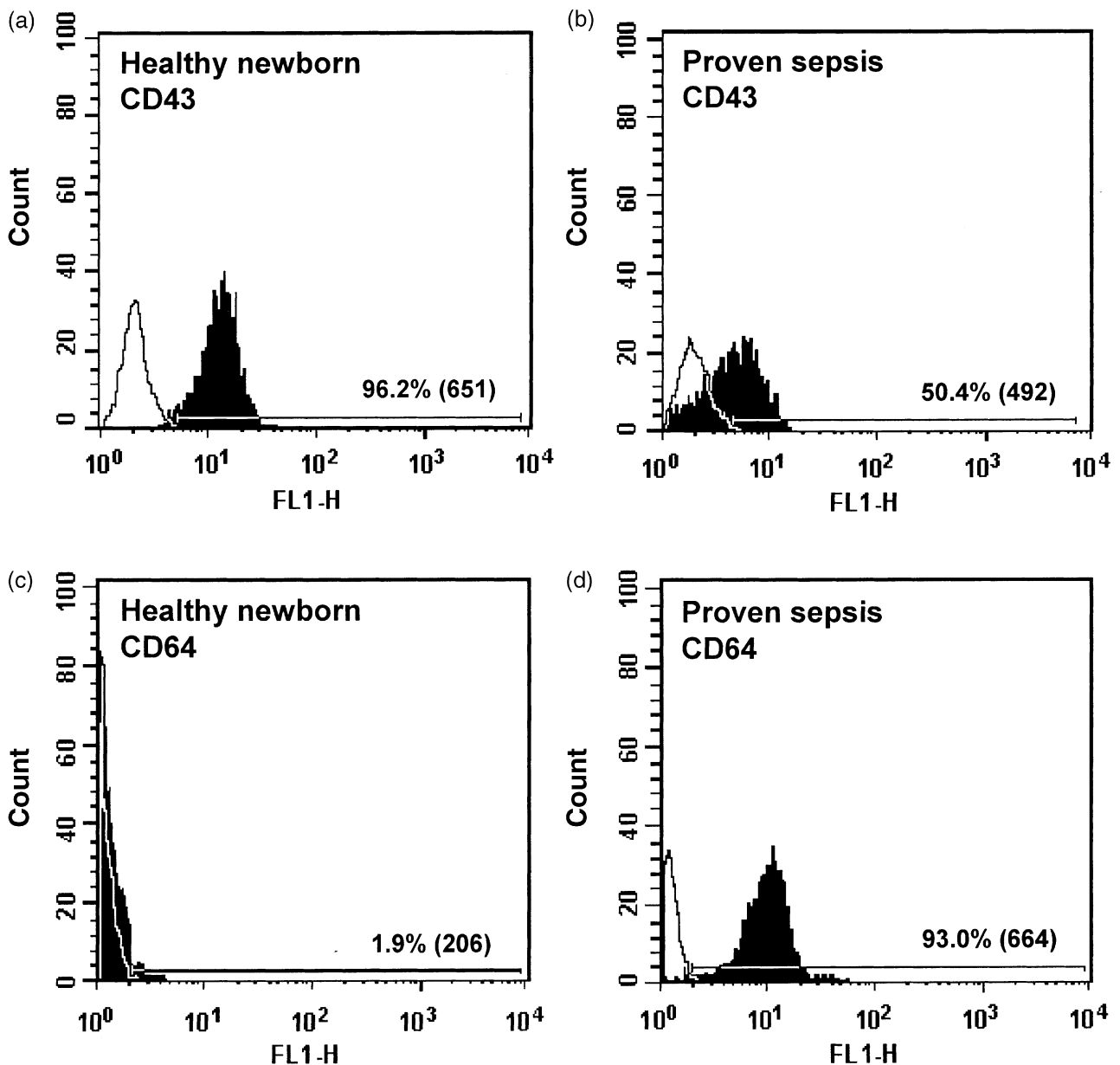


Fig. 3. Flow cytometry analysis of CD43 and CD64 expression by neutrophils from four neonates. The normal expression of CD43 and CD64 in healthy newborns are shown in panels (a) and (c), whereas the decreased and increased expression of CD43 and CD64 in patients with neonatal sepsis are shown in panels (b) and (d), respectively. Empty histograms correspond to the isotype-matched controls. The percentages of positive cells are shown and the mean fluorescence intensity (MFI) values are indicated within parentheses.

raised plasma levels of TNF- α for the diagnosis of neonatal sepsis were 60% and 84%, and positive and negative predictive values were 69% and 78%, respectively. No significant differences in plasma levels of sCD62L were found among the four groups studied (Fig. 2a).

Expression of leucocyte-differentiation antigens by peripheral blood PMNs

In accordance with the results on the plasma levels of sCD62L, the expression of this adhesion

receptor on the surface of PMNs was similar in the four groups studied (data not shown). On the other hand, neutrophils from some patients with proven sepsis showed a decreased expression of CD43 (Fig. 2b, and Fig. 3a, 3b). However, statistical analysis revealed no significant differences. In contrast, when normal levels of CD64⁺ cells were defined as the arithmetic mean + 3 SD of the values observed in healthy newborns, eight patients with proven or clinical sepsis, but only one with disease without infection, showed raised percentages of CD64⁺ cells ($p < 0.05$, chi-square

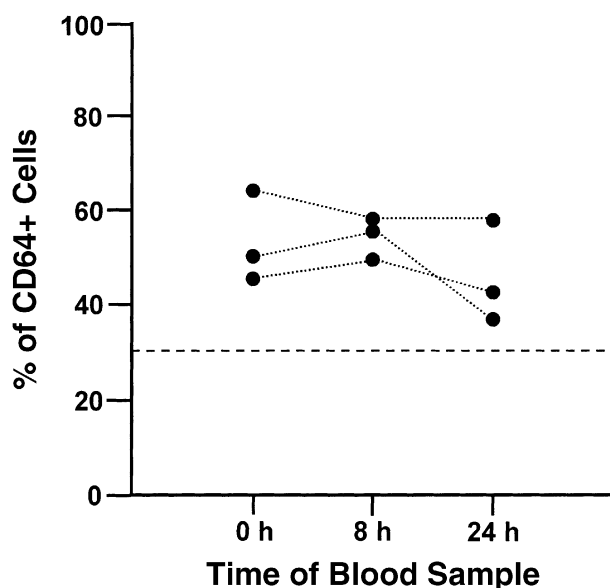


Fig. 4. Sequential determinations of CD64⁺ neutrophils in three patients with neonatal sepsis (NS). Blood samples were obtained from three newborns at 0, 8, and 24 h of clinical suspicion of NS. The percentage of CD64⁺ neutrophils was determined using flow cytometry, as described in the Materials and methods. The dashed line corresponds to the cut-off value (arithmetic mean + 3 SD of the percentage of CD64⁺ cells found in normal neonates), which was arbitrary and considered as the top normal level.

test, Fig. 2c, and Fig. 3c, 3d). This laboratory parameter showed a high specificity and positive predictive value for sepsis (96.8 and 88.8%, respectively), although with a low sensitivity (25.8%) and an intermediate negative predictive value (57.4%). Interestingly, when repeated determinations of CD64⁺ neutrophils were performed (at 0, 8 and 24 h), a sustained increase of the percentage of CD64⁺ cells was found in the three patients with sepsis (Fig. 4). Lastly, we found no significant differences in the expression of Mac-1, CD44, and CD50 among the four groups studied (data not shown).

Discussion

An early laboratory marker with high diagnostic sensitivity and specificity for NS would be a valuable tool for therapeutic decision-making, thus avoiding the unnecessary use of antibiotics in those patients without infection but in whom sepsis is suspected on a clinical basis (1). In this study, we attempted to identify novel immunological indicators that, through a single and rapid determination, were useful for the diagnosis of NS. We found that the enhanced expression of CD64 by neutrophils has a high specificity and positive predictive value for NS, but a low sensitivity. It is worth mentioning that the

analysis of CD64 expression is performed with a small sample (25–50 µl of whole blood), is very rapid (≈ 20 min), and highly reliable. In addition, our data indicate that the high specificity of raised levels of CD64 for NS is achieved through a single determination and the high percentage of CD64⁺ cells seen in NS is maintained in these patients for at least 24 h. Therefore, CD64 expression may be useful (although of low sensitivity) for the diagnosis of NS. In contrast, our data indicate that other leucocyte-differentiation antigens that are up-regulated (Mac-1 or CD11b/CD18), or down-regulated (CD43, CD50, CD62L) upon neutrophil activation do not seem to be useful for the diagnosis of NS, at least using a single determination.

It is worth mentioning that the neonates with NS included in this study were older than control neonates (Table 1), and it is possible that this difference might have an influence on CD64 expression. However, it is very unlikely that the observed differences in CD64 expression are related to disparity in the age of the groups of neonates studied. In this regard, it has been described that the expression of CD64 by neutrophils only occurs upon cell activation (16). In addition, preliminary results of our laboratory indicate that there are no significant differences in the expression of this Fc receptor in healthy neonates 3–4 days of age compared with healthy neonates 7–14 days of age.

As is not easy to clinically diagnose sepsis in newborns, several groups of researchers have attempted to characterize a laboratory indicator that is useful for the early diagnosis of NS. Some immunological markers such as serum levels of IgG, IgM, C3, and C4 have been evaluated, but they did not seem to have significant value for the early diagnosis of NS (17). In addition, it has been found that the plasma levels of soluble receptors of TNF only show small variations in NS, and therefore are not useful as indicators of this condition (18). In contrast, the plasma levels of the IL-1 receptor antagonist (IL-1RA) are significantly increased in NS (19). Furthermore, it has been claimed that thromboelastography (20) and a polymerase chain reaction assay for the detection of bacterial 16S rRNA (21) are sensitive indicators of NS. However, as these laboratory parameters have been described only recently, their value has not been confirmed and they have not been widely used to date.

In the present study, we have corroborated that there is a significant increase in the plasma levels of IL-6 and TNF- α in patients with sepsis compared with healthy newborns or patients without infection. In contrast, we did not detect

significant differences in the levels of IL-1 β or soluble L-selectin in the four groups of newborns studied. There are different reports on the plasma levels and gene expression of IL-1 β , IL-6, and TNF- α in NS and it has been proposed that these immune parameters are sensitive indicators of systemic infection (2,5–9,22,23). It is not evident why our patients with sepsis did not show raised levels of IL-1 β , but it is feasible that this finding could be related to the immaturity of blood leucocytes described previously in neonates with infectious complications (24). In addition, our results on IL-1 β plasma levels are consistent with those of Atici et al. (25), who found that the plasma concentration of IL-1 β is not increased in neonates with sepsis. On the other hand, our results on normal plasma levels of soluble L-selectin in NS are in accordance with previous works (26,27) in which a low basal expression and a diminished down-regulation of L-selectin in neutrophils from healthy neonates was observed. In contrast, a significant, low L-selectin expression on umbilical cord blood granulocytes and monocytes from newborns with acute bacterial infection has been reported (28). However, there are no previous reports on soluble L-selectin plasma levels in NS. Therefore, the expression of L-selectin by neutrophils from newborns, and the plasma levels of the soluble form of this adhesion receptor in NS, remain controversial and their possible value as indicators of this condition needs to be examined further.

The disparity between the high diagnostic sensitivity reported by Messer et al. (18) for IL-6 plasma levels and NS, and the values of the present study, could be the result of differences at the time at which blood samples were taken, for IL-6 has a short half-life in plasma (29). In this regard, Panero et al. (2) made two separate determinations of IL-6 plasma levels in each patient with NS, and found that additional measurements are helpful for detecting infection in the neonate. It is important to note that we only obtained one blood sample from each patient because the aim of our study was to identify a laboratory parameter that was useful with a single and rapid determination (i.e. flow cytometry analysis of a whole blood sample). On the other hand, it is worth remembering that IL-6 is the main stimulus involved in the induction of the acute-phase reaction and enhancement of C-reactive protein (CRP) synthesis. Thus, it is expected that the expensive and time-consuming determination of the IL-6 plasma concentration could be replaced by the simpler and more cost-effective analysis of CRP levels.

Interestingly, patients with proven sepsis appeared to fall into two different subgroups according to their plasma levels of cytokines, suggesting that some neonates seem to have a greater capability to synthesize pro-inflammatory cytokines. In this regard, it has been reported that a polymorphic variant of the promoter region of the TNF- α gene is associated with an increased production of this cytokine, and that this genetic trait could be associated with an increased risk for some inflammatory diseases (30). Therefore, it is feasible that some neonates have an increased capability to release TNF- α in response to bacterial products, and that these patients exhibit very high serum levels of cytokines during infectious episodes such as sepsis. It is also possible that the enhanced expression of CD64 seen in some infected newborns is related to an increase in the plasma levels of pro-inflammatory cytokines such as TNF- α . In this regard, we found a significant correlation between IL-6 plasma levels and CD64 expression ($r=0.48$, $p<0.05$), but not between TNF- α levels and the expression of this Fc receptor ($r=0.26$, $p>0.05$). These results may be owing to differences in the kinetics of cytokine synthesis, neutrophil activation, and neutrophil half-life (31).

We found no differences in the expression of Mac-1 by neutrophils among the study groups. These results are in accordance with reports on a diminished ability of translocation of Mac-1 in neonatal neutrophils (26). However, Rebuck et al. (27) did not find any deficit in the ability of neonatal neutrophils to up-regulate their Mac-1 expression upon stimulation, and they suggested that the deficit found in previous studies was a result of the cell isolation procedure. Our data do not support this point, as we studied Mac-1 expression in whole blood, without any isolation procedure. On the other hand, one study has reported an increase in neutrophil expression of Mac-1 in all neonates with confirmed infection, in 94% of newborns with suspected infection, and in none with no infection (32). These contradictory findings could be a result of differences in the genetic background of the patients studied which might affect both the basal expression of Mac-1 and the capability of neutrophils to respond to different bacterial stimuli. Further studies are necessary to elucidate this point.

The expression of CD64 is enhanced on activated neutrophils, and it has been shown that leucocytes from patients with streptococcal infection have an increased expression of this Fc receptor (16,33). In addition, Fjaertoft et al. (34) found, in a small sample of newborns, that the expression of CD64 was significantly enhanced in

infected newborns compared to healthy neonates. We found that eight patients with proven or clinical sepsis, but only one with disease without infection, had an increased percentage of CD64⁺ cells. These data therefore indicate that the levels of expression of this antigen may be a specific *in vivo* indicator of neutrophil activation, and the presence of infection. In addition, our results suggest that the enhancement of CD64⁺ cells in peripheral blood is an immune parameter with a high specificity for NS. However, as in our hands CD64 expression had a low sensitivity for NS, it will be necessary to perform a multicenter prospective study to define the real value of this parameter in the early and rapid diagnosis of NS. Finally, we also evaluated the expression of CD43, CD44 and CD50, and no significant differences were found between infected and uninfected newborns. These results could also be caused by the intrinsic characteristics of neonatal neutrophils, probably associated with their functional immaturity.

In this study, we attempted to identify a novel immunological indicator of NS. We found that, like most clinical data, there was a significant degree of overlap in the majority of laboratory parameters evaluated among the groups studied. Thus, the immunological parameters studied by us do not seem to fulfil the proposed characteristics of an ideal indicator of NS (18), such as early appearance, persistence during the overall course of sepsis as well as a high sensitivity and specificity. However, our data indicate that, in addition to plasma levels of TNF- α and IL-6, the expression of CD64 by neutrophils could be useful as an indicator of NS. It is worth remembering that the analysis of expression of CD64 is made on a small blood sample and by a rapid assay, providing a reliable and prompt result for the clinician. Obviously, this analysis can be combined with additional, time-consuming determinations, such as ELISA measurements of TNF- α and IL-6 to improve the probability of NS detection. Furthermore, additional novel markers of NS, also detected by flow cytometry, should be explored. An interesting possibility is the expression of chemokine receptors by neutrophils, because it has been described that CXCR1 and CXCR2 are down-regulated on human neutrophils after stimulation with bacterial lipopolysaccharide (LPS) (35). Finally, it is highly probable that our results on CD64 expression may be of assistance in the early management of neonates with suspicion of NS. In such cases, a high expression of CD64 would strongly suggest the presence of infection and

indicate that antibiotic administration is necessary.

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