Brief communication

Mutations in the gene for toll-like receptor 4 and multiple sclerosis

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Key words:
DNA-polymorphism; multiple sclerosis; toll-like receptor 4

Acknowledgments:
This work was carried out in partial fulfillment of the course 'Research in Biology' (JI).

Abstract: Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system with heterogeneous pathological features, disease courses and genetical backgrounds. In this study we determined whether genetic variants of toll-like receptor (TLR) 4, which confer substantial differences in the inflammation elicited by bacterial lipopolysaccharide, are related to the development of MS. We found no differences in the frequencies of the cosegregating TLR4 Asp299Gly and Thr399Ile polymorphisms between Austrian MS patients (11.6%) and age-matched controls (13.7%). Furthermore, we could not detect any influence of these mutations on clinical parameters and serum levels of soluble adhesion molecules of MS patients. Our data indicate that these TLR4 polymorphisms have no influence on the incidence, progression and inflammatory parameters of MS.

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Received 29 August 2002, revised 27 September 2002, accepted for publication 3 October 2002

Tissue Antigens . 0001-2815

Tissue Antigens 2003 **61**: 85–88
Printed in Denmark .

Neuropathological and immunological findings in multiple sclerosis (MS) assume that autoimmune mechanisms based on genetic susceptibility and environmental triggers contribute to the etiopathogenesis of this disease (1). Briefly, a T-cell-dependent autoimmune reaction mediates the initial inflammatory process, which is extended and amplified by a cascade of inflammatory effector cells such as macrophages, microglia, cytotoxic T cells and B cells. Subsequently, demyelination and tissue destruction occurs, mediated by a variety of inflammatory mechanisms. As healthy donors have similar numbers of myelin-reactive T cells as MS patients, additional factors are clearly needed to promote (auto)-reactivity of T cells within the central nervous system (CNS). Recent experimental studies have shown that various infectious and proinflammatory agents such as lipopolysaccharide (LPS) are capable of reversing the tolerant state of T cells (2).

Lipopolysaccharide is recognized by toll-like receptor (TLR) 4, a transmembrane receptor that initiates the innate immune response to common Gram-negative bacteria such as *Chlamydia pneumoniae*

(3–5) and induces a signal transduction cascade leading to increased expression of cytokines as well as reactive oxygen species. Toll-like receptor 4 is expressed on a variety of cell types throughout the body, from macrophages to airway epithelial cells. Recently it was demonstrated that within the CNS, expression of TLR4 was highest in microglia, whereas astrocytes and oligodendrocytes expressed none (6). Moreover, in this study it was also shown that TLR4 is essential for LPS-induced oligodendrocyte cell death *in vitro* and *in vivo*.

An additional possible role for TLR4 signaling in MS comes from the frequent, although controversial, observation that *Chlamydia pneumoniae* and other bacterial infections may play a role in the pathogenesis of MS (1,7). *Chlamydia pneumoniae* and LPS may, either directly by LPS/TLR4-mediated activation of monocytes and/or microglia, or indirectly by the activation of autoreactive T cells in the periphery, be involved in MS disease pathogenesis. This idea is supported by the observation that the activation of autoreactive T cells is crucially dependent on the presence of both a stimulatory agent and the specific (auto)-antigen (8). Further, research on inflammatory properties of bacterial antigens in brain tissue has shown that bacteria injected into the brain parenchyma are able to induce inflammatory responses only after peripheral sensitization of T cells (9).

These findings raise the question whether LPS and its receptor TLR4 are involved in the pathogenesis of MS. We therefore decided to analyze MS patients for two well-described cosegregating mutations in TLR4 originally identified by their association with a decreased response to inhaled endotoxin (10). These mutations exchange a highly conserved asparagine residue at position 299 to a glycine (Asp299Gly) and a less conserved threonine at position 399 to an isoleucine (Thr399Ile). In central Europeans these mutations occur most commonly as cosegregating alleles (6–14% of the general population) and differing genotypes have been hypothesized to alter the susceptibility of the carriers to infections (10,11). Carriers of these mutations were also shown to have an increased risk for Gram-negative septic shock (12) and a higher incidence of premature birth (13). Most recently it could be demonstrated that these mutations are associated with a decreased atherosclerosis risk (14). We therefore hypothesized that an attenuated innate immune defense caused by these TLR4 mutations may have an influence on the severity and course of MS and on the inflammatory parameters in this disease.

In this study we have analyzed for the first time the occurrence of TLR4 mutations in MS patients and controls and correlated these mutations with clinical parameters. These mutations could either modulate MS susceptibility or disease course as it has been shown in several genome wide screens for other genes such as the human leukocyte antigen (HLA) gene locus (15–18). Moreover, as TLR4 mutations have an influence on the serum levels of soluble adhesion molecules (10,14) and the latter are biological markers for disease activity in MS (19), we also analyzed whether TLR4 mutations have any influence on the serum levels of soluble adhesion molecules in MS.

For this study we used blood samples (EDTA-treated whole blood and serum obtained with informed consent between 1996 and 2001) of 190 patients from our department with clinically definite MS available from a larger collective of the Austrian MS study group described in a previous study (20). These patients (126 women and 64 men) had a mean age of 40 ± 11 years. Only individuals with disease durations of at least 3 years were included. The clinical data included (mean ± standard deviation) were the age at onset of MS (28 ± 10 years), disease duration (12 ± 8 years), disease severity according to Kurtzke's Expanded Disability Status Scale (EDSS; 3 ± 2; 21), the number of relapses (5 ± 3) and the annual relapse rate (0.6 ± 0.4). The course of MS was relapsing-remitting (RR, $n = 122$, 64.2%), secondary chronic progressive (SP; $n = 52$, 27.4%) or primary chronic progressive (PP; $n = 16$, 8.4%). Examiners also recorded the time of transition to secondary progressive MS and any treatment of MS.

As a healthy control group from a comparable geographic region we included 95 anonymized age-matched organ donors from the General Hospital and University Clinics, Innsbruck, Austria. These individuals (40 women and 55 men) had a mean age of 45 ± 18 years.

DNA was isolated from leukocytes by the salting-out method (GenomicPrep Blood Kit, Amersham Pharmacia Biotech, Uppsala, Sweden) and TLR4 genotyping was performed by polymerase chain reaction-restricted fragment length polymorphism (PCR-RFLP) as previously described for both the Asp299Gly and Thr399Ile polymorphisms (22). Human leukocyte antigen typing was performed for class II genes using PCR amplification with sequence-specific primers in a low-resolution kit (Olerup SSP-Combi Tray, Saltsjöbaden, Sweden). Polymerase chain reaction products were analyzed by agarose gel electrophoresis and the Helmberg SCORE Software (Geno Vision, West Chester, PA, USA). Serum levels of soluble E-selectin, soluble intercellular adhesion molecule 1 (sICAM-1) and soluble vascular cellular adhesion molecule (sVCAM) were measured by sandwich enzyme-linked immunosorbent assay according to the manufacturer's instructions (Bender MedSystems, Vienna, Austria). Dilution of sera was 1: 5 for E-selectin, 1:50 for sVCAM and 1:100 for sICAM-1.

Comparisons between the Asp299Gly and Thr399Ile alleles of MS patients and controls were made using Fisher's exact test. The Mann–Whitney *U*-test, Student's *t*-test, Fisher's exact test and the Chi-square test were used to compare clinical and immunological data. All stat-

istical analyses were performed using the GraphPad InStat and Prism statistical analysis programs (GraphPad Software, San Diego, CA, USA). *P*-values <0.05 were considered statistically significant.

Of the 96 MS patients tested, 23 (12.1%, 14 females and nine males) were heterozygous for the TLR4 Asp299Gly allele and 22 (11.6%, 14 females and eight males) were heterozygous for the TLR4 Thr399Ile allele (Table 1). In 22 of the 23 patients with a TLR4 Asp299Gly allele a cosegregation of the TLR4 Asp299Gly and Thr399Ile polymorphisms was observed, whereas an isolated TLR4 Asp299Gly polymorphism occurred in only a single male patient. The TLR4299 and 399 wildtype alleles were observed in 167 MS patients (87.9%, 112 females and 55 males).

The frequencies of these mutations in MS patients were then compared with an age-matched control population (organ donors) of a similar geographic origin. In this group, 13 of 95 individuals (13.7%, five females and eight males) were carriers of the cosegregating Asp299Gly and Thr399Ile mutations. Thus these mutations were observed at similar frequencies in MS patients and healthy controls. In contrast, we found a highly significant association of MS with the HLA DQB1*06, DRB1*15 and DRB5 alleles (all $P<0.001$). Thus this well-recognized association between MS and these DQB and DRB alleles (1) was evident in our study cohort.

We further analyzed whether MS patients with the TLR4 Asp299Gly and Thr399Ile alleles have an altered MS disease course as compared with wild-type carriers. For this purpose we compared the disease courses (RR, SP and PP), age at disease onset, disease duration and the annual relapse rate of MS patients with the wildtype and mutated TLR4 alleles. However, as can be seen in Table 1, we did not find any disease modifying influence of the TLR4 Asp299Gly and the Thr399Ile alleles on MS. Finally we determined whether the TLR4 mutations influence the serum levels of sE-Selectin, sVCAM and sICAM-1. As can be seen from Table 1 there were no significant differences in the serum levels of soluble E-selectin, sVCAM, and sICAM-1 for the Asp299Gly and Thr399Ile mutations.

The TLR4 Asp299Gly and Thr399Ile mutations investigated in this study affect the extracellular domain of the receptor. The Asp299Gly variant appears to be biologically more important as it, even in a heterozygous state, markedly attenuates human responsiveness to inhaled endotoxin *in vivo* and interrupts TLR4-mediated LPS signaling in cellular transfection studies (10). Furthermore, most recently we have shown that these TLR4 alleles were associated with lower levels of circulating proinflammatory molecules and conferred a lower risk of atherosclerosis development (14).

Here we report that the cosegregating Asp299Gly and Thr399Ile mutations are equally frequent in MS patients from Western Austria (11.6%) and in a comparable control population (13.7%). These mutations were observed at a comparable frequency (12–16%) in healthy individuals from Finland (13) and the USA (10,23), whereas two other studies found lower allelic frequencies of the cosegregating TLR4 mutations in 810 individuals of the general community of Bruneck (6%) (14) and a large French population (7%) in the CEPH Study (24). However, the lower frequencies found in the latter studies may be explained by differences in the age distribution of the study populations, as the common TLR4 Asp299Gly poly-

Influence of toll-like receptor 4 mutations on the clinical course and serum levels of soluble adhesion molecules of multiple sclerosis patients from Western Austria

	Asp299Gly Asp/Asp	Asp/Gly	Thr399Ile Thr/Thr	Thr/Ile
Total	167 (87.9%)	23 (12.1%)	168 (88.4%)	22 (11.6%)
RR	107 (64%)	15 (65%)	108 (64%)	14 (64%)
SP	47 (28%)	5 (22%)	47 (28%)	5 (23%)
PP	13 (8%)	3 (13%)	13 (8%)	3 (14%)
Age at MS onset (y)*	28 ± 10	30 ± 10	28 ± 10	30 ± 10
Disease duration (y)*	11 ± 8	14 ± 8	11 ± 8	14 ± 8
Annual relapse rate*	0.6 ± 0.4	0.5 ± 0.3	0.6 ± 0.4	0.5 ± 0.4
EDSS*	3.3 ± 2.1	2.8 ± 2.5	3.3 ± 2.1	2.9 ± 2.5
sE-Selectin (ng/ml)*	30 ± 9	33 ± 10	30 ± 9	31 ± 8
sICAM-1 (ng/ml)*	550 ± 314	688 ± 404	560 ± 326	623 ± 350
sVCAM (ng/ml)*	701 ± 203	743 ± 229	701 ± 203	739 ± 234

RR, relapsing-remitting disease course; SP, secondary chronic progressive disease course; PP, primary chronic progressive disease course; y, years; EDSS, Kurtzke's Expanded Disability Status Scale.
*Mean ± standard deviation.
Overall, no statistically significant differences were detected (at $P<0.05$).

Table 1

morphism enhances the risk of severe bacterial infections (10,14) and predisposes persons to septic shock with Gram-negative bacteria (12), both affecting the long-term survival of the carriers of this TLR4 mutation.

Multiple sclerosis, like many other autoimmune diseases mainly affects females and therefore females were over-represented in our MS study-population (66%). However, we found no significant influence of gender on the Asp299Gly and Thr399Ile alleles in MS patients and controls. This was also seen in our recent study using 810 individuals of the Bruneck control population, where the gender distribution was approximately in balance for carriers of both mutations (14).

Our clinical data indicate that the Asp299Gly and Thr399Ile TLR4 alleles have no influence on neurological parameters in MS. Furthermore, we could not find any influence of these TLR4 mutations on the levels of the circulating adhesion molecules sE-selectin, sICAM-1 and sVCAM, although they are biological markers for disease activity in MS (19). Therefore it seems to be rather unlikely that the TLR4 gene plays a role as a disease modifier of MS.

In conclusion we have shown that TLR4 Asp299Gly and Thr399Ile mutations have no influence on the incidence of MS in patients from Central Europe. Moreover, we found no effects of these mutations on MS disease progression and serum levels of soluble adhesion molecules.

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