A Functional Polymorphism of Toll-like Receptor 4 Is Not Associated with Likelihood or Severity of Meningococcal Disease

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Human Toll-like receptor 4 (TLR4) transduces proinflammatory cytokine release by human cells in response to lipopolysaccharide (LPS). This study tested the hypothesis that, if TLR4 is rate limiting for a successful response to bacterial LPS in humans, a human gene polymorphism that results in the amino acid substitution Asp299Gly and causes reduced expression and function of TLR4 should influence susceptibility to or severity of natural gram-negative infection. The allele frequency of the Asp299Gly polymorphism was 5.9% among 879 blood donors, 6.5% among 1047 patients with microbiologically proven meningococcal disease, and 4.1% among 86 patients who died of meningococcal disease. No significant differences were observed, including those analyzed after stratification of the infected population by age and by meningococcal serogroup. Therefore, this functional TLR4 polymorphism does not influence susceptibility to or severity of meningococcal disease.

Successful defense against gram-negative infection relies on an innate response to bacterial determinants, including lipopolysaccharide (LPS), during which proinflammatory cytokines such as tumor necrosis factor and interleukin-1 and -6 are secreted by mononuclear cells [1]. However, maladaptive and excessive release of proinflammatory cytokines can result in gram-negative septic shock and sepsis syndrome [2]. Evidence for genetic control of responses to LPS was provided in studies of C3H/HeJ [3] and C57BL/10ScCr [4] mouse strains, which are highly resistant to the deleterious biologic effects of LPS. This resistance is due to defects in the gene encoding the transmembrane Toll-like receptor 4 (TLR4) [5]. TLR4 is expressed in humans and is a critical sensor of LPS [6], and it requires the cofactor MD2 [7].

Human polymorphisms of TLR4 were described recently [8]. One of these, a common adenine for guanine substitution 896 nt downstream of the transcription start site (+896) of TLR4, results in replacement of an aspartic acid residue with glycine at amino acid 299 (Asp299Gly). This missense polymorphism is in the fourth exon of TLR4 and alters the extracellular domain of the receptor. Airway epithelial cells from patients who carry the Asp299Gly polymorphism are hyporesponsive to LPS and exhibit reduced expression of TLR4, which can be restored by transfection of cells with vectors containing the wild-type gene [8].

In human meningococcal disease, disease severity correlates with plasma LPS concentrations [9]. Wild-type Neisseria meningitidis signals via TLR4 and its cofactor MD2, but an LPS-deficient mutant cannot signal via this pathway, which is consistent with a critical role of TLR4 in response to LPS, at least in vitro [10]. In mice, a genetic defect of TLR4 expression results in greatly prolonged bacteremia after experimental infection [11]. Therefore, we reasoned that humans with the Asp299Gly polymorphism of TLR4 should be more susceptible to meningococcal disease or its severe manifestations if TLR4 is critical to host defense during natural gram-negative infections.

Patients and Methods

Patients. We genotyped TLR4 derived from a large number of patients with microbiologically proven meningococcal disease. From June 1998 through November 1999, we archived 1047 consecutive anonymous whole-blood samples that were sent to the Meningococcal Reference Unit for England and Wales for diagnostic polymerase chain reaction (PCR) amplification of meningococcal DNA. All originated from white patients with culture- or PCR-proven meningococcal disease. Of these DNA samples, 86 were from patients who died and 961 were from survivors. Once clinical data were collated, samples were coded so that patients remained anonymous. DNA from a population of 879 northern English white blood donors (age range, 18–65 years) was used as a noninfected control sample.
**Laboratory methods.** DNA was extracted by standard methods. We probed for the Asp299Gly polymorphism of TLR4 by using a validated 5' nuclease assay (TaqMan allelic discrimination test; Perkin Elmer Biosystems) [12] that used 20 ng of genomic DNA as a template for amplification. Probes, primer sequences, and cycling conditions are shown in figure 1. We used “1” to denote the frequent allele at position +896 and “2” to denote the rare allele. Homozygosity is indicated as “1/1” or “2/2” and heterozygosity as “1/2,” per standard nomenclature.

**Statistical analysis.** The strength of the association between clinical outcome and Asp299Gly polymorphism was expressed as an odds ratio (OR) together with a 95% confidence interval (CI).

**Results**

**Patients and microbiological data.** Of the 1047 samples from persons with meningococcal disease, 86 were from patients who died and 961 were from survivors. Patients who died were significantly older (mean, 23.18 ± 2.46 years) than the survivors (mean, 12.9 ± 0.48 years) (\(P = .001\), Mann-Whitney). The median age in both cases was <1 year. The serogroups of N. meningitidis detected by culture or PCR of samples from patients who survived were B (442 patients), C (354 patients), and W135 (5 patients), and samples from 160 patients were nongroupable. Serogroups in samples from those who died were B (32 patients), C (44 patients), and W135 (1 patient), and samples from 9 patients were nongroupable.

**TLR4 genotyping.** Table 1 shows genotyping results. Overall, the allelic frequency of the Asp299Gly polymorphism was 5.9% among control patients and 6.5% among patients with meningococcal disease. There was a small but nonsignificant excess of frequency of carriage of the rare allele among patients with meningococcal disease (OR, 1.14; 95% CI, 0.86–1.52). When patients who survived were compared with those who died there was a small but nonsignificant excess of frequency of carriage in the survivors (OR, 1.55; 95% CI, 0.70–3.44). Therefore, the common polymorphism Asp299Gly of TLR4 does not appear to influence susceptibility to or severity of meningococcal disease. To confirm the veracity of the TaqMan genotype technique, a 79-bp segment encompassing the polymorphic locus was sequenced in 25 subjects of whom 10 were 1/1, 9 were 1/2, and 6 were 2/2. In every case, the TaqMan probe genotype was confirmed by direct sequencing.

Because patient ages and infecting serogroups were not randomly distributed among patients who died or survived meningococcal disease, we investigated a possible interaction between TLR4 genotype and age or infecting serogroup by repeating the analysis after stratification of patients into groups infected with either serogroup B or serogroup C and into groups by ages 0–3 years, 4–13 years, and 14–70 years. No significant association of the Asp299Gly polymorphism with susceptibility to or severity of meningococcal disease was observed in any of these groups.

**Discussion**

We found no association of the human TLR4 polymorphism (Asp299Gly) with likelihood or severity of meningococcal disease. This was surprising because TLR4 is considered to be the most important mammalian sensor of LPS, and the polymorphism...
phism we investigated results in clearly altered TLR4 function in humans [8]. A number of possible conclusions can be drawn from the absence of an association between the Asp299Gly polymorphism and meningococcal disease. First, the polymorphism may not be functional. This is unlikely because it is clearly associated with reduced function and expression in vitro, albeit in epithelial cells, and heterozygotes for this allele exhibit a blunted response to LPS inhalation on lung function testing [8]. The second possible conclusion is that inefficient TLR4 function is not rate limiting during human response to severe infection by gram-negative organisms such as *Neisseria meningitidis.* Ten human TLRs have been described. These differ in microbial pattern–recognition specificities and cofactor requirements [13]. In the case of *Neisseria meningitidis,* TLR2 is capable of transducing a proinflammatory response to this organism even in the absence of LPS [10, 14]. Thus, it is possible that TLR2 and perhaps other members of the TLR family or their cofactors contribute to effective innate immunity in humans even if TLR4 function or expression is impaired.

The methods used in this study were appropriate. We genotyped patients with microbiologically proven meningococcal disease. The control population of white blood donors was derived from a similar geographic region. A subset of patients who died was used as a cohort with severe disease, because fatal outcome is easily defined and verified. We recognize that within the spectrum of meningococcal disease there may be other severe but survivable clinical events that are potentially initiated by LPS (e.g., purpura fulminans). We did not analyze subgroups of survivors because of the difficulty of verification. However, if the Asp299Gly polymorphism influences disease severity, we should have seen a change in allele frequency among the patients who died.

It is possible that other, as-yet-unidentified polymorphic loci within TLR4 can influence susceptibility to gram-negative infection. In experimental infection of mice with *Neisseria meningitidis,* intraperitoneally inoculated wild-type mice develop transient bacteremia that is cleared within hours; however, *lps*Δ mice develop prolonged bacteremia [11]. In C3H/HeJ mice, a point mutation within exon 3 modifies TLR4 within the cytoplasmic domain of the protein, creating an inhibitory effect on LPS signal transduction [5]. We performed conformation-sensitive gel electrophoresis of a 500-bp segment across the equivalent region in 51 patients with microbiologically proven meningococcal disease and in 3 healthy control patients. No evidence of sequence variation was found (data not shown), suggesting that these persons with evidence of severe gram-negative infection do not have polymorphisms in this critical region of the gene. Others directly sequenced the same region of human *TLR4* in healthy volunteers and found no sequence variants [8].

In summary, we found that a common functional polymorphism of the gene encoding TLR4 is not over- or underrepresented among patients with an important gram-negative infection, meningococcal disease, including those with lethally severe manifestations. This suggests that effective TLR4 function may not be a rate-limiting component for containment of natural gram-negative infection by the human innate immune system.

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**References**