Immune Status in Very Preterm Neonates

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ABBREVIATIONS: hCA—histological chorioamnionitis, IL—interleukin, IUGR—intrauterine growth restriction, LPS—lipopolysaccharide, MHC—major histocompatibility complex, PPROM—preterm premature rupture of membranes, PTL—preterm labor, TNFα—tumor necrosis factor α

Drs Peebles and Klein conceived and designed the study; Drs Azizia and Lloyd acquired consent and recruited women, extracted the clinical data, collected the samples, and performed assays; Drs Allen and Azizia did data analysis including statistical analysis; Drs Azizia, Klein, and Peebles interpreted the data; and Drs Azizia, Lloyd, Allen, Klein, and Peebles drafted and approved the manuscript.

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WHAT’S KNOWN ON THIS SUBJECT: The very preterm neonate is more susceptible to bacterial infection; this is thought to be due to immaturity of the innate immune response.

WHAT THIS STUDY ADDS: Monocytes have an anti-inflammatory profile at birth and are hyporesponsive to inflammatory stimuli in fetuses born very prematurely. This reflects the response to the pro-inflammatory events leading to preterm birth as well as gestational immaturity.

abstract

OBJECTIVES: Preterm neonates are at increased risk of sepsis compared with those born at term. We investigated immune status at birth and early neonatal life in very preterm neonates and its association with short-term outcomes.

METHODS: Prospective observational study conducted at a university hospital recruiting 113 preterm neonates (23–32 weeks) and 78 controls. Monocyte major histocompatibility complex (MHC) class II expression, serum, and ex vivo lipopolysaccharide stimulated levels of six cytokines (tumor necrosis factor α, interleukin (IL)-1β, IL-6, IL-8, IL-10, and IL-12p70) were measured in umbilical cord blood and over the first 7 days. The presence of neonatal sepsis and histologic chorioamnionitis was recorded.

RESULTS: Prematurity (preterm labor and preterm premature rupture of membranes cohorts), neonatal sepsis, and histologic chorioamnionitis were associated with significant reduction in monocyte MHC class II expression. Neonates who had evidence of subsequent protracted sepsis had low levels of MHC class II expression at birth. Serial monocyte MHC class II expression revealed a fall by day 2, in all preterm neonates, with the degree being influenced by both prematurity and sepsis, and incomplete recovery by day 7, suggesting immunoparalysis in preterm premature rupture of membranes and preterm labor cohorts. Whole blood lipopolysaccharide stimulation assay showed significantly lower tumor necrosis factor α values in preterm neonates who subsequently developed sepsis indicating a degree of immunoparalysis.

CONCLUSIONS: Our data support the concept that fetal exposure to inflammation before preterm delivery leads to subsequent endotoxin hyporesponsiveness (immunoparalysis), which increases the risk of subsequent sepsis and associated organ dysfunction.

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Preterm birth remains 1 of the main causes of perinatal mortality and long-term morbidity despite advances in neonatal intensive care. Sepsis has a large contribution to perinatal mortality and morbidity in this setting. Susceptibility to bacterial infections is thought to be due to immaturity of the innate immune system and organs. Recently it has been shown that the immune response to a microbial stimulus is biphasic with an early pro-inflammatory phase and a later anti-inflammatory phase, thought to help regulate the potentially deleterious effects of the initial inflammatory reaction. The second phase, also termed immunoparalysis, can be profound and is characterized by decreased monocyte major histocompatibility complex (MHC) class II expression, suboptimal functional response of monocytes to endotoxin (tumor necrosis factor α [TNFα]), and elevated anti-inflammatory cytokines, including interleukin (IL)-10. We and several other groups have previously described the presence and effects of immunoparalysis in several clinical settings including sepsis, severe trauma, stroke, post–cardiac surgery, burns, pancreatitis, and preterm labor. Also we and others have shown that bacterial colonization of the choiodecidual space leads to chorioamnionitis and that chorioamnionitis is associated with preterm labor (PTL), preterm premature rupture of membranes (PPROM), and development of subsequent neonatal sepsis. On this basis, we hypothesized that exposure to the pro-inflammatory environment associated with preterm birth would lead to fetal and neonatal immune paralysis, rendering the neonate more susceptible to bacterial infection. To investigate this hypothesis, we measured monocyte MHC class II expression and circulating cytokine levels in very preterm infants from birth to 7 days of age. The influence of gestation, antenatal inflammation (histologic chorioamnionitis), and mode of delivery on monocyte function and class II expression were assessed and related to clinical and laboratory indices of neonatal sepsis.

**METHODS**

**Patient Selection**

One hundred ninety-one infants were recruited in this prospective observational study conducted at University College London Hospitals (October 2003 to September 2007) approved by the University College London/University College London Hospitals Research Ethics Committee (the study groups are defined in Table 1). There were no major changes in the clinical indications for preterm delivery over this time period. Recruitment and the assays were performed by a clinical research fellow who was independent of the clinicians caring for infants or mothers and the pathologist performing routine clinical tests. These individuals were in turn blinded to the cytokine and cord monocyte MHC class II expression data. We excluded neonates with known congenital anomalies (except feticide cohort) or with a history of maternal complications, namely, immunosuppressive disorders, gestational diabetes mellitus, or preexisting diabetes mellitus and retroviral infection. Clinical signs and laboratory evidence (white cell count, C-reactive protein, and blood cultures) of infection were routinely sought in preterm infants. Neonates were classified as having sepsis (presumed or proven) at 3 times (early onset 0–3 days, onset within 0–7 days, and late onset 7–28 days of life) by using recently defined consensus criteria. In summary, sepsis is defined as evidence of a systemic inflammatory response in the presence of at least 2 of 4 altered physiologic variables (temperature, heart rate, respiratory rate, leukocyte abnormalities) of which 1 must be either abnormal temperature or leukocyte count. Infection is either suspected (clinical syndrome associated with a high probability of infection, positive findings on examination, imaging, or laboratory test including elevated C-reactive protein) or proven (positive bacteriological culture). Neonates were classified as having sepsis at 3 times...
Measurement of Monocyte MHC Class II Expression

A previously optimized dual staining technique was used to determine monocyte MHC class II expression. Briefly, monocytes were flow sorted after staining with R-phycocerythrin conjugated antibody to CD14, fluorescein isothiocyanate conjugated—conjugated antibody to MHC class II and mouse immunoglobulin G1 antibody raised against keyhole limpet hemocyanin for MHC class II (Dako, Ely, United Kingdom).

Cytokine Measurement

Whole blood was spun at 1200 g for 10 minutes, and plasma was stored at −70°C in aliquots. Frozen aliquots were thawed immediately before analysis. TNFα, IL-1β, IL-6, IL-8, IL-10, and IL-12p70 were assayed by using a commercially available BD Cytometric Bead Array Human Inflammation Kit (BD Bioscience, Oxford, United Kingdom) per manufacturers’ recommendation (test performance available from manufacturer). Data were analyzed by using manufacturers supplied BD CBA software (BD Bioscience). The sensitivity of the assay was 3 pg/mL for TNFα, IL-6, IL-8, IL-10, and IL-12p70, and 100 pg/mL for IL-1β.

Whole Blood Lipopolysaccharide Stimulation Assay

Whole blood and controls were incubated with an equal volume of RPMI-1640 with L-glutamate and 100 ng/mL of Escherichia coli 0111:B4 lipopolysaccharide (Sigma, Poole, United Kingdom) for 24 hours at 37°C in 5% CO2. Supernatant was removed and frozen at −70°C in aliquots until later use to avoid interference with assay results from repeated freeze-thaw cycles. Cytokine concentrations were assayed by using the cytometric bead array kit as described earlier. Whole blood lipopolysaccharide (LPS) stimulated cytokine response is a surrogate marker of monocyte cytokine response.

Statistics

Data analysis was performed by using Microsoft Excel/Access and Statistical Package for the Social Sciences version 14 (Chicago, IL) and Sigmaplot program version 9 (SYSTAT, Washington, DC). Unless specified, nonparametric Mann-Whitney U test was used to identify statistical significance. \( \chi^2 \) test was used for testing the association of variables in a 2 by 2 table format.

RESULTS

Patient Characteristics

One hundred and ninety-one infants were recruited to the study; data on 10 were not included because early death meant that insufficient samples were taken (7 PTL and 3 PPROM neonates). The demographic characteristics and gestational ages of the participants are shown Table 1. Histologic chorioamnionitis (hCA) was more common in the PPROM cohort compared with the PTL (\( P < .05 \)).

Expression of MHC Class II on Cord Blood Monocytes

At term, there was no effect of mode of delivery (elective caesarean delivery and vaginal delivery) on monocyte MHC class II on cord blood. It is of note that even in these control groups, there was a relatively wide range of expression (from 99.9% to 26.8%; Fig 1).

It was difficult to assess the effect of gestation on monocyte class II expression because the circumstances of birth for most premature infants are not normal and include factors, independent of gestation that might influence class II expression. We therefore studied a control cohort of fetuses having blood taken during feticide for fetal abnormality. Monocyte MHC class II expression was found to be significantly lower in feticides compared with term infants. The percentage of monocytes expressing MHC class II was also significantly lower in both PTL and PPROM groups when compared with preterm controls (feticides). There was no difference between PPROM and PTL groups (Fig 1).

Cord Blood Monocyte MHC Class II Expression and hCA

Histologic chorioamnionitis, the presence of an inflammatory infiltrate in fetal membranes, acts as a marker of prenatal exposure to an inflammatory/infectious stimulus. Cord blood monocyte MHC class II expression was studied in preterm neonates who were born with or without evidence of hCA (see Fig 2). Median monocyte MHC class II expression was significantly lower in infants with hCA compared with those without (\( P < .05 \)). Interestingly, even the preterm group without chorioamnionitis had lower MHC class II expression than the feticide control group (\( P < .05 \)). The cohort with the lowest percent expression at birth was those preterm infants that had evidence of hCA and then went on to develop sepsis in the first 3 days of life (\( n = 13 \), data not shown). In contrast, those infants with no evidence of hCA and who did not develop early-onset sepsis (\( n = 13 \)) had high expression, with levels comparable to feticide controls (\( P < .05 \)).

Cord Blood Monocyte MHC Class II Expression and Evidence of Neonatal Sepsis

Infants with evidence of sepsis during the first week of life already had lower expression of MHC class II antigen in their cord blood monocytes than infants without sepsis (see Fig 3). Of the 58 infants delivered in the term control groups, 7 were unexpectedly admitted...
to the neonatal unit and treated with antibiotics for presumed sepsis; these infants had lower MHC class II expression than nonseptic control infants ($P < .01$). A similar pattern was seen in the preterm groups (PTL and PPROM), with septic infants showing significantly lower MHC class II expression on cord monocytes than those infants that showed no evidence of sepsis during the first week of life ($P < .01$; see Fig 3).

The link between low monocyte MHC class II expression in cord blood and neonatal sepsis was strengthened by additional data looking at the length of antibiotic use as a surrogate marker of protracted clinical sepsis. Overall, preterm neonates requiring more than 10 days of antibiotic ($n = 27$) median 34.5% (27.9–56.0) vs 70.4% (61.8–81.8, $P = .0001$); the findings were similar when PTL and PPROM groups were analyzed individually.

**Postnatal Changes in Monocyte MHC Class II Expression**

Monocyte MHC class II expression was determined serially at birth (cord blood) and on days 1 to 2 (24–48 hours) and day 7 after delivery (Fig 4). The majority of neonates had ≥2 measurements in the first week of life. Fetecides served as gestational matched controls for cord samples. Because serial samples from fetecides are not possible, serial samples from growth-retarded preterm neonates (IUGR) were used for comparison. IUGR neonates showed comparable cord expression to controls (feticides), followed by a modest fall in monocyte MHC class II expression at 24 to 48 hours; numbers at day 7 ($n = 6$) were small, but there appeared to be a trend toward subsequent recovery of expression to baseline levels. Both preterm cohorts revealed a postnatal drop in monocyte MHC class II expression at 24 to 48 hours of birth that had not returned fully to baseline levels by day 7. The effect of sepsis, at any given time point, was to lower the monocyte MHC class II expression (data not shown).

**Raised Cord Cytokines in Preterm Neonates**

Levels of cytokines were determined in cord blood in all term and preterm groups. In the preterm cohorts (PTL and PPROM, $n = 46$), the cord IL-6 levels were significantly higher (median: 41 pg/mL; interquartile range: 9.5–192.8) compared with gestation matched (1.7 pg/mL [1–4.3] and term controls 6.8 pg/mL [1–23.4], $P = .0001$). Additional analysis of the preterm cohorts showed significantly elevated cord IL-6 levels in those with hCA (77.4 pg/mL; 34.4–525.7) compared with those without hCA (9.8 pg/mL; 7.8–28.6; $P = .002$). Likewise, preterm neonates who went on to develop sepsis (proven or suspected) within the first week of life had a tendency to have high cord IL-6 at
birth (97.7 pg/mL; 14.4–242) compared with those who did not (18.3 pg/mL; 7.9–59.5; \( P = .08 \)). Interestingly, preterm neonates with no evidence of hCA still had significantly elevated cord IL-6 (9.8 pg/mL; 7.8–28.6) when compared with gestation-matched controls (1.7 pg/mL; 1.0–4.3; \( P = .001 \)). Also, preterm neonates with no evidence of sepsis in the first week of life still had significantly elevated cord IL-6 when compared with gestation matched controls (18.3 pg/mL; 7.9–59.5 vs 1.7 pg/mL; 1–4.3, \( n = 22 \) and 16, respectively, \( P < .01 \)).

The cord levels of IL-8 and IL-10 closely mirrored the pattern seen with IL-6. Low range variations not attaining statistical significance were seen in the other cytokines tested (IL-12, TNF\( \alpha \), IL1\( \beta \)).

**Whole Blood Response to Endotoxin Stimulation**

Having demonstrated decreased levels of monocyte MHC class II expression in the cord blood of fetuses born after PPROM and PTL, as well as elevated cord IL-6, we sought to investigate whether the circumstances leading to preterm birth affected the functional competency of cord blood monocytes. This was done by using an ex vivo whole blood LPS stimulation assay. Feticides had low levels of TNF\( \alpha \) (Fig 5A) and IL-6 (Fig 5B) compared with term cord blood after LPS stimulation. Preterm neonates (PPROM and PTL) also have reduced TNF\( \alpha \) output compared with term, but IL-6 production was similar.

TNF\( \alpha \) production was reduced in septic preterm neonates when compared with feticide (median: 41.3 pg/mL; 31.4–127.9 versus 265.9 pg/mL; 100.7–389.3, \( P = .045 \)). In contrast, cord IL-6 production was not impaired in infants that went on to develop sepsis in the first week. This suboptimal selective TNF\( \alpha \) cytokine response to LPS stimulation suggests immunoparalysis in preterm neonates (PPROM and PTL) with sepsis.
Neonatal Survival and Cord Blood Monocyte MHC Class II Expression

A complete data set was available for 41 preterm neonates (PTL and PPROM, <32 weeks) of whom there were 36 survivors (>28 days) and 5 neonatal deaths. In this small cohort, those neonates who died had a nonsignificant trend toward lower monocyte MHC class II expression than survivors (48.1%; 35.7–80.9 vs 63.5%; 49.3–80.8).

DISCUSSION

It has long been thought that complications observed in preterm neonates occur because of immature organ development. However, the identification of a fetal inflammatory response syndrome, characterized by the presence of pro-inflammatory cytokines in fetal blood, and the demonstration that this correlates with the incidence of major neonatal morbidity suggests that factors other than immaturity may be important in determining the outcome of very preterm infants. In this study, we show that even at birth, preterm infants (born before 32 weeks’ gestation) have a lower expression of MHC class II antigen on their monocytes than those from term infants; they also have increased circulating levels of IL-6 and impaired ex vivo production of TNFα in response to LPS. These findings indicate a degree of immune incompetence in preterm fetuses.

A number of studies suggest that the innate immune system of preterm infants is immature. Our finding that MHC class II expression is lower in blood taken from fetuses in the second trimester before termination for fetal abnormality (feticides) than in those born at term would be consistent with this conclusion. Although not a perfect control group because these fetuses had a structural abnormality, they were not delivered preterm. This enabled us to explore the relative contributions of immaturity and preterm labor on monocyte MHC class II expression. The process of preterm birth appears to have an additional impact on monocyte MHC class II expression because both PTL and PPROM groups had even lower expression than the gestation matched feticide group (Fig 1).

A clue was provided by finding lower MHC class II expression (Fig 2) in cord blood from fetuses with evidence of an inflammatory response in the placenta/membranes (hCA).

Our observation that MHC class II expression is lower in the cord blood of fetuses born after PTL or PPROM than gestation matched feticide controls or iatrogenic preterm birth (IUGR) is consistent with monocytes having been
exposed to an inflammatory stimulus. In a study in sheep fetuses, Kramer et al demonstrated that after intra-amniotic injection of endotoxin, a known cause of chorioamnionitis, the expression of MHC class II antigen on fetal blood monocytes was reduced after 1 day and was even lower after 3 days\(^ {30,31}\). We showed that MHC class II expression was lower 24 to 48 hours after preterm birth than in cord blood (Fig 4) and may reflect a developing monocyte hypo-responsiveness after exposure to infection/inflammation around the time of preterm birth.

Two pieces of evidence suggest that low monocyte MHC class II expression is of functional significance. First, we have shown that neonates born with low monocyte MHC class II expression were more likely to be diagnosed with sepsis and to require treatment with antibiotics for >10 days. It is particularly striking that this was also observed in the term group, with 7 of 10 infants born with MHC class II expression in the lowest quartile being unexpectedly admitted for observation on the neonatal unit for suspected sepsis. Second, we showed that when whole blood was stimulated with bacterial endotoxin ex vivo, the production of TNF\(\alpha\) was reduced, suggesting monocyte hypo-responsiveness. Other groups have demonstrated a similar hyporesponsiveness to LPS after exposure to sepsis\(^ {32–34}\) and suggested that in combination with low monocyte MHC class II expression, this could indicate a degree of immune paralysis. The timing of MHC class II reduction and impaired ex vivo cytokine production would be consistent with an inflammatory insult around the time of delivery. It is tempting to relate these observations to the presence of bacteria in the fetal membranes and amniotic fluid found in women who deliver preterm.\(^ {24}\) However, we do not know that noninfectious causes of premature labor (eg, placental abruption, multiple gestation) may not lower monocyte MHC class II expression.

Additional studies are required to fully understand our observations. Our findings that monocyte MHC class II expression is reduced in some infants for at least the first 48 hours of life and that these infants are more at risk for being diagnosed with sepsis suggest that measurement of monocyte MHC class II expression in cord blood could be used to identify a group of infants that might be particularly at risk for infection and may be suitable for antibiotics and/or immune modulation. A similar relationship between late-onset neonatal sepsis and monocyte HLA-DR expression has recently been described in term infants.\(^ {15}\)

### CONCLUSIONS

These data support the concept of in utero origins of neonatal sepsis at least in the setting of preterm birth. Monocyte MHC expression in cord blood may have prognostic value as a robust marker of fetal exposure to inflammation.

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