CONCISE COMMUNICATION

Human Toll-Like Receptor 4 Mutations but Not CD14 Polymorphisms Are Associated with an Increased Risk of Gram-Negative Infections

Doreen M. Agnese,[a,b] Jacqueline E. Calvano,[a] Sae J. Hahm, Susette M. Coyle, Siobhan A. Corbett, Steve E. Calvano, and Stephen F. Lowry

Division of Surgical Sciences, Department of Surgery, University of Medicine and Dentistry New Jersey–Robert Wood Johnson Medical School, New Brunswick

Human toll-like receptor 4 (hTLR4) and CD14 are known to be components of the lipopolysaccharide receptor complex. Our study investigated the association between TLR4 mutations (Asp299Gly and Thr399Ile) and CD14 polymorphism(s) with outcome in an intensive care unit (ICU) population at risk for sepsis. By use of a polymerase chain reaction–based restriction fragment–length polymorphism analysis technique, the hTLR4 gene was altered in 14 (18%) of 77 ICU patients (all positive for systemic inflammatory response syndrome) and in 5 (13%) of 39 volunteers. There was a significantly higher incidence of gram-negative infection among patients with the mutations (11 [79%] of 14), compared with that in the wild-type population (11 [17%] of 63; $P = .004$). No association between CD14 polymorphism(s) and the incidence of infection or outcome was observed. These findings indicate that hTLR4 mutations are associated with an increased incidence of gram-negative infections in critically ill patients in a surgical setting.

Despite major advances in the understanding of sepsis and the inflammatory response, sepsis remains a significant problem worldwide. Septic shock, the most severe form of sepsis, results in >175,000 deaths annually in the United States. Approximately one-half of the cases of sepsis and septic shock are caused by gram-negative bacteria [1]. Lipopolysaccharide (LPS), a major component of the outer cell wall of gram-negative bacteria, is a key factor in eliciting the systemic inflammatory response that can lead to sepsis, organ failure, and septic shock [2]. Immune cell recognition of LPS involves an LPS receptor complex, of which CD14 and toll-like receptor 4 (TLR4) are important components [3].

Recently, base-pair changes in the genes for both CD14 and TLR4 have been described in humans. Arbour et al. [4] demonstrated 2 cosegregating missense mutations in the extracellular domain of the receptor of the human TLR4 (hTLR4) gene. The first is an A→G substitution at nucleotide 896 from the start codon of the hTLR4 gene, which results in an aspartic acid–to–glycine substitution at position 299 of the amino acid sequence (Asp299Gly). A cosegregating point mutation that results in a threonine-to-isoleucine substitution at position 399 of the amino acid sequence (Thr399Ile) also was identified. These mutations are associated with functional changes, as demonstrated by decreased airway responsiveness after LPS stimulation [4]. A polymorphism in the promoter region of the CD14 gene also has been described. This polymorphism involves a C→T substitution at base-pair −159 of the 5′ flanking region of the CD14 gene [5]. Genotypes include the CC homozygote, the CT heterozygote, and the TT homozygote. The TT homozygote has been associated with increased circulating soluble CD14 levels and a higher density of the monocyte CD14 receptor [5, 6].

Because of the important roles that hTLR4 and CD14 play with respect to LPS binding and signaling, we hypothesized that mutations in the hTLR4 gene and polymorphisms in the CD14 promoter may be important factors for determining susceptibility to gram-negative infections and may be predictive of outcome. In the present study, we investigate the association between hTLR4 mutations and CD14 polymorphisms and subsequent outcomes in a surgical intensive care unit (ICU) population. We describe the prevalence of these genetic variations in the surgical ICU populations and in healthy volunteers. In addition, we correlate the presence or absence of the mutation with the risk of gram-negative infection and death.

Patients, Materials, and Methods

Patients. After obtaining informed written consent, blood samples were collected from 77 sequential patients admitted to the

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Informed consent was obtained from all patients or a designated relative. Human experimentation guidelines of the Institutional Review Board of the University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School were followed in the conduct of this research.

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[a] These authors contributed equally to this work.

[b] Present affiliation: Division of Surgical Oncology, Department of Surgery, Ohio State University, Columbus.

Reprints or correspondence: Dr. Stephen F. Lowry, Dept. of Surgery, UMDNJ–Robert Wood Johnson Medical School, 125 Paterson St., New Brunswick, NJ 08903 (lowrysf@umdnj.edu).

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surgical ICU at the Robert Wood Johnson University Hospital who, by virtue of their primary disease process, were judged to be at risk for extended (>48 h) ICU stay. All ICU patients manifested ⩾2 of the systemic inflammatory response syndrome criteria (heart rate >90 beats/min, temperature >38°C or <36°C, respiratory rate >20 breaths/min or mechanical ventilation, and white blood cell count >12,000 or <4000 cells/mm^3). Patients receiving steroids were excluded from participation, because of the well-established immunomodulatory effects of steroids and their possible effects on outcome. There were 53 men and 24 women in this group, with ages ranging from 19 to 90 years. APACHE II and sequential organ failure assessment (SOFA) scores were calculated at the time of enrollment in the study.

Infection was defined as the presence of a positive culture from any site that, in the opinion of the treating intensive care physician, was of sufficient concern to initiate specific antibiotic therapy. Quantitative cultures were not used routinely.

Samples were obtained from 39 healthy volunteers over the same time period to serve as control samples. There were 27 men and 12 women, ranging in age from 19 to 51 years.

DNA purification. Whole blood was collected from the study subjects, and genomic DNA was extracted by use of the phenol/chloroform method, which was followed by ethanol precipitation.

hTLR4 mutation analysis. Screening for the hTLR4 gene mutations was accomplished by use of 50 ng of genomic DNA and a polymerase chain reaction (PCR)–restriction fragment–length polymorphism (RFLP) assay, as described by Lorenz et al. [7]. This technique alters the forward primers to create *Nco*I and *Hin*fI restriction sites in the mutant alleles and allows distinction between wild-type and mutant TLR4 alleles based on the presence of these restriction enzyme recognition sites. A portion of the resulting PCR product was digested overnight with the appropriate restriction enzymes (*Nco*I for Asp299Gly and *Hin*fI for Thr399Ile) and was visualized on 3% NuSieve gels (BioWhittaker Molecular Applications) stained with ethidium bromide. To verify the presence of the mutation, the wild-type primers were used to amplify genomic DNA from each of the samples. The appropriate-sized fragments were gel purified by use of the Qiagen gel purification kit and were sequenced by use of the Model 3100 automated sequencer (Applied Biosystems).

CD14 polymorphism analysis. Screening of the polymorphism at bp −159 of the 5′ flanking region of the CD14 gene was assessed by use of an AvaII PCR-based RFLP technique described by Baldini et al. [5]. The same subjects who were screened for hTLR4 also were screened for the CD14 genotypes. By use of primers that generated a 497-bp product of CD14, 100 ng of genomic DNA from each individual was amplified. Each product was gel purified and was digested with the restriction enzyme AvaII. All products were visualized on 2% agarose gels stained with ethidium bromide, to determine the appropriate genotype of each individual. To validate this method, 7 individuals were sequenced, as described above.

Statistical analysis. Data were analyzed by use of the 2-tailed Fisher's exact test. Significance was set at $P < .01$, to correct for multiple comparisons.

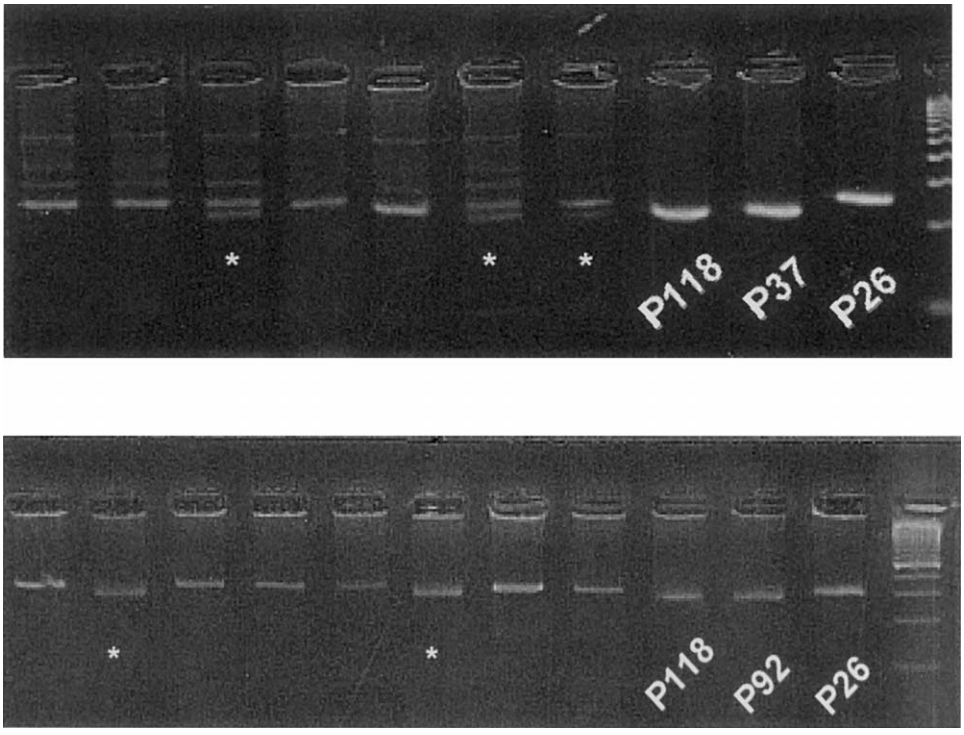

Figure 1. Representative digestions showing the Asp299Gly *(A)* and Thr399Ile *(B)* mutations. P118 is a plasmid that contains both mutations, P37 is a plasmid that contains the 299 mutation, P92 is a plasmid that contains the 399 mutation, and P26 is a plasmid that contains the wild type. Asterisks indicate individuals heterozygous for the respective mutations.

Results

Representative digestions demonstrating heterozygotes for the Asp299Gly and Thr399Ile mutations, as well as their plasmid controls, are shown in figure 1. All mutations were confirmed by direct sequencing.

The characteristics of the study population and relationship to TLR4 mutations are shown in table 1. The TLR4 gene was altered in 14 (18%) of 77 ICU patients and in 5 (13%) of 39 healthy volunteers. All individuals with TLR4 mutations were heterozygous for both the Asp299Gly and the Thr399Ile allele.

For these patients in the surgical ICU, there was a significantly higher rate of subsequent gram-negative infections in the hTLR4 mutant group (11 [79%] of 14), compared with that in the nonmutant group (for gram-negative infection only, 11 [17%] of 63; $P = .004$; for gram-negative and mixed infections, 14 [22%] of 63; $P = .015$). There was a trend toward an increase in 28-day all-cause mortality among the ICU patients with the mutation (5 [36%] of 14) versus no mutation (7 [11%] of 63; $P = .077$). However, the specific mortality for the patients with gram-negative infections, including mixed infections, was 45% (5 of 11) in the mutant group versus 36% (5 of 14) in the nonmutant group (P was not statistically significant). In the population screened for the CD14 polymorphism, 26% were CC homozygotes, 52% were CT heterozygotes, and 22% were TT homozygotes. No association between the CD14 polymorphism and the incidence of subsequent infection or outcome was observed.

Table 1. Characteristics of patients with and without toll-like receptor 4 (TLR4) mutations (Asp299Gly and Thr399Ile).

Characteristic	With TLR4 mutation ($n = 14$)	Without TLR4 mutation ($n = 63$)	P
Age, mean years	62.5	62.4	NS
Male	10 (71)	43 (68)	NS
Race/ethnicity			
White	12	46	NS
Black	1	8	NS
Hispanic	1	5	NS
Asian	0	3	NS
Other	0	1	NS
Mean SOFA score	7.5	6.2	NS
Mean APACHE II score	18	16.8	NS
Diagnosis			
Trauma	3 (21)	22 (35)	NS
Postoperative	11 (78)	41 (65)	NS
Thoracic/cardiac	3 (21)	18 (29)	NS
General abdominal	4 (29)	11 (17)	NS
Vascular	3 (21)	10 (16)	NS
Neurosurgical	1 (7)	2 (3)	NS
Primary site of infection			
Lung	6 (43)	16 (25)	NS
Urine	2 (14)	2 (3)	NS
Blood	2 (14)	4 (6)	NS
Other	1 (7)	2 (3)	NS
Multiple	1 (7)	2 (3)	NS
Microorganism			
Gram negative	11 (79)	11 (17)	.004
Gram positive	1 (7)	10 (16)	NS
Mixed gram positive and gram negative	0	3 (5)	NS
Other	0	2 (3)	NS
Mortality	5 (35)	7 (11)	NS

NOTE. Data are no. (%) of patients, unless otherwise indicated. NS, not significant; SOFA, sequential organ failure assessment.

Discussion

One significant challenge in the identification and appropriate treatment of sepsis is identifying the patient who is at an increased risk for sepsis or who, in fact, is infected but without culture documentation. Several approaches to this dilemma have been undertaken, including efforts to correlate soluble mediator or receptor levels to clinical outcomes. For instance, soluble proteins induced by systemic inflammation, such as C-reactive protein, may be useful for identifying patients with bacterial infections [8]. Inflammatory cytokines, such as interleukin (IL)–6, also may serve as markers of systemic inflammation and may identify patients at risk for complications [8]. Likewise, changes in cell-surface proteins have been used as markers for clinical course and outcome. Monocytes with persistently low levels of HLA-DR have been linked to a state of immune paralysis, which may identify a subset of patients at increased risk for the development of sepsis [9]. Our laboratory also has demonstrated that levels of monocyte tumor necrosis factor receptor may help distinguish survivors from nonsurvivors, once the diagnosis of severe sepsis is entertained [10]. Although markers can be shown to change with the clinical status of the patient and therefore may be useful for disease monitoring, they do not, per se, serve as useful markers for determining the risk of subsequent infection. Consequently, the identification of risk factors that correlate with the subsequent occurrence of infection and sepsis may promote earlier detection and treatment strategies.

Membrane CD14 forms a complex with LPS and LPS-binding protein; however, CD14 has no transmembrane domain and therefore cannot initiate intracellular signaling [11]. Recent evidence has suggested that TLR4 is a critical link between LPS/CD14 and subsequent intracellular signaling, making up that portion of the LPS receptor complex that enables intracellular signaling [12].

TLR4 is an evolutionarily conserved receptor that belongs to the toll-like/IL-1 receptor superfamily. The importance of TLR4 in the response to LPS was initially demonstrated by the observation that LPS-hyporesponsive C3H/HeJ mice have a point mutation in the TLR4 gene [13]. Subsequently, Arbour et al. [4] demonstrated the presence of a TLR4 mutation in humans and further demonstrated the importance of TLR4 by demonstrating an association of the specific nucleotide substitution with hyporesponsiveness to inhaled LPS.

The data reported here support the clinical importance of TLR4 in the response to LPS. In addition, these data provide the first evidence that human TLR4 mutations are associated with an increased incidence of gram-negative infection in an at-risk critically ill population.

A recent study investigated whether the presence of altered TLR4 alleles in patients with septic shock are associated with a more severe disease and less favorable outcome [14]. Ninety-

one patients with septic shock and 73 healthy control subjects were genotyped. A similar prevalence of the double mutation in both patient populations was noted. Gram-negative infection was identified in 50% of the patients with the wild-type alleles and in 100% of the patients with the Asp299Gly mutation. No significant differences were observed between the TLR4 genotype and mortality. Of note, that study did not address a population at risk for infection, because all the patients enrolled had septic shock at the time of study entry.

Hubacek et al. [15] have demonstrated that the CD14 polymorphism is not associated with an increased incidence of sepsis or mortality. The data presented here support this finding, because no difference in ICU infection or mortality incidence was detected among any of the CD14 genotypes.

In conclusion, the ability to identify subgroups of patients at risk for developing infection may be a critically important factor that will lead to improvements in the management of infection in at-risk patients. Because of the persistently high mortality associated with severe gram-negative infections in critically ill patients, awareness of such genetic predisposing factors may promote heightened surveillance and preventive measures. Doubtless, further studies will identify other genetic factors that are associated with risk for and response to infection.

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