Polymorphism in Tumor Necrosis Factor Genes Associated with Mucocutaneous Leishmaniasis

By Maira Cabrera,*‡ Marie-Anne Shaw,* Claire Sharples,* Hazel Williams,* Marianella Castes,‡ Jacinto Convit,‡ and Jenefer M. Blackwell*

From the *Department of Medicine, University of Cambridge Clinical School, Addenbrooke's Hospital, Cambridge CB2 2QQ, United Kingdom; and the ‡Instituto de Biomedicina, Caracas 1010A, Venezuela

Summary

Recent studies have shown that mucocutaneous leishmaniasis (MCL), a severe and debilitating form of American cutaneous leishmaniasis (ACL) caused by *Leishmania braziliensis* infection, is accompanied by high circulating levels of tumor necrosis factor (TNF)-α. Analysis of TNF polymorphisms in Venezuelan ACL patients and endemic unaffected controls demonstrates a high relative risk (RR) of 7.5 (P <0.001) of MCL disease in homozygotes for allele 2 of a polymorphism in intron 2 of the TNF-β gene, especially in females (RR = 9.5; P <0.001) compared with males (RR = 4; P <0.05). A significantly higher frequency (P <0.05) of allele 2 at the -308-basepair TNF-α gene polymorphism was also observed in MCL patients (0.18) compared with endemic control subjects (0.069), again associated with a high relative risk of disease (RR = 3.5; P <0.05) even in the heterozygous condition. Because both the TNF-α and TNF-β polymorphisms have previously been linked with functional differences in TNF-α levels, these data suggest that susceptibility to the mucocutaneous form of disease may be directly associated with regulatory polymorphisms affecting TNF-α production.

The reason some individuals infected with *Leishmania braziliensis* go on to develop mucocutaneous leishmaniasis (MCL),[1] sometimes months to years after a simple localized cutaneous lesion, is not clearly understood. Clinically advanced MCL has traditionally been associated with strong skin test delayed-type hypersensitivity to leishmanial antigens in vivo, and with high IFN-γ/IL-2–producing T cell proliferative responses in vitro (1–3). Analysis of MCL lesions has demonstrated mRNA for a mixture of Th1 (IFN-γ, IL-2, TNF-β) and Th2 (IL-4, IL-5, IL-10) cytokines (4, 5), with TNF-α mRNA also present. Recently we observed that active MCL disease was also associated with high circulating levels of TNF-α (6), which might, as has now been demonstrated for cerebral malaria (7), be related to genetic regulation of cytokine production. The TNF-α gene itself lies in the class III region of the MHC (8). Although previous studies (9) had demonstrated association between class II DRβ alleles and MCL disease, it was possible that these associations had arisen through linkage disequilibrium between class II genes and variable genetic elements in the class III region known to control TNF-α

production. Two such polymorphisms have been described, at position -308 in the promoter region of the TNF-α gene (10), for which allele 2 is associated with higher constitutive and inducible levels of TNF-α (11), and in intron 2/exon 3 of the TNF-β gene (12), with the two polymorphic alleles variably associated with high and low levels of TNF-α secretion by mononuclear cells, depending on the population under investigation (12–14). The latter polymorphism involves an NcoI polymorphism in intron 2 and is also always associated with a substitution at amino acid position 26 encoded in exon 3 of the TNF-β gene (12). Precisely how this polymorphism influences TNF-α secretion is not known, but may be caused by linkage disequilibrium with other elements within the TNF-α gene itself.

To determine whether these polymorphisms at the TNF loci influence susceptibility to different forms of American cutaneous leishmaniasis (ACL), case/control analysis was undertaken comparing MCL patients or localized cutaneous leishmaniasis (LCL) patients with healthy endemic control subjects. Our results demonstrate a high relative risk (RR = 7.5; P <0.001) of MCL disease in homozygotes for allele 2 (=TNFB*1 [12–14]) of the intron 2/exon 3 polymorphism in the TNF-β gene, especially in females (RR = 9.5; P <0.001) compared with males (RR = 4; P <0.05). A significantly higher frequency (P <0.05) of allele 2 at the

−308-bp TNF-α gene polymorphism was also observed in MCL patients (0.18) compared with endemic controls (0.069), again associated with a high relative risk (RR = 3.5; P <0.05) of disease even in the heterozygous condition. These results suggest that susceptibility to the mucocutaneous form of disease may be directly associated with regulatory polymorphisms affecting TNF-α production.

Materials and Methods

Study Design. Genomic DNA was extracted from EBV-transformed cell lines prepared from 49 ACL patients (age range 11–48 yr) and 43 control subjects (age range = 13–45 yr). As before (1–3), patients were classified on the basis of clinical, parasitological, and histopathological criteria (15) as LCL (n = 24; 16 males, 8 females; mean age = 26.7 ± 8.6 yr) or MCL (n = 25; 14 males, 11 females; mean age = 30.4 ± 10.6 yr). The sample was drawn from patients routinely attending clinic at the Instituto de Biomedicina in Caracas, Venezuela. Patients travel to the clinic from periurban and rural regions surrounding Caracas, mostly from Miranda state. All MCL and the majority of LCL disease in this region is caused by *L. braziliensis* (15). The higher proportion of male to female patients presenting with *L. braziliensis* infection in Venezuela is thought to be related to higher occupational exposure to the vector sandfly. There are no longitudinal data available on the relative proportions of males to females progressing to MCL disease. To test for association between TNF or HLA-DR and disease, ACL patients (MCL alone, LCL alone, and pooled) were compared with 43 endemic volunteers selected as age-matched (mean age 24.9 ± 8.8 yr; 12 males, 31 females) control subjects from a recent vaccine trial carried out in Miranda state, Venezuela (16, 17). A larger set of 64 individuals (18 males, mean age = 18.9 ± 6.5 yr; 46 females, mean age 22.5 ± 8.2 yr) from the vaccine trial were used in testing for linkage disequilibrium between the TNF genes and HLA-DRβ. Volunteers were selected into the trial on the basis of a double skin test–negative response to mycobacterial purified protein derivative and to leishmanial antigen. They had no scars from previous leishmanial lesions or Bacille Calmette-Guérin vaccination, and no other clinical history of leishmaniasis.

Typing of HLA-DRβ, TNF-α and TNF-β Genes. DRβ typing (18) was carried out by Southern blot analysis of RFLPs. This method does not distinguish between DR3 and DRw6, or between DR7 and DRw9. DR15 and DR16 subtypes of DR2 were pooled for analysis. For PCR amplification of TNF-α and TNF-β regions, 1 μg of DNA was added to 40 μl of reaction mixture containing 100 ng of each primer (TNF-α: 5′ AGGCA-ATAGGTTTTGAGGGCCAT 3′, 5′ TCCTCCCTGCTCCGAT-TCCG 3′; TNF-β: 5′ CCGTGCTTCGTGCTTTGGACTA 3′, 5′ AGAGCTGGTGGGACATGTCTG 3′), 0.25 mM of each dNTP, 1.5 mM MgCl₂, and 1 U Taq polymerase (Cetus Corp., Berkeley, CA). Cycling conditions for TNF-α were as follows: 1 cycle of 94°C for 3 min, 60°C for 1 min, 72°C for 1 min; 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min; 1 cycle of 94°C for 1 min, 60°C for 1 min, 72°C for 5 min. Cycling conditions for TNF-β were as follows: 95°C for 6 min, 35 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min. Products of 107 and 740 bp were generated for TNF-α and TNF-β, respectively. For TNF-α, primers were designed to incorporate a polymorphic site at a position −308 bp of the TNF-α gene into an NcoI restriction site. Restriction digests generated products of 87 and 20 bp for allele 1 and 107 bp for allele 2. The

740-bp fragment amplified across intron 2 of the TNF-β gene also incorporates a polymorphic NcoI site, which generates fragments of 740 bp for allele 1 (=TNFB*2 or the 10-kb allele of previous workers [12–14]), and 555 plus 185 bp for allele 2 (=TNFB*1 or the 5.5-kb allele [12–14]).

Statistical Analyses. The χ² test with Yates correction or Fisher's exact test were used to test for significant associations between disease phenotype (LCL, MCL, or pooled ACL against endemic controls) and HLA-DRβ, TNF-α, or TNF-β genotypes or alleles. Relative risks associated with a particular allele (e.g., allele 2) were calculated (19) using a χ² distribution: $RR = n_1.n_4/n_2.n_3$, where n_1 is the proportion of patients carrying allele 2, n_2 is the number of controls with allele 2, and n_3 and n_4 are the corresponding proportions of individuals in patient and control groups not carrying allele 2. The test statistic is calculated as $(1/V)(\log_e RR)^2$, where $V = 1/n_1 + 1/n_2 + 1/n_3 + 1/n_4$. The value is compared with a χ² distribution with one degree of freedom and must be >3.84 to attain statistical significance (P <0.05). To test for linkage disequilibrium between DRβ and the TNF loci, the distribution of DRβ alleles among individuals homozygous at the TNF-α or TNF-β genes was compared in all ACL patients plus control subjects (using the larger vaccine volunteer group). Associations with disease type were also analyzed using computer-generated haplotype frequencies obtained by an iterative procedure based on the gene counting technique (20). This computer-assisted analysis was carried out using the linkage utility program ASSOCIATE on-line to the HGMP Resource Centre (Cambridge, UK).

Results and Discussion

Association between DRβ and Disease. Table 1 shows allele frequencies for DRβ in the three patient groups (ACL and the two subgroups, LCL and MCL) and age-matched controls. As with previous studies (9), DR2 showed a significantly reduced frequency in MCL patients (0.04), and in ACL patients overall (0.09), compared with control subjects (0.18). DR7/DRw9, which had a very low frequency (0.01) in the control population, showed a significantly higher frequency in LCL patients (0.11), and in ACL patients overall (0.08), compared with control subjects. Both effects were sufficiently strong to cause significant differences when the total ACL group was compared with control subjects, even though LCL patients did not themselves show a significant decrease in DR2, and MCL patients did not show a significant increase in DR7/DRw9.

Association between Disease and Polymorphisms at the TNF Loci. Table 1 also shows allele frequencies for the two TNF genes in the three patient groups and control subjects. The distribution of genotypes for the two TNF loci among control and patient groups is shown in Table 2. For the TNF-α promoter region polymorphism, where the rarer allele 2 had previously been shown to be associated with increased risk of cerebral malaria in Gambian children (7), only two homozygotes were observed in the total population sample, within the LCL group. Nevertheless, the gene frequency for allele 2 was significantly (P <0.05) higher in MCL patients (P <0.18) compared with control subjects (P <0.07). The overall gene frequency for this allele in patients plus control subjects was 0.12, similar to the fre-

Table 1. *HLA-DRβ, TNF-α, and TNF-β Allele Frequencies (f) in Controls Subjects, Age-Matched ACL Patients, and Age-Matched ACL Patients Stratified by Disease Phenotypes LCL and MCL*

Locus/allele	Controls (n = 43) f	ACL (n = 49) f	ACL (n = 49) P	LCL (n = 24) f	LCL (n = 24) P	MCL (n = 25) f	MCL (n = 25) P
DR1	0.08	0.06	NS	0.07	NS	0.04	NS
DR2 (15/16)	0.18	0.09	<0.05	0.13	NS	0.04	<0.05
DR3/DRw6	0.26	0.23	NS	0.16	NS	0.31	NS
DR4	0.20	0.24	NS	0.22	NS	0.27	NS
DR5	0.17	0.26	NS	0.27	NS	0.26	NS
DR7/DRw9	0.01	0.08	<0.05	0.11	<0.02	0.04	NS
DRw8	0.05	0.04	NS	0.04	NS	0.04	NS
DR10	0.01	0	NS	0	NS	0	NS
DRw8	0.04	0	NS	0	NS	0	NS
TNF-α1	0.93	0.84		0.85		0.82	
TNF-α2	0.07	0.16	NS	0.15	NS	0.18	<0.05
TNF-β1	0.69	0.51		0.60		0.42	
TNF-β2	0.31	0.49	<0.025	0.40	NS	0.58	<0.01

Fisher's exact test was used to determine whether significant differences (P values) in gene frequencies were observed when each patient group (ACL or LCL and MCL subgroups) were compared with endemic control subjects. n, number of individuals in each group; 2n, number of chromosomes scored to determine allele frequencies. Similar results were obtained when the larger control group was compared with the patient groups, i.e. without precise age matching.

quency of 0.16 maintained in the Gambian population despite the increased risk of death or neurological sequelae from cerebral malaria. The relative risk of MCL disease associated with allele 2, even in the heterozygous condition, was 3.5 (P <0.05). For the TNF-β polymorphism, a high relative risk (RR = 7.5; P <0.001) of MCL disease was observed in individuals homozygous for allele 2. Stratification by sex revealed a higher relative risk in homozygous females (RR = 9.5; P <0.001) compared with homozygous males (RR = 4.0; P <0.05). The relative risk of MCL associated with allele 2 in the heterozygous or homozygous condition was 3.2 (P <0.05): 2.9 (P <0.05) in females and 4.0 (P <0.001) in males; thus, for females the risk is higher when allele 2 is homozygous, whereas for males an equivalent risk occurs when one or two copies of allele 2 are present.

Evidence That TNF-α Is the Functionally Important Locus in Determining MCL Disease. The observation that active cases of MCL have high circulating TNF-α levels (6) suggests that the functional basis of the associations between disease phenotype and HLA class II and class III genes observed here may result from linkage disequilibrium with regulatory elements influencing TNF-α transcription. The −308-bp polymorphism at the TNF-α gene has already been shown to be a functionally important promoter region element influencing TNF-α transcription (11). This polymorphism might therefore be directly responsible for MCL disease in individuals bearing at least one copy of allele 2.

Polymorphism at the TNF-β locus was also significantly associated with MCL disease. Although significant (P = 0.004) allelic association between TNF-α and TNF-β genes was demonstrated in the total population sample, case/control ratios for haplotype frequencies in ACL, LCL,

Table 2. *Distribution of TNF-α and TNF-β Genotypes in Control Subjects, Age-matched ACL Patients, and Age-matched ACL Patients Stratified by Disease Phenotypes LCL and MCL*

Locus/genotype/ haplotype	Controls (n = 43)	ACL (n = 49)	LCL (n = 24)	MCL (n = 25)
TNF-α*				
1/1	37	35	19	16
1/2	6	12	3	9
2/2	0	2	2	0
TNF-β‡				
1/1	19	15	10	5
1/2	21	20	9	11
2/2	3	14	5	9

*$\chi^2_{(4)}$ = 11.41, P <0.05 overall.
‡$\chi^2_{(4)}$ = 10.45, P <0.05 overall; $\chi^2_{(2)}$ = 10.25, P <0.01 for control subjects versus MCL; $\chi^2_{(2)}$ = 7.25, P <0.05 for control subjects versus ACL.
n, number of individuals in each group.
The subscripted (2) and (4) represent degrees of freedom (df).

Table 3. *Computer-generated TNF-α.TNF-β Haplotype Frequencies in Control Subjects, ACL Patients, and ACL patients Stratified by Disease Phenotypes LCL and MCL*

Haplotype	Haplotype frequencies							
	Control subjects (2n = 128)	ACL (2n = 92)		LCL (2n = 46)		MCL (2n = 46)	
TNF-α1.TNF-β1	0.69	0.42	(0.6)	0.49	(0.7)	0.35	(0.5)
TNF-α1.TNF-β2	0.24	0.42	(1.7)	0.36	(1.5)	0.48	(1.9)
TNF-α2.TNF-β1	0.01	0.06	(5.9)	0.07	(7.4)	0.04	(4.1)
TNF-α2.TNF-β2	0.06	0.10	(1.7)	0.08	(1.3)	0.13	(2.2)
$\chi^2_{(3)}$		17.23*		9.07‡		16.34*	

*P <0.001, ‡P <0.005.
$\chi^2_{(3)}$ tests for significant differences in the distribution of TNF-α. TNF-β haplotypes between controls and each of the three patient groups. Case/control ratios for haplotype frequencies are shown in parentheses. 2n, number of chromosomes scored to determine haplotype frequencies. The subscripted (3) represents df.

and MCL patients compared with control subjects suggest an equivalent risk (case–control ratios for TNF-α1.TNF-β2, TNF-α2. TNF-β1, or TNF-α2.TNF-β2 all >1.0) in individuals bearing either TNF-α allele 2 or TNF-β allele 2 (Table 3). Hence, there is a significant negative risk associated with the haplotype TNF-α1.TNF-β1 (case/control ratios <1.0) for both LCL (RR = 0.45; P <0.05) and MCL (RR = 0.24; P <0.01) groups. Using the computer-generated haplotype frequencies, a significant difference in the distribution of haplotypes was observed (Table 3) in ACL (χ^2 = 17.23; P <0.001), LCL (χ^2 = 9.07; P <0.05), or MCL (χ^2 = 16.34; P <0.001) compared with control subjects. The significance levels reflect the intermediate magnitude of serum TNF-α levels observed in LCL patients (68 ± 34 pg/ml) compared with MCL patients (94 ± 13 pg/ml) and control subjects (12 ± 3 pg/ml) (6). Because MCL patients arise as a subset of initially LCL patients, it is likely that the LCL patient group will contain a subset of high TNF-α producers predisposed to MCL disease.

The fact that TNF-β allele 2 is independently associated with MCL disease suggests that it is in linkage disequilibrium with other regulatory elements for TNF-α production. In previous studies, the two alleles of the TNF-β gene have been shown to be in linkage disequilibrium with different DRβ alleles, and both DRβ and TNF-β have been shown to be associated with functional differences in LPS-induced TNF-α secretion by mononuclear cells (12, 14, 21, 22), or with TNF-α secretion by nonstimulated EBV cell lines (13). Two of these studies (21, 22) demonstrated that mononuclear cells from DR2-positive individuals exhibit low TNF-α production in response to LPS, whereas DR3- and DR4-positive individuals show high levels of TNF-α production. In our total study population (Table 4), DR2 was in linkage disequilibrium (P = 0.03) with the higher frequency allele 1 of the TNF-β gene (=TNFB*2 [12–14]), and both DR2 and the TNF-β1 allele were significantly reduced in the MCL patient group (Table 1). Conversely, DR3 and DR4 were each associated

(P <0.05) with allele 2 of the TNF-β gene (TNFB*1 [12–14]) (Table 4), which was significantly increased in individuals with MCL disease (Table 1). Although DR3 and DR4 were not individually associated with MCL disease (Table 1), haplotype frequencies (Table 5) for DR3.TNF-β2 (0.23 in MCL patients compared with 0.08 in control subjects) and DR4.TNF-β2 (0.18 in MCL patients compared with 0.09 in control subjects) made up a large component of the overall TNF-β2 disease association. For DR3, case/control ratios of >1.0 (Table 5) for DR3.TNF-β2 and non-DR3.TNF-β2 indicate that the risk of MCL lies with TNF-β2 independently of DR3. For DR4, case/control ratios >1.0 were associated with either DR4 or TNF-β2, suggesting that susceptibility to MCL is associated with an element in linkage disequilibrium with both. In previous studies, the particular allele at the TNF-β gene associated with TNF-α secretion varies according to the population

Table 4. *Tests for Linkage Disequilibrium between DRβ and TNF-β Alleles in the Total Population Sample*

DRβ	TNF-β homozygotes		χ^2	p_1	p_2
	1/1	2/2			
DR2	18	3	4.11	<0.05	0.03
Non-DR2	66	39			
DR3	17	16	4.29	<0.05	0.07
NON-DR3	65	26			
DR4	10	10	2.97	0.07	0.05
NON-DR4	74	32			

Associations (χ^2, p_1) between DRβ and TNF-β alleles are calculated using only individuals homozygous at the TNF-β locus. These associations were confirmed (p_2) using the computer-generated haplotype frequencies.

Table 5. *Computer-generated DR.TNF-β Haplotype Frequencies in Control Subjects, ACL Patients, and ACL Patients Stratified by Disease Phenotypes LCL and MCL*

Haplotype	Haplotype frequencies							
	Control subjects (2n = 128)	ACL (2n = 92)		LCL (2n = 46)		MCL (2n = 46)	
DR2.TNF-β1	**0.15**	0.06	(0.4)	0.10	(0.6)	**0.02**	(0.1)
DR2.TNF-β2	0.03	0.03	(1.0)	0.04	(1.2)	0.02	(0.8)
Non-Dr2.TNF-β1	0.54	0.42	(0.8)	0.47	(0.9)	0.37	(0.7)
NON-Dr2.TNF-β2	**0.28**	0.49	(1.8)	0.40	(1.5)	**0.59**	(2.1)
$\chi^2_{(3)}$		12.63*		2.85[§]		16.07[‡]	
Dr3.TNF-β1	0.15	0.07	(0.5)	0.06	(0.4)	0.07	(0.5)
DR3.TNF-β2	**0.08**	0.18	(2.2)	0.13	(1.6)	**0.23**	(2.8)
Non-DR3.TNF-β1	0.55	0.41	(0.8)	0.50	(0.9)	0.32	(0.6)
Non-DR3.TNF-β2	**0.22**	0.34	(1.5)	0.30	(1.4)	**0.38**	(1.7)
$\chi^2_{(3)}$		12.06*		3.88[§]		14.31*	
DR4.TNF-β1	0.10	0.12	(1.3)	0.10	(1.1)	0.16	(1.6)
DR4.TNF-β2	**0.09**	0.16	(1.7)	0.11	(1.3)	**0.18**	(2.0)
Non-DR4.TNF-β1	0.60	0.37	(0.6)	0.46	(0.8)	0.25	(0.4)
Non-DR4.TNF-β2	**0.21**	0.36	(1.7)	0.32	(1.5)	**0.41**	(1.9)
$\chi^2_{(3)}$		11.91*		2.95[§]		16.40[‡]	

*$P <0.01$, [‡]$P <0.001$; [§] NS.

$\chi^2_{(3)}$ test for significant differences in the distribution of DR.TNF-β haplotypes between controls and each of the three patient groups. Case/control ratios for haplotype frequencies are shown in parentheses. 2n, number of chromosomes scored to determine haplotype frequencies.

The subscripted (3) represents df. The boldface numbers represent the differences between MCL cases and control subjects, as discussed in the text.

under investigation. Hence, Messer et al. (12) and Pociot and coworkers (14) found TNFB*2, the equivalent of our TNF-β1 allele, to be associated with high TNF-α release in individuals with the DR4.TNFB*2 haplotype. Conversely, Abraham and co-workers (13) found high TNF-α secretion to be associated with TNFB*1, the equivalent of our TNF-β2 allele, in individuals bearing the DR3. TNFB*1 haplotype. Although further analysis is required to provide the functional link between regulatory elements and TNF-α levels in Venezuelan MCL patients, our MHC associations appear to concur with the low TNF-α secretion associated with DR2 and the TNFB*2 allele, and high TNF-α secretion associated with DR3.TNFB*1 and DR4.TNFB*1 haplotypes.

Overall, the results of this study indicate a strong relative risk of MCL disease associated with particular alleles at the TNF-α and TNF-β genes in the class III region of the MHC. Together with the recent observation (7) that increased risk of cerebral malaria is similarly associated with functional polymorphisms influencing TNF-α secretion, our results highlight the possible impact that polymorphism at this locus might have in determining susceptiblity to severe clinical forms of infectious disease, as well as in autoimmune disease (12, 14, 21–24). Further analysis of the TNF-α/TNF-β gene region is required to identify all polymorphisms associated with disease, to test their role in TNF-α regulation, and to determine the positive balancing selection that must operate to maintain these apparently deleterious alleles in the population.

Address correspondence to J. M. Blackwell, Department of Medicine, Level 5, Addenbrooke's Hospital, Cambridge CB2 2QQ, UK.

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