LAH 433

Neutrophil CD11b expression as a diagnostic marker for early-onset neonatal infection

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Objectives: To determine whether neutrophil surface expression of CD11b predicts early-onset infection or suspected infection in at-risk infants.

Study design: CD11b expression on peripheral blood neutrophils was determined by flow cytometry of whole blood samples. Blood (0.1 ml) was obtained from a convenience sample of at-risk infants admitted to the neonatal intensive care unit, stained with antibodies detecting CD11b and CD15, chilled, and analyzed within 8 hours. Blood for culture, blood counts, and C-reactive protein (CRP) determination was obtained simultaneously. Subjects were grouped on the basis of culture results and clinical signs, and investigators were blinded to CD11b level.

Results: Of 106 subjects, seven had positive bacterial or viral cultures ("confirmed infection"), 17 had clinical signs of infection but negative cultures ("suspected infection"), and 82 had negative cultures and no clinical signs ("no infection"). Neutrophil CD11b was elevated in all infants with confirmed infection, 94% with suspected infection, and none with no infection. The negative and positive predictive values, sensitivity, and specificity were 100%, 99%, 96%, and 100%, respectively, for diagnosis of neonatal infection at initial evaluation. CD11b levels correlated with peak CRP ($r^2 = 0.76$, $p < 0.0001$); however, CD11b was elevated at the time of admission in all five infants with proven bacterial infection, whereas CRP was normal until the second day in the neonatal intensive care unit in three of these five. Both infants with positive viral cultures had elevated CD11b, but the CRP levels remained within normal limits. The negative predictive value of neutrophil CD11b for identifying suspected or confirmed infection was 99%.

Conclusion: This assay for neutrophil CD11b is a promising test for exclusion of early-onset neonatal infection. If validated prospectively, this assay may reduce hospital and antibiotic use in the population of neonates at risk for early-onset infection. (J Pediatr 1998;132:445-51.)

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Every year, 300,000 newborns are treated for infection, at a cost of over $800 million.[1,2] Treatment is often initiated empirically because of nonspecific clinical signs or maternal risk factors, resulting in treatment of 17 infants for each infant who has a confirmed infection.[1-6] The prevalence of severe bacterial infection in the newborn infant ranges from 1 to 10 per 1000 live births.[1,2] Although the prevalence is low, the mortality rate is high (20% to 75%) and has not decreased despite the development of potent antibiotics over the last decade.[4-6] Multiple immunologic impairments in neonates such as low levels of IgA, IgM, and some IgG subclasses, a smaller neutrophil reserve pool than adults (10% of adult levels at birth), and functional deficiencies in circulating neutrophils[7-13] put them at risk for serious infection on exposure to pathogens. Thus clinical practice defines a series of risk factors, which dictates clinical decisions aimed at prevention or anticipatory treatment of neonatal infection.[14-16]

CRP	C-reative protein
FACS	Fluorescence-activated cell sorter
FU	Fluorescence units
LPS	Lipopolysaccharide
NICU	Neonatal intensive care unit

Serum C-reactive protein[17,18] has been used to exclude the diagnosis of infection. Three negative CRP measurements on consecutive days, in the absence of clinical signs of infection, provide evidence that an infant is not infected. However, CRP levels in serum may be increased nonspecifically, as seen in cases of trauma and other forms of physiologic stress.[19,20] Also, the time delay between bacterial exposure and elevated serum CRP

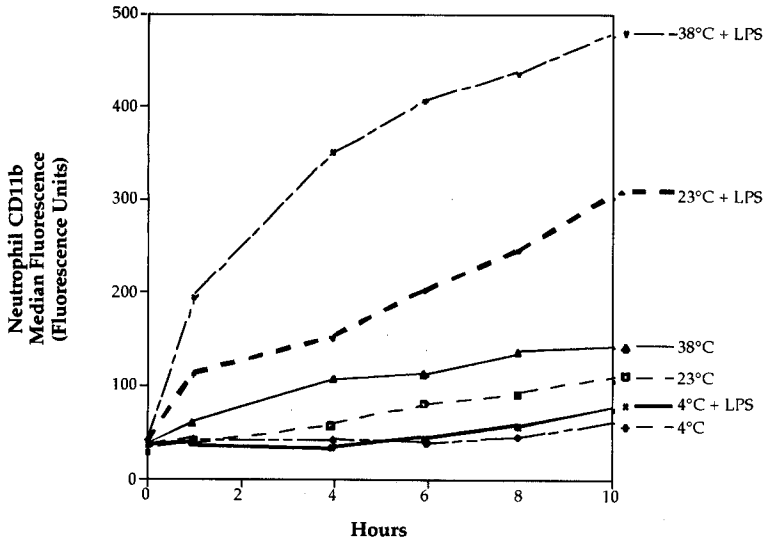

Fig. 1. Effect of time and temperature on neutrophil surface expression of CD11b, with and without LPS stimulation. Neutrophil CD11b does not increase appreciably on the neutrophil surface when maintained at 4° C for up to 8 hours. However, a slight increase is noted in LPS-stimulated and unstimulated cells after 8 hours. Neutrophil surface CD11b does increase on cells stored at room temperature for more than 2 hours and on cells stored at 38° C almost immediately. Cells stimulated with LPS at room temperature and at 38° C showed the largest magnitude increase in neutrophil surface expression of CD11b.

Table I. Demographics of sample population by outcome group

	No infection (mean ± SEM)	Suspected infection (mean ± SEM)	Confirmed infection (mean ± SEM)
Gestational age (wk)	36.3 ± 0.65	37.4 ± 1.34	36.4 ± 0.86
Weight (gm)	2692.0 ± 119	3010 ± 129	2741 ± 471
Gender (%)			
Male	54	49	57
Female	46	51	43
WBC (%)	16.6 ± 1.12	16.04 ± 1.57	18.2 ± 2.83
Segmented neutrophils (%)	39.2 ± 2.21	41.3 ± 4.11	43.1 ± 6.8
Band neutrophils (%)	8.3 ± 1.09	9.09 ± 2.68	20.7 ± 8.8
Lymphocytes (%)	40.2 ± 2.57	33.3 ± 3.70	24.8 ± 7.65
Apgar at 1 min	7 ± 1	7 ± 1	8 ± 1
Apgar at 5 min	8 ± 1	7 ± 1	7 ± 1
Grunting/flaring at admission (%)	35%	43%	38%
Maternal age	26.4 ± 1.31	27.6 ± 1.56	26.4 ± 1.53
Prenatal care beginning at 2 mo (%)	69%	72%	67%

WBC, White blood cells.

levels may range from hours to days. Because of the delay in elevation and lack of specificity, a CRP determination can help to exclude infection retrospectively, supporting discontinuation of antimicrobial therapy, but does not identify infants for whom antibiotic treatment may be safely withheld.[19,20]

CD11b (Mac-1, CR3), a member of the β-integrin family of adhesion proteins,[21] is expressed in very low levels on the surface of unstimulated neutrophils but is primarily stored in secretory granules within the cell.[22] CD11b increases on the neutrophil surface within 5 minutes of exposure to bacterial products such as endotoxin (lipopolysaccharide) in vitro, and expression peaks within 30 minutes.[23,24] CD11b is increased on neutrophils in vivo in settings of infection and may be an early postsurgical predictor of sepsis in adults.[25] Neutrophils of neonates generally contain less intracellular CD11b than do neutrophils of adults.[26,27] However, in vitro studies demonstrate that neonatal neutrophils stimulated with LPS increase surface expression of CD11b proportionately to that of adults, to an upregulated level in infected neonates, which is detectable by flow cytometry. In this study neutrophil CD11b was assessed as a marker for neonatal infection.

METHODS

Subjects

Eligible subjects included neonates born at greater than 28 weeks' gestation who were admitted, on the first day of life, to the neonatal intensive care unit at Lucile Salter Packard Children's Hospital between August 1994 and January 1995. Subjects were enrolled if the reason for admission to the NICU was perceived risk for infection, either because of maternal intrapartum risk factors or infant symptoms. The decision to admit an infant to the NICU and determination of risk for infection were made by the attending pediatrician, not affiliated with this study, in accordance with the NICU guidelines. To avoid misclassification of infants with symptoms of infection in the suspected infection group, those with noninfectious diagnoses that accounted for symptoms were not included. This study was approved by the Stanford University Human Subjects Committee. Because this study did not affect infant treatment or outcome and because samples were obtained only as waste from clinically indicated blood draws, the requirement for informed consent was waived.

Samples for CD11b measurement

were obtained on the day of admission, concurrently with blood drawn for routine bacterial culture, blood counts, and CRP measurement by the hospital's diagnostic facility. Gestational age and weight were recorded at this time. Cultures for isolation of viruses were performed as clinically indicated. Results of serum CRP determination and blood, urine, and cerebrospinal fluid cultures were collected at least 7 days later. Clinical signs of infection were recorded in the infant's chart by NICU nursing staff members not affiliated with the study. Risk factors, maternal pretreatment with antibiotics, discharge diagnoses, and clinical signs of infection (as noted in the infant's chart) were recorded by a party blinded to CRP and CD11b results.

Subjects were assigned to three groups: *confirmed infection* (positive bacterial or viral culture), *suspected infection* (negative cultures but elevated CRP or white blood cell count or strong and persistent clinical signs of infection), and *no infection* (negative cultures, normal CRP over 3 days, no clinical signs of infection). Assignment to all outcome groups was carried out by a party blinded to CD11b results. Clinical signs of infection leading to placement in the suspected infection group included one or more of the following conditions recorded over more than 8 hours or two or more noted for more than 4 hours: fever, hypothermia, exanthem, or respiratory distress. Infants were included in this group only when a noninfectious cause could not be identified to account for clinical signs (Table I).

Table II. Data summary

Outcome group	Total in each group	No. cultured	No. positive by culture	Neutrophil CD11b >60	Peak serum CRP >1
Infection confirmed*	7			7	5
Bacterial	5	106	5	5	5
Viral	2	11	2	2	0
Infection suspected	17			16	15
No infection	82			0	2
Total	106			23	22

Assay for CD11b

This study presents a novel technique: flow cytometric determination of neutrophil CD11b in whole blood. Experiments here were performed at 4° C, because CD11b can upregulate spontaneously on neutrophils in peripheral blood samples at room temperature.[28-30] CD11b levels do not change if samples are kept at 4° C for up to 8 hours (Fig. 1) but do increase slightly thereafter. Whole blood (0.1 ml) was obtained from each subject and was diluted with 2.5 ml of cold staining medium. Staining medium was composed of deficient RPMI-1640 medium (Applied Scientific, Irvine, Calif.), 1% fetal calf serum (Gemini Bioproducts, Calabasas, Calif.) with 1 mol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, pH 7.2 (Sigma, St. Louis, Mo.). Sodium azide stock solution (10%) was added to a concentration of 0.02%. If stored, samples were maintained at 4° C for up to 8 hours. Samples were washed once with staining medium, resuspended in 0.5 ml of staining medium, and stained for 15 minutes with fluorescein isothiocyanate—conjugated CD15 (to identify neutrophils) and phycoerythrin-conjugated CD11b mouse anti-human monoclonal antibodies (Becton-Dickinson, Milpitas, Calif.). Samples were then washed three times and resuspended in 1% paraformaldehyde/staining medium. Before staining, the fluorescein isothiocyanate—conjugated anti-CD15 was diluted with unconjugated anti-CD15 antibody to bring the neutrophil CD15 staining into mid range on the flow cytometry instrument (FACScan, Becton Dickinson). The instrument was adjusted so that the fluorescence of standard beads (3.2 μm Sphero Rainbow Fluorescent Particles; Sphertech, Inc., Libertyville, Ill.) in each fluorescence channel was exactly the same for each run.

Samples were analyzed on the FACScan with a threshold set such that only cells with the correct range of CD15 fluorescence for neutrophils were accepted as events. This threshold excludes erythrocytes and all other leukocytes. The median fluorescence of neutrophil CD11b was determined for each sample on the basis of the distribution of CD11b fluorescence per 30,000 cells counted and ranged from 1 to 475 fluorescence units. Cultured U-937 cells (ATCC, Rockville, Md.), which express low levels of CD11b and CD15,[31] were stained and analyzed concurrently with patient samples to verify the staining procedure. Over the period of this study, U-937 cells were analyzed 100 times, with a mean ± SD of 2.00 ± 0.11 for CD11b fluorescence.

Serum CRP

The CRP assay performed by the hospital's diagnostic facility has a lower limit of sensitivity of 0.4 mg/dl. CRP levels below this threshold are reported categorically (as "<0.4 mg/dl") rather than quantitatively. Levels greater than 1 were defined as abnormal in accordance with clinical protocol at Lucile Packard Children's Hospital at Stanford.

Statistical Analysis

Statistical analysis was performed by using JMP software (SAS Institute, Cary, N.C.), version 3.1. Differences in outcome groups were assessed by using the G-test of independence for 2×3 and 2×2 contingency tables, as appropriate for these data. Correlation between CD11b and peak CRP levels was assessed for combined categorical and continuous data by using the Spearman

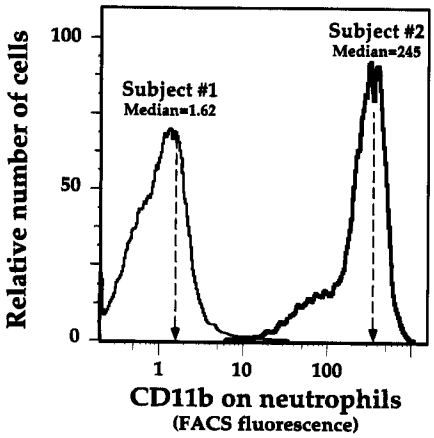

Fig. 2. Example of FACS fluorescence plots. Example of fluorescence plots for neutrophil CD11b expression in an uninfected infant with low CD11b expression (median fluorescence = 1.62) and in an infant infected with Group B streptococci, showing elevated CD11b (median fluorescence = 245.00). Each plot shows CD11b expression for a single patient sample (30,000 cells counted). Median CD11b fluorescence is calculated from this distribution. Note that the fluorescence scale is logarithmic. The sample CD11b fluorescence is distributed tightly, indicating little interference from nonspecific binding.

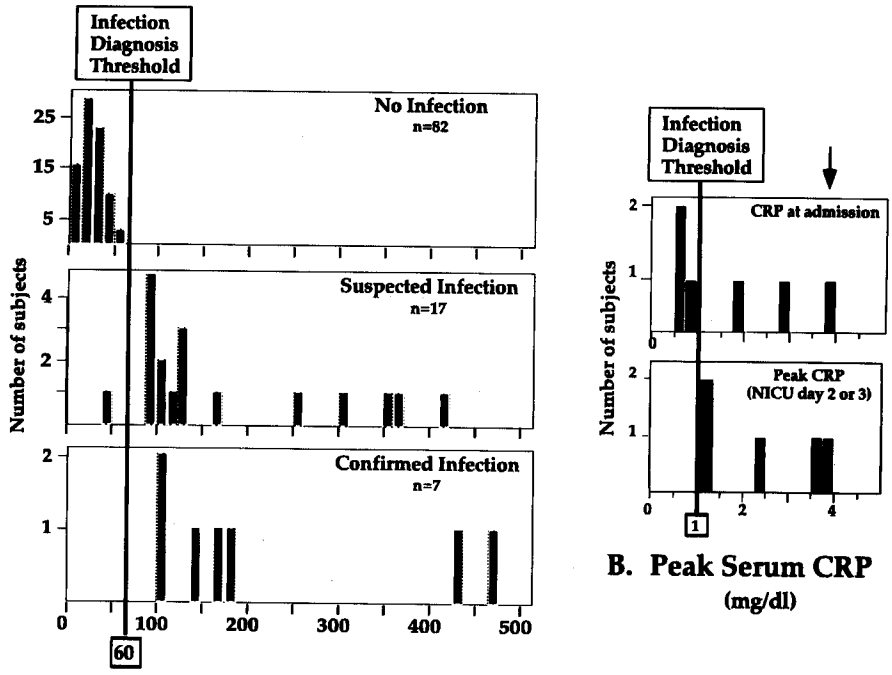

Fig. 3. Distribution of neutrophil CD11b by clinical outcome group. **A,** Distribution of median neutrophil CD11b for each clinical outcome group. All infants in the confirmed infection group had CD11b levels greater than 100 FU. All but one infant in the suspected infection group had CD11b levels greater than 60 FU. All infants in the no infection group had CD11b levels less than 60 FU. The "Infection Threshold" is defined as CD11b greater than 60 FU on the basis of distribution of values. **B,** Distribution of serum CRP for patients in whom bacterial infection was confirmed by positive culture. CRP elevation was delayed until the second day after admission in three of five infected neonates. CD11b was elevated on the day of admission in all infected neonates.

Rho measure of association; correlation of continuous data was assessed by using a standard linear regression.

RESULTS

Fig. 2 shows sample FACS plots for two neonates, one with low and one with high neutrophil CD11b. Each contour represents 5% of the sample of 30,000 cells. The distributions are tight, indicating little nonspecific artifact. Although a hard gate was set on the FACScan so that only neutrophils were counted as events, further analysis gates were not necessary, because the samples clearly represent only one population of cells.

Neutrophil CD11b was high in all 106 infants with a positive bacterial culture (Table II) (3 cases of group B streptococci, 1 case of group D streptococci, and 1 case of *Enterococcus faecalis*) and was also elevated in two of 11 subjects with positive viral cultures tested for virus (herpes simplex virus and Coxsackie B virus). CRP levels were elevated by day 3 in those infants with positive bacterial cultures but was not elevated in those infants with positive viral cultures (Table III). In 82 of 106 infants (77%), infection was excluded by negative cultures and by the absence of clinical signs of infection. None of these infants had high CD11b.

Fig. 3, *A*, shows the distribution of neutrophil CD11b levels for each outcome group. CD11b was greater than 100 median FU in all subjects in the confirmed infection group and less than 60 FU in the no infection group. In the suspected infection group, neutrophil CD11b was greater than 60 FU in all but one subject. Infection was suspected in this subject because of persistent hypothermia. CRP levels were low in this infant as well.

Fig. 3, *B*, shows the distribution of CRP on day of admission and peak CRP in neonates with positive bacterial cultures; all blood samples used for cultures were drawn on hospital day 1. All patients in this group had high neutrophil CD11b. However, the majority of these infants did not have an elevated serum CRP level until at least hospital day 2. CD11b elevation preceded CRP elevation in 60% of neonates with bacteremia. In the confirmed infection group, two subjects had positive viral cultures. These subjects had low CRP levels but elevated CD11b.

Fig. 4 demonstrates that elevated CD11b on neutrophils is correlated with peak CRP ($r^2 = 0.76$, $p < 0.0001$). The CRP measurement included in this figure was the highest value obtained over a 3-day period, whereas the CD11b measurement was obtained on admission. Subjects with CRP less than 0.4 were omitted from this regression, because the CRP assay has a limit to sensitivity of 0.4. However, the entire data set of 106 subjects was analyzed by using the Spearman-Rho measure of association, which allows inclusion of the categorical data ("<0.4"). This test yielded $r^2 = 0.54$, $p < 0.0001$.

Table IV shows the sensitivity, specificity, and positive and negative predictive

Table III.

Cultured organism	Median neutrophil CD11b	Serum CRP
Group B streptococci	426	4
	468	3.5
	146	2.4
Group D streptococci	100	1
Enterococcus faecalis	110	1.6
Coxsackie B virus	184	<0.4
Herpes simplex virus	174	0.5

values of the CD11b assay in detecting infection for the following groups of infants: (1) suspected or confirmed infection (n = 17, 96% sensitive and 100% specific); (2) bacterial or viral infection confirmed (n = 7, a subset of group 1, 100% sensitive and 83% specific); (3) bacterial infection confirmed (n = 5, a subset of groups 1 and 2, 100% sensitive and 81% specific). According to the G statistic, these values were all significant at a p value of less than 10^{-4}. The negative predictive value of the suspected or confirmed infection grouping is 99%, with a 95% confidence interval of 96.4% to 100%.

No significant relationship was found between elevation or reduction in white blood cell subpopulations measured (e.g., segmented neutrophils, immature neutrophils, lymphocytes) and positive cultures, clinical signs of infection, or elevation of CD11b or CRP. No relationships were found between Apgar scores, birth weights, or gestational ages and CD11b, CRP level, positive bacterial cultures, or clinical outcome.

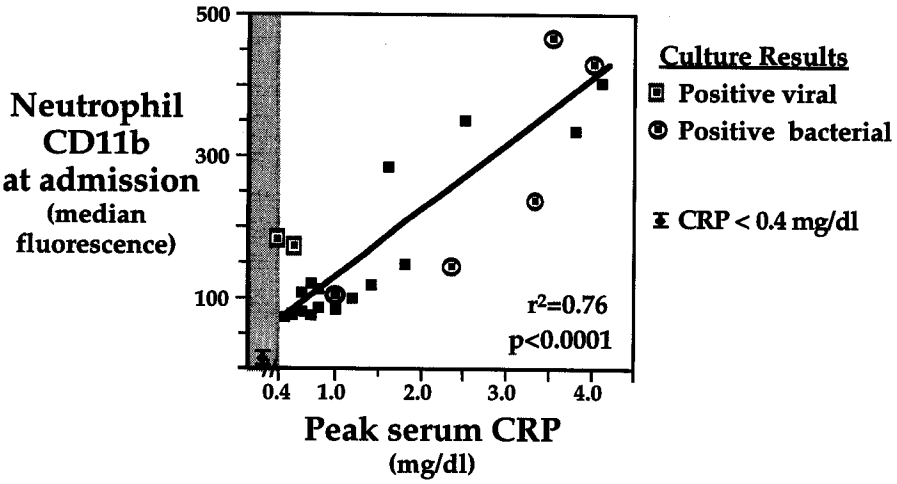

Fig. 4. Relationship between neutrophil CD11b and highest measured serum CRP in infants being evaluated for infection. This figure shows the correlation between neutrophil CD11b and serum CRP. The CD11b measurement graphed here was made at time of admission to the NICU. The CRP measurement was the highest obtained during the first 3 days of hospital stay. For n = 26 observations, r^2 = 0.76, p < 0.0001. Values mutually negative for CRP and CD11b were excluded from this linear regression because negative CRP values are reported categorically as "CRP < 0.4". These data are shown on the graph as the mean with error bars at CRP = 0.3. However, these data do not participate in the linear regression shown. The entire data set of 106 subjects was assessed by using a nonparametric Spearman-Rho test for association, with a resulting r^2 = 0.54, p < 0.0001.

Table IV. CD11b assay is sensitive and specific for identifying infection in infants

Outcome group	n	G-test	p Value	Sensitivity	Specificity	NPV	PPV
Infection suspected or confirmed	17	98.99	3×10^{-23}	96%	100%	99%	100%
Bacterial or viral infection confirmed	7	20.87	5×10^{-6}	100%	83%	100%	30%
Bacterial infection only confirmed	5	13.8	2×10^{-4}	100%	81%	100%	22%

*G-test for 2 × 3 contingency table yields G of 91, $p = 1.5 \times 10^{-20}$
NPV, Negative predictive value; *PPV*, positive predictive value.

DISCUSSION

Studies on this population of neonates at risk for early-onset infection show that CD11b is elevated on neutrophils in those with proven infection and in nearly all of those in whom clinical signs indicate infection. In contrast, CD11b is low in at-risk neonates for whom infection is later excluded. Because of the strength of association between detection of this marker and the clinical and microbiologic diagnosis of infection, confirmation of these results in a multicenter study is warranted.

CD11b is a β-integrin involved in neutrophil adhesion, diapedesis, and phagocytosis. It is the receptor for C3bi, a component of the complement cascade,[32,33] and is stored in primary and secondary intracellular granules within unstimulated neutrophils and certain other leukocytes.[22,24,34] It is expressed on a variety of cells; however, it is normally expressed at very low levels on neutrophils and becomes elevated when neutrophils encounter pathogens or their products. This rapid shift after exposure to pathogens makes neutrophil surface CD11b an excellent, early index of infection.*

Neutrophil upregulation of CD11b on stimulation with LPS has been shown to

*References 23, 24, 32, 33, and 35-40.

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