Association between the Tumor Necrosis Factor Locus and the Clinical Outcome of *Leishmania chagasi* Infection

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A periurban outbreak of visceral leishmaniasis (VL) caused by the protozoan *Leishmania chagasi* is ongoing outside Natal, northeast Brazil. Manifestations range from asymptomatic infection to disseminated visceral disease. Literature reports suggest that both genetic and environmental factors influence the outcome of infection. Due to the association of the tumor necrosis factor (TNF) locus with other infectious diseases, we examined whether polymorphic alleles at this locus are associated with the outcome of *L. chagasi* infection. Neighborhoods with ongoing transmission were identified through patients admitted to local hospitals. Altogether, 1,024 individuals from 183 families were classified with the following disease phenotypes: (i) symptomatic VL, (ii) asymptomatic infection (positive delayed-type hypersensitivity [DTH+]), or (iii) no evidence of infection (DTH−). Genotypes were determined at a microsatellite marker (MSM) upstream of the *TNFB* gene encoding TNF-β and at a restriction fragment length polymorphism (RFLP) at position −307 in the promoter of the *TNFA* gene encoding TNF-α. Analyses showed that the distribution of *TNFA* RFLP alleles (TNF1 and TNF2) and the TNF MSM alleles (TNFa1 to TNFa15) differed between individuals with VL and those with DTH+ phenotypes. TNF1 was transmitted more frequently than expected from heterozygous parents to DTH+ offspring ($P = 0.0006$), and haplotypes containing TNF2 were associated with symptomatic VL ($P = 0.0265$, transmission disequilibrium test). Resting serum TNF-α levels were higher in TNF1/2 heterozygotes than in TNF1/1 homozygotes ($P < 0.05$). These data led us to hypothesize that an individual's genotype at the TNF locus may be associated with whether he or she develops asymptomatic or symptomatic disease after *L. chagasi* infection. The results preliminarily suggest that this may be the case, and follow-up with larger populations is needed for verification.

The *Leishmania* spp. are obligate intracellular protozoa that cause a spectrum of diseases, including cutaneous, mucocutaneous, and visceral leishmaniasis (VL), in tropical or subtropical countries. In Latin America, the form of leishmaniasis leading to most fatalities, VL, is caused by *Leishmania chagasi* (44). The outcome of *L. chagasi* infection ranges in severity from asymptomatic infection to a severe progressive wasting disease called VL, which has 10% mortality even with medical treatment (5, 33, 34, 44). The factors that determine whether an individual will develop asymptomatic or symptomatic disease after *L. chagasi* infection are not defined.

Malnutrition, young age, and gender in those over age 10 predispose individuals to develop VL (21, 31). In addition, studies of inbred mice have led investigators to hypothesize that genetic factors may also contribute to the outcome of human disease (10, 12). Supporting this hypothesis, several groups of researchers have independently documented familial aggregation of either symptomatic VL or positive delayed-type hypersensitivity (DTH) to leishmania antigen, a measure of asymptomatic infection (1, 12, 19, 28, 34). The results of seg-

regation analysis of symptomatic VL in multicase families from northern Brazil were consistent with the hypothesis that susceptibility to active disease is influenced by an additive dominant single gene (11). As reported in a previous study, comparison of siblings of VL patients with the general population showed that the sibling relative risk for VL was 33.6 (43). The results of a second segregation analysis of individuals with positive DTH skin test reactivity to leishmania antigen in families from a region where leishmaniasis is endemic were also consistent with the hypothesis that genetic factors contribute to asymptomatic *L. chagasi* infection in Brazilians; the data were consistent with a contribution from a recessive or additive gene(s) (28). These data led us to hypothesize that an individual's genetic makeup may determine in part how he or she will respond to *L. chagasi* infection.

Leishmaniasis is a vector-borne disease transmitted by *Phlebotomus* or *Lutzomyia* sp. sand flies, insects with a short flight pattern and focal habitat (44, 63). Identification of humans who are exposed is difficult with such a vector-borne disease. A periurban outbreak of VL outside the city of Natal, northeast Brazil, allowed us to identify neighborhoods in which there was recent or ongoing transmission of *L. chagasi* infection. Unlike prior studies which have focused on either patients with VL or individuals with asymptomatic infection (positive skin test result for DTH to leishmania antigen), our study differentiated among individuals with active disease, individuals with asymp-

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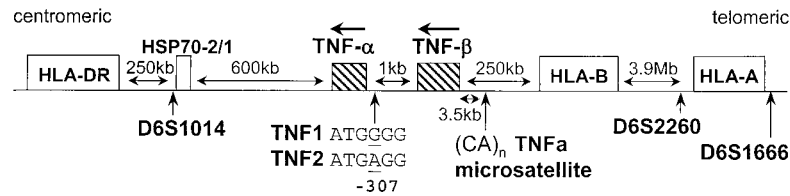
FIG. 1. Map of the human TNF locus and HLA loci based upon the studies of Wilson et al. (61) and Zanelli et al. (64). The drawing indicates the relative positions of the *TNFA* and *TNFB* genes encoding TNF-α and TNF-β, respectively, with respect to HLA regions on human chromosome 6p. Rectangles indicate genes, and horizontal arrows above genes show the direction of transcription. Vertical arrows indicate the positions of markers used in this study. These include the polymorphic TNF MSM repeat upstream of *TNFB*; the RFLP marker for TNF1 and TNF2 alleles in the *TNFA* promoter, located at position −307 (called −308 in the literature) with respect to transcription; and three MSMs in flanking HLA regions. HSP70-2/1 indicates the location of the *HSP70-2* (centromeric) and *HSP70-1* (telomeric) genes. Distances between regions, marked with double-headed arrows, are indicated in kilobases or megabases. The figure is not drawn to scale.

tomatic infection, and individuals living in households with a high chance of exposure but who remained disease free. The tumor necrosis factor (TNF) locus, which includes genes encoding TNF-α and TNF-β, has been associated with the outcome of several infectious diseases, including malaria and mucosal leishmaniasis (20, 24, 29, 40, 56, 58). In addition, VL is accompanied by high levels of TNF-α (6). The purpose of the present study was to assess the contribution of polymorphic markers at the TNF locus to the different clinical outcomes of *L. chagasi* infection.

MATERIALS AND METHODS

Study design. Individuals with active VL were identified after admission to one of three public hospitals in Natal. Criteria for a diagnosis of VL included symptoms of leishmaniasis, a clinical response to antimony therapy, and either a bone marrow smear containing leishmania amastigotes or a positive serologic reaction to total leishmania and k39 antigens (52). Diagnoses of VL were confirmed through hospital records. Families of patients and families living in adjacent households (neighborhood families) were enrolled in the study. Neighborhood families were chosen by selection of the household containing a nuclear family with children that was living nearest to the index family. The neighborhood families were excluded prior to recruitment if they lived more than 500 m away. Most neighboring families lived between 2 and 10 m away from index families. If two neighboring families lived equally close to the dwelling of the VL index family, then more than one neighborhood family was enrolled for that particular index family.

After obtaining informed consent, family members were interviewed in a group setting to determine exposure, medical history, and family pedigree. Subjects underwent a physical examination. Blood was drawn for complete blood count, white blood cell differential count, leishmania serology testing, and DNA extraction. A Montenegro (*L. chagasi* antigen) DTH skin test (kindly provided by Steven Reed, Infectious Diseases Research Institute, Seattle, Wash.) was performed. An area of induration of ≥5 mm at 48 to 72 h after testing was considered a positive result. Any medical conditions discovered, including symptoms and serology suggesting VL, were treated or referred for appropriate medical care.

Subjects and pedigrees. Phenotypes and genotypes were determined for 1,024 individuals from 183 families. Eighty-three families were selected because one or more members had a case of VL; 100 families were neighborhood families. Of the 183 families, 85 contained a member with current VL or a history of VL and 98 had no members with VL. Family size ranged from 1 to 27 members (mean, 6.9). Disease phenotypes were defined as follows: (i) VL, active or prior VL (104 individuals); (ii) DTH+, asymptomatic infection indicated by a positive Montenegro skin test result but without a history of VL (381 individuals); and (iii) DTH−, negative leishmania serology and a negative Montenegro test result but presumably exposed to VL due to residence for ≥4 years in or near households where individuals had acquired VL (513 individuals). Twenty-six individuals with positive serology but negative Montenegro test results and no history of VL were excluded from genetic studies, since seropositivity is usually transient and these individuals could resolve into any of the other phenotypes. Eighteen families had more than one case of VL; one family had three cases.

Serology was measured by using published enzyme-linked immunosorbent

assay (ELISA) protocols, using both whole *L. chagasi* promastigote antigen and recombinant k39 (rk39; kindly provided by Steven Reed) (17, 27, 52). Briefly, 96-well ELISA plates were coated with 200 μg of *L. chagasi* lysate or 50 μg of rk39, followed by incubation in patient serum at a dilution of 1:500. After the blocking and washing steps, the reaction was developed with protein A-horseradish peroxidase (Boehringer Mannheim) and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as previously reported (17). ELISA plates included positive control VL patient sera and negative control sera from unexposed individuals (26). *L. chagasi* antigen was prepared from a local isolate of *L. chagasi*.

DNA was extracted from 10 ml of anticoagulated whole blood (EDTA) by erythrocyte lysis in 70 μg of $NH_4H_2CO_3$/ml and 7.0 mg of NH_4Cl/ml followed by lysis of leukocytes in 1% sodium dodecyl sulfate, 100 mM EDTA, and 200 mM Tris (pH 8.5) and precipitation in isopropanol.

Genotyping methods. Genotypes were determined for the TNF dinucleotide microsatellite marker (TNF MSM), a marker located just upstream of the *TNFB* gene encoding TNF-β (Fig. 1) (61). This TNF MSM is the most polymorphic of the five microsatellites (designated TNFa to TNFe) in the TNF locus. PCR was performed on 40 ng of genomic DNA with primers TNFB.IR2 (5′-GCCCTCT AGATTTCATCCAGCCACA-3′) and TNFB.IR4 (5′-CCTCTCTCCCCTGCA ACACACA-3′) (GDB:574102; Integrated DNA Technologies, Iowa City, Iowa) (42). Fifteen alleles of 101 to 125 bp containing CA repeats, which were detected by silver staining of 6% denaturing polyacrylamide gels, corresponded to the reported TNFa1 to TNFa15 alleles (30, 35).

TNF-α −307 promoter polymorphism. We evaluated a restriction fragment length polymorphism (RFLP) reported at position −307 in the promoter of the *TNFA* gene encoding TNF-α (20) (Fig. 1). A 107-bp fragment of DNA was amplified using primer pairs 5′-AGGCAATAGGTTTTGAGGGCCAT-3′ and 5′-TCCTCCCTGCTCCGATTCCG-3′ (GDB:196368). Digestion of the TNF1 (−307*G) allele with *Nco*I yielded fragments of 87 and 20 bp on a 3% agarose gel, whereas TNF2 (−307*A) remained uncut (107 bp) (30, 60). For RFLP analysis, 751 individuals were chosen because they could be included in the transmission disequilibrium test (TDT) or case-control analyses.

HLA region markers. MSMs near the flanking major histocompatibility complex (MHC) class II (between *TNFA* and HLA-DR) and MHC class I (located telomeric to HLA-B) regions were evaluated (Fig. 1). The trinucleotide repeat marker D6S1014 (CHLC.GCT4B05) between the HLA-DR region and *HSP70-2* was amplified using primers 5′-GGGTCTGACCACTGAGACAC-3′ and 5′-CA GTGAGAGCTCTGAGGGTC-3′. The Cooperative Human Linkage Center (CHLC) marker GATA43E06 (D6S2260) located telomeric to HLA-B was amplified using primers 5′-TCTGTATACTTTCCCTGCAGG-3′ and 5′-CCTGGG CTTAAGAAAGCTTT-3′ (64). The dinucleotide repeat marker D6S1666 (AFMc019yc5) located 0.25 Mb telomeric to the HLA-A region was amplified with primers 5′-CTGAGTTGGGCAGCATTTG-3′ and 5′-ACCCAGCATTTT GGAGTTG-3′.

TNF ELISA. TNF-α in the serum was detected by ELISA. The reagents used were 4 μg of monoclonal antibody MAB610/ml (capture), 200 ng of biotinylated BAF210 goat antiserum/ml (detection), streptavidin horseradish peroxidase (Zymed Laboratories, San Francisco, Calif.), and ABTS developing reagent (Kirkegaard & Perry Laboratories, Gaithersburg, Md.). A standard curve was generated with recombinant human TNF-α (R&D Systems, Minneapolis, Minn.). The sensitivity was 5 pg/ml.

Statistical analyses. Allele frequencies were estimated using unrelated individuals from different pedigrees (excluding probands) or within pedigrees when related only by marriage (Table 1). The association between genetic markers and case-control status, with different definitions of "case" and "control" as shown in

TABLE 1. TNF MSM and TNF-α promoter RFLP
allele frequencies

Marker and allele[a]	Frequency[b]	
	Total sample	Unrelated individuals
TNF MSM		
TNFa1	0.031	0.032
TNFa2	0.228	0.208
TNFa3	0.006	0.003
TNFa4	0.079	0.075
TNFa5	0.072	0.051
TNFa6	0.208	0.193
TNFa7	0.104	0.168
TNFa8	0.012	0.008
TNFa9	0.015	0.013
TNFa10	0.137	0.151
TNFa11	0.077	0.070
TNFa12	0.007	0.003
TNFa13	0.021	0.023
TNFa14	0.001	0.000
TNFa15	0.002	0.002
TNF-α promoter RFLP		
TNF1	0.848	0.860
TNF2	0.152	0.140

[a] Fifteen polymorphic alleles of the TNF microsatellite dinucleotide repeat marker upstream of the *TNFB* gene and two alleles of the RFLP marker at position −307 in the TNF-α promoter were detected.
[b] Shown are the frequencies of alleles in the entire population (Total sample) and in 301 unrelated individuals.

Table 2, was investigated using contingency table analysis as implemented in the CLUMP program (51). This program compares the frequency of each allele for a particular phenotype with the frequency of all other alleles clumped together. Monte Carlo simulations were conducted with the CLUMP program to determine asymptotic *P* values that account for sparse cells in contingency tables. The T3 test statistic reported here resulted from the identification of a single allele which, when compared to all other alleles clumped together, provided a two-by-two table with the largest chi-square statistic (lowest *P* value) for the data.

An extended TDT for multiallele marker loci was used as implemented in the ETDT program (50). This method evaluates the pattern of allele transmission across genotypes by using logistic regression. The program considers all alleles across families, so that only alleles preferentially transmitted to multiple affected children are significant. Only unrelated trios were included in the analysis. In some cases, this exceeded the total number of pedigrees when nuclear families were connected by marriage. Haplotypes were determined by examination of three-generation families in which it was evident which TNF MSM and TNF-α

alleles were syntenic. The extended TDT was also used to assess disequilibrium in the transmission of these 30 haplotypes.

SIMLINK was used to estimate the power to detect linkage in these families (14).

RESULTS

Allele frequencies. Microsatellite amplification identified 15 different TNF MSM alleles in 1,024 individuals (Table 1). The 751 individuals that could be included in association analyses were also genotyped for the −307 polymorphism in the *TNFA* promoter (TNF1 and TNF2). It was found that 596 individuals were homozygous for TNF1 (72%), 216 were heterozygous (TNF1/2; 26%), and 18 were homozygous for TNF2 (2%). The frequency of the less common TNF2 allele was 0.152, similar to that of other populations (20, 40, 46, 59, 61). The distribution of the TNF MSM alleles and the distribution of TNF-α alleles were consistent with a Hardy-Weinberg equilibrium. This was true both when all individuals were considered in the analysis and when only unrelated individuals were included.

The TNF MSM polymorphism and the *TNFA* promoter RFLP are only 3 kb apart from each other on chromosome 6p. As expected, these markers are tightly associated in linkage disequilibrium ($P = 0.0007$, chi-square test). TNF2 occurred relatively more frequently among individuals bearing the TNFa5 allele (32.4%). In contrast, TNF2 occurred less frequently among individuals bearing the TNFa11 or TNFa13 allele (9.3 and 5.0%, respectively).

Case-control analyses. There were differences in the distributions of the TNF MSM and *TNFA* promoter RFLP alleles between unrelated individuals in the different phenotype groups (Table 2). TNFa5 was more common in VL patients than in DTH+ individuals, whereas TNFa11 and TNFa13 were more common in DTH+ individuals than in those with other phenotypes. The *TNFA* promoter RFLP allele TNF2 occurred more frequently in VL patients (17.1%) than in DTH+ (6.3%) individuals.

ETDT. A TDT (ETDT program) was used to assess the pattern of transmission of *TNFA* promoter RFLP alleles and the 15 TNF MSM alleles from heterozygous parents to their offspring with either the VL or DTH+ phenotype (50). Only unrelated trios were included in the analysis. The results indi-

TABLE 2. Case-control analyses of the distribution of alleles in unrelated individuals with different phenotypes

Marker and allele	P value from analysis of marker or individual alleles (allele frequencies)[a]		
	VL vs DTH+	VL vs DTH−	DTH+ vs DTH−
TNFA promoter polymorphism	0.026	0.59	0.27
TNF1	0.026 (0.829 vs 0.937)	0.59 (0.829 vs 0.858)	0.27 (0.898 vs 0.856)
TNF2	0.026 (0.171 vs 0.063)	0.59 (0.171 vs 0.142)	0.27 (0.102 vs 0.144)
TNF MSM[b]	0.029	0.420	0.031
TNFa5	0.009 (0.100 vs 0.024)		
TNFa11	0.005 (0.033 vs 0.146)		0.004 (0.118 vs 0.045)
TNFa13	0.086 (0.011 vs 0.055)		0.031 (0.049 vs 0.014)

[a] *P* values result from chi-square analysis (CLUMP program). The CLUMP program compares each specific allele with all other alleles grouped together. The values shown are from T3 analysis, in which each allele is compared with all other alleles clumped together in a two-by-two table. The value showing the largest chi-square or lowest *P* value is shown. *P* values were not adjusted for multiple hypothesis tests. For the comparison of VL and DTH+, 90 individuals were in the VL group and 164 were in the DTH+ group; for the comparison of VL and DTH−, 90 were in the VL group and 230 were in the DTH− group; and for the comparison of DTH+ and DTH−, 246 were in the DTH+ and 222 were in the DTH− group. These numbers differ between groups since only unrelated individuals connected by marriage within each pedigree were analyzed.
[b] The three TNF MSM alleles listed are those for which the *P* values were <0.10.

TABLE 3. Transmission disequilibrium analysis of the association of TNF MSM (15 alleles) and *TNFA* promoter RFLP (2 alleles) with the three phenotypes[a]

Phenotype	*P* value[b] (no. of heterozygous parents included in analysis)		
	TNF MSM	*TNFA* RFLP	Haplotype
VL	0.0886 (118)	0.3157 (25)	0.0265 (59)
DTH+	0.0797 (183)	0.0006 (36)	0.0040 (101)
DTH−	0.1353 (390)	0.3824 (84)	0.3255 (199)

[a] Only unrelated trios were included in the analysis.
[b] *P* values were obtained from genotype-wise analysis and indicate the strength of the association with the phenotypes.

cated an excess frequency of transmission of specific alleles at each locus (Tables 3 and 4). Notably, TNF1 was passed 28 of 36 times from heterozygous parents to DTH+ offspring (*P* = 0.0006) compared with 38 of 84 times to DTH− offspring (*P* = 0.3824) (Table 4). This suggests an association between TNF1 and the tendency to develop asymptomatic infection. TDT results for passage of *TNFA* promoter RFLP alleles to VL offspring did not meet statistical significance, possibly because of an insufficient number of trios with heterozygous parents (*n* = 25). Nonetheless, ETDT analysis of the transmission of TNF MSM/*TNFA* RFLP haplotypes, which include 30 alleles, to VL offspring yielded a *P* value of 0.027 (*n* = 59 trios).

Power studies. With VL defined as affected, DTH+ defined as unaffected, and DTH− defined as unknown, SIMLINK analysis of the families with two or more cases of VL (14) indicated that the power of finding a lod score of >1.0 for these families was 0.46 at a θ value of 0.0 or 0.23 at a θ value of 0.10. This result suggested that we had little power to detect linkage to a susceptibility gene by using linkage methods of analysis. This was not a surprise since a simple Mendelian model of inheritance must be designated to perform nonparametric linkage, and such a model is not appropriate for this complex disease. Therefore, we relied upon association methods as the most appropriate method of analysis for this population.

ETDT and case-control analysis of HLA-DR and HLA-B markers. Because the TNF locus is in linkage disequilibrium with HLA, it is possible that the low *P* values resulting from the TDT analysis were actually due to strong linkage disequilibrium with nearby HLA genes. We therefore determined the genotype by using markers in flanking regions toward both the class II and class I HLA regions. Case-control and TDT analyses were performed on the same trios used above for analysis of the VL and DTH+ phenotypes (Table 5). Six alleles were

TABLE 4. Frequencies with which *TNFA* promoter alleles were passed from heterozygous parents to offspring with the different phenotypes in unrelated trios

Phenotype	No. of cases in which allele was passed			*P* value[a]
	TNF1	TNF2	Total	
VL	15	10	25	0.3157
DTH+	28	8	36	0.0006
DTH−	38	46	84	0.3824

[a] The *P* values were obtained from TDT allele-wise analysis.

TABLE 5. Case-control (CLUMP program) and ETDT analysis of microsatellites in the HLA-DR (D6S1666) and HLA-B (D6S2260) regions

Phenotype(s)	*P* value resulting from each comparison			
	Case-control analysis[a]		ETDT analysis[b]	
	HLA-DR	HLA-B	HLA-DR	HLA-B
VL vs DTH+	0.361	0.336		
VL vs DTH−	0.379	0.901		
DTH+ vs DTH−	0.231	0.070		
VL			0.0201	0.4818
DTH+			0.2271	0.1541

[a] Data from the CLUMP program, which compares each specific allele to all other alleles grouped together, are the results of T3 analysis.
[b] ETDT results are from a genotype-wise analysis. Only VL and DTH+ trios were examined.

detected at the D6S1014 marker between HLA-DR and HSP70-2, which in turn lies between HLA-DR and the TNF locus (Fig. 1). Five alleles were detected at the D6S2260 marker that lies telomeric to HLA-B. Thirteen alleles were detected at the D6S1666 (AFMc019yc5) marker which is further in the direction of the telomere, near HLA-A. The results of case-control comparisons (CLUMP program) were not significant for any of the phenotypic comparisons with these markers. TDT analysis (ETDT program) of the D6S2260 marker nearest to HLA-B yielded high (nonsignificant) *P* values as well. However, analysis of the D6S1014 marker (near HLA-DR) in DTH+ trios by using ETDT yielded a *P* value of 0.0223, in contrast to a value of 0.6110 for VL trios. Analysis of the D6S1666 marker (telomeric to HLA-A) that lies further from the TNF locus in VL trios yielded a *P* value of 0.0201 compared with a value of 0.2271 for DTH+ trios. Further studies are needed to verify whether there is an association between the DTH+ phenotype and the HLA-DR marker as well as an association between the VL phenotype and markers near HLA-A.

Serum TNF-α levels. Sera were drawn at the time of study entry. TNF-α levels were determined for all individuals for whom there were remaining serum samples after leishmania serology testing. Basal serum levels of TNF-α, measured by ELISA, for individuals with different *TNFA* promoter RFLP genotypes were compared. Attempts were made to include only individuals who were healthy, since TNF-α may transiently rise during acute illness.

There is an increase in TNF-α during active VL (6). Our VL group included individuals at different stages of treatment as well as those cured of prior infection. Furthermore, we had serum from only a subset of VL individuals, in whom the TNF-α serum levels were as follows: 124 ± 121 pg/ml (mean ± standard deviation) for TNF1/1 individuals (*n* = 4; values, 0, 0, 487, and 10); 31 ± 19 pg/ml for TNF1/2 individuals (*n* = 10); and 47 pg/ml for TNF2/2 individuals (*n* = 1). The extreme variability in TNF-α serum levels among VL patients was likely due to the small number of samples and the varied times at which serum was drawn with respect to treatment. Since there was no way to correct for these parameters, we excluded the VL group from the overall statistical analysis shown in Table 6. Including healthy persons only, therefore, individuals with the

TABLE 6. Serum TNF-α levels in DTH+ or DTH− individuals
with different genotypes

Phenotype(s)	Mean serum TNF-α level (pg/ml) ± SEM in individuals with genotype[a]		P value[b]
	TNF1/1	TNF1/2	
DTH+	26.8 ± 9.1 (20)	53.4 ± 17.8 (13)	0.087
DTH−	13.4 ± 4.1 (21)	38.6 ± 13.7 (29)	0.223
DTH+ and DTH−	20.0 ± 5.0 (41)	44.2 ± 11.1 (42)	0.049[c]

[a] The numbers of individuals with the indicated genotypes and phenotypes are shown in parentheses. The number of those with the TNF2/2 genotype ($n = 3$) was too small to provide meaningful data.
[b] Statistical analysis was performed with the Mann-Whitney rank sum test since the data were not normally distributed.
[c] $P < 0.05$.

TNF1/1 genotype had significantly lower levels of TNF-α than did TNF1/2 heterozygotes (Table 6).

DISCUSSION

Recent literature includes many reports of genetic factors that influence the outcome of human infection with intracellular pathogens such as *Mycobacterium* spp., *Salmonella* spp., and *Plasmodium falciparum* (3, 4, 7, 8, 25, 36, 40, 48, 57). Familial aggregation studies and segregation analyses have also suggested that genetic factors contribute to the outcome of human VL (10–13, 28). In the present study, we investigated whether genetic variability at or near the TNF locus is associated with the development of symptomatic or asymptomatic *L. chagasi* infection. This study was unique in that we were able to differentiate symptomatic from asymptomatic infection in individuals living in neighborhoods where there was ongoing transmission of *L. chagasi*. The TNF locus was studied as a candidate susceptibility gene locus for three reasons. First, TNF-α levels are elevated in acute VL (6). Second, polymorphisms at the TNF locus have been associated with a large number of autoimmune and some infectious diseases (24, 29, 40, 56, 58). Third, a case-control study of 46 patients with mucocutaneous leishmaniasis, a hyperergic disease most often caused by *L. braziliensis*, suggested an association with the TNF2 allele (20). Visceral and mucocutaneous leishmaniasis lie at opposite poles of the spectrum of leishmaniasis. Immune responses are suppressed and the parasite load is high during VL, whereas there are few parasites but a vigorous immune response during mucocutaneous leishmaniasis. We hypothesized that distinct and opposite immune factors may predispose individuals to develop each of these diseases. However, our results suggested that the same genotype at the TNF locus is associated with development of these contrasting forms of disease.

Analysis of 1,024 subjects living in neighborhoods where leishmaniasis is endemic suggested an association between the outcome of *L. chagasi* infection and alleles at the TNF locus. The strongest association was found between asymptomatic infection (DTH+) and a polymorphism in the TNF-α promoter at position −307 with respect to the transcription start site (erroneously labeled −308 in the literature [62]). The CA repeat at the TNF MSM was also associated with the DTH+ phenotype although not as strongly. There was not complete association between the two markers that we tested. This could

be due to historical recombination between the two markers, although it is more likely that there have been mutations of the CA repeats since they lie in noncoding regions of the genome. Analysis of extended haplotypes, considering genotypes at both the TNF MSM and the TNF-α RFLP markers, also suggested an association between TNF alleles and symptomatic VL. According to the results of case-control analysis, the genetic differences were greatest between individuals with symptomatic and those with asymptomatic *L. chagasi* infection (VL versus DTH+). Due to the use of multiple testing in this study (multiple markers, phenotypes, and analytical methods), the chance of a type I error was increased. As the most significant *P* values (0.0006 for TDT) were greater than the value of 0.0001 expected of a genome-wide linkage scan, the results must be regarded as preliminary but suggestive that further study with larger populations is worthwhile. Furthermore, due to the smaller number of VL cases compared with cases of asymptomatic infection, the association between TNF and VL warrants further studies with a larger sample size.

Analysis of the same population by using MSMs in the HLA-B and HLA-DR regions did not reveal as strong an association as that with TNF, and there was a lack of association with the marker nearest to HLA-B. The lowest *P* values were obtained in association analyses of asymptomatic infection (DTH+) with the D6S1014 marker near HLA-DR ($P = 0.022$) or in analyses of symptomatic disease (VL) with the D6S1666 marker telomeric to HLA-A ($P = 0.020$). The negative results for an association between the D6S1666 marker and disease suggest that the TNF locus association results are probably not caused by a stronger association with MHC class I genes. The *P* values for the D6S1014 marker and DTH+ phenotype warrant follow-up, but again, these data are weaker rather than stronger than the data for an association of TNF with asymptomatic disease. Taken together, these analyses favor a model in which TNF1 is associated with the development of asymptomatic *L. chagasi* infection detectable by a positive Montenegro skin test exam, independent of a possible association with MHC class I and class II genes.

A case-control study with Gambian children suggested that TNF2/2 homozygotes had a sevenfold increased risk of death or neurological sequelae from malaria than did heterozygotes (40). A previous study of individuals with or without VL in Belém, northern Brazil, did not document linkage with the TNF locus (12). However, by distinguishing those with active VL from those with asymptomatic infection and by distinguishing each of these from the population that is DTH− but likely exposed, we were able to detect an association that might have been inapparent when disease-negative individuals were analyzed as a single group.

Numerous polymorphisms at the *TNFA* locus have been reported, with conflicting reports of functional significance. Promoter polymorphisms cluster in repeating 4-base G/A motifs (49). Whereas some studies show that the −307 promoter polymorphism influences the amount of TNF-α produced by stimulated peripheral blood mononuclear cells (15, 39), others suggest that there is no effect (24, 32, 41, 47). Whether the results are significant depends in part upon the number of subjects examined and the stimulus chosen (2). Studies using reporter genes under the control of the TNF-α promoter have also produced conflicting results (16, 18, 38, 53, 55, 62), which

differ depending on the length of the 5′ untranslated region used and whether a 3′ untranslated region was cloned adjacent to the reporter gene (2). Those studies that have reported a difference in expression, whether or not the difference reached statistical significance, have consistently shown that transcription and/or TNF-α levels are increased in individuals with the rarer −307*A (TNF2) allele. Consistently, TNF1/1 homozygotes in the population we studied had significantly lower basal TNF-α serum levels than did TNF1/2 heterozygotes. It is possible that this result was due to a sequence tightly linked to the −307 polymorphism. Nonetheless, the data suggest that a polymorphic sequence(s) at or near the −307 locus influences TNF-α levels and disease outcome.

The data discussed above led us to hypothesize that the propensity of an individual to develop asymptomatic infection versus symptomatic disease after infection with *L. chagasi* is associated in part with his or her genotype at the TNF locus. If this hypothesis is true, then the symptoms of VL would be due in part to the level of TNF-α achieved (6, 22, 45, 54). Moderate levels of TNF-α, as would be expected among TNF1 homozygotes, would facilitate control of intracellular infection. Higher levels of TNF-α in individuals bearing the TNF2 allele would predispose them to clinical manifestations of VL including fever, wasting, anorexia, pancytopenia, and polyclonal B-cell activation (6, 9). Among persons infected with *Plasmodium falciparum*, the TNF2 allele is found more frequently in individuals who developed the severe cerebral form of disease (40). In an analogous manner, our hypothesis would indicate that heterozygotes in our study population who harbor the TNF2 allele are more likely to develop the severest visceral form of disease after exposure to *L. chagasi* rather than asymptomatic or limited infection. The above-mentioned data do not distinguish whether the *TNFA* RFLP is a functional polymorphism or whether it is in linkage disequilibrium with a true functional polymorphism. Nonetheless, they do lead to the hypothesis that genetically determined high-level TNF-α responses to *L. chagasi* infection might predispose individuals, in part, to development of the severest visceral form of disease due to this intracellular protozoan. It may be reasonable to study agents that can lower TNF-α levels, such as thalidomide or pentoxifylline, as adjuvants to therapy for VL (23, 37).

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