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The Role of Mannose-Binding Lectin in Susceptibility to Infection in Preterm Neonates

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ABSTRACT: Preterm neonates are susceptible to infection due to a combination of sub-optimal immunity and increased exposure to invasive organisms. Mannose-binding lectin (MBL) is a component of the innate immune system, which may be especially important in the neonatal setting. The objective of this study was to investigate the impact of MBL on susceptibility and severity of infection in preterm neonates during their first month of life. One hundred fifty eight preterm neonates were genotyped for MBL mutations by heteroduplex analyses. Consecutive serum MBL levels were measured by ELISA and clinical and laboratory data, including blood cultures, were collected for each baby. A third of the premature neonates had genetically determined MBL deficiency. In addition, MBL levels were also low in the first week of life and lower in neonates with a wild type genotype who were less than 28 wk gestation or a birth weight of less than 1000 g, thereby increasing the number of neonates with a low MBL level at birth. MBL deficiency was associated with an increased risk of sepsis ($p < 0.01$). This study indicates that MBL levels are low in neonates at birth and renders premature neonates to an increased risk of infection. **(*Pediatr Res* 63: 680–685, 2008)**

Despite advances in perinatal care, neonatal infection remains an important cause of morbidity and mortality, particularly among very low birth weight (VLBW) preterm infants (1,2). Reported rates of infection vary considerably, but may be higher than 20% in low gestational ages (3). It would appear that this figure has not changed dramatically in the last decade.

Numerous factors are responsible for the high rate of infection in this patient population. Neonates of low gestational age and birth weight are particularly at risk of infection, in part because of the intensity of supportive care required such as mechanical ventilation, parenteral feeding, and the requirement for prolonged i.v. access. In addition, there are immunologic reasons why infection occurs. The absence of passively derived maternal antibody such as that directed against Group B *Streptococcus* appears to be important (4,5). Indeed premature neonates of less than 32 wk of gestation have fetal IgG concentrations of less than 50% of maternal levels (6,7). Many elements of the acquired immune system are also either low or function sub-optimally (8,9). It is in these circumstances, that the innate immune system may be particularly important in providing protection against infection.

Mannose-binding lectin (MBL) is a circulating pattern recognition molecule of the innate immune system (10–12). MBL recognizes carbohydrate structures on the surface of a wide range of microorganisms including bacteria, viruses, yeasts, protozoa, and parasites (11,13,14). This lectin can mediate phagocytosis and can activate the complement pathway in an antibody and C1-independent manner (15–17). Initiation of this lectin pathway follows binding, *via* a carbohydrate recognition domain (CRD), to mannose, *N*-acetyl glucosamine, fucose, and glucose residues present in the orientations and densities commonly found on microorganisms (12–14).

Circulating MBL concentrations are correlated with genetic variations in the structural and promoter regions of the *MBL-2* gene (18). Three single nucleotide polymorphisms in codons 52, 54, and 57 (*D*, *B*, and *C* variants, respectively) in exon-1 of the MBL gene lead to reduced MBL concentrations (18–20). Polymorphisms in the promoter region at −221 (variants X/Y), and −550 (variants H/L), also modulate MBL concentrations (20,21). MBL deficiency is common, affecting approximately a third of the population. More severe deficiency, less than 400 ng/mL, occurs in about 10% of the white population (10,20). Individuals deficient in MBL are more susceptible to infections, particularly in the context of another "immune defect" (22–26).

Numerous studies have now looked at MBL in the neonatal setting. Terai and Kobayashi reported that in term neonates, MBL levels increased during the first 5 days after birth (27). Thiel *et al.* showed that MBL levels at term were only a third of those seen at 3 mo (28). It would appear from subsequent

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Abbreviations: ELBW, extremely low birth weight; **MBL,** mannose-binding lectin; **SIRS,** systemic inflammatory response syndrome; **VLBW,** very low birth weight

publications that MBL levels are lower in preterm than term neonates and is related to gestational age rather than birth weight (29–31). However, it is unclear if MBL deficiency is clinically important in neonates. The present study was designed to investigate if low MBL levels predispose preterm neonates to infection.

PATIENTS AND METHODS

Patients. One hundred and sixty-six premature neonates of gestational age less than <37 wk were prospectively recruited for this study between September 2002 and June 2005. Sixty-seven were from the University College London Hospitals, NHS Trust, London, UK and 99 were from the Polish Mother Memorial Hospital, Lodz, Poland. Patients were randomly selected on the basis of availability of the research staff to obtain consent, collect and process the sample. Most patients were recruited between 2003 and 2004. Patients were randomly selected on the basis of availability of the research staff to obtain consent, collect and process the sample and are representative of the total preterm population admitted. Ethical approval was granted by the ethics committees of The Polish Mother Memorial Hospital-Research Institute, Lodz, Poland and UCLH in London. Parental consent was obtained before patient enrollment. Neonates with major congenital abnormalities or requiring surgical intervention were excluded.

MBL genotype and protein analyses. All neonates included in the study had blood taken for MBL phenotyping and genotyping. Blood was collected on days 1–3, 7–10, 14–18, and 27–30 and analyzed for MBL levels by ELISA (MBL oligomer Elisa Kit, Antibody Shop, Denmark). DNA was isolated from whole-blood samples using a QIAamp DNA blood mini kit (Crawley, UK). The genetic variants within exon-1 of the MBL gene at codon 52 (D), 54 (B) and 57 (C) were determined by polymerase chain reaction (PCR) followed by heteroduplex analysis utilizing polyacrylamide gel electrophoresis (PAGE) (32–34). Wild type alleles were denoted as A and variant alleles as O. Promoter polymorphisms at −221 (X/Y) of the MBL gene were also determined using a similar method (32). The three *MBL-2* structural gene mutations, B, C, and D are in linkage disequilibrium with the promoter region polymorphism X/Y, so that only Y associates with variant alleles (20).

Infection. Each neonate was followed up during the study period (days 0–30) for evidence of infection. Blood cultures were performed when infection was suspected. Definitive sepsis was diagnosed if they had the combination of a positive blood culture and clinical and/or laboratory evidence of sepsis. Clinical markers of sepsis included poor circulation (pallor, decreased perfusion, hypotension, tachycardia or bradycardia), increased oxygen requirement or ventilation parameters, temperature instability, lethargy or irritability, abdominal distension, feeding intolerance and jaundice. Laboratory markers included abnormal leukocyte count, increased immature-to-total neutrophil ratio, low platelet count, and raised C-Reactive Protein (CRP). Positive blood cultures in the absence of clinical or laboratory evidence of sepsis were considered to be contaminants and excluded from the study. Neonates were "presumed" to have sepsis if they had clinical or laboratory evidence of sepsis but without positive blood cultures. The clinical data were collected prospectively and a diagnosis of sepsis was recorded. However, to provide a more consistent evaluation of this diagnosis, the designation of a sepsis diagnosis was made by two clinicians who reviewed the notes retrospectively. They were blinded from the MBL data. In the UK cohort, the duration of antibiotic therapy used in the first 30 d of life was also recorded as a proxy marker of sepsis. This information was not recorded in the Polish cohort.

Statistical analysis. Patients were classified according to their MBL exon-1 mutations and their corresponding promoter allotypes as follows: Patients with either YA/YA, YA/XA, XA/XA genotype were summarized as wild-type (A/A), patients with YA/YD, YA/ YB+YC, XA/YD or XA/ YB+YC as heterozygous (A/O) and patients with YO/YO MBL genotype were defined as homozygous (O/O). Genotype (or allele) frequencies were analyzed by the χ^2 test. Differences between groups (MBL serum levels) were compared using the Mann-Whitney *U* Test or Kruskal-Wallis test. Changes in mean MBL values were analyzed using a mixed effect model that took into account correlations between repeated measurements within an individual (35). All models included an intercept for measurements performed on days 1–3, a first slope for the change to 7–10 d and then a second slope for the change from 7–10 d to the end follow-up. Then, the effect of the genotype, LBW, and low gestational age at birth on baseline and evolution of mean MBL were tested. The analyses of the risk of sepsis according to MBL level, birth weight, and gestational age at birth were performed using logistic regression.

Probability values were two-sided, and statistical significance was defined as $p < 0.05$.

RESULTS

Patients' characteristics. One hundred sixty-six premature neonates (99 Polish and 67 British) were enrolled into the study. One hundred fifty-eight were included for further analysis (see below). Within this group, the mean gestational age was 30 wk (range: 24–36 wk) and the mean birth weight was 1399 g (range: 510–2958 g). Ninety-five of 158 (60%) neonates had a birth weight ≤1500 g and were classified as very low birth weight (VLBW) and 45 of 158 (28%) had a birth weight ≤1000 g (extremely low birth weight, ELBW). Forty-six of 158 (29%) were neonates of gestational age ≤28 wk, and 81% of these were ELBW. There were no significant differences between the British and Polish cohorts in terms of sex ($p = 0.93$), gestational age ($p = 0.39$) and birth weight ($p = 0.37$).

Analysis of MBL genotype. *MBL-2* genotypes were successfully determined in 158 (97 Polish and 61 British) neonates. In eight neonates, a genotyping sample was either not obtained ($n = 5$) or was insufficient ($n = 3$) for MBL genotyping. Table 1 shows the frequency of MBL genotypes for British and Polish neonates. There was no significant difference in the frequency of MBL genotypes between the two populations ($p = 0.44$). The distribution of MBL genotypes was similar between neonates of different gestational age and birth weight (data not shown).

MBL genotype and sepsis. Forty-seven neonates from the 158 with known MBL genotypes (29.7%) had a single positive blood culture during the study period which was associated with clinical or laboratory evidence of sepsis (definitive sepsis): 16 (26.2%) were from the British cohort and 31 (31.9%) were from the Polish population. Nine neonates had a second episode of sepsis with *Coagulase negative staphylococci* $n = 3$; *Enterobacter* sp., $n = 1$, *Klebsiella* sp., $n = 2$, *Candida albicans*, $n = 3$. These were not included in the analyses. The organisms isolated during the first episode of sepsis are presented in Table 2. In both populations, the majority of positive blood cultures were caused by *Coagulase negative staphylo-*

Table 1. *Frequency of MBL Exon-1 and X/Y promoter polymorphisms*

MBL genotype	British neonates *n* (%)	Polish neonates *n* (%)
YA/YA	22 (32.8)	34 (34.4)
YA/XA	14 (20.9)	29 (29.3)
XA/XA	0 (0.0)	4 (4.0)
Wildtype (A/A)	36 (53.7)	67 (67.7)
YA/YD	0	5
YA/YB + YC	17	17
XA/YD	1	0
XA/YB + YC	5	5
Heterozygous (A/O)	23 (34.3)	27 (27.3)
YO/YO	2 (3.0)	3 (3.0)
Homozygous (O/O)	2 (3.0)	3 (3.0)
Missing	6 (9.0)	2 (2.0)
Total	67 (100)	99 (100)

Patients with either YA/YA, YA/XA, XA/XA genotype were summarized as A/A; YA/YD, YA/YB+YC, XA/YD or XA/YB+YC patients were defined as A/O and patients with YO/YO MBL genotype as O/O.

Table 2. *Microorganisms associated with the first episode of bacteraemia*

Organisms	British	Polish	Total
Gram-positive	15	26	41
Coagulase negative *Staphylococcus*	13	22	35
Staphylococcus aureus	1	3	4
Group B *Streptococcus*	1	1	2
Gram-negative	1	4	5
Escherichia coli	0	1	1
Klebsiella sp.	1	0	1
Enterobacter sp.	0	1	1
Haemophilus influenzae	0	1	1
Corynobacterium macginleyi	0	1	1
Fungi	0	1	1
Candida albicans	0	1	1
Total	16	31	47

The table shows the number of individual neonates in the studied cohort with positive blood cultures.

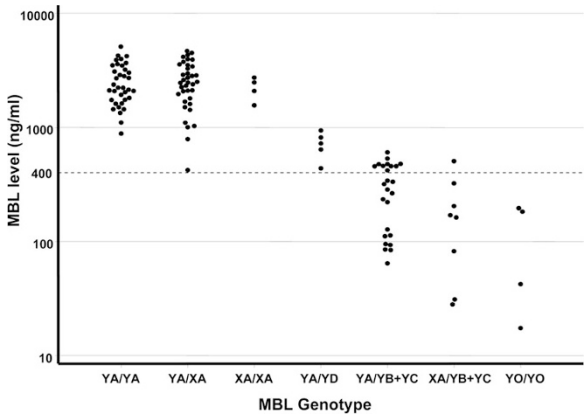

Figure 1. Correlation between MBL levels and MBL genotype. The horizontal dashed line represents MBL level of 400 ng/mL.

cocci. Only five blood cultures were positive for Gram-negative organisms and one for *Candida albicans*. No significant association was found between definitive sepsis and MBL genotype for the neonatal population as a whole ($p = 0.46$). Of the 47 positive blood cultures, 42 were from VLBW neonates. Of these, 17 had variant alleles and 25 were WT. There were 53% of the VLBW neonates with variant alleles who had a definitive sepsis compared with 39% in the WT VLBW population and this just failed to reach significance ($p = 0.053$). Analyses of the effect of MBL genotype, gestational age ≤28 wk and birth weight ≤1000 g also did not show a statistically significant association with sepsis. Interestingly, 4 of the 5 patients who were found to be homozygous for MBL variant alleles had definitive sepsis. There was no apparent relationship between MBL genotype and susceptibility to any specific micro organism.

In total, 80 of 158 (50.6%) preterm neonates were diagnosed with sepsis, both definitive ($n = 47$) and presumed ($n = 33$). A total of 54.6% of neonates with *MBL-2* variants had sepsis compared with 48.6% with a wild type genotype (A/A). This was not significant ($p = 0.47$).

Ninety-five neonates were classified as VLBW. Of these, 69 (72.6%) had a septic episode, definitive or presumed. Twenty-seven out of 32 (84.3%) neonates with variant alleles had a septic episode compared with 42 of 63 (66.6%) with a wild type genotype. This just failed to reach significance ($p = 0.056$). Analyses of the effect of MBL genotype, gestational age ≤28 wk and birth weight ≤1000 g also did not show a statistically significant association with sepsis, definitive or presumed.

MBL phenotypic analysis. MBL levels at birth and throughout the 30-d study period were measured and related to *MBL-2* genotype, gestational age, and birth weight. One hundred thirty neonates had a sample taken within the first 3 d of life and 38 neonates had 4 samples collected (completed serial measurements). As expected, baseline serum MBL levels were significantly related to *MBL-2* genotype ($p < 0.001$) (Fig. 1). MBL levels were also determined by postnatal age. Levels increased between the 1–3 d sample and the 7–10 d sample (Figs. 2 and 3). The increase between the first two samples was larger in WT neonates (+243 ng/mL/d 95% confidence interval [CI] = +196; +291) than in neonates with MBL mutations (+43 ng/mL/d [CI] = −17; +103, $p < 0.001$). This increase remained stable for the subsequent two samples. In WT neonates, baseline MBL levels were significantly lower in babies born at 28 wk of gestational age or less (2077 *versus* 2627 ng/mL, $p = 0.022$) whereas they were not statistically significant in neonates with MBL mutations (214 *versus* 440 ng/mL, $p = 0.43$). Interestingly, the rate of change of MBL levels between the first and subsequent samples was marginally influenced by gestational age at birth during the first week, tending to be larger in neonates with lower gestational age (+82 ng/mL/d $p = 0.12$). MBL levels in WT neonates born at less than 28 wk therefore reached those born at greater than 28 wk at 4 wk postpartum (3900 *versus* 3956 ng/mL, $p = 0.84$). The difference in baseline MBL level between neonates with very low birth weight (≤1500 g) and the others were not significant either in WT ($p = 0.73$) or neonates with variant alleles ($p = 0.57$). However, in WT

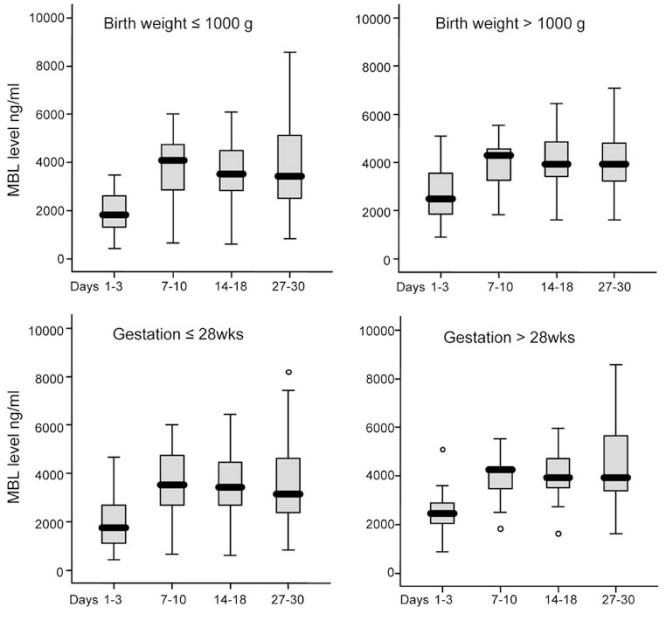

Figure 2. MBL levels in neonates with a wild-type MBL genotype in relation to postnatal age. The box plots show MBL levels (ng/mL) for birth weight (≤1000 and >1000 g) and gestational ages (≤28 and >28 wk). Outliers are shown as black dots. Levels are expressed as mean ± 2SE.

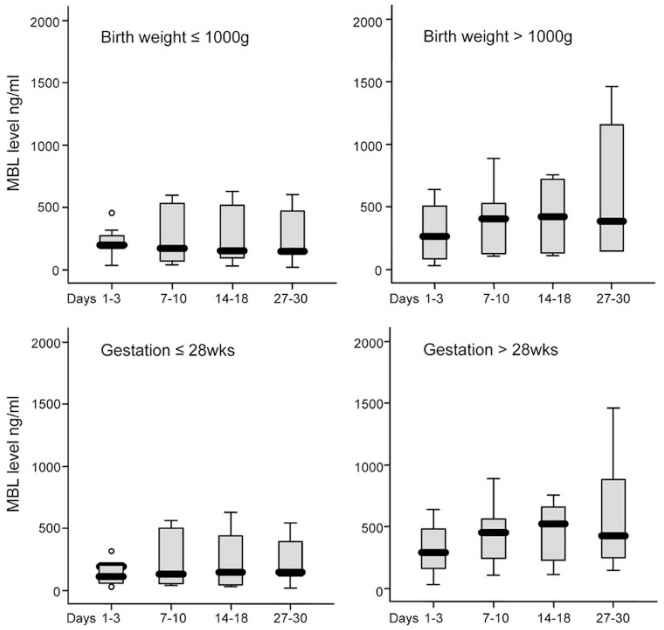

Figure 3. MBL levels in neonates with MBL variant alleles in relation to postnatal age. The box plots show MBL levels (ng/mL) for birth weight (≤1000 and >1000 g) and gestational ages (≤28 and >28 wk). Outliers are shown as black dots. Levels are expressed as mean ± 2SE.

neonates baseline MBL levels were significantly lower in neonates with a birth weight ≤1000 g (2099 *versus* 2654 ng/mL, $p = 0.015$), or with gestational age ≤28 wk. This effect was not observed in neonates with MBL mutations (216 *versus* 441 ng/mL, $p = 0.43$). As with gestational age, the rate of change of MBL levels between the first and subsequent samples was marginally influenced by birth weight (+62 ng/mL/d, $p = 0.23$). The independent effects of each variable were not distinguishable because birth weight and gestational age at birth were too highly correlated ($r = 0.83$, $p < 0.0001$).

MBL phenotype and risk of sepsis. We looked at the risk of definitive sepsis in the 120 neonates with samples available for both MBL phenotyping and genotyping from the first 3 d after birth. From this group, 37 had definitive sepsis. In concordance with the analysis of the entire cohort, MBL genotype was not significantly associated with the risk of developing sepsis (Table 3). Phenotypic analysis, however, revealed that 52% of neonates with an MBL level of ≤400 ng/mL ($n = 27$) (first quartile of the distribution), had sepsis compared with 26%, OR = 3.1, $p = 0.01$, in neonates with MBL levels of greater than 400 ng/mL. The effect of low birth weight and low gestational age at birth in relation to MBL levels were then analyzed through a multivariable logistic regression model (Table 3). MBL level, low birth weight, and low gestational age remained independently associated with the risk of sepsis. The probability of sepsis in a neonate of ≤28 wk or ≤1000 g with MBL levels ≤400 ng/mL was 70%. With MBL levels above 400 ng/mL, the risk of sepsis was 47% in both groups. Because of the high correlation between birth weight and gestational age, their independent effect could not be checked.

When looking at the risk of sepsis, definitive and presumed, there was still a trend toward a higher risk of sepsis in those with low MBL but this did not reach statistical significance (73% if MBL <400 ng/mL *versus* 57% if MBL was ≥400 ng/mL, OR = 2.0, $p = 0.11$).

MBL status and duration of antibiotic therapy. In the British neonates, the duration of antibiotic therapy used in the first 30 d of life was recorded. Forty-seven neonates had both MBL genotype and phenotype measured in the first 3 d of life. Nineteen neonates received more than 10 d of antibiotics. Of these, 10 had one or more variant alleles. Of the 28 who received antibiotics for 10 d or less, 9 had A/O or O/O genotypes ($p = 0.08$). When antibiotic duration was related to MBL status, as determined by phenotype (Fig. 4), median MBL levels were found to be significantly lower in patients treated with antibiotics for more than 10 d compared with neonates receiving antibiotics for 10 d or less (422 ng/mL *versus* 1618 ng/mL, $p = 0.02$). Three of the 9 neonates who had a WT genotype and who received more than 10 d of antibiotics had levels of less than 1000 ng/mL. This was double the proportion (3 of 19) of WT neonates with MBL levels of 1000 ng/mL or less and who received antibiotics for less than 10 d.

DISCUSSION

This is the first study to look in detail at MBL genotype, MBL levels, and susceptibility to infection in premature neonates. The most important finding was that babies who were

Table 3. *Risk of definitive sepsis depending on MBL genotype, phenotype, gestational age (GA) and birth weight (BW)*

Factor	Patients ($n = 120$)	Definitive sepsis (%)	Unadjusted	Multivariable model 1	Multivariable model 2
MBL genotype			($p = 0.33$)		
A/A	77	29	1		
A/O and O/O	43	37	1.48		
MBL phenotype			($p = 0.01$)	($p = 0.03$)	($p = 0.04$)
>400 ng/ml	93	26	1	1	1
≤400 ng/ml	27	52	3.1	2.9	2.7
BW			($p = 0.0006$)	($p = 0.001$)	—
>1000 g	83	22	1	1	
≤1000 g	37	54	4.2	4.1	
GA			($p < 0.001$)		($p < 0.003$)
>28 wks	87	23	1		1
≤28 wks	33	55	4.0		3.7

Uni- and multivariable analysis; $n = 120$.

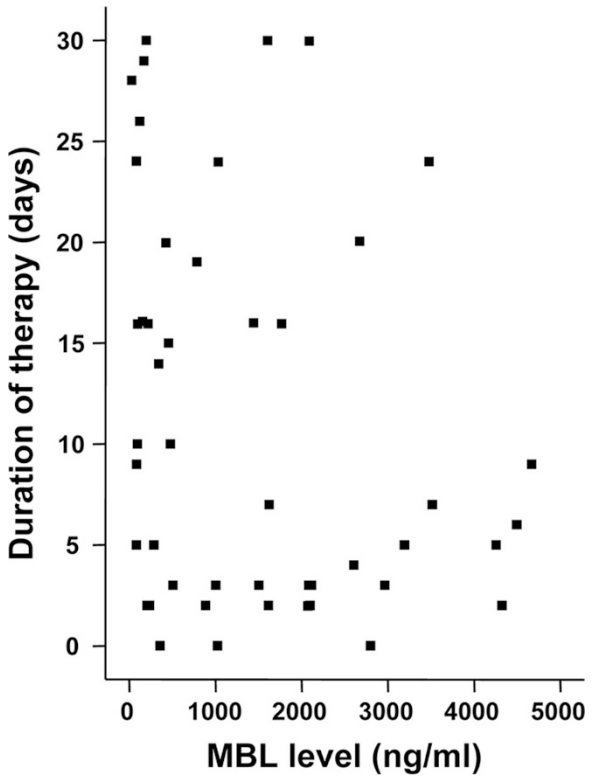

Figure 4. Relationship between MBL levels and duration of antibiotic therapy in the British cohort MBL levels were lower in neonates treated with antibiotics for more than 10 d compared with neonates receiving antibiotics for 10 d or less ($p = 0.02$).

less than 1000 g or born at less than 28 wk who had low MBL levels (<400 ng/mL) were more likely to suffer from sepsis.

A recent article has also found an association between low MBL levels and an increased risk of sepsis (36). In this study, nearly half of the positive blood cultures grew *Klebsiella pneumoniae*. The rate of proven sepsis to non-*Coagulase negative staphylococci* organisms in our study was much lower. This may be due to differences in the environment, the microbial etiology of sepsis and supportive care practice between centers. However, it would seem that MBL is still operating to protect neonates from sepsis in these different settings. In both studies, the key observation was that MBL was influencing the rate of bacteraemia. In our study, the significance of the MBL effect was lost when presumed sepsis (*i.e.*, without a positive blood culture) was included in the analyses. This indicates that MBL is likely to be influencing host bactericidal properties, probably through activation of complement pathways (37). Such an explanation is thought to explain why MBL deficient mice are more likely to succumb to *Staphylococcus aureus* and *Pseudomonas* infections (38). In addition, MBL may also be modulating the host's response to a bacterial challenge. In a recent study of children with systemic inflammatory response syndrome (SIRS), MBL deficiency was highly correlated with the development of systemic inflammatory response syndrome (26). This was not apparently due to an increase in invasive bacterial infections. It has now been established that MBL can modulate cytokine production *in vitro* and in *vivo* and this may influence the response of neonates to even minor infections (39).

In previous studies, gestational age and birth weight were found to markedly influence MBL levels (29–31). We found that the most important effect was postnatal age. As Figures 2 and 3 demonstrate, MBL levels were low at birth and increased significantly in WT neonates during the first week of life. There was also an effect of gestational age or birth weight, but only in wild type neonates of less than 1000 g or of 28 wk gestation. MBL is synthesized by hepatocytes and seems to show a similar developmental pattern to that of other proteins synthesized by the liver. The significance of this finding, also highlighted by Frakking, is that even neonates with a WT genotype may be deficient in MBL at birth (31). Indeed, in our study, genotype alone did not significantly influence the rate of sepsis. It was only when MBL levels were analyzed, that a correlation between low MBL levels and sepsis emerged. We chose a level of 400 ng/mL as our cutoff for two reasons. First, in a study of MBL levels and genotypes from over 500 children from the Avon longitudinal study of Parents and Children, individuals who were either YO/YO or XA/YB or XA/YC all had levels of less than 400 ng/mL (unpublished data). Second, in previous studies we found that MBL levels below 400 ng/mL did not activate complement binding and did not enhance MBL-mediated opsonophagocytosis (37).

The reasons why genotype analyses did not reveal a significant association with sepsis are because it underestimated the number of neonates who had levels of less than 400 ng/mL. Indeed the number of neonates who were YO/YO, XA/YB or XA/YC, (*i.e.*, the genotypes associated with levels below 400 ng/mL in healthy children), was 10% (12 of 120). However, the proportion of neonates with levels of less than 400 ng/mL was higher at 23% (27 of 120). This is because nearly half of the neonates with YA/YO had levels below 400 ng/mL (Fig. 3). This significantly increased the number of premature neonates who were phenotypically extremely MBL deficient at birth.

The clinical effect of having low MBL levels at birth was also demonstrated in the analysis of antibiotic usage within WT neonates in the UK cohort. Six of the 28 WT neonates had MBL levels of less than 1000 ng/mL. Half of these received more than 10 d of antibiotics. We consider antibiotic usage to be a marker of sepsis, as a course of at least 10 d of antibiotics was always administered for proven or presumed sepsis in the UK cohort. As such, it would seem that low levels of MBL even within the WT population could predispose neonates to sepsis.

This study demonstrates that MBL levels below 400 ng/mL treble the chances of developing sepsis. The combination of prematurity, gestation of ≤28 wk or birth weight ≤1000 g and MBL levels ≤400 ng/mL increase the risk of sepsis to 70%. Whereas, in the population of neonates with MBL levels above 400 ng/mL, the risk of sepsis was less than 50%. MBL will become available as a therapeutic agent in the near future and from our results, may be a useful adjunct to the care of premature neonates (40).

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