The cytokine synthesis by heterozygous carriers of the Toll-like receptor 4 Asp299Gly polymorphism does not differ from that of wild type homozygotes

Michael Heesen[1], Brunhilde Bloemeke[2], Dagmar Kunz[3]

[1] Department of Anesthesia, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1100 DD Amsterdam, The Netherlands; [2] Department of Dermatology; [3] Department of Clinical Chemistry and Pathobiochemistry, University Hospital of Aachen, Pauwelsstr. 30, 52057 Aachen, Germany

Correspondence: Priv.-Doz. Dr. Michael Heesen, Department of Anesthesia, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1100 DD Amsterdam, The Netherlands. Tel.: + 31205662533; Fax: + 31206979441; E-mail: m.heesen@amc.uva.nl

ABSTRACT. Previous studies have found that heterozygosity for the A896G mutation of the endotoxin receptor TLR4 confers susceptibility to Gram-negative infections and septic shock. To evaluate the underlying mechanisms, we studied the association of the TLR4 polymorphism with endotoxin-induced cytokine synthesis in human whole blood. Monocyte CD14 density and monocyte count were also determined. Healthy individuals were genotyped by means of a real-time polymerase chain reaction. Plasma concentrations of TNF-α, IL-6, and IL-8 were measured by chemiluminescence. No significant differences in cytokine synthesis were observed between heterozygous individuals and homozygous carriers of the wild type allele. Our study suggests that heterozygosity for this TLR4 mutation is not a major factor determining the cytokine response to endotoxin.

Keywords: Toll-like receptor 4, tumor necrosis factor-α, interleukin-6, endotoxin, genetic polymorphism, polymerase chain reaction

INTRODUCTION

Endotoxin, a cell wall component of Gram-negative bacteria binds to Toll-like receptor 4 and CD14 [1]. Activation of this receptor complex leads to the synthesis and release of cytokines. The crucial role of TLR4 is underlined by mouse strains that are hyporesponsive to endotoxin. These mice bear a mutation of TLR4 [2]. Recently, a mutation of the human TLR4 gene was identified [3]. This mutation is located at position 896 and consists of a single nucleotide polymorphism (A→G) that is translated into a substitution of aspartic acid by glycine at position 299 of the protein sequence [3]. This site co-segregates with another point mutation (threonine-to-isoleucine substitution at position 399 of the amino acid sequence) [3]. The rare G allele confers hyporesponsiveness to inhaled endotoxin [3]. Individuals with the G allele displayed lower basal plasma concentrations of several markers of inflammation after overnight fasting [4]. They also had a lower risk of carotid atherosclerosis than wild type homozygotes. The relevance of this TLR4 mutation has also been elucidated in patients with Gram-negative infections. Two groups independently studied the association of the mutant G allele with susceptibility to Gram-negative infections and septic shock. Heterozygous carriers of the G allele had a higher incidence of Gram-negative infections [5] and a higher susceptibility to septic shock [6]. However, the reasons for this are not yet understood. We therefore carried out a study of the cytokine synthesis capacity of human whole blood after endotoxin activation in heterozygotes (AG carriers) versus A (wild type) homozygotes.

METHODS

After approval by the local ethics committee and written informed consent, healthy, Caucasian blood donors were studied. Venous blood samples were taken.

Genotyping for the TLR4 A896G mutation

Genotyping for the TLR4 A896G mutation was performed by means of real-time polymerase chain reaction (PCR) assay using specific fluorescence-labeled hybridisation probes (LightCycler™, Roche Diagnostics, Basel, Switzerland). Details of the method have been published elsewhere [7]. Two μmoL/L of the primers (sense: 5'-aag aaa tta ggc ttc ata agct, antisense: 5'-acc ctt tca ata gtc aca ctc a), as well as 0.2 μmoL/L of the detection probe specific for the Aallele (5'-LC Red640-act acc tcg atg ata tta ttg act tat t-fluorescein)), and the anchor probe (5'-aat tgt ttg aca aat gtt tct tca ttt tcc- p; the 3'-end was phosphorylated to block extension) were used. The PCR contained 1 μL reaction buffer (LightCycler DNA master hybridization probes 10 C buffer, 1.75 mmoL/L, Roche Diagnostics), 2.5 mmol/L MgCl2 in a total volume of 10 μL. The PCR conditions were: initial denaturation at 95 °C for 120 s, followed by 50 cycles of denaturation (95 °C for 5 s, 20 °C/s), annealing (50 °C for 5 s), and extension (72 °C

for 10 s). The melting curve consisted of 1 cycle at 95 °C for 5 s, 45 °C for 10 s, and then increasing the temperature to 90 °C at the rate of 0.1 °C/s.

Monocyte CD14 expression

CD14 expression was analyzed using the phycoerythrin (PE)-labeled anti-CD14 antibody (MøP9, Becton Dickinson, Heidelberg, Germany), as described previously (8). The instrument was calibrated using the QuantiBRITE™ phycoerethrin (PE) beads according to the manufacturer's instructions (Becton Dickinson, Heidelberg). The QuantiBRITE™ tube contains a lyophilized pellet of beads conjugated with four levels of PE: 1 700, 14 000, 39 000 and 133 000 molecules per bead. These beads and the sample were measured with the same instrument settings in the PE fluorescence channel. The beads were used to calculate the number of antigen molecules per cell, expressed as antibody binding capacity (ABC).

Ex vivo *lipopolysaccharide stimulation of whole blood cytokine release*

The tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-8 responses of human whole blood to lipopolysaccharide (LPS)-stimulation were assessed as previously described [9]. Heparin-anticoagulated venous blood was diluted 1:1 (v/v) with RPMI 1640 (Gibco BRL, Karlsruhe, Germany). 100 ng/ml endotoxin (*E.coli* O5:B55) were added for 4 hrs of incubation at 37 °C. The cytokine concentrations were determined by measuring immunoreactivity by means of chemiluminescence (Immulite™, DPC Biermann, Bad Nauheim, Germany).

Statistical analysis

Values are given as median (interquartile range, 25^{th}-75^{th} percentile). Due to the non-parametric distribution of the cytokine and CD14 data, the Wilcox Mann Whitney test was used to compare the cytokine concentrations between the genotypes. The 2*2 table Chi square test served to compare expected and observed genotype frequencies. P values < 0.05 were considered as statistically significant.

RESULTS

Ninety-three individuals were included, 83 (89%) were A homozygous, 10 (11%) were carriers of the heterozygous genotype. The observed and the expected genotype frequencies did not differ (p = 0.157), indicating that the study population was in Hardy-Weinberg equilibrium.

Table 1
Cytokine concentrations (pg/mL) after *ex vivo* endotoxin stimulation of human whole blood according to TLR4 A896G genotypes. Values are median (interquartile range, 25^{th}-75^{th} percentile)

	TLR4 A896G		P value
	AA n =83	AG n =10	
TNF-α	2559 (1975-4411)	3191 (1909-5245)	0.661
IL-6	3839 (2508-5600)	4397 (2966-6560)	0.597
IL-8	1275 (806-2022)	2170 (1444-3018)	0.134

The density of monocyte CD14 was $5.82*10^6$ abc (3.68-$7.10*10^6$ abc) in TLR4 A896G AA carriers and $6.35*10^6$ abc (2.33-$7.82*10^6$ abc) in AG carriers (p = 0.639). Cytokine concentrations for the 2 TLR4 genotypes are given in Table 1. No significant differences were found.

DISCUSSION

The frequencies of the TLR4 A896G genotypes were in accordance with previous reports [3, 4]. The mutated G allele is rather rare and only 0.24% were G homozygous [4]. Interestingly, two studies [5, 6] found that heterozygosity for this mutated TLR4 allele conferred susceptibility to Gram-negative infections or septic shock in intensive care unit patients.

When comparing the levels of TNF-α, IL-6, and IL-8, elicited after endotoxin stimulation of whole blood, no statistically significant differences were found in our study. The CD14 density of monocytes and the monocyte count were included as confounding parameters which, however, did not differ between the TLR4 genotypes. We used a whole blood assay that obviates the adherence-induced expression of cytokines, associated with the isolation of certain cell populations. In addition, whole blood probably represents the most physiological environment for cytokine-producing cells. Our result is in line with the findings by Schmitt *et al.* [10], when comparing TLR4 A896G heterozygotes and carriers of two wild type alleles. This group used a completely different methodological approach. Monocytes were isolated and the TNF-α mRNA production was analyzed. Moreover, a range of endotoxin concentrations was tested whereas we used only one concentration. Our assay with a concentration of 100 ng/mL endotoxin is a standard assay that has been used by us and others to study the cytokine response [11-16]. This assay was suitable for detecting significant associations of TNF haplotypes and of an IL-6 promoter polymorphism, with the synthesis of the respective cytokines [9, 11].

Kiechl and colleagues [4] found a significant difference in the plasma levels of IL-6 and various other inflammatory mediators between heterozygous carriers of the mutated allele and wild type homozygotes. It has to be pointed out that basal levels were assessed whereas we measured endotoxin-stimulated concentrations, reflecting the cytokine synthesis capacity after stimulation with the TLR4 ligand endotoxin. Moreover, IL-6 is produced by a variety of cells [17], and many more cell types may contribute to the basal plasma IL-6 levels measured by Kiechl *et al.* [4] as compared to the IL-6 levels only produced by blood cells in our experimental setting. However, we think it is unlikely that the effect of the TLR4 polymorphism is cell- or tissue-specific. An example of a cell type-dependent effect is the IL-6 promoter polymorphism – 174 G/C. The – 174 C allele was associated with a lower IL-6 synthesis by HeLa cells [18], a finding that could not be replicated when using ECV304 cells [19]. This discrepancy was attributed to the creation of a binding site for the transcription factor NF-1 by the C allele. NF-1 is a suppressor of gene transcription in HeLa cells [20], but not in ECV304 cells [21]. It has to be emphasized that the A896G mutation of TLR4 is not located within the promoter of the gene, but in the coding region. It affects the extracellular domain of the molecule. Replacement of aspartic acid by glycine at position 299 of the amino acid chain induces a

disruption of the a -helical protein structure [22]. In addition, the mutation can also affect the transport of the molecule to the cell membrane or ligand binding, as speculated by Arbour *et al.* [3].

Among the reasons for the lack of association between cytokine levels and the heterozygous TLR4 A896G genotype, genetic polymorphisms of the studied cytokines have to be mentioned. The TNF gene carries various polymorphic sites that have been related to TNF-α production. These include the TNF-α-308 G/A substitution [23], the TNF-β NcoI polymorphism [24], and a recently identified TNF-α-863 C/A polymorphism [25]. We described a linkage disequilibrium between the TNF-α-308 and TNF-β NcoI sites and the association of the haplotypes with the TNF-α synthesis [11]. The IL-6-174 G/C site has already been mentioned above [9, 18, 19]. It could be speculated that these cytokine genetic variations might outweigh the effect of a defective TLR4 molecule. Moreover, it can not be excluded that in TLR4 heterozygotes, the effect of one mutated allele is compensated for by the wild type allele. This could result in a surface density of TLR4 molecules that does not differ from that found in wild type homozygotes. Flow cytometric analysis could be helpful in answering this question.

Heterozygosity for TLR4 A896G was found to confer susceptibility to Gram-negative infections and septic shock in some studies [5, 6, 26], a finding that was not replicated by others [27]. Accordingly, a recent study by Erridge did not find differences in the cytokine response to endotoxin from various bacterial strains [28], supporting our finding but contrasting with *in vitro* studies that clearly showed a role for TLR4 in endotoxin signaling [29]. Alternatively, additional molecules might play a role in the host response to endotoxin. The latter speculation has gained some support by the findings of Arbour and colleagues [3] that not all of the hyporesponsive individuals were carriers of the mutated TLR4 allele, and conversely, that not all carriers were endotoxin hyporesponsive. Moreover, we used a commercially available endotoxin preparation that contained 1.4% protein according to the manufacturer's information. Traditional preparation methods do not remove substantial amounts of other substances that could have influenced our results [30, 31].

In summary, our results suggest that heterozygosity for the TLR4 A896G substitution is not associated with a cytokine-response to endotoxin that differs from wild type homozygotes.

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