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Increased CD11b-Density on Circulating Phagocytes as an Early Sign of Late-Onset Sepsis in Extremely Low-Birth-Weight Infants

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ABSTRACT

Late-onset hospital-acquired sepsis is common in extremely low birth-weight (<1000g) (ELBW) infants. The diagnosis is difficult since, at early stages of sepsis, routine laboratory tests are neither specific nor sensitive. In term infants with sepsis neutrophil surface expression of CD11b/CD18, a β_2-integrin, is significantly increased. Here we studied whether increased CD11b/CD18 density on blood neutrophils and monocytes serves as an early sepsis marker in ELBW infants. Blood samples were obtained from 30 ELBW infants on a daily basis for 3–4 postnatal weeks, and neutrophil and monocyte CD11b/CD18 expression was determined by flow-cytometry. Patients were assigned one of 3 groups: 1) an infected group, comprised of infants who had blood culture-positive sepsis and/or necrotizing enterocolitis, 2) a non-infected group, and 3) a potentially infected group, comprised of infants in whom infection was suspected but could not be confirmed microbiologically. One patient had blood culture contamination and was excluded from the analysis. In the infected group, CD11b expression gradually increased during the three days preceding sampling for blood culture. At the day of sampling, median expression of CD11b in neutrophils and monocytes was higher in the infected group than in the control group. For neutrophils the sensitivity and specificity were 1.00 and 0.56, respectively, and for monocytes, 0.86 and 0.94, respectively. From these data, we conclude that determination of CD11b/CD18 density on neutrophils and monocytes may improve diagnosis of late-onset sepsis in ELBW infants. (***Pediatr Res* 57: 270–275, 2005**)

Abbreviations

ACD, acid-citrate-dextrose
AUC, area under curve
CI, confidence interval
CRP, C-reactive protein
ELBW, extremely low birth-weight
IQR, interquartile range
NEC, necrotizing enterocolitis
NICU, Neonatal intensive care unit
NPV, negative predictive value
PE, phycoerythrin
PPV, positive predictive value
RDS, respiratory distress syndrome
RFU, relative fluorescence unit
ROC, receiver operating curve
WBC, white blood cell count

Extremely low (<1000g) birth-weight (ELBW) infants are at increased risk of developing life threatening late-onset (>72 h after birth) nosocomial infections. In such infants, the incidence of late-onset sepsis is remarkable, 16–21%, and correlates inversely with birth weight (1,2). The clinical picture of late-onset infection is variable. The most common microbes growing in blood culture are Gram-positive bacteria. They account for 53–70% of blood stream infections (1,2), with the predominance of *Staphylococcus epidermidis*, commonly associated with the presence of i.v. lines. Gram-negative bacteria account for 18–22% of the preterm infant blood stream infections, which are associated with higher mortality than Gram-positive infection, 36% *versus*. 11% (1). Fungal organisms cause 12–15% of late-onset blood stream infections (1,2), whose mortality is high, 32% (1). Necrotizing enterocolitis (NEC), a neonatal bowel disease whose incidence among preterm infants is approximately 10%, is also an important predisposing condition to blood stream infections. Indeed,

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17–23% of the preterm infants with NEC develop blood culture positive sepsis (3), which are caused predominantly by intestinal Gram-negative bacteria (4).

Clinical signs of sepsis in ELBW infants are nonspecific, and routine laboratory markers, such as C-reactive protein (CRP), or immature/total neutrophil ratio, are of limited value in deciding on antimicrobial therapy (5–7). The need for early detection of infection and immediate introduction of antimicrobials is further supported by recent findings indicating that most deaths associated with late-onset infections in ELBW infants occur during the first few days after sampling for blood culture (1). It is therefore recommended that antimicrobial therapy is started on the basis of clinical signs after sampling for blood culture (8). However, this strategy renders many infants unduly susceptible to side effects of the drugs, increases hospital costs, and induces microbial resistance in the hospital (9). To reduce the use of antimicrobials, novel markers are needed to improve the diagnosis of sepsis in ELBW infants.

We and others have recently found that an increase in neutrophil surface density of CD11b/CD18 (Mac-1, $\alpha_{M\beta2}$, CR3), a β_2-integrin adhesion molecule, is a promising sepsis marker in term neonates (10,11). In adults CD11b/CD18 expression is increased on circulating phagocytes in patients with systemic inflammation triggered by infection or noninfectious insults (12–17). We hypothesized that in ELBW infants, as in infants born at term, CD11b/CD18 might serve as a marker of sepsis. To address this question we studied prospectively on a daily basis CD11b/CD18 density on the surface of blood neutrophils and monocytes in ELBW infants during their stay at the neonatal intensive care unit (NICU).

METHODS

Subjects. The local institutional review boards approved the study protocol. Parents gave their informed consent. All ELBW infants (birth weight <1000g) born and treated in the tertiary level NICU at the Hospital for Children and Adolescents, University of Helsinki, Finland, were eligible for the study. Between December 2000 and April 2002, 30 infants were enrolled during the first week of life. The follow-up period of each infant was 21 d. A total of seven infants had the blood culture test positive for microbial growth. One of them was excluded from the analysis since her blood culture, positive for *Staphylococcus epidermidis*, was considered a contamination due to the following reasons. Firstly, the reason for obtaining the blood culture was an increase in the infant's oxygen requirement, which proved to be transient lasting for a few hours only and did not result in starting of antimicrobial therapy. Secondly, the daily CRP-values remained negative (<5 mg/L). Thirdly, microbial growth was slow (detectable at 48 h) and was confined to one of the two culture bottles. For these reasons, the infant was excluded from the analysis. Seventeen healthy adult volunteers were recruited for the validation of the sampling method.

Clinical data. According to the clinical practice in our NICU, a blood sample for culture was taken immediately after birth, and a combination therapy with ampicillin (200 mg/kg/d) and netilmycin (5 mg/kg/d) was started. If the clinical condition proved to be stable and the CRP-levels remained low, the antimicrobials were withdrawn within the first week of life. The clinical infection screening in these patients included daily blood CRP and white blood cell count (WBC) measurements, and these parameters were collected from the patient records.

Patient grouping. According to clinical findings, the study patients were retrospectively assigned in three groups: infection group, possible infection group and infants without infection.

The Infection group consisted of infants ($n = 7$) who had clinical signs of infection (hypotension, increased oxygen requirement, lethargy, or abdominal distension), and who had blood culture positive for microbial growth ($n = 6$), surgery for NEC ($n = 3$), or both, during the study period (Table 1). The day of sampling for blood culture or of surgery for NEC is referred to as Day 0.

The Possible infection group consisted of infants meeting the following criteria: two or more clinical signs of infection, as listed above, and the blood culture test negative for microbial growth ($n = 4$) or no blood sample for culture taken ($n = 2$). The day of sampling for blood culture or emergence of clinical signs of infection is referred to as Day 0.

To create a group of age-matched controls, we first determined the postnatal age (days) of each infant with infection ($n = 7$) on Day 0. Second, among the infants without infection ($n = 16$) we found a control infant with equal postnatal age and no infection for each infant with infection. These seven infants without infection are referred to as the control group. The matching was done because postnatal age may affect the phagocyte CD11b expression level (18). The characteristics of the infants in infection group, possible infection group and control group are presented in Table 2.

Blood samples. A 25-μL blood sample was obtained from neonates on a daily basis from an arterial catheter, or from blood obtained with a heel lancet designed for newborns (Tenderfoot, ITC, Edison, NJ, USA). The blood was collected into pyrogen-free tubes (Falcon Polystyrene Round-Bottom Tube, Becton Dickinson, Franklin Lakes, NJ, USA) containing 5 μL of acid-citrate-dextrose (ACD) in 25 μL of PBS as anticoagulant. To avoid phagocyte activation *in vitro*, the samples were cooled immediately after sampling on ice and subsequently kept at 4°C. One of the authors (RT) or a trained research nurse was present at the time of sampling to ensure the appropriate handling of the samples. To validate the heel lancet sampling method, blood samples (0.1 mL) from 17 healthy adult volunteers were obtained simultaneously by venipuncture, the routine sampling system in studies of CD11b/CD18, and by fingertip pricking with the newborns' heel lancet. After pricking, three consecutive drops of blood were developed and a 25-μL sample was collected from each drop; the three consecutive samples and the one obtained by venipuncture were treated and analyzed further in parallel.

Table 1. *Characteristics of and phagocyte CD11b expression in ELBW infants with infection for 3 d before d 0, denoted as day of sampling for blood culture or surgery for NEC*

Patient no.	Age at d 0 (d)	Microbe in blood culture	Clinical data	CD11b expression from d −3 to d 0*							
				Neutrophils				Monocytes			
				−3	−2	−1	0	−3	−2	−1	0
1†	11	*Proteus vulgaris*	NEC surgery at d 0	173	184	255	248	235	284	441	461
2	4	*Staphylococcus epidermidis*	NEC surgery at d 0	179	137	368	372	145	103	414	485
3†	13	*Candida parapsilosis*		132	136	90	141	139	191	105	183
4	8	*Serratia marcescens*	Death at d 0	133	118	191	466	255	227	340	701
5	12	*C. parapsilosis*		69	155	88	157	95	179	124	310
6	17	*C. parapsilosis*		74	93	157	161	51	90	244	276
7	6	(No blood culture)	NEC surgery at d 0	119	120	101	219	88	104	103	229

* CD11b expression given in relative fluorescence units.

† Treated with corticosteroids between d −3 and d 0.

CD11b expression. CD11b density on neutrophils and monocytes was determined by whole-blood flow cytometry, as described (19,20). In brief, 25-μL aliquots of blood were labeled within 24 h from sampling with fluorescent anti-CD11b (phycoerythrin, PE) and anti-CD14 (FITC, FITC) antibodies (BD Biosciences, San Jose, California, USA) and incubated in dark at 4°C. Red blood cells were lysed with FACS lysing solution (BENEX Limited, BD Biosciences, Shannon, Country Clare, Ireland) and the cells were collected by centrifugation at 4°C. The samples were resuspended in 1% formalin and kept at 4°C for up to 24 h until analysis. The acquisition and analysis of the data were done using a FACSort flow cytometer and CellQuest Pro analysis software (both BD Biosciences, San Jose, California, USA). Neutrophils were identified by their light scatter pattern and monocytes by their light scatter pattern and CD14 positivity. CD11b expression is reported in relative fluorescence units (RFU). To study the effect of 24 h storage of the blood sample, we conducted a prelimary experiment, in which neutrophil and monocyte CD11b expression levels were determined in a neonate without infection immediately after sampling and 24 h later from the rest of the sample retained in cold. Coefficients of repeatability in samples from 6 infants were for neutrophils 27 RFU, and monocytes 19 RFU, indicating that there is a 95% expectation that neutrophil values will differ by less than 27 RFU and monocyte values by less than 19 RFU between samples stored in cold up to 24 h.

Statistical analysis. The results were expressed as mean or median and SD (SD), or interquartile range (IQR) and 95% confidence intervals (95% CI). Comparison of clinical data of infection group, possible infection group and controls, was done using analysis of variance (ANOVA), Kruskall-Wallis test with exact significance and Fisher-Freeman-Halton test. CD11b expression at Day 0 in infection group and control neonates was compared by the Mann-Whitney U-test with exact significance. In addition, the changes during the 3 consecutive days preceding Day 0, *i.e.*, Days -3, -2, and -1, were evaluated by the Page test for ordered alternatives with exact significance. Hodges-Lehmann estimate with 95% CI was used to evaluate the change from Day -3 to Day 0. Receiver-operating characteristics (ROC) curves were used for determination of the threshold value for the infection group compared with the infants without infection, and the respective areas under the curve (AUC) with 95% CI were calculated with bias-corrected accelerated bootstrap (5000 replications). In ROC calculations, CD11b expression value at day 0 was used for patients with infection ($n = 7$) and the maximum CD11b expression of the follow-up samples for patients without infection ($n = 16$), because median values would underestimate the control CD11b expression levels. The infants in the possible infection group ($n = 6$) were not used in the analysis, since they could not be allocated with certainty to either infection group or the group of infants without infection.

RESULTS

Validation of the sampling technique. In healthy adult volunteers, a significant agreement in CD11b density existed between the sample obtained by venipuncture [median 150 RFU (range: 66–225) in neutrophils and 151 RFU (74–183) in monocytes] and the 3 other samples obtained by fingertip pricking [median 152 RFU (75–223) in neutrophils, 142 RFU (85–184) in monocytes]. The intraclass correlation coefficients with 95% confidence intervals were for neutrophils 0.91 (95% CI: 0.84 to 0.97) and monocytes 0.82 (95% CI: 0.67 to 0.92).

Patients. The mean (SD) birth weight of the study patients was 810g (116) and the mean gestational age 26.4 wk (1.8). Respiratory distress syndrome (RDS) occurred in 25/29 infants, all of whom received surfactant. The CRP levels and WBC on Day 0 in infection group, possible infection group and control group are presented in Table 1.

In the infection group, one patient had blood culture positive for *Staphylococcus epidermidis*, two for Gram-negative bacteria, and three for *Candida parapsilosis* (Table 1). Three infants had surgery for NEC, and from two of them, blood samples were obtained for culture on the day of operation before surgery.

Two infants (numbers 1 and 3) received treatment with corticosteroids during the study period. Of the study infants, two died, one due to infection (number 4) and another due to respiratory insufficiency without infection at the age of 7 d.

CD11b expression. In the infection group, there was a monotonic increase in CD11b expression from Day -3 to Day 0 in both neutrophils (p for trend; $p = 0.006$) and monocytes ($p < 0.001$) (Fig. 1). The highest CD11b density occurred in the patient 4 with fatal disease (Table 1). The median change from Day -3 to Day 0 was for neutrophils 97 RFU (95% CI: 48 to 216) and for monocytes 225 RFU (95% CI: 129 to 340). At Day 0, median CD11b density was higher in infection group than in controls on both neutrophils [219 RFU (range: 141–466) *versus*. 75 RFU (45–146); $p = 0.001$] and monocytes [310 RFU (183–701) *versus*. 69 RFU (43–125); $p < 0.001$]. In the infants without infection, the median of the number of the follow-up samples of the 16 infants without infection was 8 (range 4–17). The median of the maximum values of the follow-up samples was 137 RFU (86–205) for neutrophils and 106 RFU (72–257) for monocytes.

ROC analysis served for the determination of the best threshold for patients with infection ($n = 7$) *versus* infants without infection ($n = 16$). In neutrophils, at the cut-off point

Table 2. *Clinical data of ELBW infants with infection, with possible infection and controls*

	Infection ($n = 7$)	Possible infection ($n = 6$)	Controls ($n = 7$)	p Value between groups
Birth weight (g)*	757 (132)	732 (104)	853 (102)	0.15
Gestational age (wk)*	24.9 (1.0)	26.6 (2.2)	26.8 (1.6)	0.097
Male sex	4	1	4	0.33
PROM	3	1	0	0.16
Chorionamnionitis	4	0	1	0.075
Pre-eclampsia	1	4	1	0.15
Cesarean section	1	5	4	0.058
Apgar score at 1 min†	3 (1–7)	7 (2–9)	5 (2–6)	0.026
Cord blood pH†	7.34 (0.04)	7.33 (0.08)	7.26 (0.14)	0.32
RDS	6	6	6	1.00
CRP (mg/L) at d 0†	19 (<5–59)	7 (<5–9)	<5 (<5–12)	0.020
WBC (10^9/L) at d 0†	15.4 (4.5–31.0)	19.3 (11.2–38.5)	14.3 (4.3–39.9)	0.52

* Data given as mean (SD).

† Data given as median (range).

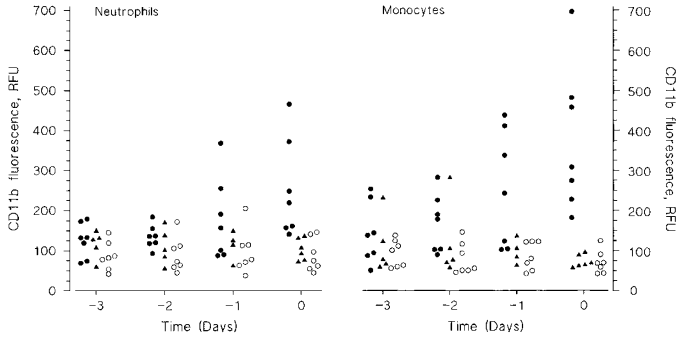

Figure 1. CD11b expression on neutrophils and monocytes in extremely low birth-weight infants with infection (*filled circle*), possible infection (*filled triangle*) and without infection (*open circle*). Time: Days preceding Day 0, which denotes sampling for blood culture or surgery for necrotizing enterocolitis; RFU, relative fluorescence units

of 139 RFU, sensitivity was 1.00 (95% CI: 0.59 to 1.00), specificity 0.56 (95% CI: 0.30 to 0.80), positive predictive value (PPV) 0.50 (95% CI: 0.22–0.77), negative predictive value (NPV) 1.00 (95% CI: 0.66–1.00) and AUC 0.83 (95% CI: 0.57 to 0.96). In monocytes, at the cut-off point of 225 RFU sensitivity was 0.86 (95% CI: 0.42 to 1.00), specificity 0.94 (95% CI: 0.70 to 1.00), PPV 0.86 (95% CI: 0.42–1.00), NPV 0.94 (95% CI: 0.70–1.00) and AUC 0.95 (95% CI: 0.72 to 1.00). In the possible infection group, CD11b expression was similar to that of controls (Fig. 1).

A total of 10 blood culture tests and 347 CD11b tests were performed. During the follow-up the CD11b expression level rose from normal level over the threshold-value on neutrophils in 20 tests in infection group, 16 tests in possible infection group, and 9 tests among healthy infants, and on monocytes in 16 tests in infection group, 5 tests in possible infection group, and once among healthy infants.

DISCUSSION

The study shows that determination of CD11b expression may be useful in early identification of ELBW infants with late-onset sepsis. At the time of sampling for blood culture, both neutrophil and monocyte CD11b expression levels were significantly higher in ELBW neonates with infection than in control neonates. Of note, all neonates in the infection group showed higher monocyte CD11b expression than did control neonates.

Another novel finding was that in the infection group, at least in some infants the neutrophil and monocyte CD11b expressions were elevated up to 3 d before the onset of symptoms, which made the clinician to order the blood culture test. However, there was individual variation in the CD11b profiles. In patient 1, CD11b expression increased consistently from Day –3 to Day 0 (Table 1), whereas in some patients it increased sharply on Day –1 (patient 2) or Day 0 (patients 4, 5, 6 and 7). The reason for these differences is not known, but it may involve differences in the course of systemic inflammation associated with underlying disease. To our knowledge, there are no longitudinal studies of the emergence and course of systemic inflammation in late-onset sepsis and NEC in ELBW infants during the time period preceding the appearance clinical symptoms. In concert, the findings in the present study suggest that prospective CD11b measurements may enable, at least in some infants, early diagnosis of infection However, several aspects of the use of the CD11b test as a diagnostic tool in ELBW infants remain to be explored in detail, as discussed below.

When interpreting the data it is important to recognize that high CD11b expression is not specific for infection-triggered inflammation and may, at least in adults, be induced by a variety of noninfectious conditions, such as trauma and autoimmune diseases (15–17,21–25). In the present study, specificity and sensitivity of the CD11b test for sepsis were reasonably high in monocytes, but the specificity was quite poor in neutrophils. The poor specificity inevitably increases the need for blood cultures, which can cause additional stress to the infants and increase costs. Indeed, if an abnormal result in the CD11b test in the present study would have been a reason to order the blood culture test, neutrophil and monocyte CD11b expression levels would have caused ordering of 35 and 12 extra blood culture tests, respectively.

The method may be susceptible to false positive results due to improper sample handling (19,20), but false negative results are unlikely to occur, providing that saturating amounts of antibodies are used. However, the number of patients in the present study was so low that the results need to be interpreted cautiously. In addition, fluorescence intensity was quantified using relative fluorescence units, which makes it difficult to compare the data obtained in different laboratories. Finally, in the present study, only one patient had sepsis due to *Staphylococcus* epidermidis, the major cause of the blood stream infection in ELBW infants (1,2), and as many as three patients had sepsis due to *Candida parapsilosis*. Indeed, during the study period, an outbreak of *Candida parapsilosis* hospital infections was detected. Since the results of the present study were obtained in an unusual epidemiologic setting, CD11b test needs to be evaluated further under nonepidemic conditions. Furthermore, the CD11b expressions seemed to increase less in *Candida parapsilosis* sepsis than in bacterial sepsis (Table 1). If confirmed by other studies, this finding may be of interest in terms of the mechanisms of phagocyte activation.

Increased CD11b expression may be generated as an *ex vivo* artifact if the precautionary measures in sample handling have failed (19,20). However, in the present study, control infants had consistently low level of CD11b expression, suggesting that, (i) the method itself was stable and the data reproducible, and, (ii) invasive treatment strategies such as intravascular catheters, parenteral nutrition, and mechanical ventilation do not promote CD11b expression.

We recently found that CD11b expression is transiently enhanced in preterm infants with RDS and returns to normal within the first week of life (18). Because this may interfere with the interpretation of the results, in the present study we used controls age-matched by day. The present data and the results of the previous study (18), suggest that when measured prospectively on daily basis during the first week of life, CD11b density should decline, and a consequent rise should raise suspect of emerging infection. Differentiating the CD11b

up-regulation due to RDS and due to late-onset infection is challenging during the first week of life and needs to be explored in detail.

Corticosteroids also interfere with increases in CD11b/CD18 expression *in vitro* (26) and *in vivo* both in adults (24,27) and in neonates (28). In accordance with this, the responses in CD11b expression during infection were lower in neonates receiving corticosteroids compared with the 5 other infants in the infection group (Table 1). Evidently, the patterns of CD11b expression during infection in ELBW infants treated with corticosteroids need to be further evaluated.

In a recent study (29), neutrophil CD11b expression failed to differentiate preterm infants with late-onset infection from noninfected infants. The failure may not result from immaturity of the neutrophils, since CD11b expression enhanced by fmlp, a neutrophil agonist, is comparable in preterm and term infants (18). Rather, the failure may involve the methods used. Firstly, the blood samples were exposed to temperature changes *in vitro* (29), which is known to promote phagocyte CD11b expression (20,30–33). Secondly, in the presence of EDTA as an anticoagulant, the CD11b-antibody used by the authors (29) does not bind to its epitope optimally (20).

To date, clinicians have no reliable means to identify patients with sepsis at the time of sampling for blood culture. Although CRP has been used in screening for infection in neonates, low serum levels do not exclude the possibility of emerging sepsis (5,6). Unlike CRP, CD11b up-regulation does not need *de novo* protein synthesis (34) and occurs promptly *ex vivo* (30,35) and *in vivo* (17). Other benefits of the CD11b test are small blood sample volume per test, 25 μL, and rapid performance, and the fact that the results are independent of the sampling method, *i.e.*, capillary, venous or arterial sampling, as shown in the present data. These properties make the CD11b test well suited for daily screening test in ELBW infants.

The clinical practice of treating infants with suspected infection with broad-spectrum antimicrobials increases the risk of invasive fungal infections, and promotes development of resistant bacterial strains, such as vancomycin-resistant *Staphylococcus aureus* (36). If the present findings are conformed in larger studies, the CD11b test may provide a means to reliably reduce the use of antimicrobial drugs in preterm infants.

Our results indicate that in ELBW infants with late-onset sepsis CD11b density may increase before the arousal of the clinical suspicion of sepsis. The possibility that earlier diagnosis of sepsis might also improve infant outcome is attractive. Several other markers have been studied in these patients and some seem to be promising (29,37–41). The results in the present study suggest that daily CD11b expression measurements might prove useful in the earlier diagnosis of late-onset infection in ELBW infants, but larger multi-center trials with sufficient number of patients are needed to explore potential utility of such markers.

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