Asp299Gly and *Thr399Ile* Genotypes of the *TLR4* Gene Are Associated With a Reduced Prevalence of Diabetic Neuropathy in Patients With Type 2 Diabetes

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OBJECTIVE — To establish whether single nucleotide polymorphisms (*Asp299Gly* and *Thr399Ile*) of the toll-like receptor 4 have an association with late diabetic complications.

RESEARCH DESIGN AND METHODS — The study was conducted in 246 type 1 and 530 type 2 diabetic patients. The alleles of both polymorphisms were detected using PCR and subsequent cleavage by *Nco*I and *Hin*fI restriction endonucleases.

RESULTS — No difference was found between type 1 and type 2 diabetic patients in the prevalence of alleles of the *Asp299Gly* and *Thr399Ile* polymorphisms. In most cases, the alleles *Gly299* and *Ile399* occurred in a co-segregatory manner. The prevalence of the *Gly299/Ile399* haplotype was 10.6 and 12.1% in type 1 and type 2 diabetic patients, respectively ($P = 0.63$). No association with diabetic nephropathy or diabetic neuropathy was found in type 1 diabetic patients. In type 2 diabetic patients, however, heterozygote carriers of the *Asp299Gly* and *Thr399Ile* genotypes had a significantly reduced prevalence of diabetic neuropathy (odds ratio 0.35 [95% CI 0.19–0.61]; $P = 0.0002$); no association with diabetic nephropathy was found.

CONCLUSIONS — Our data indicate that *Asp299Gly* and *Thr399Ile* genotypes of the *TLR4* gene are associated with reduced prevalence of diabetic neuropathy in type 2, but not in type 1, diabetes. Thus different mechanisms may be involved in the pathophysiology of diabetic neuropathy in type 1 and type 2 diabetes.

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Recently, our understanding of type 2 diabetes has changed considerably. Levels of C-reactive proteins have been shown to predict the occurrence of type 2 diabetes. Studies in animal models as well as humans have suggested that type 2 diabetes might be associated with changes in the innate immune response (1–5). Furthermore, experiments in which the mitogen-activated protein kinase or inhibitor κB-kinase pathways are genetically controlled have shown that activator protein-1 and nuclear factor (NF)-κB are central regulators not only of inflammatory reactions (6), but also of the insulin response and glucose metabolism (7,8). In addition, one of the receptors important for developing late diabetic complications, the receptor for advanced glycation end products (RAGE), has been shown to participate in the innate immune response and behave as a pattern recognition receptor (9,10). This implies that factors regulating the innate immune response might be also involved in late diabetic complications. One of the central regulators of the innate immune response is the toll-like receptor (TLR)-4 (11). The recognition of microbial components by mammalian TLRs plays an important role in activation of the innate immune response and subsequent proinflammatory reactions. In addition to binding lipopolysaccharide (LPS), TLR-4 also interacts with endogenous ligands such as oxLDL, heat shock proteins 60 and 70, fibrinogen, and fibronectin (11,12), which are also elevated in diabetes (13–17).

Two common single nucleotide polymorphisms have been found in the coding region of the human *TLR4* gene at exon 3 that are in a tight linkage disequilibrium (18). They lead to amino acid exchanges in positions 299 (*Asp299Gly*) and 399 (*Thr399Ile*). It has been shown that the presence of *Gly299* and *Ile399* alters the structure of the extracellular domain of TLR-4 (19), which might influence the ligand binding. The functional significance of these polymorphisms has recently been demonstrated in experiments showing an association of hyporesponsiveness to inhaled LPS (19). The carriers of the *Asp299Gly* genotype have reduced

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Abbreviations: AF, allelic frequency; CR, carrier rate; LPS, lipopolysaccharide; NF, nuclear factor; RAGE, receptor for advanced glycation end products; TLR, toll-like receptor.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

levels of proinflammatory cytokines, acute phase reactions, soluble adhesion molecules, and an increased risk of severe infections but at the same time have a lower risk of carotid atherosclerosis (12).

These data suggest that the *Asp299Gly* polymorphism of *TLR4* might also be of importance in noninfectious diseases associated with inflammatory reactions. Because the accumulated evidence suggests that acquired immunity and inflammation as well as oxidative stress and apoptosis may play a crucial role in the pathogenesis of late diabetic neuropathy (20–24), we studied whether the modulated innate immune response caused by these polymorphisms of *TLR4* has any association with neuropathy and nephropathy in type 1 and type 2 diabetes.

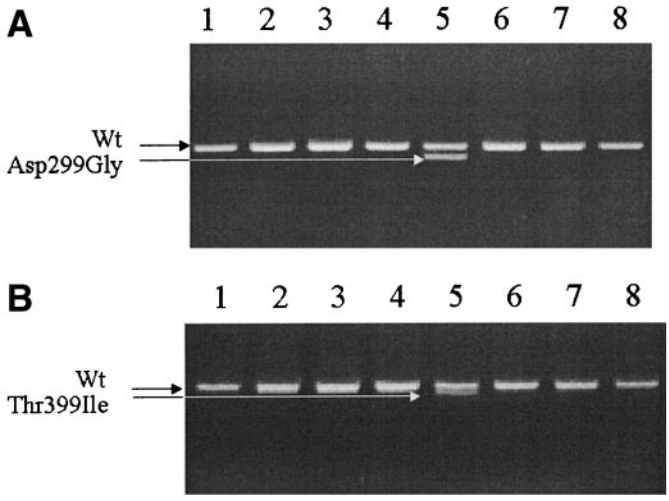

Figure 1—*Electrophoretic separation of PCR fragments containing* Asp299Gly *and* Thr399Ile *polymorphic sequences after restriction endonuclease cleavage (type 2 diabetic patients) on a 3% agarose gel.* A*: Alleles of the* Asp299Gly *polymorphism of* TLR4 *in eight type 2 diabetic patients. A heterozygous carrier is shown in* lane 5. B*: Alleles of the* Thr399Ile *polymorphism of* TLR4 *in eight type 2 diabetic patients. A heterozygous carrier is shown in* lane 5.

RESEARCH DESIGN AND METHODS

— The study groups consisted of 776 Caucasian subjects, 246 with type 1 diabetes and 530 with type 2 diabetes. Patients were consecutively enrolled between January 1998 and October 2002. The patients were recruited from the outpatient clinic of the Department of Endocrinology (University of Heidelberg, Germany). Because the study was performed as a monocentric study, care was taken to avoid that any carriers of the polymorphisms studied were related to each other. To make that determination, the study subjects were questioned about whether they had diabetic family members being treated in the recruiting center; those who answered affirmatively were excluded from the study.

Diagnostic criteria

Diabetes was diagnosed according to American Diabetes Association criteria. Mean duration of diabetes was 11.5 ± 0.8 years. Diabetic nephropathy was defined as microalbuminuria >20 mg/l in two or three samples of morning urine obtained within 12 months. Patients with urinary infections were excluded. The absence or presence of neuropathy was defined according to Diabetes Control and Complications Trial criteria (25). It was diagnosed by decreased or missing ankle reflex and symptoms, reduced vibration perception with symptoms, or neuropathic foot ulcer. Symptoms were defined as numbness, dysesthesias or paresthesias, hypersensitivity to touch, burning pain, aching, or stabbing pain in hands and/or feet. Patients with ischemic-related diabetic foot disease were excluded. Patients were not stratified according to the severity of diabetic complications.

Genotyping

Genomic DNA was prepared from peripheral blood using the QIAmp Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The alleles of *TLR4*, the *Asp299Gly* and *Thr399Ile* polymorphisms, were detected using PCR and subsequent cleavage by *Nco*I and *Hin*fI restriction endonucleases (26) (Fig. 1). The *TLR4* alleles were confirmed by sequencing, as previously described (26).

Statistical analysis

Group characteristics were presented by type of diabetes, expressed as mean values and asymptotic 95% CI for continuous data. For binary data, the rate and asymptotic 95% CIs were given. This description was for the investigation of homogeneity of groups. All *P* values given in Table 1 were for exploratory use only. The two primary outcomes (neuropathy and nephropathy) were analyzed with a multivariable logistic regression model. Covariates were included for adjustment of potential confounding factors (age [years], sex, diabetes duration [years], hypertension status, HbA_{1c} [percent]). Sensitivity analysis was used to categorize the three continuous covariates using the quartiles. Because of the two primary outcomes, an α level of 2.5% was used to guarantee a global error rate of 5%. This analysis was done separately because of the different pathogenetic backgrounds of type 1 and type 2 diabetes. For justification, a model with the interaction terms diabetes type and *TLR4* was calculated. Because of the low power of this statistical method, $P < 0.20$ was considered to indicate an interaction between diabetes type and *TLR4* polymorphisms. The statistical packages SPSS (release 11.0) and SAS (release 8.1) were used.

RESULTS

— The study was performed as a monocentric cross-sectional pilot study, in which 776 Caucasian patients with type 1 ($n = 246$) or type 2 diabetes ($n = 530$) were analyzed.

In the type 1 diabetic group, 27 of the 246 patients were heterozygous for the *Asp299Gly* polymorphism (carrier rate [CR] 11.0%, allelic frequency [AF] 5.5%), 27 patients were heterozygous for the *Thr399Ile* polymorphism of *TLR4* (CR 11.0%, AF 5.5%), and 26 patients carried both heterozygous genotypes (CR 10.6%). In the type 2 diabetic group, 68 of the 530 patients were heterozygous for the *Asp299Gly* polymorphism (CR 12.8%, AF 6.4%), 67 patients were heterozygous for the *Thr399Ile* polymorphism of *TLR4* (CR 12.6%, AF 6.3%), and 65 patients carried both heterozygous genotypes (CR 12.3%). Only 1 patient with type 2 diabetes was homozygous as a car-

Table 1—*Baseline characteristics of type 1 and type 2 diabetic patients with different genotypes of the TLR4 polymorphisms*

	Type 1 diabetic patients			Type 2 diabetic patients		
	Asp/Asp and *Thr/Thr* genotypes	*Asp/Gly* and *Thr/Ile* genotypes	*P*	*Asp/Asp* and *Thr/Thr* genotypes	*Asp/Gly* and *Thr/Ile* genotypes	*P*
n	218	26	—	460	65	—
Diabetes duration (years)	18.7 (17.1–20.4)	20.8 (15.4–26.2)	0.44*	11.5 (10.6–12.3)	10.7 (8.4–12.9)	0.44*
Female (%)	50 (35.5–64.5)	43 (24–62]	0.54†	44 (39.4–48.4)	46.2 (34.0–58.3)	0.79†
Age (years)	46.5 (44.9–48.2)	49.0 (42.8–55.3)	0.51*	65.6 (64.6–66.5)	63.8 (61.2–66.4)	0.14*
Nicotine use (%)	26 (19.1–32.9)	25 (8–42)	1.00†	25.5 (20.6–30.5)	26.3 (12.3–40.3)	1.00†
Hypertension (%)	32.7 (19.1–46.3)	26.9 (24.6–29.2)	0.66†	71.8 (67.5–75.9)	66.2 (54.7–77.7)	0.38†
HbA_{1c} (%)	7.3 (7.1–7.5)	7.3 (6.8–7.7)	0.58*	7.5 (7.4–7.7)	7.2 (6.8–7.5)	0.08*
Cholesterol (mg/dl)	193 (188–198)	194 (179–209)	0.90*	197 (193–201)	193 (180–206)	0.49*
Triglycerides (mg/dl)	94 (84–103)	81 (63–98)	0.53*	171 (161–180)	173 (138–207)	0.82*
BMI (kg/m^2)	25.4 (24.9–25.9)	25.0 (23.9–26.2)	0.79*	30.2 (29.7–30.7)	30.4 (29.1–31.7)	0.49*

Data are means (asymptotic 95% CI). *Mann-Whitney *U* test; †Fisher's exact test.

rier for both genotypes (*Gly299Gly* and *Ile399Ile*).

The prevalence of the *Gly299* allele (without the *Ile399* allele) was very low (0.5%), and the prevalence of the *Ile399* allele (without the *Gly299* allele) was similarly low (0.4%) in the tested groups. There was no difference in the cosegregation prevalence in type 1 versus type 2 diabetes ($P = 0.63$).

The frequencies of the genotypes were not significantly different between the type 1 and type 2 diabetic patients (Table 1). Furthermore, the prevalence of different genotypes in groups with and without nephropathy ($P = 0.81$) and neuropathy ($P = 1.00$) showed no significant difference in patients with type 1 diabetes (Table 2). In type 2 diabetic patients, the prevalence of different genotypes in groups with and without nephropathy was also not significantly different ($P = 0.59$). In contrast to type 1 diabetic patients, however, type 2 diabetic patients demonstrated a strong association of the *Asp299Gly/Thr399Ile* polymorphisms with diabetic neuropathy. Although 50.9% of the carriers of the *Asp299Asp/Thr399Thr* genotypes had diabetic neuropathy, only 26.2% of the carriers of the *Asp299Gly/Thr399Ile* genotypes were affected ($P = 0.0002$, OR 0.35 [CI 0.19−0.61]) (Table 2).

Interaction terms were modeled to elucidate whether the effect of the *TLR4* polymorphisms on nephropathy or neuropathy was dependent on diabetes type. These showed no statistical interaction of the *Asp299Gly/Thr399Ile* genotype with diabetic nephropathy ($P = 0.42$), but did indicate an interaction with diabetic neuropathy ($P = 0.19$).

The sensitivity analysis using age, HbA_{1c}, and diabetes duration categorized in quartiles gave the same result for the association of neuropathy and *Asp299Gly/Thr399Ile* genotype in type 2 diabetic patients ($P = 0.0007$) (data not shown).

Age- and diabetes duration−adjusted analysis with a multiple logistic regression model showed an independent association of the presence of the cosegregation alleles with diabetic neuropathy in type 2 diabetes ($P = 0.0005$) (Table 3). However, there was no association of neuropathy with type 1 diabetes or any association of nephropathy with either diabetes types (data not shown).

CONCLUSIONS — Our results showed a strong association of the *Asp299Gly/Thr399Ile* genotypes of *TLR4* with reduced prevalence of peripheral neuropathy in type 2 diabetic patients (but not in type 1 diabetic patients). These data suggest that the pathogenesis of diabetic neuropathy may partly differ between type 1 and type 2 diabetes, as previously discussed (27). A type-related effect between *TLR4* and type 2 diabetes (but not type 1) was indicated by using an interaction term model. Thus these results raise the question of whether the innate immune response may be involved in the pathogenesis of neuropathy in type 2 diabetes (1–3).

The amino acid polymorphisms in positions 299 and 399 determine the differences in the structure of the extracellular domain and thus the pattern recognition of the TLR-4 receptor isoforms (19,28). However, little is known about the influence of these polymorphisms on the ligand binding to the receptor (12,19,26,28). One might suggest that an abnormality in the TLR regulation might increase the susceptibility for diseases because of a reduced defense against

Table 2—*Frequencies and rates of genotypes of TLR4 polymorphisms in type 1 and type 2 diabetic patients with nephropathy and neuropathy*

	Type 1 diabetes				Type 2 diabetes			
	Asp/Asp and *Thr/Thr* genotypes	*Asp/Gly* and *Thr/Ile* genotypes	*P*	OR	*Asp/Asp* and *Thr/Thr* genotypes	*Asp/Gly* and *Thr/Ile* genotypes	*P*	OR
Nephropathy (%)	51 (23.2%)	5 (19.2%)	0.81	0.79 (0.28–2.20)	183 (39.4%)	23 (35.4%)	0.59	0.84 (0.49–1.45)
Neuropathy (%)	64 (29.1%)	7 (26.9%)	1.00	0.90 (0.36–2.24)	236 (50.9%)	17 (26.2%)	0.0002	0.34 (0.19–0.61)

Data are means (asymptotic 95% CI) unless otherwise noted. *P* calculated with Fisher's exact test.

Table 3—*Multivariate logistic regression analysis assessing the independent association of* Asp299Gly *and* Thr399Ile *genotypes of* TLR4 *gene and the presence of peripheral neuropathy in type 2 diabetic patients*

	OR (95% CI)	*P*
Sex (male)	1.40 (0.94–2.09)	0.0963
Hypertension	1.40 (0.89–2.19)	0.1485
Age	1.05 (1.03–1.07)	<0.0001
HbA_{1c}	1.14 (0.99–1.31)	0.0637
Diabetes duration	1.05 (1.02–1.07)	0.0004
TLR-4 polymorphisms	0.31 (0.16–0.60)	0.0005

An OR <1 means an inverse association with diabetic neuropathy.

invading neurotrophic bacteria (28). However, this condition would not play a role in diabetic neuropathy, which is caused by a metabolic disorder. Therefore, it is more likely that in diabetic neuropathy a mutation in the ligand binding region might protect against binding of endogenous ligands such as oxLDL (11,12). These ligands are known to induce oxidative stress (29), which might contribute to late diabetic complications. A reduced receptor-ligand interaction with endogenous ligands might therefore result in decreased susceptibility for diabetic neuropathy.

To date, no data are available concerning the function and expression of *TLR4* in diabetic complications and it is not known whether TLR-4 expression is influenced by hyperglycemia. Therefore, we cannot determine whether the differences observed in this study were caused by a protective effect of the *Asp299Gly/Thr399Ile TLR4* alleles or an increased susceptibility of the predominant *Asp299Asp/Thr399Ile TLR4* alleles. Ligation to the pattern recognition receptor TLR-4 is known to activate the proinflammatory transcription factor NF-κB and subsequent gene expression of NF-κB–regulated genes such as cytokines and leukocyte adhesion molecules. Another pattern recognition receptor, RAGE, has also been demonstrated to induce NF-κB activation and NF-κB–dependent gene expression. Moreover, recent studies have shown for the first time that RAGE-dependent NF-κB activation is involved not only in the pathogenesis of diabetic neuropathy, but also in the direct molecular link of pain perception (30). Furthermore, a combined treatment with two NF-κB–specific inhibitors, pDTC (pyrrolidine dithiocarbamate) and TLCK (*N*-α-tosyl-L-lysine chloromethyl ketone), not only reduced NF-κB activation in sciatic nerves of diabetic rats, but also improved nerve conduction velocities in these animals (31). Consistent with the observations in experimental diabetic neuropathy, the expression of activated NF-κB has been demonstrated in epineural and endoneural vessels and the perineurium of patients with long-standing diabetes, further implying that NF-κB activation might contribute to neuronal dysfunction in diabetic neuropathy. This indicates that a TLR-4 polymorphism that prevents ligand binding and subsequent cellular signaling resulting in NF-κB activation might lower NF-κB activation and subsequent NF-κB–dependent proinflammatory gene expression and thus reduce the burden of the peripheral nerve. Hence, further studies are required to show whether the polymorphisms are associated with a reduced activation of NF-κB, cytokine expression, and expression of leukocyte adhesion molecules in nerves of type 2 diabetic patients.

Furthermore, diabetic neuropathy is influenced by risk factors such as hypertension, body weight, microalbuminuria, hyperglycemia, and nicotine use in type 2 diabetes (29, 31–37). Because these risk factors were not distributed differently in the groups in this study, it seems unlikely that the effect of the *Asp299Gly/Thr399Ile* genotype on diabetic neuropathy was caused by a profile of lower risk factors in these patients.

The interpretation of our study results is limited because the patients were recruited from just one center and the diabetes duration (type 1, 19–21 years; type 2, 11–12 years) and age distribution (type 1, 47–50 years; type 2, 66–64 years) in patients with type 1 and type 2 diabetes were different. However, when we categorized age and diabetes duration into quartiles, we found no age- or duration-dependent influence on the association of *Asp299Gly/Thr399Ile* genotypes with diabetic neuropathy.

In conclusion, the results presented here suggest that the *TLR4* gene might indeed be involved in the pathogenesis of diabetic neuropathy in type 2 diabetes, but the functional significance of this finding has yet to be confirmed in animal models with genetically controlled *TLR4* expression.

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