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Mutations of Genes Involved in the Innate Immune System as Predictors of Sepsis in Very Low Birth Weight Infants

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ABSTRACT

Mutations of genes involved in the innate immune system have been reported to be associated with an increased sepsis rate in adults. We determined the −159T mutation of the CD14 gene, the 896G mutation of the toll-like receptor 4 gene, the 3020insC mutation of the *NOD2* gene (*NOD2*-3020insC), the IL-6 174G/C promoter polymorphism (*IL6*-174G/C), and the mannose-binding lectin genotype and their association to the subsequent development of neonatal sepsis in a large cohort of very low birth weight (VLBW) infants. Fifty (14%) of 356 VLBW infants developed blood culture–proven sepsis during their stay in the hospital. VLBW infants carrying the *NOD2*-3020insC allele (n =15) and the *IL6*-174G allele (n =121) had a significantly higher rate of blood culture–proven sepsis (33% and 19.8%, respectively) than VLBW infants without these genotypes (p = 0.046 and 0.035, respectively). In a multivariate logistic regression analysis, gestational age less than 28 wk (odds ratio, 3.2; 95% confidence interval, 1.7–6.0; $p < 0.001$) and the homozygous *IL6*-174G allele (odds ratio, 1.9; 95% confidence interval, 1.0–3.9; p = 0.039) were predictive for the development of sepsis, whereas the *NOD2*-3020insC allele was only of borderline significance (odds ratio, 3.2; 95% confidence interval, 1.0–10.4; p = 0.052). VLBW infants with repeated episodes of sepsis had higher frequencies of the *NOD2*-3020insC and *IL6*-174G allele. The increased sepsis rate of homozygous *IL6*-174G carriers was especially related to an increase in Gram-positive infections, and was not observed in VLBW infants who received prophylaxis with teicoplanin (frequency of Gram-positive sepsis in homozygous *IL6*-174G carriers without prophylaxis 16.5% *versus* 2.4% in homozygous *IL6*-174G carriers with prophylaxis; p = 0.033). **(*Pediatr Res* 55: 652–656, 2004)**

Abbreviations

LPS, lipopolysaccharide
MBL, mannose-binding lectin
NF-κB, nuclear-factor kappa B
TLR, toll-like receptor
VLBW, very low birth weight

Although advances in neonatal intensive care have led to improved survival, sepsis continues to be an important cause of death among VLBW infants (1, 2). Recent advances in our understanding of the innate immune system triggered the identification of specific point mutations that are associated with an altered response of the innate immune system.

CD14 is the main receptor for bacterial LPS and is expressed on the surface of phagocytes. In adults, the homozygotic −159T mutation of the CD14 gene (*CD14*-159T) is associated with a high mortality rate in sepsis (3). The TLR4 is a coreceptor for LPS, harboring a transmembrane domain, which is important for intracellular signaling. The 896G mutation of the *TLR4* (*TLR4*-896G) leads *in vitro* to a reduced NF-κB activation after LPS stimulation, and *in vivo* to a reduced systemic inflammatory response after LPS inhalation (4). This mutation was found frequently in adults who developed septic shock (5), suggesting an association of this mutation with sepsis. *NOD2* is a gene that also confers responsiveness to bacterial LPS. The *NOD2*-3020insC mutation is associated *in vitro* with reduced NF-κB response after stimulation with several Gram-negative bacteria (6) and is associated with Crohns' disease (6–8). IL-6 is an important proinflammatory cytokine. The homozygous −174G allele of the *IL6*-174G/C promoter polymorphism is associated with reduced IL-6 levels

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in healthy newborns (9). MBL is participating in the innate immune defense by opsonizing various microorganisms for phagocytosis. Heterozygous mutations of the MBL gene (*MBL* B/C/D) are associated with reduced MBL serum levels and an increased sepsis rate in immunosuppressed patients (10).

Because a reduced response of the innate immune system in carriers of these mutations might be associated with a higher rate of bacterial infections in VLBW infants, the purpose of our study was to evaluate the usefulness of these genetic markers in predicting blood culture–proven sepsis in VLBW infants.

METHODS

Between December 1999 and November 2002, 535 VLBW infants were enrolled in an open multicenter study concerning the influence of genetic factors on the clinical course of VLBW infants. In November 2002 follow-up was completed in 372 VLBW infants. Because polymorphism frequencies differ widely among populations, we decided to exclude infants of African and Far Eastern descent ($n = 16$). The remaining 356 VLBW infants formed our study population.

Follow-up of prospectively enrolled VLBW infants was complete before discharge from the hospital. All parents had given informed written consent. Sepsis in VLBW infants was defined as blood culture–proven sepsis (clinical signs of sepsis and positive blood culture) at any time during the stay in the hospital. The clinical data of all patients were documented and coded before mutation analysis.

DNA samples of the infants were extracted from buccal swabs. DNA was extracted with a commercially available kit (Qiagen, Hilden, Germany). All polymorphisms were detected by PCR and restriction enzyme digestion. Primer and DNA sequences were selected from previously published reports (4, 7, 11–13). Primer pairs for detection of the *CD14*-159T mutation were 5'-GTG CCA ACA GAT GAG GTT CAC-3' and 5'-GCC TCT GAC AGT TTA TGT AAT C-3' (*Ava*II digest); for the *TLR4*-896G mutation, 5'ATA CTT AGA CTA CTA CCT CCA-3' and 5'CTT TGT TGG AAG TGA AAG TAA GCC-3' (*Nco*I digest); for the *NOD2*-3020insC -mutation, 5'-CTG AGC CTT TGT TGA TGA GC-3' and 5'-TCT TCA ACC ACA TCC CCA TT-3' (*Nla*IV digest); for the *IL6*-174G/C polymorphism, 5'TGA CTT CGA CTT TAC TCT TGT-3' and 5'-CTG ATT GGA AAC CTT ATT AAG-3' (*Nla*III digest); and for the *MBL* genotype, 5'-AGT CGA CCC AGA TTG TAG GAC AGAG-3' and 5'-AGG ATC CAG GCA GTT TCC TCT GGA AGG-3' (*Bam*I and *Mbo*II digest), and 5'-CAT CAA CGG CTT CCC AGG CAA AGA CGC G-5' and 5'-AGG ATC CAG GCA GTT TCC TCT GGA AGG-3' (*Mlu*I digest).

Our power calculation was based on the assumption that the expected frequency of sepsis in VLBW infants is 20% and that the carrier status of a specific mutation is associated with an increased risk of 50%. The expected carrier frequencies for the homozygous *CD14*-159T mutation, the heterozygous or homozygous *TLR4*-896G and *NOD2*-3020insC mutations, homozygous −174G-*IL6* promoter polymorphism, and the *MBL* B/C/D genotype were 20% (14), 12% (4), 8% (8), 37% (12), and 40% (15), respectively. On the basis of these assumptions, a number of 230 infants would be sufficient (α, 0.05; power, 0.8; two-sided test) to test our hypothesis.

All parts of the study were approved by the local committee on research in human subjects of the Medical University of Lübeck. Hypotheses were evaluated with Fisher exact test, Mann-Whitney *U* test, and multivariate logistic regression models.

RESULTS

Ten participating centers included 356 patients (median, 21; range, 1–136). The clinical data and the frequencies of homozygous *CD14*-159T allele, heterozygous or homozygous *TLR4*-896G allele, heterozygous or homozygous *NOD2*-3020insC allele, homozygous *IL6*-174G allele, and heterozygous or homozygous *MBL* B/C/D genotype in VLBW infants are given in Table 1. We observed no statistical differences in clinical data between infants with and without specific mutations.

The frequency of blood culture–proven sepsis was 14% in the whole group ($n = 356$), ranging from 0% (none of 28 infants) to 100% (1 of 1 infant) among different centers. The frequency of blood culture–proven sepsis in two centers that included the majority of patients (110 and 136 infants) was 19.1 and 7.4%, respectively.

The frequencies of blood culture–proven sepsis in VLBW infants with and without mutations of the innate immune system are given in Figure 1. VLBW infants who did not carry any of the mutations ($n = 119$) had a lower frequency of blood culture–proven sepsis than infants who carried any of the mutations tested ($n = 237$); however, this difference was not significant (11.8 *versus* 15.2%, $p = 0.42$, Fisher's exact test). In VLBW infants carrying mutations of the innate immune system, the amount of increase in sepsis rate was quite different, reaching statistical significance for carriers of the homozy-

Table 1. *Clinical data of VLBW infants with and without mutations of the innate immune system*

	All ($n = 356$)	No mutation ($n = 105$)	*CD14*-159T ($n = 72$)	*TLR4*-896G ($n = 34$)	*NOD2*-3020insC ($n = 15$)	*IL6*-174G ($n = 121$)	*MBL* B/C/D ($n = 116$)
Birth weight (g)	1073 (295)	1057 (296)	1041 (296)	1111 (311)	1048 (337)	1101 (284)	1105 (298)
GA < 28 wk	38	40	40	35	40	40	32
Male	48	44	47	44	47	53	50
Multiple birth	36	39	35	38	47	40	30
Antenatal steroids	64	67	67	65	73	67	58
Frequency of mutation	Ref.	29.5	20	9.6	4.2	34	33

Birth weight is given as mean (SD); all other data are given as percentages. There were no significant differences between infants with and without mutations of the innate immune system.

Abbreviation used: GA, gestational age.

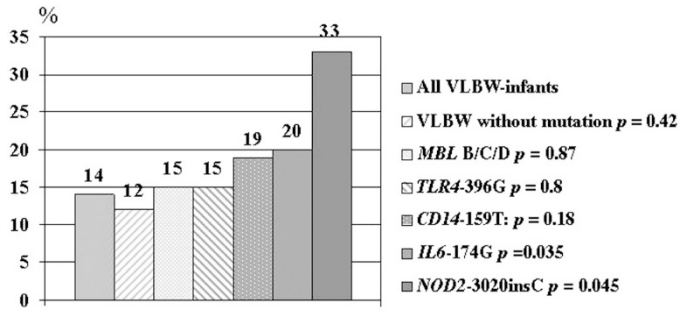

Figure 1. Frequency of blood culture–proven sepsis in 356 VLBW infants with or without mutations of the innate immune system. Probability values are given for frequencies of blood culture–proven sepsis in infants with a specific mutation *vs* infants without the mutation (Fisher's exact test, two-sided).

gous *IL6*-174G mutation (n =121, sepsis rate 19.8% *versus* 11.1% in noncarriers of the mutation, $p = 0.035$, Fisher's exact test) and carriers of the *NOD2*-3020insC mutation (n =15, sepsis rate 33% *versus* 13.2% in noncarriers of the mutation, $p = 0.045$, Fisher's exact test).

To rule out possible confounding, we performed a multivariate logistic regression analysis including gestational age, sex, multiple birth, antenatal treatment with glucocorticoids, and all mutations studied as independent variables and blood culture–proven sepsis as the dependent variable (Table 2). After stepwise exclusion of nonsignificant independent variables, only gestational age less than 28 wk (odds ratio, 3.2; 95% confidence interval, 1.7–6.0; $p < 0.001$) and homozygous carrier status of the *IL6*-174G mutation (odds ratio, 1.9; 95% confidence interval, 1.0–3.6; $p = 0.039$) were significant predictors of blood culture–proven sepsis, whereas the carrier status of the *NOD2*-3020insC mutation was of only borderline significance (odds ratio, 3.2; 95% confidence interval, 1–10.4; $p = 0.052$).

Furthermore, we analyzed the frequency of blood culture–proven sepsis in infants with and without the *IL6*-174G or *NOD2*-3020insC mutation; 306 of 356 infants had no sepsis, 42 infants one sepsis, seven infants two episodes of sepsis, and one infant suffered three events of blood culture–proven sepsis. The frequency of the *IL6*-174G or *NOD2*-3020insC mutation in these four groups was 34%, 48%, 57%, and 100%, demonstrating a statistically significant trend toward higher rates of repeated episodes of sepsis in infants carrying the *IL6*-174G or *NOD2*-3020insC mutation ($p = 0.026$, Mann-Whitney U test).

The type of infection in VLBW infants carrying the *IL6*-174G or the *NOD2*-3020insC mutation is given in Table 3. The increased sepsis rate in carriers of the homozygous *IL6*-174G mutation is related to an increase of sepsis caused by Gram-positive bacteria. Two of the participating centers instituted routine prophylaxis against Gram-positive infections (16), consisting of daily administration of 6 mg teicoplanin/kg body weight per day if an i.v. catheter is necessary in all infants with a birth weight below 1500 g (center 4, n = 136 infants) or infants with a birth weight below 1000 g (center 10, n = 1 infant). Therefore, we evaluated the rate of different types of infections in VLBW infants with and without teicoplanin prophylaxis stratified to their *IL6*-174G genotype. Because there were large differences among the rates of infection in different centers, we furthermore present data of the two largest centers, numbers 4 (routine teicoplanin prophylaxis for all VLBW infants) and 6 (no prophylaxis; Table 4). The higher rate of blood culture–proven sepsis in carriers of the homozygous *IL6*-174G allele was related to the high rate of Gram-positive infections in VLBW infants who did not received teicoplanin prophylaxis. Furthermore, homozygous carriers of the *IL6*-174C genotype had a relatively lower risk of Gram-positive sepsis than carriers of the heterozygous *IL6*-174G/C genotype. The trend toward higher rates of Gram-positive infections in homozygous carriers of the *IL6*-174G allele was detectable, even when only one large center was analyzed. No differences between carriers of different *IL6*-174 alleles were observed with regard to Gram-negative infections and in VLBW infants who were treated with teicoplanin prophylaxis (Table 4).

Table 3. *Type of infection in infants with specific mutations of the innate immune system*

	Gram-positive infections	Gram-negative infections	Other infections
All VLBW (n = 356)	6.7	2.5	5.3
IL6-174G (n = 121)	**11.6**	3.3	5.8
NOD2-3020insC (n = 15)	13.3	0.0	**20**

All data are given as percentages of all infants or percentages of infants with specific mutations. Gram-positive infections include infections with *Staphylococcus aureus* (n = 11), *Staphylococcus epidermidis* (n = 12) and *Streptococci* (n = 6); Gram-negative infections include infections with *Escherichia coli* (n = 5), *Klebsiella* (n = 1), *Enterobacter* (n = 2), *Serratia* (n = 0), *Proteus* (n = 0), and *Pseudomonas* sp. (n = 1). Other infections include *Listeria* (n = 0), anaerobic infections (n = 0), fungi (n = 4), and other Gram-negative or Gram-positive infections not listed above (n = 17). Significant differences between carriers and noncarriers of the mutation are given in bold ($p < 0.05$, Fisher's exact test, two-sided).

DISCUSSION

Recent advances in understanding the molecular basis of the innate immune system led to the identification of certain point mutations of genes involved in the early recognition of bacteria and associated with a reduced host response (3–6, 9, 10). The

Table 2. *Multivariate logistic regression analysis: gestational age and homozygous IL6-174G mutation as independent predictors of blood culture–proven sepsis*

Independent variable	VLBW with sepsis (n = 50)	VLBW without sepsis (n = 306)	OR	95% CI	p value
Gestational age < 28 wk	62	34	3.2	1.7–6.0	<0.001
Heterozygous *NOD2*-3020insC	10	3.3	3.2	1.0–10.4	0.052
Homozygous *IL6*-174G	48	32	1.9	1–3.6	0.039

Frequencies of independent variables in VLBW infants with and without blood culture–proven sepsis are given as percent values.
Abbreviations used: OR, odds ratio; CI, confidence interval.

Table 4. *Frequency of blood culture–proven sepsis in VLBW infants with different IL6-174 genotypes stratified to type of infection and teicoplanin prophylaxis*

Type of infection, subgroup	Frequency of blood culture–proven sepsis in infants according to *IL6*-174 genotype				
	All infants	*IL6*-174GG	*IL6*-174G/C	*IL6*-174CC	*p* value*
All kind of infections, all infants	50/356 (14%)	24/121 (19.8%)	21/198 (10.6%)	5/37 (13.5%)	0.035
Gram-negative and other, all infants	26/356 (7.3%)	11/121 (9.1%)	14/198 (7.1%)	3/37 (8.1%)	0.54
Gram-positive, all infants	24/356 (6.7%)	14/121 (11.6%)	8/198 (4%)	2/37 (5.4%)	0.013
Gram-positive, all infants with teicoplanin prophylaxis	3/137 (2.2%)	1/42 (2.4%)	1/82 (1.2%)	1/13 (7.7%)	1.0
Gram-positive, all infants without teicoplanin prophylaxis	21/219 (9.6%)	13/66 (16.5%)	7/116 (6%)	1/24 (4.2%)	0.015
Gram-positive, study center 4, teicoplanin prophylaxis	3/136 (2.2%)	1/42 (2.4%)	1/81 (1.2%)	1/13 (7.7%)	1.0
Gram-positive, study center 6, no teicoplanin prophylaxis	11/110 (10%)	8/37 (21.6%)	3/52 (5.8%)	0/21 (0%)	0.006

* *p* values are given for infants carrying the −174GG genotype *vs* infants carrying the −174G/C or −174C/C genotype (Fisher's exact test, two-sided).

scope of our study was the association of these mutations with blood culture–proven sepsis in a large cohort of VLBW infants. Although the sepsis rate was higher in infants carrying any of the mutations studied, these differences were statistically significant only for the *NOD2*-3020C mutation and the *IL6*-174G mutation.

The *NOD2*-3020insC mutation was first described in 2001 as a risk factor for Crohn's disease. *NOD2* is expressed in monocytes and carries a binding domain for bacterial LPS (17). The 3020insC mutation resulted in a frameshift at the second nucleotide of codon 1007 followed by a premature stop codon. The truncated *NOD2* protein contained 1007 amino acids instead of 1040 amino acids of the wild-type *NOD2* protein. *In vitro* studies using HEK293T cells transfected with wild-type or mutant *NOD2* receptor demonstrated that LPS from various bacteria induced NF-κB activation in wild-type but not in cells transfected with *NOD2*-3020insC (6). The frequency of the heterozygous *NOD2*-3020insC mutation in our cohort of preterm infants was 4.2%, which is in line with previous reports (6–8). At this time there are no studies concerning the association of the *NOD2*-3020insC mutation and septic events in patients of any age.

The *IL6*-174G/C polymorphism and its association with septic events in adults was studied recently. Allele frequencies did not differ significantly between patients with or without sepsis, and median systemic IL-6 levels were not associated with the genotype. However, in patients who finally died because of sepsis, significantly fewer carriers of the homozygous *IL6*-174G genotype were observed (18). Studies concerning the effect of different *IL6*-174 genotypes on IL-6 plasma and C-reactive protein levels gave conflicting results. Some groups report no or only moderate effects (19, 20), whereas others report lower IL-6 plasma levels in healthy carriers of the *IL6*-174C allele (12) and—in contrast to these data—higher baseline C-reactive protein levels in carriers of the C allele (21). The only study concerning the genotype-dependent IL-6 production in healthy newborns reported significantly higher IL-6 plasma levels in carriers of the *IL6*-174C allele compared with infants homozygous for the *IL6*-174G allele (9). We therefore decided to evaluate the hypothesis that VLBW infants carrying the homozygous *IL6*-174G genotype are at an increased risk for the development of sepsis.

We observed a significant association between the heterozygous *NOD2*-3020insC and the homozygous *IL6*-174G genotype and the development of blood culture–proven sepsis in VLBW infants. The latter association was not influenced by other known or possible risk factors for the development of sepsis in VLBW infants. Furthermore, VLBW infants with repeated episodes of septicemia were more frequently carriers of the mutations than infants without or with only one blood culture–proven sepsis. The increased sepsis rate of homozygous carriers of the *IL6*-174G mutation was especially related to an increase of Gram-positive infections. Infants receiving prophylaxis with teicoplanin had a low sepsis rate even if they were carriers of the homozygous *IL6*-174G mutation, whereas noncarriers of the mutation had a low sepsis rate even if they had not had any prophylactic treatment. The number of infants needing to be treated with teicoplanin prophylaxis to prevent one blood culture–proven Gram-positive sepsis calculated on the basis of our data would be seven infants in carriers of the mutation, but 28 infants in noncarriers of the mutation.

CONCLUSIONS

Our data suggest that teicoplanin prophylaxis stratified to the individual genetic risk of a given VLBW infant to develop Gram-positive sepsis might be useful in reducing the number of unnecessary prophylactic antibiotic treatments. However, it must kept in mind that this association study is not proof of a causal relationship between the *IL6*-174G allele and Gram-positive infections, but might be related to linkage to other polymorphisms. Furthermore, inasmuch as frequencies of blood culture–proven sepsis varied widely among different centers of our study, a possible association between *IL6*-174G and susceptibility to Gram-positive sepsis must be evaluated by randomized controlled trials, because the aim of our study was the association between specific mutations of the innate immune system and the development of sepsis in VLBW infants, and the differences observed between VLBW infants with and without prophylactic antibiotic treatment might be the result of other confounding factors.

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APPENDIX

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