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Polymorphisms in Tumor Necrosis Factor Genes and Susceptibility to Visceral Leishmaniasis in Moroccan Children

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Abstract

In this study we examine two polymorphic alleles -308 *TNFα* and +252 *TNFβ*, to determine their implication in the genetic predisposition to visceral leishmaniasis (VL) caused by *Leishmania infantum* in Moroccan children. We used PCR-RFLP method to genotype these two polymorphisms in 102 patients with VL, and 132 subjects with no history of Leishmania infection: 92 asymptomatic subjects with a positive skin test delayed type hypersensitivity (DTH+), and 40 healthy controls with a negative skin test delayed type hypersensitivity (DTH-). Statistical analysis showed no significant association between polymorphisms of *TNFα* when comparing with VL and DTH+ groups (p>0.05). The associations were detected between VL and DTH- groups for the heterozygote genotype (P=0.021), the recessive model: 1/2+2/2 (P=0.044) and the minor allele 2 (P=0.019). The resistance to VL was found to be under the recessive model 1/2+2/2 of tumor necrosis factors-β, when comparing with VL and DTH+ (odds ratios: 0.558, 95%; confidence interval: 0.316-0.987; P=0.044). Data provide that these preliminary results warrant further study with larger populations.

Keywords: Visceral leishmaniasis; *Leishmania infantum*; -308 *TNFα*; +252 *TNFβ*; Moroccan children.

Introduction

Visceral leishmaniasis (VL) is the most severe form of leishmaniasis caused by *Leishmania donovani* and *Leishmania infantum* (chagasi). Annual incidence of VL is approximately 500 000 cases and the mortality rate in most endemic countries is almost 10%, even when treatment is available [1,2]. In Morocco, VL is exclusively caused by *L. infantum* [3,4]. Each year, 150 cases are recorded, 93% mostly in children under 10 years old [4]. On the other hand, the incidence of LV in Morocco is low and the number of clinical cases does not reflect the reality of the parasite infection, it seems that asymptomatic carriage is the rule in Leishmania infection, this suggests the existence of other factors including genetics that could influence the outcome of Leishmania infection.

Tumor necrosis factor-α (*TNFα*) (cachetin) is an inflammatory cytokine primarily produced by activated macrophages and lymphocytes T and B. It is involved in the innate phase of the immune response with a central role in the defense against intracellular pathogens [5]. The tumor necrosis factor-β (*TNFβ)* is a Th1 cytokine, primarily produced by activated lymphocytes T and B. *TNFβ* (lymphotoxin α) is also a key mediator in the initiation of a local vascular inflammatory response. Genetic polymorphisms in TNFα and *TNFβ* locus affect expression level of their genes [6]. The transition G to A at base pair 308 in the promoter region of TNFα has been identified (termed the A allele) [7]. A polymorphism in first intron of the *TNFβ* at position +252 (A>G) leads to two allelic forms; the common allele is *TNFβ A* and *TNFβ G* is variant allele [8]. Variant alleles of *TNF2* and *TNFβ* G seem to have a strange transcriptional activation, which leads to their higher serum levels [9]. In this study we aimed to examine whether polymorphic alleles at these two loci are involved in the susceptibility versus resistance to VL in children living in Moroccan leishmaniasis endemic area.

Materials and Methods

We involved 102 children with active VL, diagnosed clinically by serological and parasitological examinations. Two groups of controls comprised 132 unrelated children from the same endemic region: 92 asymptomatic healthy volunteers, with no history of leishmaniasis and positive leishmanin skin test (DTH+); 40 healthy volunteers, with no history of leishmaniasis and negative leishmanin skin test [negative skin test delayed-type hypersensitivity (DTH-)]. Leishmanin skin test was performed by intradermal injection of 0.1 ml. Approval for the study was provided by the Ethical Committee of the Institute Pasteur of Morocco and with children's parents consent. There were no significant differences in the distribution of the mean age and sex between cases and controls (P>0.05), suggesting that the matching based on these two variables was adequate. Molecular identification of the causative agent of VL was done by ITS1 PCR-RFLP for 55 patients; all were found to be infected by *L. infantum*. Genotyping of the –308 *TNFα* and the +252 *TNFβ* was performed by PCR-RFLP (18).

Briefly, 0.5 µg of DNA was added to 20 µL of reaction mixture containing 20 pmol of each primer, (*TNF-*: αα: 3'AGGCAATAGGTTTTGAGGGCCAT5'; 3'TCCTCCC TGCTCCGATTCCG5'; *TFN-* 3'CCGTGCTTCGTGCTTTGGACTA5'; 3'AGAGCT GGTGGGACATGTCTG5') with an annealing temperature of 60°C for *TNFα* and 65°C for *TNFβ* . In a final volume of 15 µL, 10 µL of the PCR products was digested by 5 IU of NcoI (BioLabs, New England), for 3h at 37°C. Restriction fragments were separated in 3% agarose gel electrophoresis. For *TNFα*, an amplified product of 107 bp, containing the G to A transition at position -308,

was obtained and restriction digests generated products of 87 and 20 bp for *TNF1* allele and 107 bp for *TNF2* allele. For *TNFβ* amplification followed by NcoI digestion generated a fragment of 740 bp for allele 1 (*TNFβ 2*) and 555 plus 185 bp for allele 2 (*TNFβ1*). Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA).

Results

All SNPs were in Hardy-Weinberg equilibrium. Associations were detected between group with active VL and DTH- control group for the heterozygote genotype (P=0.021), the recessive model: 1/2+2/2 (P=0.006) and the minor allele 2 (P=0.019) (Table 1). The minor allele 2 frequencies in *TNFα* promoter region are 21.6% in VL, 15.8% in DTH+ and 10.3% in DTH- groups. Regarding the polymorphism of *TNFβ,* the frequencies of the minor allele 2 are 29.4% in VL, 20.1% in DTH+ and 22.5% in DTH- groups. According to our statistical results, the allelic frequencies distribution in this SNP did not significantly differ between all groups. By using 1/1 genotype as the reference group, we showed a significant association under a recessive model, when comparing VL patients with DTH+ group (OR 0.558 [0.316-0.987]; P=0.044). The recessive genotype 2/2 was associated with VL patients compared to DTH- group (OR 0.245 [0.065- 0.925]P=0.023).

TLRs polymorphisms		119 VL	95 DTH+	43 DTH-	P VL *Vs* DTH+	OR VL *Vs* DTH+	P VL *Vs* DTH-	OR VL *Vs* DTH-
-308 *TNFα* **Genotypes**	1/1	64.71%	72.83%	82.05%	-	1	-	1
	1/2	27. 45%	22.83%	15. 38%	0.336	0.726 [0.377– 0.140]	0.021	0.440 [0.216-0.896]
	2/2	7.84%	4.34%	2.56%	0.190	0. 445 [0.128– 0.448]	0.065	0.297 [0.076-1.165]
-308 *TNFα* **Allelic frequencies**	1	0.784 ± 0.056	0.842 ± 0.053	0.897 ± 0.067	-	1	-	1
	2	0.216 ± 0.056	0.158 ± 0.053	0.103 ± 0.067	0.279	0.675 [0.331–1.379]	0.019	0.394 [0.176–0.883]
-308 *TNFα* **Recessive model**	1/1	64.71%	72.83%	82.05%	-	1	-	1
	1/2 + 2/2	35.29%	27.17%	17.94%	0.221	0.687 [0.376– 1.256]	0.006	0.408 [0.212–0.785]
+252 *TNFβ* **Genotypes**	1/1	51.96 %	66.30%	57.5%	-	1	-	1
	1/2	37.25%	27.17%	40%	0.076	0.575 [0.311–1.063]	0.916	0.969 [0.541–1.736]
	2/2	10.78%	6.52%	2.5%	0.177	0.501 [0.182–1.383]	0.023	0.245 [0.065–0.925]
+252 *TNFβ* **Allelic frequencies**	1	0.706 ± 0.063	0.799 ± 0.058	0.775 ± 0.092	-	1	-	1
	2	0.294 ± 0.063	0.201 ± 0.058	0.225 ± 0.092	0.138	0.612 [0.319–1.176]	0.313	0.722 [0.383–1.362]
+252 *TNFβ* **Recessive model**	1/1	51.96 %	66.30%	57.5%	-	1	-	1
	1/2 + 2/2	48.03%	33.69%	42.5%	0.044	0.558 [0.316–0.987]	0.440	0.803 [0.460–1.401]

Table 1: Genotypes, allelic and recessive model frequencies distribution of -308 TNFα and +252 TNFβ polymorphisms in the VL, DTH+ and DTH– groups (CI, confidence interval; OR, odds ratio).

Discussion

The pro-inflammatory cytokine *TNF* plays a role in innate and adaptive immune responses, and is also implicated in a wide variety of infectious and autoimmune human diseases. In the present work, no association was found in genotypic and allelic frequencies of *TNF-α* and *TNF-β* between patient group and asymptomatic infected group (DTH+). Associations were detected between VL patients and DTH- control group for the heterozygote genotype, the minor allele2 and the recessive model for *TNF-α*, and for homozygote genotype 2/2 of *TNF-β*, but due to smaller number of DTH- cases, this result must be regarded as preliminary.

A few studies with controversial results have been performed to evaluate the *TNF* polymorphisms in different clinical types of leishmaniasis. In Brazil, an association was found between the outcome of *Leishmania chagasi* infection and alleles at *TNF* locus. The strongest association was found between asymptomatic infection DTH + and a polymorphism in the *TNF* locus and haplotypes containing *TNF2* were associated with symptomatic VL [10]. In agreement with this finding, a case control study of 46 patients with mucocutaneous leishmaniasis caused by *Leishmania braziliensis* suggested that the frequency of allele 2 at the -308 *TNF-α* gene polymorphism is significantly higher in patient than asymptomatic group [11]. Other studies showed that *TNF* polymorphisms are not responsible for the resistance versus susceptibility to cutaneous leishmaniasis [11,12]. In Tunisia, Meddeb-Garnaoui failed to find associations between either the -308 of *TNF-α*, gene polymorphism or the first intron of *TNF-β* gene polymorphisms and susceptibility to Mediterranean VL [13]. Although controversial, the majority of the data support a direct role for the -308 *TNF2* allele in the elevated *TNF* levels observed in *TNF2* homozygotes [9], many arguments support the central role of *TNF* in the anti-Leishmania immune response has been strengthened by observations in *Leishmania major* (*L. major*) (strain BNI) infected B6. *TNF* –/– mice, which were not able to develop an efficient immune response against parasites and died 6–8 weeks after infection from visceral leishmaniasis [14]. Conversely, Ritter et al. showed that infection of B6. *TNF* –/– mice with *L. major* (strain FRIEDLIN) resulted in an attenuated form of disease, even though the animals were not able to resolve the local lesions but developed a chronic form of cutaneous leishmaniasis. Thus, in addition to the extensively studied host factors, the biological properties of *Leishmania* strains play an important role in the outcome of leishmaniasis [15]. Considering numerous genetic variations, that influence the apparently divergent findings, our data show that -308 *TNF-α* and +252 *TNF-β* genotypes do not influence susceptibility versus resistance to visceral leishmaniasis. However, some associations were detected between VL patients and DTH- control group, but due to smaller number of DTH- cases, these results should be interpreted with caution. These associations however, point out the need for further studies with a larger sample size of control groups, to understand the specific role of *TNF* that confer protection against leishmaniasis.

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