Polymerisms in tumour necrosis factor genes and susceptibility to visceral leishmaniasis in Moroccan children

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Objective: To examine whether polymorphic alleles at these two loci are involved in the susceptibility to visceral leishmaniasis (VL) in Moroccan children.

Methods: We have genotyped polymorphisms by PCR-restricted fragment length polymorphisms in 102 patients with VL, 92 asymptomatic carriers (positive skin test delayed-type hypersensitivity (DTH+)) and 40 healthy controls (negative skin test delayed-type hypersensitivity), with no history of Leishmania infection.

Results: Regression analysis showed no significant association between polymorphisms of tumor necrosis factors-α when comparing VL and DTH+ group (P > 0.05). The associations were detected between VL and negative skin test delayed-type hypersensitivity 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 VL and DTH+ group (odds ratios: 0.558, 95% confidence interval: 0.316-0.987; P = 0.044).

Conclusions: These results must be regarded to preliminary but suggestive that further study with larger populations is worthwhile.

KEY WORDS
Visceral leishmaniasis, Susceptibility, TNF-α, TNF-β, Morocco

1. Introduction

Visceral leishmaniasis (VL) is the most severe form of leishmaniasis caused by Leishmania donovani and Leishmania infantum (L. infantum)chagasi. An 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]. Importantly, 80%–90% of human infections are subclinical or asymptomatic, usually associated with strong cell-mediated immunity [positive skin test delayed-type hypersensitivity (DTH+)]3]. Leishmaniasis due to L. infantum infection is a zoonotic disease presented mainly in Mediterranean basin, central Asia and Brazil. Signs and symptoms of VL include prolonged fever, fatigue, weakness, anemia, enlarged lymph nodes, splenomegaly, and hepatomegaly; if left untreated, it is almost always fatal[4,5].

In Morocco, VL is exclusively caused by L. infantum[6,7]. The
endemic area extends throughout the Rif Mountains, the pre-Rif plateau, and other parts of the country. The disease continues to occur sporadically and scatter. The current active foci are located mainly in the following provinces: Fes, Taounate, Zouagha and Moulay Yacoub, Al Houcema, Chefchaouen, Sefrou, Taza and Meknes. Expansion of arid areas and an increase in temperatures are thought to be synergistic risk factors for increasing the incidence of leishmaniasis in these regions[7]. Each year, 150 cases are recorded, 93% mostly in children under 10 years old[7]. Asymptomatic infection is prevalent in the endemic areas and was evaluated to 11.4%(8). It seems obvious that the asymptomatic infection is the rule, however, this fact still remains unclear. One hypothesis is that differences in genetic background could elicit different immune response resulting in either resistance or susceptibility to the disease[3]. Tumor necrosis factor-α (TNF-α) 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[9]. This cytokine exerts a wide range of biological activities including proliferation and differentiation, apoptosis, cytotoxicity, inflammation and immunomodulation[10]. In fact, serum TNF-α level has been detected in patients with VL and its presence has been related to disease gravity[11]. The tumor necrosis factor-β (TNF-β) is a Th1 cytokine, primarily produced by activated lymphocytes T and B. TNF-β (lymphotoxin α) is also a key mediator in the initiation of a local vascular inflammatory response.

The TNF-α and the TNF-β genes are both located on the short arm of chromosome 6 between the class I and class II regions of the human leukocyte antigen complex. A striking feature of the entire human leukocyte antigen complex is a high degree of genetic variation. Indeed, genetic polymorphisms in TNF-α and TNF-β locus affect expression level of their genes[12]. A guanine to adenosine transition at position –308 (G to A) of TNF-α gene is performed by PCR-restricted fragment length polymorphisms[18]. Briefly, 0.5 µg of DNA was added to 20 µL of reaction mixture containing 20 pmol of each primer (TNF-α: 3′ AGGCAATAGGTTTGAGGCCAT 5′; 3′ TCC TCCCTGCTCGATTCG 5′; TNF-β: 3′ CGGTGCTTGCTGCT TGACTA 5′; 3′ AGAGCTGGTGGGACATGTCTG 5′), with an annealing temperature of 60 °C for TNF-α and 65 °C for TNF-β. In a final volume of 15 µL, 10 µL of the PCR products was digested by S 11 U of Ncol (BioLabs, New England), for 3 h at 37 °C.

Restriction fragments were separated in 3% agarose gel electrophoresis. For TNF-α gene, 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 TNF3 allele and 107 bp for TNF2 allele. For TNF-β gene, amplification followed by Ncol digestion generated a fragment of 740 bp for allele 1 (TNF-β 2) and 555 plus 185 bp for allele 2 (TNF-β 1).

2.2. Single nucleotide polymorphisms (SNPs) genotyping

Genomic DNA was extracted from peripheral blood leukocytes by phenol-chloroform procedure, as previously described[17]. The blood samples were submitted to digestion in sodium dodecyl sulfate/ proteinase K buffer at 37 °C for 6 to 12 h, followed by phenol and chloroform extractions. DNA was then ethanol-precipitated and resuspended in TE buffer. Genotyping of the biallelic polymorphisms in the promoter region at position –308 (G to A) of TNF-α gene and in intron 1 at position +252A/G of TNF-β gene was performed by PCR-restricted fragment length polymorphisms[18]. Briefly, 0.5 µg of DNA was added to 20 µL of reaction mixture containing 20 pmol of each primer (TNF-α: 3′ AGGCAATAGGTTTGAGGCCAT 5′; 3′ TCC TCCCTGCTCGATTCG 5′; TNF-β: 3′ CGGTGCTTGCTGCT TGACTA 5′; 3′ AGAGCTGGTGGGACATGTCTG 5′), with an annealing temperature of 60 °C for TNF-α and 65 °C for TNF-β. In a final volume of 15 µL, 10 µL of the PCR products was digested by S 11 U of Ncol (BioLabs, New England), for 3 h at 37 °C.

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2.3. Statistical analysis

The Hardy-Weinberg equilibrium was tested for each group, using the Chi-square test. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA). The odds ratio (OR) and P values were calculated for each allele and genotype among case and control groups. Allelic, genotypic frequencies and the recessive model were compared by Fisher’s exact test, ORs and 95% confidence interval (CI) were estimated.

3. Results

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-restricted length polymorphism for 55 patients: all were found to be infected by *L. infantum*, the only species responsible for visceral form of leishmaniasis in Morocco (data not shown).

The observed genotypes and alleles frequencies for TNF-α and TNF-β genes in the present survey are shown in Tables 1, 2 and 3. All SNPs were in Hardy-Weinberg equilibrium. Table 1 shows the distribution of genotype and allele frequencies in TNF loci among cases and asymptomatic carriers DTH+. No significant difference was noted in genotype or allele frequencies of TNF-α and TNF-β gene polymorphisms between patients and DTH+ control group. Regarding
the association between TNF-α promoter variants with the outcome of Leishmania infection, 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 2). A minor significant association was found between DTH+ and DTH- for the heterozygote genotype 1/2 (P = 0.021) (Table 3). The minor allele 2 frequencies in TNF-α promoter region are 21.6% in VL, 15.8% in DTH+ and 10.3% in DTH- groups.

### Table 1

Distribution of allelic and genotypic frequency of the -308 TNF-α polymorphisms in the VL versus DTH+ and DTH- groups.

| TNF-α Polymorphism | VL | DTH+ | OR (CI) 95% | P |
|--------------------|----|------|-------------|---|
| 1/1                | 66 (64.71%) | 67 (72.83%) | 1 | |
| 1/2                | 28 (27.45%) | 21 (22.83%) | 0.726 (0.377–0.140) | 0.336 |
| 2/2                | 8 (7.84%)   | 4 (4.34%)   | 0.445 (0.128–0.448) | 0.190 |
| Recessive          | 1/1 | 66 (64.71%) | 67 (72.83%) | 1 |
| Model              | 1/2 + 2/2 | 36 (35.29%) | 25 (27.17%) | 0.687 (0.376–1.256) | 0.221 |
| Allelic frequency  | 1   | 0.784 ± 0.056 | 0.842 ± 0.053 | 1 |
| frequency          | 2   | 0.216 ± 0.056 | 0.158 ± 0.053 | 0.675 (0.331–1.379) | 0.279 |

### Table 2

Distribution of allelic and genotypic frequency of the -308 TNF-α and +252 TNF-β polymorphisms in the VL versus DTH- groups.

| TNF-α Polymorphism | VL | DTH- | OR (CI) 95% | P |
|--------------------|----|------|-------------|---|
| 1/1                | 66 (64.71%) | 32 (82.05%) | 1 | |
| 1/2                | 28 (27.45%) | 6 (15.38%) | 0.440 (0.216–0.896) | 0.021 |
| 2/2                | 8 (7.84%)   | 1 (2.56%)   | 0.297 (0.076–1.165) | 0.065 |
| Recessive          | 1/1 | 66 (64.71%) | 61 (66.30%) | 1 |
| Model              | 1/2 + 2/2 | 36 (35.29%) | 25 (27.17%) | 0.575 (0.311–1.063) | 0.076 |
| Allelic frequency  | 1   | 0.784 ± 0.056 | 0.842 ± 0.053 | 1 |
| frequency          | 2   | 0.216 ± 0.056 | 0.158 ± 0.053 | 0.394 (0.176–0.883) | 0.044 |

### Table 3

Distribution of allelic and genotypic frequency of the -308 TNF-α and +252 TNF-β polymorphisms in the DTH+ versus DTH- groups.

| TNF-α Polymorphism | DTH+ | DTH- | OR (CI) 95% | P |
|--------------------|------|------|-------------|---|
| 1/1                | 67 (72.83%) | 32 (82.05%) | 1 | |
| 1/2                | 21 (22.83%) | 6 (15.38%) | 0.607 (0.293–1.257) | 0.021 |
| 2/2                | 4 (4.34%)   | 1 (2.56%)   | 0.668 (0.145–3.083) | 0.065 |
| Recessive          | 1/1 | 67 (72.83%) | 61 (66.30%) | 1 |
| Model              | 1/2 + 2/2 | 25 (27.17%) | 7 (17.94%) | 0.593 (0.302–1.165) | 0.126 |
| Allelic frequency  | 1   | 0.842 ± 0.053 | 0.897 ± 0.067 | 1 |
| frequency          | 2   | 0.158 ± 0.053 | 0.103 ± 0.067 | 0.583 (0.251–1.357) | 0.205 |

### Table 4

Distribution of allelic and genotypic frequency of the -308 TNF-α and +252 TNF-β polymorphisms in the DTH+ versus DTH- groups.

| TNF-α Polymorphism | DTH+ | DTH- | OR (CI) 95% | P |
|--------------------|------|------|-------------|---|
| 1/1                | 61 (66.30%) | 23 (57.5%) | 1 | |
| 1/2                | 25 (27.17%) | 16 (40%)  | 1.668 (0.923–3.079) | 0.087 |
| 2/2                | 6 (6.52%)   | 1 (2.5%)   | 0.488 (0.923–1.973) | 0.297 |
| Recessive          | 1/1 | 61 (66.30%) | 23 (57.5%) | 1 |
| Model              | 1/2 + 2/2 | 31 (33.69%) | 17 (42.5%) | 1.439 (0.813–2.549) | 0.211 |
| Allelic frequency  | 1   | 0.799 ± 0.058 | 0.775 ± 0.092 | 1 |
| frequency          | 2   | 0.201 ± 0.058 | 0.225 ± 0.092 | 1.179 (0.600–2.318) | 0.632 |

Regarding the polymorphism of TNF-β, 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. No association was found in genotypic frequencies between patients and asymptomatic group (DTH+). 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 (ORs: 0.558, 95% CI: 0.316–0.987; P = 0.044). The recessive genotype 2/2 was associated with VL patients compared to DTH+ group (ORs: 0.245, 95% CI: 0.065–0.925; P = 0.023).

### 4. Discussion

Molecular studies could contribute to a better understanding of pathogenic processes that cause major infectious diseases. TNF, an important proinflammatory cytokine, plays a role in innate and adaptive immune responses, and is also implicated in a wide variety of infectious and autoimmune human diseases. Several studies have attempted to show links between susceptibility to leishmaniasis and TNF gene polymorphisms. Controversial results from these studies prompted us to search for eventual associations of TNF variants with susceptibility versus resistance to L. infantum infection. In the present work, we analysed, thus, two biallelic polymorphisms at position -308 (G: TNF-α allele to A; TNF-2 allele) of TNF-α and in intron 1 at position +252 ofTNF-β. 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. These associations however, point out the need for further studies with a larger sample size of control groups. A few studies with controversial results have been performed to evaluate the TNF polymorphisms in different clinical types of leishmaniasis. In Brasil, 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 TNF-2 were associated with symptomatic VL[19]. 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[20]. Other studies showed that TNF polymorphisms are not responsible for the resistance versus susceptibility to cutaneous leishmaniasis[20,21]. 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[22].

A number of groups have set out to determine whether the polymorphism -308 TNF-α could affect TNF transcription and expression levels. Many studies showed that the rare allele 2 TNF-α is strongly associated with elevated serum TNF levels and a more severe outcome in infectious diseases, such as cerebral malaria and mucocutaneous leishmaniasis[20,23]. In addition, individuals homozygous for this allele are more likely to die or suffer severe neurological complications due to cerebral malaria. This allele...
is overrepresented in diseases where increased TNF-α levels are associated with poor prognosis[23]. Contrariwise, other studies have failed to show any functional difference between the two allelic forms[24,25], but although controversial, the majority of the data support a direct role for the -308 TNF-2 allele in the elevated TNF levels observed in TNF2 homozygotes[15].

Concerning the +252 TNF-β, Naresh et al. reported that the genotype-phenotype analysis of TNF-β revealed a higher expression levels in patients with GG and AA genotypes as compared to controls, while, the genotype TNF-β +252A/G was associated with vitiligo susceptibility and influence the TNF-β expression[26]. TNF seems to be especially important during infection with intracellular pathogens. In order to assess the importance of TNF for protection against leishmaniasis, several studies were reported both on humans and on experimental models. Indeed, in some rheumatic disease patients under anti-TNF therapy, the consequence of blocking TNF was a reoccurrence of the clinical symptoms of leishmaniasis[27-29]. Other argument supporting 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[30]. 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[31]. Oliveira, et al. showed that L. major-infected TNF-receptor-1 KO mice (C57BL/6) can control parasite replication, but fail to resolve lesions. Indeed, the intense inflammatory process was observed even after 20 weeks of infection, while wild-type mice completely heal[32]. On the other hand, no significant difference in the levels of TNF mRNA expression was found in both strains. The information resulting from these investigations supports the notion that, in vivo, TNF is not the decisive factor responsible for the resistant versus susceptible phenotype in Leishmania infection.

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. Well-designed studies with larger population are needed to understand the specific role of TNF that confer protection against leishmaniasis.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

VL is the most severe form of leishmaniasis caused by L. donovani and L. infantum (chagasi). A usual incidence of VL is approximately 500 000 cases and the mortality rate in most endemic countries is almost 10%, even when treatment is available. The signs and symptoms of VL include prolonged fever, fatigue, weakness, anemia, enlarged lymph nodes, splenomegaly, and hepatomegaly; if left untreated, it is almost always fatal. TNF (cachectin) 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.

Research frontiers

This study examine the implication of the polymorphisms in the -308 position of the promoter region of gene TNF-α and in intron 1 of +252 TNF-β genes in the susceptibility versus resistance to VL in children living Moroccan leishmaniasis endemic area. The study involved 102 children with active VL admitted between 2005 and 2007. 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 (DTH-).

Related reports

No significant difference was noted in genotype or allele frequencies of TNF-α and TNF-β gene polymorphisms between patients and DTH+ control group. The polymorphism of TNF-β and the frequencies of the minor allele 2 are 29.4% in VL, 20.1% in DTH+ and 22.5% in DTH- groups. A few studies with controversial results were reported to evaluate the TNF polymorphisms in different clinical types of leishmaniasis. However, this study in agreement with Cabera et al. (1995), 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.

Innovations & breakthroughs

Their data show that -308 TNF-α and +252 TNF-β genotypes do not influence susceptibility versus resistance to VL. 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. TNF seems to be especially important during infection with intracellular pathogens.

Applications

To examine whether polymorphic alleles at these two loci are involved in the susceptibility to VL in children. These results must be regarded as preliminary but further study with larger populations is very important and worthwhile. VL in Morocco is exclusively caused by L. infantum, but can be apply in other counties also.
Peer review
This is a good study in which the authors evaluated VL is the most severe form of leishmaniasis. Also they studied TNF production and with susceptibility to intracellular infectious diseases. This study aimed to examine whether polymorphic alleles at these two loci are involved in the susceptibility to VL in Moroccan children.

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