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Lack of association between TLR4 polymorphism and severe gram-negative bacterial infection in neonates

Aim: Gram-negative microorganisms are responsible for a significant percentage of systemic bacterial infections in neonates, which tend to progress to sepsis. Toll-like receptor 4 (TLR4), one of the receptors in the innate immune system, binds to the lipopolysaccharide (LPS) present on gram-negative bacteria. Following ligation, a cascade of cellular signals leads to activation of NFκB, resulting in the generation of such proinflammatory cytokines as TNFα, IL-1, IL-2, and IL-6. These are the mediators of inflammation, which is the cellular response to activation of the innate immune system. As such, TLR4 appears to be a very likely candidate involved in the immediate immune response to gram-negative bacteria. Two polymorphisms in the TLR4 gene, Asp299Gly and Thr399Ile, cause a reduction in inflammatory cytokine response to LPS. We sought to determine if there was any correlation between these polymorphisms and severe gram-negative infection in neonates.

Materials and Methods: The study included 17 neonates with severe gram-negative infection and 70 healthy controls.

Results: No polymorphism was noted in the patient group. In all, 4 Asp299Gly and 7 Thr399Ile heterozygous polymorphisms were observed in the control group, and 4 subjects had both polymorphisms.

Conclusion: There appeared to be no correlation between the 2 polymorphisms–Asp299Gly and Thr399Ile– and gram-negative infection.

Key Words: Neonate, TLR4, gram-negative infections

Yenidoğanlarda TLR4 polimorfizmi ile ciddi gram negatif bakteriyel enfeksiyonlar arasındaki ilişki

Amaç: Yenidoğanlarda gram (–) mikroorganizmalar sepsise varan sistemik bakteriyel enfeksiyonların önemli yüzdesinden sorumludur. Toll-like reseptör 4 (TLR4) immune sistemin reseptörlerinden biridir, ve gram (–) bakterilerin üzerindeki lipopolisakkaritlere bağlanır. Bağlanmadan sonra hücresel sinyal kaskadı NFκβ'nın aktivasyonuna ve dolayısıyla TNFα, IL1, IL2 ve IL6 gibi proinflamatuar sitokinlerin oluşmasına yol açar. Bu mediatörler inflamasyonun hücresel cevabını aktive ederler. Bu yüzden TLR4, gram (–) bakterilere karşı immün cevapta önemli rol oynar. TLR4 geninin iki polimorfizmi vardır. Bunlar; Asp299Gly ve Thr399Ile'dir. Bu polimorfizmler lipopolisakkaritlere karşı azalmış inflamatuar sitokin cevabına yol açarlar.

Biz yenidoğanlarda şiddetli gram (–) enfeksiyonlarla bu polimorfizm arasında bir korelasyon olup olmadığını araştırdık.

Yöntem ve Gereç: Çalışmaya 17 şiddetli gram (–) enfeksiyonlu yenidoğan hasta ile 70 sağlıklı kontrol dahil edildi.

Bulgular: Hasta grubunda polimorfizm saptanmadı. Kontrol grubunda dört vakada heterozigot Asp299Gly ve 7 Thr399Ile polimorfizmi saptandı.

Sonuç: Gram (–) enfeksiyonlar ile iki polimorfizm arasında korelasyon görülmedi.

Anahtar Sözcükler: Yenidoğan, TLR4, gram negatif enfeksiyonlar

Introduction

The innate immune system is the frontline defense mechanism against foreign insults. The response is mediated by phagocytic- and antigen-presenting cells, and a group of host defense molecules, such as complement system components and pattern recognition receptors (PRRs). PRRs bind to pathogen-associated molecular patterns (PAMPs) expressed in pathogenic organisms. A more recently recognized PRR is toll-like receptor (TLR) 4, which appears to be a highly likely candidate for involvement in the immediate immune response to gram-negative bacteria. TLR4 is known to bind to the lipopolysaccharide (LPS) on gram-negative bacteria. Following ligation, a cascade of cellular signals leads to activation of NFκB, which causes the generation of such proinflammatory cytokines as TNFα, IL-1, IL-2, and IL-6. These cytokines are the mediators of inflammation, which is the cellular response to activation of the innate immune system (1).

Gram-negative bacteria are often responsible for severe, life-threatening infections in neonates. These infections usually progress to sepsis, which is the most common cause of death in newborns and may even cause neonatal mortality of 60% in developing countries (2).

A number of genetic polymorphisms have been reported to affect the immune response to pathogenic insult. Two polymorphisms of the TLR4 gene–a substitution of aspartic acid to glycine at position 299 and substitution of threonine to isoleucine at position 399 of amino acid sequence–lead to hyporesponsiveness to an inhaled LPS challenge and a reduction in inflammatory cytokine response to LPS (3,4). In addition, these 2 polymorphisms were correlated with an increased susceptibility to gram-negative infection in pregnant women and premature infants (5,6). The present study aimed to determine if there is an association between TLR4 polymorphism and susceptibility to gram-negative infection in neonates.

Materials and Methods

Patients

The study group included neonates with severe gram-negative bacterial infections that were followed-up at the Hacettepe University, İhsan Doğramacı Children's Hospital Neonatology Unit between January 2004 and January 2005. The control group was composed of healthy neonates with no known risk factors for sepsis whose families provided informed consent. Patients were scored according to Tollner's scoring system (7). When there was a positive blood and/or cerebrospinal fluid culture, it was defined as proven sepsis. Patients with localized infection and a positive blood culture were considered to have a severe gram-negative infection. One investigator that was blinded to the diagnoses of the participants performed the molecular analyses.

Asp299Gly polymorphism results in substitution of A with G at position 896; therefore, AA denotes the normal genotype, and AG and GG indicate heterozygous and homozygous polymorphisms, respectively. Thr399Ile polymorphism leads to substitution of C with T at position 1196. The normal genotype is CC; heterozygosity is indicated as CT, whereas the TT genotype represents homozygotes.

Methods

From each individual a 2-ml blood sample was drawn into a 0.1 M EDTA tube and stored at 4 °C. DNA was extracted from 200 μl of peripheral blood using a QIA-Amp Blood Mini Kit (Qiagen, Hilden, Germany) and was stored at –20 °C. We performed sequence analysis for each sample. For sequencing we used a common primer for both Asp299gly and Thr399Ile. The forward primer was F5'-AGTTTGACAAATCTGCTCTA-3' and the reverse primer was R5'-TGTTCTAGTTGTTCTAAGCC-3'. PCR reactions were run at 95 °C for 4 min, followed by 30 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final cycle at 72 °C for 5 min. A PCR product of 466 bp was obtained. These products were labeled using a BigDye Terminator Cycle Sequencing Kit v.3.1 (Perkin Elmer Applied Biosystems Division, Foster City, CA, USA) and were analyzed using an ABI PRISM® 310 Genetic Analyzer capillary electrophoresis system (Perkin Elmer Applied Biosystems Division, Foster City, CA, USA).

Results

The study included 17 patients, 16 with proven sepsis and 1 with severe gram-negative infection (3 male and 14 female). Median gestational age of the patients was 37 weeks (range: 28-40 weeks). Median birth weight was 2900 g (range: 860-3420 g). The control group was composed of 70 neonates (37 male and 33 female) with a median gestational age of 38 weeks (range: 34-41 weeks) and a median birth weight of 3200 g (range: 2190-4560 g). In the proven sepsis patient group, blood cultures revealed Klebsiella in 8 samples, E. coli in 6, and Neisseria in 2 samples. E. coli was isolated in 1 patient with severe gram-negative infection, which was localized. Overall, 17 positive blood cultures were obtained.

We did not observe any polymorphism in the patient group; therefore, Asp299Gly and Thr399Ile polymorphisms were not correlated with gram-negative infection. In the control group there were 4 Asp299Gly and 4 Thr399Ile heterozygous polymorphisms (Figures 1 and 2). Thr399Ile accompanied all Asp299Gly polymorphisms and the incidence of Asp299Gly and Thr399Ile polymorphisms was 2.9% and 5.1%, respectively.

Discussion

Newborns are known to be susceptible to bacterial infection and sepsis due to the physiological immaturity of the immune system. In addition, the diagnosis of sepsis is challenging, as the inflammatory response in newborns differs greatly from that in adults. Therefore, identifying patients with an increased risk for sepsis might facilitate earlier detection and initiation of treatment.

TLR ligands are bacterial motifs that are recognized by innate immune response elements. LPS, a glycolipid component of the outer membrane of gram-negative bacteria, exhibits the most potent immunostimulating activity of the TLR ligands. LPS is known to play a central role in both eliciting inflammatory responses and causing septic shock during gram-negative bacterial infection (8).

The TLR4 gene encodes a highly conserved transmembrane receptor crucial to endotoxin detection and initiation of the systemic inflammatory responses observed in sepsis. As such, single nucleotide polymorphisms of this protein might have an effect on functioning at the protein level, resulting in defective recognition and clearance of microorganisms. The inability of C3H/HeJ TLR4-deficient mice to eradicate invading pathogens highlights the importance of TLR4 in the recognition of gram-negative bacteria (9). There are 2 known missense single nucleotide polymorphisms (SNPs) that lead to alteration in the extracellular domain of the TLR4 receptor. These SNPs, namely Asp299Gly and Thr399Ile, have been shown to attenuate the response to endotoxin exposure in vivo and in vitro (4,10,11). There are variable findings related to those polymorphisms and predisposition to infection. Two subsequent reports indicated associations between those 2 SNPs and the incidence of gram-negative infection and septic shock in intensive care unit (ICU) patients (12,13). In one of those reports the TLR4 gene was altered in 14 (18%) of 77 ICU patients, and in 5 (13%) of 39 healthy volunteers. In the other study 91 patients with septic shock and 73 healthy controls were genotyped, and the prevalence of the 2 mutations in both groups was similar (13). Gram-negative infection was identified in 50% of patients with wild-type alleles and in 100% of patients with the Asp299Gly mutation. The relationship between the TLR4 genotype and mortality was not significant. Montes et al. reported that the Asp299Gly polymorphism in the TLR4 gene was associated with an increased risk of gram-negative infection and hematogenous osteomyelitis (14). In another study 355 patients with meningococcal sepsis were evaluated for those polymorphisms and Asp299Gly was observed in 14 of the patients (15). In very low birth weight infants Asp299Gly polymorphism was not predictive of sepsis (16). In a study by Sackesen et al. Asp299Gly polymorphism was observed in 2.5% of the healthy individuals of Turkish ethnic background and Thr399Ile polymorphism was noted in 4% (17). We also obtained similar results; however, there was no correlation between Asp299Gly or Thr399Ile polymorphism, and gram-negative infection in the neonates we studied.

The present study has some limitations that should be considered. Firstly, it is difficult to

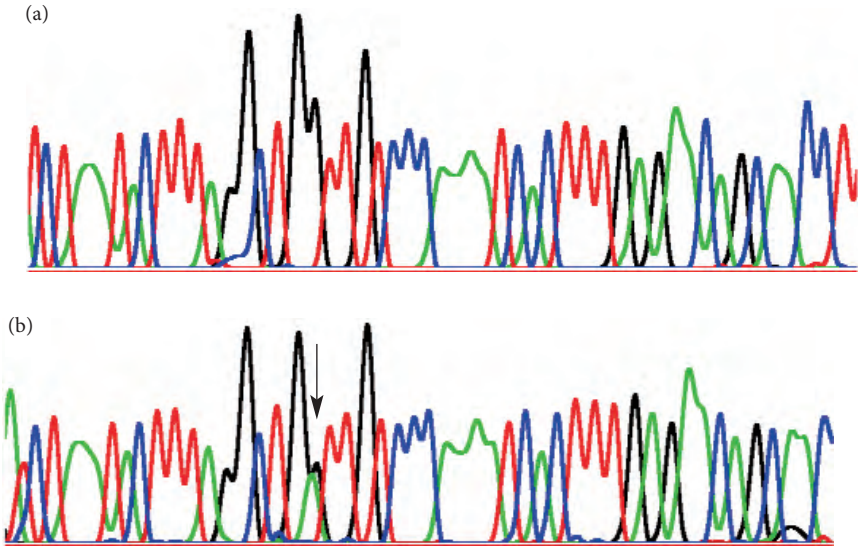

Figure 1 a. Sequence of the TLR4 gene without polymorphism.
b. Sequence of a control showing the heterozygous polymorphism of the substitution of A with G at position 896 in the TLR4 gene (Asp299Gly).
Green indicates adenine, Red indicates thymidine, Blue indicates cytosine, Black indicates guanine.

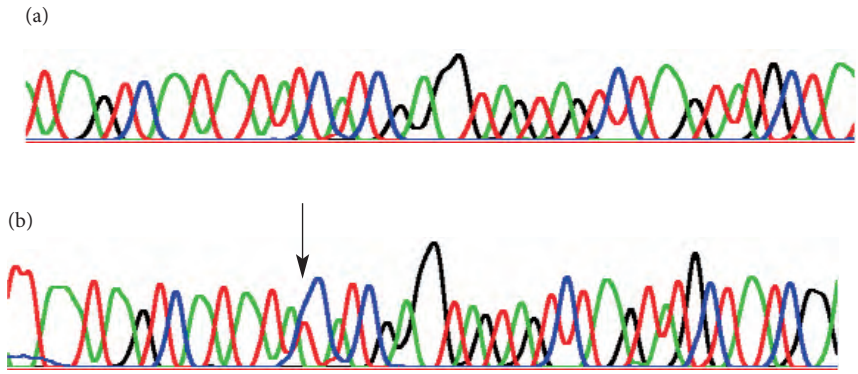

Figure 2 a. Sequence of the TLR4 gene without polymorphism.
b. Sequence of a control showing the heterozygous polymorphism of the substitution of C with T at position 1196 in the TLR4 gene (Thr399Ile).
Green indicates adenine, Red indicates thymidine, Blue indicates cytosine, Black indicates guanine.

generalize the findings due to the small number of patients that had gram-negative infection. Secondly, it is possible that other SNPs exist in both the TLR4 gene and nearby genes, and that related genes probably function in the signaling pathway. As recent studies have suggested, LPS recognition is complex and involves TLR4, CD14, and MD2 (18); therefore, there is a need for further investigation of the polymorphisms of CD14 and MD2 genes, and of other potential factors that may have a role in susceptibility to gram-negative infection in neonates.

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