Toll-like receptor 4 (TLR4) polymorphisms in Iranian patients with visceral leishmaniasis

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Abstract The role of Toll-like receptor (TLR) 4 in visceral leishmaniasis (VL), a disease caused by an obligate intracellular protozoan parasites belonging to the genus *Leishmania,* has been shown in the recent leishmaniasis experimental studies. As genetic host factors play an important role in the susceptibility and/or resistance to VL, the association between TLR4 gene mutations [A896G and C1196T single nucleotide polymorphisms (SNPs)] and VL was investigated. Genotyping of A896G (Asp299Gly) and C1196T (Thr399Ile) SNPs was performed in the patients with VL ($N = 122$) and ethnically matched controls ($N = 155$) using polymerase chain reaction–restriction fragment length polymorphism method. When VL patients and the controls were compared, no statistically significant differences were observed in A896G and C1196T alleles and genotypes ($P > 0.05$). The TLR4 A896G and C1196T were in moderate linkage disequilibrium in the controls and patients ($r^2 = 0.497$, 0.548 and $D' = 0.705$, 0.808, respectively), and haplotypes reconstructed from these SNPs were not significantly different between the aforementioned study groups. In conclusion, based on the results, TLR4 gene polymorphisms at the positions 896 and 1196 cannot be regarded as the major contributors to VL susceptibility among the Iranian population.

Keywords *Leishmania infantum* · Visceral leishmaniasis · TLR4 · Genetic polymorphism

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Introduction

Visceral leishmaniasis (VL), primarily caused by *Leishmania (L) donovani*, *L. infantum,* and *L. chagasi* and is endemic in the Middle East and Mediterranean regions. Northwestern and southern Iran are the primary foci for VL, mainly affecting children; moreover, it is clarified that *L. infantum* is the dominant *Leishmania* strain in Iran [1, 2].

Leishmania is an obligate intracellular parasite infecting macrophages of the reticuloendothelial tissues [3]. The control and protection of VL is the result of the type 1 immune response and the best known cytokine of this kind of immunity, IFN-γ (gamma interferon), induces innate and adaptive cellular immune responses [4].

Toll-like receptors (TLRs), type 1 transmembrane proteins, are triggered by pathogen-associated molecular pattern molecules (PAMPs) which are the characteristics of various groups of pathogens such as bacteria, fungi, viruses and parasites [5, 6]. TLR-induced activation culminates the production of proinflammatory cytokines and the upregulation of costimulatory molecules, linking innate immune responses to the adaptive ones [7–10]. There is some supporting evidence on the role of TLRs in the host response to the infection with eukaryotic parasites [11–14]. TLRs and their associated molecules have been shown to be important in the recognition of *Leishmania* species as well [15]. Both TLR2 and TLR4 have been implicated in the recognition of *L. major* promastigotes [11, 16, 17]. The other supporting observation results show that TLR2, TLR3, TLR4 and TLR9 can recognize various *Leishmania* species and effectively induced powerful immune responses [11, 16, 18, 19]. Among TLRs, TLR-4 has a vital role in triggering innate immunity, orchestrating the acquired immune responses in leishmaniasis and is required for controlling *Leishmania* parasite in mice [11]. Probably complex organisms like *Leishmania*

parasites (eukaryotic) express several PAMPs, recognized by immune receptors such as TLR4 which contributes to the immunity against *L. major* infection. Moreover, TLR4 deficient mice cannot efficiently control *L. major* replication and resolve cutaneous lesions [5].

Although direct interaction between *Leishmania* derived molecules and TLR4 has not been reported in in vitro, in vivo multiple innate immune recognition receptors may interact during pathogen recognition [20, 21]. In fact, in vitro transfection systems do not necessarily reflect the complexity of in vivo host–parasite interactions [11].

Among the great number of studies carried out on the association of single nucleotide polymorphisms (SNPs) with several infectious diseases [22–25], frequent SNPs have been described for TLR2, TLR4 and TLR9 which alter susceptibility to infectious and inflammatory diseases [26, 27]. Human TLR-4 gene is located on the long arm of chromosome 9 in which two polymorphic point mutations at the positions A896G and C1196T have been identified in the fourth exon of its gene [28, 29]. These mutations encode amino acid changes i.e., Asp299Gly and Thr399Ile in the extracellular region of TLR-4 and they in turn may affect TLR-4 expression, transportation to the membrane or the detection of ligands [30]. It has been proposed that these polymorphisms lead to a reduced cytokine response and an increased susceptibility to Gram negative infection [31]. Furthermore, these two frequently cosegregating polymorphisms (A896G and C1196T) were observed to reduce the reactivity to the inhaled lipopolysaccharide (LPS) [32]. There are many reports investigating the potential impact of TLR-4 SNPs (A896G and C1196T) on the incidence and course of infectious diseases, reviewed by Schröder and Schumann [26]. This effect is supported by other observations showing the association of TLR4 SNPs with increased risk of mortality from systemic inflammatory response syndrome, gram negative bacterimia, respiratory syncytial virus bronchiolitis and legionnaires disease [33, 34]. Previous studies have revealed that A896G and C1196T SNPs increase the susceptibility of individuals to cutaneous leishmaniasis (CL) induced by *L. major* [27], and also the risk of severe malaria in African children [26]. Taking into account the importance of TLR4 involvement in leishmaniasis and genetic background for the susceptibility and/or resistance to infectious diseases, the present study aimed to assess the association between TLR-4 SNPs (A896G/C1196T) and VL.

Materials and methods

Subjects

One hundred and twenty-two Iranian pediatric patients (67 males, mean age $\pm$ SD = 4.8 $\pm$ 11.6 years and 55 females,

mean age $\pm$ SD = 3.3 $\pm$ 3.4 years) suffering from VL were enrolled in this study. All of the patients were from Fars province, southern Iran, an endemic area for VL which was diagnosed based on the clinical signs and symptoms (fever, splenomegaly, hepatomegaly, substantial weight loss, progressive anemia, pancytopenia, hypergammaglobulinemia), serological test (IFA $\geq$ 1/128) and direct observation of Leishman body in bone marrow aspiration stained smears. The control group consisted of 155 randomly selected healthy individuals (96 males and 59 females; mean age $\pm$ SD = 12.9 $\pm$ 3.4 years) from the same area. Blood samples were collected after obtaining informed written consents from all the participants and from the parents of children under 18 years. The study design was approved by the Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran.

Genotyping

Genomic DNAs from both patients and controls were extracted from peripheral white blood cells using the salting out method. The genotypes of TLR4 at the positions A896G (Asp299Gly) and C1196T (Thr399Ile) were determined by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP). PCR was performed in volume of 10 μl containing 1 μl of 10$\times$ PCR buffer (Cinnagen-Iran), 250 ng genomic DNA, 200 mM of each of dNTPs (Cinnagen-Iran), 1 unit of *Taq* DNA polymerase (Cinnagen-Iran), different concentrations of MgCl$_2$ (Table 1) and 0.5 pM of each primer (Table 1) under the following conditions: a denaturation step for 5 min at 95 °C followed by 35 cycles of a denaturation for 30 s at 95 °C, annealing for 30 s at different temperatures based on the different pairs of primers (Table 1), extension for 30 s at 72 °C and a final extension for 5 min at 72 °C in a thermocycler (5530 Mastercycler, Eppendorf, Germany). The PCR products were digested with the related restriction enzymes (Table 1). The digested products were separated on 3 % agarose NA (GE Healthcare, USA) gel and studied on UV transilluminator after being stained with ethidium bromide. The specific primers for genotyping of TLR4 A896G (Asp299Gly) [35] and C1196T (Thr399Ile) [36] amplified the segments of 139 and 407 bp, respectively. The PCR products containing adenine at TLR4 A896G (wild type) yielded two 112 and 27 bp DNA fragments as a result of sensitivity to *Bsa*BI restriction enzyme [35], while the presence of mutated allele of guanine retained the intact PCR product (139 bp) due to insensitivity to *Bsa*BI (Fig. 1). However, the restriction enzyme *Hin*fI cut the PCR products C1196T containing mutated allele T to 376 and 31 bp DNA fragments [36], whilst the PCR products with wild type allele C remained intact (407 bp), as shown in Fig. 1.

Table 1 The primer sequences, PCR conditions and restriction enzymes used for genotyping of TLR4 gene polymorphisms

TLR4 SNPs	PCR primers (5′–3′)	AT^a (°C)	$MgCl_2$ (mM)	RE^b	Fragment sizes (bp)
Asp299Gly (A896G)	F: TTAGAAATGAAGGAAACTTGGAAAAG	51	2	*Bsa*BI	GG: 139
	R: TTTGTCAAACAATTAAATAAGTGATTAATA				AA: 112,27
					AG: 139,112, 27
Thr399Ile C1196T	F: GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA	55	2.5	*Hin*fI	CC: 407
	R:ACCTGAAGACTGGAGAGTGAGTTAAATGCT				TT: 376,31
					CT: 407, 376, 31

[a] Annealing time

[b] Restriction enzyme

Fig. 1 Different patterns of PCR products of TLR4 gene polymorphisms at the positions 896 (**a**) and 1196 (**b**) after *Bsa*BI and *Hin*fI digestion, respectively. *M* 100 bp DNA ladder (Fermentas, Lithuania)
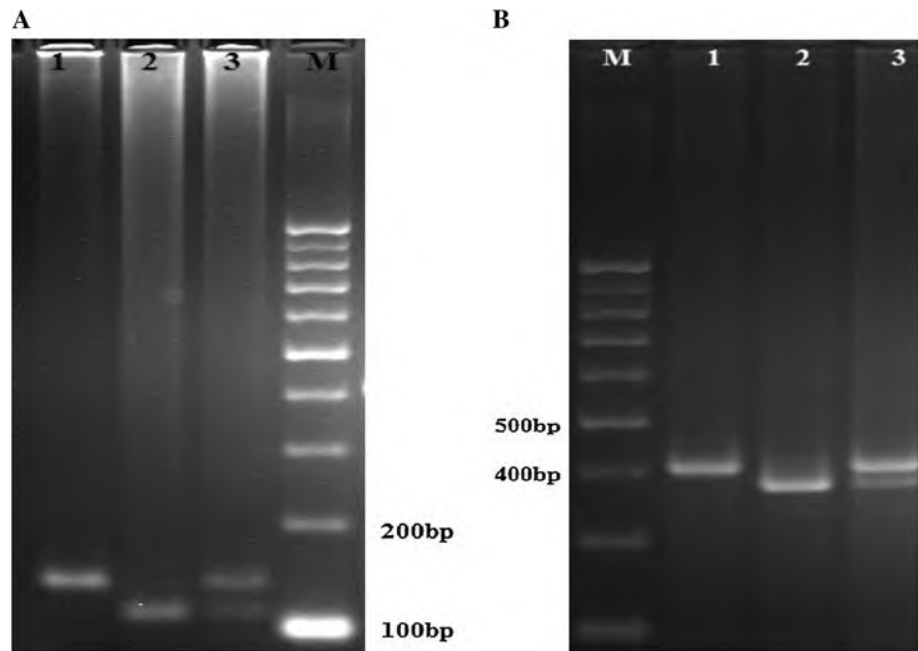
Statistical analysis

Allele and genotype frequencies were calculated for both patients and the controls by direct gene counting. Haplotype frequency and Hardy–Weinberg equilibrium were determined by Alrequin software package, version 3.1. Comparison of the distributions of the allele, genotype, and haplotype frequencies were made using the χ^2 test by EPI Info 2000 and SPSS software, version 16. D' and r^2 (linkage disequilibrium, LD) were measured using LD2SNPing v2.0 software (http://bio.kuas.edu.tw/LD2SNPing). P value less than 0.05 was considered significant.

Results

To investigate whether there is any relationship between TLR4 gene polymorphisms and susceptibility to VL, TLR4 gene mutations were determined in the VL patients and the controls using PCR–RFLP method. The distribution of these genotypic variants met the Hardy–Weinberg equilibrium. Although the results showed that 896AA genotype was more frequent in the patients (90.2 %) than that in the controls (88.4 %), the difference was not statistically significant ($P = 0.636$, $\chi^2 = 0.22$, OR = 1.2 and 95 % CI = 0.52–2.79, study power = 7 %). Also, the distribution of 896AG genotype didn't show any difference between the two study groups ($P = 0.48$, $\chi^2 = 0.49$, OR = 0.75 and 95 % CI = 0.32–1.77, study power = 11 %). Since the 896GG genotype was not found in the control group, the comparison between the two study groups was impossible. Furthermore, allele frequency was not significantly different between the two groups at the position 896 (Table 2).

The genotype distribution at the position 1196 was consistent with the Hardy–Weinberg equilibrium. The frequency of 1196CC genotype was greater in the patients than that in the controls (91.8 vs. 88.4 %, $P = 0.35$, $\chi^2 = 0.88$, OR = 1.47, 95 % CI = 0.61–3.58, study

Table 2 The frequencies of TLR4 gene polymorphisms in kala-azar patients and controls

TLR4 SNPs ID	Patients N (%)	Controls N (%)	χ^2	P value*	OR (95 % CI)	Study power (%)
896						
Genotype						
AA	110 (90.2)	137 (88.4)	0.22	0.636	1.2 (0.52–2.79)	7
GG	1 (0.8)	0 (0.0)	1.28	0.44**	2.28 (2.00–2.61)	17
AG	11 (9.0)	18 (11.6)	0.49	0.48	0.75 (0.32–1.77)	11
Allele						
A	231 (94.7)	292 (94.2)	0.06	0.807	1.10 (0.50–2.42)	4
G	13 (5.3)	18 (5.8)				
1196						
Genotype						
CC	112 (91.8)	137 (88.4)	0.88	0.35	1.47 (0.61–3.58)	16
TT	1 (0.8)	0 (0.0)	1.28	0.26	2.28 (2.00–2.61)	17
CT	9 (7.4)	18 (11.6)	1.39	0.24	0.61 (0.24–1.49)	22
Allele						
C	233 (95.5)	292 (94.2)	0.46	0.49	1.31 (0.57–3.02)	10
T	11 (4.5)	18 (5.8)				

* Each P value is the result of comparing corresponding row with the sum of other related rows

** P value is determined by Fisher exact test

Fig. 2 Pattern of LD within TLR4; the numbers on the X and Y-axis correspond to the two markers selected to cover the TLR4 gene in the controls and patients. D' values are shown in the *top left* and r^2 values are shown in the *bottom right*
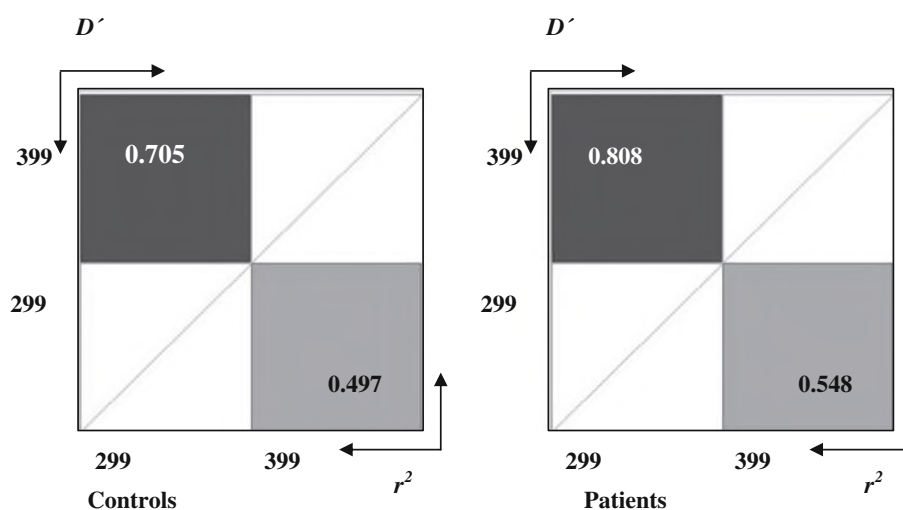
power = 16 %) and 1196CT genotype was more frequent in the controls than in the patients (11.6 vs. 7.4 %, $P = 0.24$, $\chi^2 = 1.39$, OR = 0.61, 95 % CI = 0.24–1.49, study power = 22 %). Taken as a whole, there was not any difference between the two groups in terms of the distribution of genotypes (CC and CT) and alleles (C and T). Due to the lack of TT genotype in the controls, the comparison between the two groups in this regard was impossible (Table 2).

To execute a comprehensive genetic association analysis of TLR4, we characterized the LD pattern within TLR4 gene. D' and r^2 for the aforementioned polymorphisms in TLR4 gene were calculated and four haplotypes were reconstructed according to the genotyping data in VL patients and the controls. Good LD was observed between TLR4 gene SNPs and the LD maps in the controls and patients measured by D' (0.705 and 0.808) and r^2 (0.497 and 0.548), respectively as shown in Fig. 2. The haplotype distributions of TLR4 polymorphisms in patients and the controls are presented in Table 3. The most frequent haplotype observed in VL patients and the controls was haplotype AC (93.9 and 92.6 %, respectively). No statistically significant differences were observed between the study groups in terms of this haplotype (AC) and other less frequent ones (AT, GC and GT). Similarly, the distributions of haplotype-related genotypes (diplotypes) were not

Table 3 The haplotype frequencies in kala-azar patients and healthy controls

Haplotype		Patients N (%)	Controls N (%)	χ^2	$P*$	OR (95 % CI)	Study power (%)
896	1196						
A	C	229 (93.9)	288 (92.6)	0.21	0.65	1.25 (0.43–3.72)	9
A	T	2 (0.8)	4 (1.6)	0.14	1**	0.63 (0.02–9)	14
G	C	4 (1.6)	4 (1.6)	0.06	1**	1.27 (0.13–12.86)	2
G	T	9 (3.7)	14 (4.2)	0.03	0.86	0.9 (0.24–3.27)	5

* Each P value is the result of comparing corresponding row with the sum of other related rows

** P value is measured by Fisher exact test

Table 4 Distributions of diplotype frequencies in kala-azar patients and healthy controls

Haplotypes	Patients N (%)	Controls N (%)	χ^2	P value*	OR (95 % CI)	Study power (%)
Double haplotype						
AC/AC	108 (88.52)	131 (84.54)	0.93	0.335	1.41 (0.66–3.04)	16
AC/GT	7 (5.74)	7 (4.50)	0.21	0.644	1.29 (0.39–4.22)	7
AT/AC	1 (0.82)	2 (1.29)	0.04	0.834**	0.63 (0.02–9.00)	6
AC/GC	2 (1.64)	2 (1.29)	0.07	0.790**	1.27 (0.13–12.86)	4
GC/AC	1 (0.82)	3 (1.94)	0.07	0.790**	0.42 (0.02–4.58)	13
GT/GT	1 (0.82)	0 (0.00)	0.01	0.904**	Undefined	17
AC/AT	1 (0.82)	4 (2.57)	0.41	0.523**	0.31 (0.01–3.01)	21
GT/AC	1 (0.82)	6 (3.87)	1.49	0.222**	0.21 (0.01–1.75)	41

* Each P value is the result of comparing corresponding row with the sum of other related rows

** P value is measured by Fisher exact test

significantly different between the groups and the heterozygosity according to the results obtained with the patient sample was 8.37 % (Table 4).

Discussion

The species-specific manifestations of leishmaniasis in human range from self-limited cutaneous lesion to mucosal and mucosal infections [37]. *L. infantum* infection in an endemic area can lead from asymptomatic infection to the fulminant fatal disease, VL.

Physicians have long been aware of the noticeably different immune responses of the individuals with the same exposure to inflammatory or infectious agents. Several factors can affect the outcome of the interaction between the hosts and parasites that invade the host such as the existence of immunological memory due to the prior host contact with the parasite, the quantity of parasites that invaded the host, etc. [38, 39]. One of the most important probable factors that determine the consequences of the combat between the host and parasite in infectious diseases is the genetic factor [40–43]. Different members of the TLRs family recognize diverse PAMPs of *Leishmania* species triggering the activation of innate immunity and the

subsequent development of antigen-specific adaptive immunity [11, 13, 18, 19].

Th1 cells and their IFN-γ production are involved in the protective immune response to *Leishmania* infection [44]. In this regard, in vitro experiment using TLR4 agonist, a powerful IFN-γ inducing factor, confirmed the ability of TLR4 to induce Th1 differentiation [45–47]. Based on the different results, TLR4 possesses several mechanisms for protection against *Leishmania* parasites including the induction of inducible nitric oxide syntheses in mice [11] and neutrophil elastases that induce macrophages through TLR4 to kill *Leishmania* parasite [48, 49]. Another study suggests that TLR4 recognizes P8 proteoglycolipid complex of *Leishmania pifanoi* amastigotes and thereby plays an important role in controlling the infection [15]. An experience in animal model has shown TLR4 activation during the immunity for leishmaniasis associated with an increase in the cure rate [50]. However, direct interaction between *Leishmania* derived molecules and TLR4 has not been reported in vitro [11] but the use of TLR4 transgenic mouse demonstrated that TLR4 contributes to the control of *Leishmania* growth in vivo [5].

Two polymorphic sites, Asp299Gly and Thr399Ile, have been predicted to affect ligand and co-receptor binding regions, respectively [28]. Individuals with Asp299Gly

TLR4 allele exposure have lower levels of proinflammatory cytokines, acute phase reactants and soluble adhesion molecules such as fibrinogen and IL-6 [51]. Also, it has been well recognized that the TLR4 polymorphisms influence the level of its expression [30]. Therefore, the present study aimed to determine the association of TLR4 SNPs with VL. The two polymorphisms in TLR4 gene were compared in the patients with VL and the controls. In spite of the role of TLR4 in the parasite persistence and its expected function in the immune response orchestration, there were no statistically significant differences between the two aforementioned target polymorphisms and VL in Iranian population. Since we couldn't find any study on VL and TLR4 polymorphisms, the comparison between our study and other similar ones was impossible. The haplotypes of the A896G and C1196T SNPs yielded a marker system with 6.4 % heterozygosity according to HapMap data on the CEU (Centre d'Etude du Polymorphisme Humain) sample which declared by Koch et al. [52] and the heterozygosity 8.37 % obtained according to the results of the present sample. For comparison, typing all tag SNPs suggested from HapMap data ($N = 9$), a 6-haplotype marker system with a heterozygosity of 73.1 % inferred in Kokh study. The present haplotype results captured 11 % of the genetic variance, calculated as heterozygosity, at the TLR4 locus and other polymorphisms within TLR4 may be associated with VL.

There is a study on the relationship between TLR4 polymorphisms and CL. Ajdary et al. tried to investigate the role of TLR4 mutants (Asp299Gly and Thr399Ile) in the outcome of CL. Although they found that AG (Asp299Gly) and CT (Thr399Ile) variants were more frequent among patients with chronic CL than patients with acute form of the disease, no significant differences were observed between patients with acute CL and asymptomatic ones [27].

The association of TLR4 polymorphisms with other infectious and non-infectious diseases was introduced by some researchers. Arbour and colleagues described TLR4 Asp299Gly and Thr399Ile polymorphisms which were in higher proportion among the people hyporesponsive to the inhaled LPS, compared with the controls [32]. They also found that epithelial cells derived from these SNPs both in the homozygous and the heterozygous states exhibited a decreased response to LPS stimulation in vitro [2]. Biebl et al.[53] could not observe a significant influence of TLR4 Asp299Gly polymorphism on the risk of developing invasive meningococcal disease in surviving meningococcal disease patients. Moreover, Newport et al. suggest that TLR4 Asp299Gly has no influence on monocyte responses to LPS or the susceptibility to TB in the Gambians. They also suggested that it is likely to be an ancient neutral polymorphism [54]. Ragnarsdóttir et al. [43] showed TLR4

promoter polymorphisms, as an overlooked mechanism to influence TLR4 expression and the urinary tract infection susceptibility. Na et al. [55] indicated that TLR4 polymorphisms could not be regarded as the major contributors to ankylosing spondylitis susceptibility in the Korean population. They reported that all cases and controls showed homozygous patterns (allele A for 299 and allele C for 399 variants). Rezazadeh and colleagues showed 896G allele and 896AG heterozygous genotypes of TLR4 were significantly higher in patients with brucellosis than the controls and they also indicated a sex-dependent association between TLR4 polymorphism and the susceptibility to brucellosis in Iranian population [56].

In conclusion, although this study could not find any relationship between the two aforementioned TLR4 polymorphisms and VL, it cannot exclude the probable role of the polymorphisms in the TLR4 gene or other members of TLR family which are involved in leishmaniasis like TLR2.

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