

Systemic responsiveness to lipopolysaccharide and polymorphisms in the toll-like receptor 4 gene in human beings

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Background: The response to lipopolysaccharide exposure is highly variable and might be a result of genetic diversity between individuals. The toll-like receptor 4 (TLR-4) is the principal receptor for lipopolysaccharide.

Objectives: We investigated the association between single-nucleotide polymorphisms in the *TLR4* locus and levels of systemic inflammatory markers in response to lipopolysaccharide.

Methods: Healthy subjects (n = 116) were genotyped for the most frequent polymorphisms found in the promoter and coding region of the *TLR4* gene (-2026A/T, -1607T/C, +896A/G, and +1196C/T relative to the translation start site). Subjects were challenged with 20 µg lipopolysaccharide by inhalation. **Results:** Polymorphisms at +896 and +1196 were in complete linkage disequilibrium, and no homozygotes for the less common allele, G and T respectively, were found. After lipopolysaccharide inhalation, subjects heterozygous for either TLR-4/+896 or TLR4/+1196 had significantly lower numbers of white blood cell counts and lower levels of C-reactive protein and lipopolysaccharide-binding protein compared with homozygotes with the common allele. None of the heterozygous subjects (n = 18) except 1 were high responders to lipopolysaccharide (defined as a rise in C-reactive protein > 10 mg/L), whereas 36 of 98 homozygous subjects were high responders (P < .02). No association was observed between the

TLR-4/-2026 and TLR-4/-1607 polymorphisms and lipopolysaccharide responsiveness.

Conclusion: The single-nucleotide polymorphisms at position +896 or +1196 in the *TLR-4* gene is associated with systemic inflammatory hyporesponsiveness to inhaled lipopolysaccharide. (*J Allergy Clin Immunol* 2003;112:923-9.)

Key words: Endotoxin, lipopolysaccharide, toll-like receptor-4, inflammation, C-reactive protein, leukocytosis

Innate immunity plays a primary role in infectious and noninfectious diseases and is initiated by the recognition of microbial determinants, such as gram-negative LPS, the active part of endotoxin.¹ Successful defense against gram-negative infections, such as in sepsis, relies on the innate immune system to release proinflammatory cytokines that destroy pathogens and determine the localization and extent of the challenge.² Noninfectious exposure to environmental LPS,³ in either the domestic⁴⁻⁶ or occupational^{3,7-10} setting, is associated with the development and progression of inflammatory airway diseases, such as asthma, atopy,^{5,6,11} and occupational chronic obstructive pulmonary diseases (COPDs).⁷⁻¹⁰

The toll-like receptor 4 (TLR-4) is the principal receptor for LPS. In the extracellular compartment of the body, LPS binds to the LPS-binding protein (LBP) and CD14,^{1,12} followed by a coupling to the transmembrane receptor, TLR-4.¹³ This complex initiates a complex signaling cascade, resulting in systemic inflammation mediated by monocytes and macrophages.¹⁴⁻¹⁶ Considerable differences in LPS responses are present between related species¹⁷ and within the same species.¹⁸ In humans, challenge studies show a large variability in the bronchoconstrictive and inflammatory responses to LPS given by inhalation^{14,19-23} or intravenously.^{15,16} Evidence for a genetic control of responses to LPS was initially provided in studies of C3H/HeJ and C57BL/10ScCr mouse strains.^{18,24} Mutations of *TLR4* were shown to abolish responses to endotoxin in mice,²⁵⁻²⁷ causing these animals to be hypersensitive to infection by gram-negative bacteria.^{28,29} As such, it might be expected that genetic variability in the human *TLR-4* gene could impair host responses in infectious as well as noninfectious diseases. Our group has screened the entire *TLR-4* gene by direct sequencing and found 44 polymorphisms among Euro-

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Abbreviations used:

COPD: Chronic obstructive pulmonary disease
 CRP: C-reactive protein
 FEV₁: Forced expiratory volume in 1 second
 LBP: LPS-binding protein
 PCR: Polymerase chain reaction
 TNF: Tumor necrosis factor
 TLR-4: Toll-like receptor 4
 WBC: White blood cell

pean, Hispanic, African-American, and asthmatic subjects.³⁰ Arbour and coworkers³¹ previously described 2 of the 44 polymorphisms. One of these, an A-to-G transition at +896 bp downstream of the translation start site of *TLR4*, results in replacement of an aspartic acid residue with glycine at amino acid 299. The second polymorphism was in complete linkage disequilibrium with *TLR4*/+896 and was a C-to-T transition at +1196. These polymorphisms were shown to have functional implications for LPS responsiveness. Notably, they were associated with a blunted bronchoconstrictive response to inhaled LPS.³¹

The aim of the present study was to investigate whether common polymorphisms in the *TLR-4* gene are associated with a systemic inflammatory response to small doses of LPS. Using DNA obtained from 116 subjects, we genotyped 4 common *TLR4* polymorphisms in the promoter and coding region of the gene. We subsequently evaluated these variants for evidence of association with inflammatory markers, such as peripheral white blood cells, C-reactive protein (CRP), and LBP after LPS inhalation.

METHODS

Subjects

One hundred eighteen subjects were recruited from Belgium and Sweden. A total of 77 subjects were from Belgium, 38 retrospective subjects (Belgium-1) and 37 prospective subjects (Belgium-2). The retrospective subjects had been previously challenged with LPS by the same procedure as that used in the current study and whose plasma samples had been kept frozen until analysis (see below). The remaining 41 subjects were recruited prospectively from Sweden. Inclusion criteria were healthy, nonsmoking, Caucasian adults between the ages of 18 and 50 years without any history of chronic disease, including asthma, or taking medication regularly. The Human Subjects Committees of Goteborg University and the St-Pierre University Hospital approved the protocols for challenges and for genetic determination. Written, informed consent was obtained from each subject.

LPS challenge

Subjects were exposed to LPS by inhalation challenge. Purified LPS (*Escherichia coli* O26:B6, lot-2654; Sigma Chemical Co, St Louis, Mo) was prepared in saline at a final concentration of 1 mg/mL. The LPS solution was aerosolized in dosimetric nebulizers (Mefar MB3 Inc, Belgium populations; PariBoy, Sweden population) delivering 5 μ L per aerosol over 0.6 second, corresponding to 5 μ g LPS. In immediate succession, 4 such doses were given, yielding a total inhaled dose of 20 μ g. In previous studies, this dose had

been shown to cause an inflammatory response in the absence of significant adverse symptoms.^{14,23,32} The nebulizers were calibrated regularly by the same procedure in both laboratories. Markers of systemic inflammation were assessed 6 and 24 hours after LPS challenge. These time intervals were chosen based on data from previous studies.^{14,23,32}

Clinical evaluation

A medical history, including respiratory and allergic symptoms, was obtained by questionnaire for each subject. Skin-prick tests were conducted with extracts of indoor and outdoor allergens (dust mite, cat, birch, and grass pollen from Stallergene, France). Subjects were considered to be skin-test-positive if they showed a wheal diameter ≥ 4 mm for 1 or more of the allergens.³³

Total and differential white blood cell (WBC) counts were assessed by an automated counter (H1 Counter, Technicon Instruments, Tarrytown, NY) for the Belgium populations or manual counting with Giemsa stain for the Swedish population. For technical reasons, data were not obtained for 12 individuals. Plasma concentrations of CRP for each population were determined by immunonephelometry with antibody-coated, polystyrene particles (N Latex High Sensitivity CRP, Dade Behring, Marburg, Germany). The lower detection limit of the assay was 0.175 mg/L. The plasma concentration of LBP was measured with an enzyme-linked immunosorbent assay, as reported.³⁴ Recombinant LPB was used as a standard. The detection limit of the assay was 0.2 μ g/L. The LBP was not measured 6 hours after LPS inhalation because in our experience, there is no significant change at this time.²³

To ensure consistence between the Belgium and Sweden study groups, all CRP determinations were performed in 1 laboratory (O.M.) on the same occasion, as well as LBP determinations (M.D.).

Molecular genetic analysis

A sample of whole blood was obtained from each subject. Genomic DNA was extracted by standard protocols. Genotyping of subjects for the *TLR4* polymorphisms was performed with a polymerase-chain-reaction (PCR)-based, restriction-fragment analysis assay. For detection of the *TLR4*/+896 polymorphism, PCR was performed in a final volume of 15 μ L containing 20 ng genomic DNA, 200 μ mol/L dNTP, 0.75 mmol/L MgCl₂, 1 $\times$ buffer (Life Technologies, Rockville, Md), 0.4 U platinum *Taq* polymerase (Life Technologies), and 0.1 μ mol/L of the forward primer 5'-GATTAG-CATACTTAGACTACTACCTCCAT and the reverse primer 5'-CTAAGCCCAAGAAGTTTGAACCC. Cycling conditions were 96°C for 2 minutes; 35 cycles of 96°C for 30 seconds, 62°C for 30 seconds, and 72°C for 40 seconds; and a final extension at 72°C for 10 minutes. PCR-amplified DNA was digested with 0.2 U *Nco*I (New England Biolabs Inc, Beverly, Mass) at 37°C for 16 hours. Products were separated on a 3.0% agarose gel. *Nco*I is specific for the sequence CCATGG, which is present in the PCR product only among carriers of the *TLR4*/+896G allele. The assay thus yields one 373-bp band for the AA genotype and 3 bands of 373, 347, and 26 bp for AG heterozygotes.

A similar PCR was performed for the *TLR4*/+1196 assay with the forward primer 5'-GTTCTCAAAGTGATTTGGGATGA and the reverse primer 5'-AAGAATTGCCAGCCATGATCAAG. PCR-amplified DNA was digested with 0.2 U *Bcl*II (New England Biolabs Inc) at 50°C for 16 hours. Products were separated on a 3.0% agarose gel. *Bcl*II is specific for the sequence TGATCA, which is present in the PCR product only among carriers of the *TLR4*/+1196T allele. The assay thus yields one 256-bp band for CC subjects and 3 bands of 256, 234, and 22 bp for CT heterozygotes. Primers for the *TLR4*/-1607 assay were (forward) 5'-CATTTTACA-GACCAGAAAGTAATAATACG and (reverse) 5'-CACGCGTC-

CATGAACAGACAAAT. Amplification reactions were carried out as detailed previously with an annealing temperature of 57°C. The PCR product was digested overnight with *MluI* (New England Biolabs) at 37°C, yielding fragments of 249 and 276 bp for the C and T alleles, respectively. Primers for *TLR4*-2026 assay were (forward) 5'-GTAGAACTATCTAGGACTTAGCGT and (reverse) 5'-GCAATGGTAGTCAAATTGTAC. A similar PCR was performed as described earlier with an annealing temperature of 57°C. The PCR product was digested overnight with *RsaI* (New England Biolabs) at 37°C, yielding fragments of 333 and 309 bp for the G and A alleles, respectively.

The *TLR4* locus has many rare polymorphisms. The polymorphisms genotyped for this study were selected because of either their high frequency (> 5% for the minor allele) in the proximal promoter region or their functional significance as LPS-hyporesponsive variants.

Statistical analysis

All of the results are expressed as mean $\pm$ SE. In each of the 3 studied groups of subjects, the responses in CRP, LBP, and WBCs were expressed in absolute values, and their changes were compared with their value measured before LPS inhalation. A 2-way ANOVA was performed on each parameter (factors: group and time).³⁵ Unpaired, nonnormally distributed data were tested by the Mann-Whitney nonparametric test. χ^2 tests were used to assess statistical significance in bivariate analyses. All statistical tests were 2-sided, with $P < .05$ considered significant.

RESULTS

Assessment of inflammatory markers after LPS challenge

This study comprised 3 groups of subjects (Belgium-1, Belgium-2, and Sweden). The LPS exposure was well tolerated, and no serious adverse reaction was observed. The inflammatory response to LPS inhalation was ascertained by WBC counts and plasma levels of CRP and LBP. As shown in Fig 1 (bottom), the WBC count for each subpopulation increased approximately 2-fold 6 hours after LPS inhalation ($P < .001$) but returned to baseline levels after 24 hours. The change in WBC counts (Δ WBC) 6 hours after LPS challenge was similar for each subpopulation (Table I). Notably, the plasma concentration of both CRP (Fig 1, top) and LBP (Fig 1, center) increased 3- to 4-fold and 2-fold, respectively, in response to LPS inhalation ($P < .001$), and the change (Δ CRP and Δ LBP) at 24 hours was similar for each subpopulation (Table I). The basal level of CRP was different (F test = 6.94, $P < .001$) in the 3 subgroups (Fig 1, top). We speculate that these differences might be due to the collection of samples during different seasons of the year. The amplitude of the LPS response (mean WBC counts and plasma levels of CRP and LBP) for each group was, however, not significantly different. The groups were thus pooled for further data analysis.

The demographics are shown in Table I. The characteristics were very similar for each subgroup. The male-female ratio and the proportion of atopic subjects, defined according to the skin-prick test, were the same for each subgroup, although the mean age was lower in the Swedish sample than in the Belgian sample.

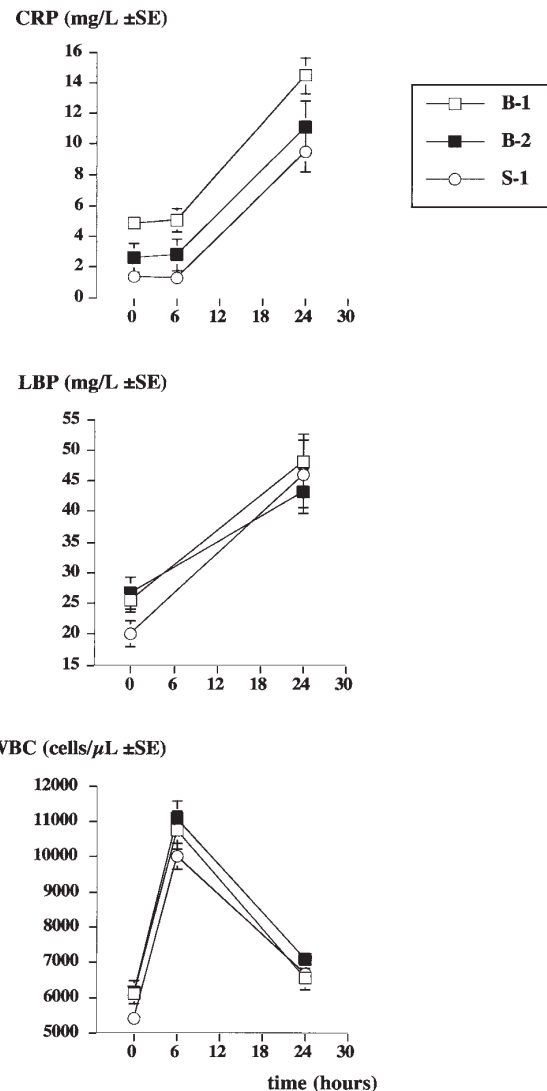


FIG 1. The systemic response before (time 0) and 6 and 24 hours after inhaled LPS. Results are mean $\pm$ SE. The responses related to time were significant (CRP: F test = 137.9, $P < .0001$; LBP: F test = 98.9, $P < .0001$; WBC: F test = 251.9, $P < .0001$). Comparison of the responses between groups was not statistically different (CRP: F test = 0.41, $P = \text{NS}$; LBP: F test = 2.02, $P = \text{NS}$; WBC: F test = 0.33, $P = \text{NS}$).

Assessment of *TLR4* single-nucleotide polymorphisms

We genotyped 116 subjects for 2 common polymorphisms in the promoter region of *TLR4*: an A-to-G transition at -2026 bp (*TLR4*-2026) and a T-to-C transition at -1607 from the translation start site. Allelic frequencies for *TLR4*-2026A and *TLR4*-1607T were 61.7% and 90.3%, respectively (data not shown). The distribution of alleles was similar in each subpopulation and in conformance with Hardy-Weinberg equilibrium.

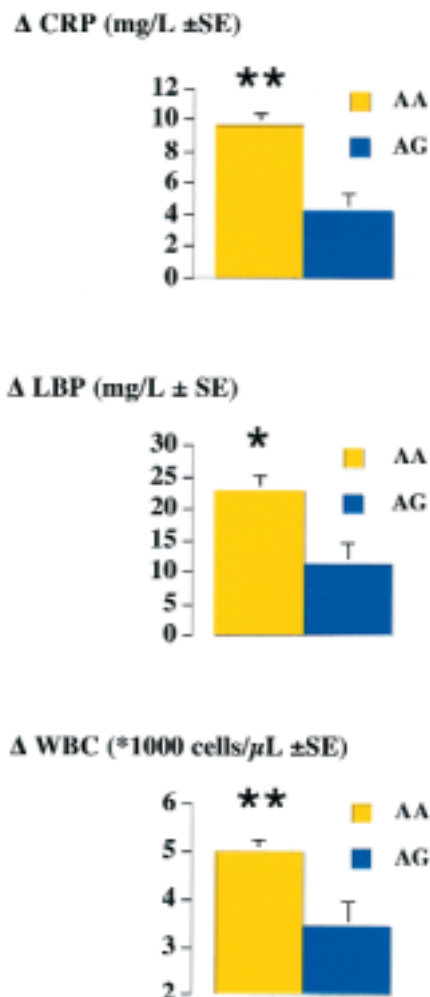


FIG 2. The amplitude of the CRP, LBP, and WBC responses to inhaled LPS in subjects with the +896 AA (AA, n = 98) compared with subjects with the +896 AG *TLR-4* allele (AG, n = 18). Mann-Whitney tests: **P* < .05, ***P* < .02. All data are mean ± SE.

Two additional missense *TLR4* polymorphisms at +896 bp (*TLR4*/+896) and +1196 bp (*TLR4*/+1196) were genotyped. The frequency of the A allele was found to be 92.3% for the entire population and similar for each subpopulation (Table II). No subjects homozygous for the G allele were detected. The polymorphism at +1196C/T was determined to be in complete linkage disequilibrium with *TLR4*/+896 by the population genetic-analysis software Arlequin 2.0 by Laurent Excoffier. In contrast, there was no significant linkage between *TLR4*/+896 and *TLR4*/-2026 or *TLR4*/-1607. In the following section, reference will thus be made to *TLR4*/+896 only.

Association between responses to inhaled LPS and *TLR4* polymorphisms

A significant relation was found between the *TLR4*/+896 variant and the effects of LPS inhalation, independent of age, sex, or atopy (Table III). Subjects heterozygous for *TLR4*/+896 were hyporesponsive to

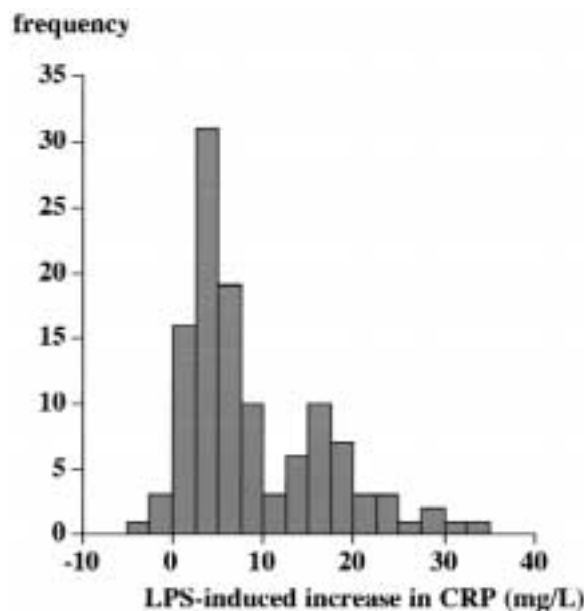


FIG 3. The histogram of the rise in blood CRP measured 24 hours after LPS inhalation.

LPS inhalation, as indicated by the decreased amplitude of the markers of inflammation (ΔCRP, ΔLBP, and ΔWBC; Fig 2). There was no significant association between the *TLR4*/-2026 and *TLR4*/-1607 polymorphisms and age, sex, and atopic status.

CRP levels, expressed as the difference between pre-exposure and 24-hour postexposure levels, showed a bimodal distribution, as shown in Fig 3. An arbitrary cut-off value for CRP (10 mg/L) was chosen to define subjects into low responders (n = 79) and high responders (n = 37). None of the heterozygous subjects (n = 18) except 1 were high responders to LPS, whereas 36 of 98 homozygous subjects were high responders (*P* < .02; Table III).

DISCUSSION

The results demonstrate that 2 common, nonsynonymous, single-nucleotide polymorphisms in the coding region of the *TLR4* gene are in complete linkage disequilibrium and are associated with low systemic inflammatory sensitivity to inhaled LPS, independent of atopic status. After LPS challenge, subjects heterozygous for either *TLR4*/+896 or *TLR4*/+1196 had significantly lower levels of WBC counts, CRP levels and LBP levels compared with homozygotes with the common allele. In contrast, the proximal promoter polymorphisms *TLR4*/-2026 and *TLR4*/-1607 were not associated with LPS-induced markers of systemic inflammation.

The amplitude of the LPS response was very similar for the 3 groups, thereby justifying amalgamation of the data of all groups for further analyses. These results con-

TABLE I. Study population characteristics

	Belgium-1 n = 38	Belgium-2 n = 37	Sweden n = 41	Total population n = 116	P-value ¶
Age (yrs ± SE)	32.6 (1.6)	35.3 (1.7)	24.6 (0.8) *	30.6 (0.5)	0.0001
Female (%)	44.7	51.3	51.2	49.2	NS
Skin-test reactivity (%)	39.5	29.7	31.7	33.6	NS
WBC (± SE)†	4.7 (0.5)	4.8 (0.5)	4.6 (0.4)	4.7 (0.2)	NS
CRP (± SE)‡	9.6 (1.2)	8.6 (1.4)	8.3 (1.3)	8.8 (0.7)	NS
LBP (± SE)§	22.5 (4.3)	16.1 (2.7)	26.3 (4.3)	21.2 (2.2)	NS

Belgium-1 and Belgium-2 are retrospective and prospective populations, respectively; Sweden is a Swedish prospective population.

*P < 0.001 for Sweden vs Belgium-1 and Belgium-2.

†WBC = increase in WBC (white blood cells × 1000 cells/μL) 6 h after LPS inhalation.

‡CRP = increase in CRP (C-reactive protein, mg/L) 24 h after LPS inhalation.

§LBP = increase in LBP (LPS-binding protein, mg/L) 24 h after LPS inhalation.

¶NS denotes nonsignificant.

TABLE II. Genotype frequency (%) of the TLR4/+896 polymorphism

TLR4/+896 Genotype	Belgium-1 n = 38	Belgium-2 n = 37	Sweden n = 41	Total n = 116
AA	86.8	81.1	85.4	84.5
AG	13.2	18.9	14.6	15.5
GG	0	0	0	0

Belgium-1 and Belgium-2 are retrospective and prospective populations, respectively; Sweden is a prospective population.

firm previous findings from studies on LPS inhalation, wherein an increase in WBCs/neutrophils, CRP, and LBP has been found.^{14,23,32} In humans, bronchial LPS instillation activates effector cells (macrophages and epithelial cells) that lead to transcription of cytokines (tumor necrosis factor [TNF], IL-1, IL-6, granulocyte colony stimulating factor, and granulocyte-macrophage colony stimulating factor) and chemokines (IL-8 and monocyte chemoattractant protein-1).³⁶ Subsequently, migration of neutrophils occurs from the blood into the lung and the airways,^{14,23,32,36} in association with an increase in blood neutrophil number.^{23,32} Cytokines IL-1, IL-6, and TNF induce the acute-phase protein response (CRP and LBP) by hepatocytes.³⁷

Markers of systemic inflammation were used as a measure of LPS response rather than pulmonary function for several reasons. Our previous findings have demonstrated a dose-response relationship between inhaled LPS and WBC, CRP, and LBP responses.^{14,32} In healthy subjects, these systemic inflammatory markers were considerably more sensitive indicators to inhaled LPS than is lung function (FEV₁). Five μg LPS was associated with a significant increase in CRP, WBC, and LBP, whereas inhalation of 50 μg is required to produce an FEV₁ response.^{23,32} Notably, responses measured with these inflammatory markers were not correlated with the degree of bronchoconstriction induced by LPS inhalation.^{22,23} In addition, the individual degree of nonspecific airway responsiveness, which is largely modulated by environmental factors,³⁹ determines the amplitude of bronchial obstruction to LPS.^{21,22,38}

Sequencing of the *TLR4* gene in humans and mice reveals that the TLR-4 is a polymorphic protein, the extracellular domain being the most variable among both strains and species.¹⁷ The results from our study by and

TABLE III. TLR4/+896 genotypes in relation with population characteristics and LPS effects

	+896 AA n = 98	+896 AG n = 18	P value
Age (yrs ± SE)	30.8 (1.0)	29.9 (2.4)	NS
Female	50 (51%)	7 (39%)	NS
Skin-test reactivity	33 (34%)	6 (33%)	NS
WBC (± SE)*	5.01 (0.25)	3.43 (0.54)	< 0.02
CRP (± SE)†	9.61 (0.82)	4.22 (1.08)	< 0.01
LBP (± SE)‡	22.8 (2.5)	11.3 (3.3)	< 0.05
LPS high responders§	36 (37%)	1 (6%)	< 0.02

*WBC = increase in white blood cells (× 1000 cells/μL) 6 h after LPS inhalation.

†CRP = increase in C-reactive protein (mg/L) 24 h after LPS inhalation.

‡LBP = increase in LPS-binding protein (mg/L) 24 h after LPS inhalation.

§LPS high responders are defined as those subjects with a ΔCRP ≥ 10 mg/L 24 h after LPS inhalation.

large confirm the findings of Arbour and coworkers.³¹ They found that 1 of 83 subjects was homozygous for *TLR4*+896G. This single subject was hyporesponsive to inhaled LPS, as measured by changes in FEV₁ (0.28% decline in FEV₁/μg of inhaled LPS) compared with 73 subjects homozygous for the common A allele (1.86% decline in FEV₁/μg of inhaled LPS). It was not reported whether the subjects homozygous for the A allele had a statistically significant difference in the amplitude of the FEV₁ responses to LPS compared with heterozygote subjects. In our population, no *TLR4*+896G homozygote was detected; however, the frequency of the A (92.3%) and G (7.8%) alleles at *TLR4*+896 were consistent with those found by Arbour et al (93.4% and 6.6%, respectively). Our present data extend these findings by demonstrating, for the first time, differences in the amplitude of systemic reactivity to LPS among subjects heterozygous for *TLR4*+896 and *TLR4*+1196.

A possible molecular mechanism to support a lower LPS responsiveness in heterozygotes has been described by Arbour and colleagues.³¹ They have shown that THP-1 transfected cells expressing the immune proteins Asp299Gly or Thr399Ile have a decrease in LPS-induced nuclear factor- κ B activity and IL-1 production.³¹ As a consequence, individuals with the *TLR4* variants might have an impaired innate immune response on LPS exposure. We have associated these *TLR4* mutations to LPS hyporesponsiveness; however, we cannot exclude the possibility that another gene(s) linked to the *TLR4* loci might control this response. Recent studies evaluating the phenotypic response to LPS in animal models suggest that genes other than *TLR4* are involved in LPS responsiveness.⁴⁰ For example *CD14*⁴¹ and *MyD88*⁴² knockout mice and the *Ran* mutation⁴³ were found to be at least 10-fold less sensitive to LPS than wild-type control animals. Consistent with these findings, we observed that most subjects with a polymorphism at *TLR4*/+896 or *TLR4*/+1196 were low responders to LPS (94%), yet 63% of subjects without the polymorphism were also low responders. This suggests that, as in animal models, a number of genes involved in the LPS signaling pathway might be important in LPS response in humans.

Humans have evolved to detect low levels of LPS in response to infection. A patient with recurrent bacterial infections was found to be hyporesponsive to LPS and IL-1 in association with a defect very early in the signal-transduction pathway.⁴⁴ In humans, the *TLR4* Asp299Gly mutation predisposes individuals to gram-negative sepsis.⁴⁵ It has also been shown during sepsis that overstimulation by LPS leads to acute respiratory distress syndrome in association with increased markers of inflammation.⁴⁶ This suggests that LPS sensing by *TLR4* is important in host defense and that mutations in the *TLR4* gene could be detrimental in some infectious diseases, whereas it could be a protective factor against the risk of developing acute respiratory distress syndrome, giving a possible explanation for this polymorphism to be so common. The same mutation does not influence susceptibility to or the severity of meningococcal disease.⁴⁷ In this case, there is evidence that the gram-negative bacterium *Neisseria meningitidis* contains components other than LPS that can elicit responses via TLR-2 or other TLR/cofactors, independent of TLR-4/MD2.⁴⁸

Exposure to environmental LPS³ has been reported as a risk factor in several acute and chronic inflammatory diseases, such as periodontitis,⁴⁹ osteoporosis, asthma,^{5,6,11} COPD,⁷⁻¹⁰ and autoimmunity.² In view of the present data, it is plausible to surmise that polymorphisms in the LPS receptor system might play a role in susceptibility to these diseases and thus, might be an important risk factor for morbidity and mortality associated with LPS exposure.

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