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Genetic polymorphisms in molecules of innate immunity and susceptibility to infection with Wuchereria bancrofti in South India

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A pilot study was conducted to determine if host genetic factors influence susceptibility and outcomes in human filariasis. Using the candidate gene approach, a well-characterized population in South India was studied using common polymorphisms in six genes (CHIT1, MPO, NRAMP, CYBA, NCF2, and MBL2). A total of 216 individuals from South India were genotyped; 67 normal (N), 63 asymptomatic microfilaria positive (MF+), 50 with chronic lymphatic dysfunction/elephantiasis (CP), and 36 tropical pulmonary eosinophilia (TPE). An association was observed between the HH variant CHIT1 genotype, which correlates with decreased activity and levels of chitotriosidase and susceptibility to filarial infection (MF+ and CP; P = 0.013). The heterozygosity of CHIT1 gene was over-represented in the normal individuals (P = 0.034). The XX genotype of the promoter region in MBL2 was associated with susceptibility to filariasis (P = 0.0093). Since analysis for MBL-sufficient vs insufficient haplotypes was not informative, it is possible the MBL2 promoter association results from linkage disequilibrium with neighboring loci. We have identified two polymorphisms, CHIT1 and MBL2 that are associated with susceptibility to human filarial infection, findings that merit further follow-up in a larger study. Genes and Immunity (2001) **2,** 248–253.

Keywords: *filariasis; polymorphism; chitotriosidase; mannose-binding lectin; innate immunity*

Introduction

Lymphatic filariasis, caused by one of the three lymph-dwelling filariae, *Wuchereria bancrofti*, *Brugia malayi*, or *Brugia timori*, infects ~129 million people throughout the tropics and subtropics with *W. bancrofti* being the most predominant.[1,2] Each of these filarial parasites goes through a complex life cycle that includes infective larvae carried by mosquitoes, adult worms found in the lymph node or in the adjacent lymphatics, and their offspring (microfilariae) that circulate in the blood, often with nocturnal periodicity. Among the varied (and occasionally overlapping) clinical manifestations of lymphatic filariasis are a subclinical condition associated with microfilaremia (asymptomatic microfilaremia; MF+), chronic lymphatic obstruction/elephantiasis or hydrocele, collectively also known as chronic pathology (CP) and the rare condition, tropical pulmonary eosinophilia (TPE). The pathogenesis of the lymphatic condition of filarial infection is complex and probably involves both inflammatory processes that are immune-mediated and secondary bacterial infections superimposed on lymphatic dysfunction.[3]

The mapping of the human genome has created a rich resource for annotating differences in populations. Many different genes with allelic variations are believed to contribute to disease outcomes, especially common infectious and autoimmune disorders.[4] Currently, there is a major effort to identify and characterize common variants in known genes, commonly known as polymorphisms.[5–7] In particular, there has been growing interest in whether human genetic polymorphisms in host defense pathways could affect susceptibility to helminth infection and the likelihood of developing particular clinical manifestations. Epidemiological studies in areas where filariasis is endemic have revealed differential susceptibilities to infection, both within entire populations as well as within families.[8] Although the cause of differential susceptibility to clinical expression of helminth infections has only been addressed in a few human studies, early studies implicated the major histocompatibility complex (MHC).[9–14] Several large-scale studies have attempted to make more compelling the argument of an underlying genetic susceptibility for disease outcome in both filarial and schistosome infection. For example, in schistosomiasis, genetic loci for susceptibility and resistance have been physically mapped.[15–21]

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Since there have been relatively few investigations of the host genetic contribution to acquisition and outcomes in human filarial infection, we performed a pilot study, utilizing a candidate gene approach. The chosen genes fulfilled the following criteria: a frequency of greater than 5% of at least one polymorphic variant in one or more populations, *in vitro* evidence of the biological significance of the variant and prior clinical studies suggesting a role in disease susceptibility or outcome. Each of the genes is a critical component of host defense microbicidal pathway, such as two components of the oxygen dependent NADPH-oxidase, p22-phox (*CYBA*)[22,23] and p67-phox (*NCF2*), myeloperoxidase (*MPO*),[24] Nramp-1 (*NRAMP*),[25] or a phagocyte specific chitotriosidase (*CHIT1*).[26,27] In addition, we studied the C-type collectin, mannose-binding lectin (*MBL2*).[28,29] In our preliminary investigation of genetic factors that influence expression of human lymphatic filariasis, we analyzed the distribution of genetic polymorphisms of the candidate genes against three major clinical outcomes following exposure to filarial parasites in a *W. bancrofti*-endemic area of South India.

Results

In the first phase, we analyzed the distribution of variant genotypes of the six candidate genes and compared the uninfected normal group (N) individually with infected individuals with either of the two major manifestations of lymphatic filarial infection (MF+ and CP) (see Table 1). For the purpose of examining susceptibility to infection, the N group was compared to MF+ and CP together; samples had been collected as part of a population based study of filarial infection in South India.[3] A significant difference was observed at the locus for *CHIT1* ($\chi^2 = 8.06$; $P = 0.018$). Analysis by individual genotypes was revealing for the comparison of N to MF+ and CP. Together, both the HH and HT genotypes differed ($P = 0.013$ and 0.034, respectively). In particular, individuals homozygous for the H allele were over-represented in the group who acquired filarial infection. Previously, the HH genotype has been shown to be associated with decreased production and enzymatic activity of the human phagocytic chitinase.[27] The HT genotype was over-represented in normal individuals. However, the *CHIT1* genotype does not influence susceptibility to one of the specific manifestations of infection with *W. bancrofti* in South India, MF+ or CP, respectively.

Analysis of two polymorphisms in the mannose-binding protein gene, *MBL2*, which have been reported to be linked, revealed an unexpected finding. The distribution of structural variants, known as B, C and D alleles of exon 1 in infected *vs* uninfected subjects did not differ statistically (see Table 2). Analysis was performed for each of the three individual variants as well as the presence of at least one of the structural variants, B, C or D (data not shown).[28] Interestingly, we examined the polymorphism in the proximal promoter region (–221 bp), known as the X/Y locus[29] and found that the XX genotype, which is correlated with significantly lower circulating levels of the mannose-binding lectin, is under-represented in the infected population ($P = 0.004$). However, we did not find a significant association for susceptibility to infection when analyzed by MBL-sufficient and MBL-insufficient haplotypes (see Table 2 and its legend). Our study did not identify an association with one of the structural variants (eg the B, C, and D alleles) nor with haplotypes (which correlate with measured MBL serum levels). Nonetheless, our analysis suggests that the promoter polymorphism could be associated with infection, either directly or in linkage with another informative locus.

Table 1 Genotype distribution of five study genes in uninfected and infected (asymptomatic microfilaremia (MF+) and chronic lymphatic obstruction/elephantiasis (CP)) subjects in South India

Genotypes	*Normal (N) (n = 67)*	*Infected group (MF+ and CP) (n = 113)*	*P values N vs (MF+ and CP)*	
			Locus	*Genotype*
CHIT1				
HH	7 (10%)	30 (27%)		0.013 (f)
HT	39 (58%)	46 (41%)	0.018	0.034
TT	21 (31%)	37 (32%)		0.98
MPO				
AA	2 (3%)	0 (0%)		0.14 (f)
AG	12 (18%)	26 (23%)	0.14	0.53
GG	53 (79%)	87 (77%)		0.89
NRAMP				
GC	13 (20%)	22 (20%)	0.98	
GG	53 (80%)	89 (80%)		
CYBA				
CC	23 (35%)	50 (45%)		0.26
TC	33 (50%)	53 (47%)	0.22	0.85
TT	10 (15%)	9 (8%)		0.22
NCF2 (–149)				
AA	0 (0%)	1 (1%)		1.0 (f)
AG	5 (8%)	10 (9%)	0.70	1.0 (f)
GG	61 (92%)	100 (90%)		0.79 (f)
NCF2 (–153)				
AA	0 (0%)	1 (1%)		1.0 (f)
AG	5 (8%)	10 (9%)	0.70	1.0 (f)
GG	61 (92%)	100 (90%)		0.79 (f)

Note: Not all samples amplified at each locus. Comparative analysis was performed at each of three loci using a χ^2 analysis (3×2 tables with 2 degrees of freedom) or a Fisher's exact test (f). MF+ indicates asymptomatic microfilaremia and CP indicates chronic lymphatic obstruction/elephantiasis or hydrocele, collectively also known as chronic pathology.

A preliminary analysis was performed examining the contribution of the two informative loci together. There was a suggestion of a significant association between the combination of the presence of the *CHIT1* genotype, HH together with the absence of the XX genotype of *MBL2* when uninfected (N) *vs* infected (MF+ and CP) (7 (11%) *vs* 30 (27%) and 59 (89%) *vs* 83 (73%); $P = 0.018$). Remarkably, no individuals, in either the control or infected groups, possessed the HH and XX genotypes together. This observation, in this exploratory analysis, suggests that the two genotypes could be acting independently. However, further studies are needed to clarify this point.

We did not observe a significant association for susceptibility to filarial infection for the other four loci studied *MPO, NRAMP, CYBA*, and *NCF2* (Table 1). Similarly, no effect was observed with respect to specific outcomes within the infected group (ie, MF+ and CP) for the other six genotypes analyzed in this study (data not shown).

A separate analysis was performed on a cohort with TPE, compared to infected individuals (MF+ and CP)

Table 2 Genotype distribution of *MBL2* in uninfected and infected subjects in South India

Genotypes	*Normal (N) (n = 67)*	*Infected group (MF+ and CP) (n = 113)*	*P values N vs (MF+ and CP)*	
			Locus	*Genotype*
MBL2				
AA	40 (61%)	69 (62%)		0.89
AO*	21 (32%)	38 (34%)	0.68	0.90
OO	5 (8%)	5 (4%)		0.50 (f)
MBL+ (–221)				
XX	11 (16%)	4 (4%)		0.004 (f)
XY	22 (33%)	40 (36%)	0.0093	0.87
YY	33 (50%)	68 (61%)		0.22
MBL2 Haplotype				
AX/AX	11 (17%)	4 (3%)		0.004 (f)
AX/AY	17 (26%)	29 (26%)		0.98
AX/OY	5 (8%)	11 (10%)		1.0 (f)
AY/AY	12 (18%)	37 (33%)		0.05
AY/OY	16 (24%)	26 (23%)		0.88
OY/OY	5 (8%)	5 (4%)		0.51 (f)
MBL-sufficient				
A/A + AY/O	56 (85%)	96 (86%)	0.87	
MBL-insufficient O/O + AX/O	10 (15%)	15 (14%)		

*O is the usual designation for variant alleles B, C, and D. The haplotypes for *MBL2* include the –221 X/Y promoter variant and variants of the coding region, known as the structural variants, B, C and D.[29] The latter are inherited independently and so far, have not been reported to occur simultaneously on the same strand. The classification of haplotypes as sufficient and insufficient is well described and separates into two groups, based upon circulating levels and functional differences *in vitro*, secondary to a structural variant (B, C or D).[39] Comparative analysis was performed at each of three loci using a χ^2 analysis (3 × 2 tables with 2 degrees of freedom) or a Fisher's exact test (f). MF+ indicates asymptomatic microfilaremia and CP indicates chronic lymphatic obstruction/elephantiasis or hydrocele, collectively also known as chronic pathology. Note: Not all samples amplified at each locus.

because the TPE group was collected separately and represented a concentration of individuals affected by a condition estimated to occur in less than 1 in 100,000. No association was observed between the six candidate genes and susceptibility to TPE (data not shown).

Discussion

In a pilot study, we examined six candidate polymorphic genes, all of which are integral components of microbicidal pathways in an effort to identify possible genetic risk factors for human filarial infection. A polymorphism in the human *CHIT1* gene, which is highly expressed in phagocytic cells, was associated with susceptibility to filarial infection in a filarial-endemic region of South India. However, the variant genotypes of *CHIT1* did not associate with the specific, common manifestations of infection, MF+ and CP. Interestingly, we also observed an association between a *MBL2* promoter polymorphism and infection with *W. bancrofti*. The circulating levels of MBL are affected by the promoter polymorphism genotype but the promoter variants do not alter the function of the protein. It is also plausible that the promoter variant is in linkage disequilibrium with another loci on chromosome 10. Follow-up studies are required to identify and confirm the importance of an additional locus (or loci). Furthermore, neither *CHIT1* nor *MBL2* nor the other four genes studied were associated with TPE, a rare complication of filariasis.

Chitinases are produced by a large number of species, including bacteria, fungi, nematodes (including filariae), and plants.[30,31] The microfilarial chitinase is an immunodominant antigen and target of current strategies to develop candidate vaccines.[32–34] The functional contribution of the microfilarial chitinase to infectious pathophysiology is still not well understood. Still, it is notable that the microfilarial sheath contains chitin, a putative target for a human chitotriosidase, encoded by *CHIT1*. The human chitotriosidase is produced by macrophages in large quantities as an acute phase reactant. *In vitro*, human chitotriosidase is capable of degrading chitin-containing pathogens.[26,30] Some authors have suggested that, on the basis of *in vitro* chitinase activity, it could play a role in microbicidal activity against complex pathogens, such as the filarial parasite whose microfilarial sheath contains chitin.[35] Recently, a mouse chitinase-like molecule (YM-1) has been shown to be highly expressed by filarial exposed alternatively activated macrophages (JE Allen, personal communication). Overall, these data suggest a role for these chitinases in modulating the immune response to helminth parasites.

In this context, we were particularly interested in the investigation of a variant of the human chitotriosidase gene, *CHIT1*. The variant H allele of *CHIT1* results in a 24-base pair duplication in exon 10 and activates a cryptic 3′ splice site, generating an mRNA with an in-frame deletion of 87 nucleotides.[27] Interestingly, there is a strong correlation between homozygosity for the 24-bp duplication (HH genotype) and chitotriosidase-deficiency; in a previously published study, individuals with the HH genotype (n = 26) demonstrated low or undetectable levels of circulating chitotriosidase. Furthermore, *in vitro* activity of the variant protein is markedly diminished.[27] In this regard, our findings suggest that an alteration in either (or both) circulating levels or altered function of the chitotriosidase, both described in HH individuals, could have important consequences for human filarial infection. Our results indicate that, as expected, uninfected individuals have a lower frequency of HH compared to infected individuals, 10% *vs* 27% (see Table 1). Although we found an over-representation of heterozygosity for this gene in the normal control group, the mechanism of protection is unknown, primarily because the biological significance of the heterozygous state is insufficiently characterized. In the course of our study, we determined the overall distribution of *CHIT1* variants and found a major difference in the allelic frequency in the South India population compared to two, large healthy control groups of North American Caucasians and African Americans in the USA. Genotype distribution of chitotriosidase among endemic normal in South India differed from healthy African American (n = 175; HH 2 (1%), HT 14 (8%), TT 159 (91%), $P < 0.0001$) and North American Caucasian populations (n = 229; HH 1 (<1%), HT 83 (36%), TT 145 (63%), $P < 0.0001$). The HH genotype is over-represented in normal South Indian populations compared to other groups studied to date. The basis for this elevation in HH genotype frequency in the South India study population is not clear.

The mannose-binding lectin, encoded by the *MBL2*

gene is a C-type collectin that participates in pathogen recognition, opsonization, phagocytosis and complement activation.[36,37] A common set of structural polymorphisms, known as the B, C and D alleles (together known as the O alleles) within a 15-bp span in exon 1 disrupt the function of MBL.[28,29] The structural change in exon 1 results in a disruption of the binding domain, critical for oligomerization. Structural variants have been associated with a wide range of infectious and autoimmune disorders.[38–40] In our study, there was no association observed between the structural variants of *MBL2* and susceptibility to filarial infection. However, we found a possible association between the promoter polymorphism at bp –221 (designated X and Y) and resistance to infection; filarial infection is less common in individuals with the XX genotype, associated with decreased circulating levels of MBL, independent of the structural allele genotype.

Although we analyzed the haplotypes generated by the linked *MBL2* sites, the total number of patients is small for this purpose; still, at the locus there might be an association for the AX/AX haplotype ($P = 0.004$). These results suggest that circulating levels of MBL could be important in the pathogenesis of filarial infection. However, we did not determine circulating serum levels of MBL because of the lack of availability of a sufficient number of samples. Published studies have divided the common haplotypes into MBL sufficient and insufficient groups based upon circulating levels as well as *in vitro* function of the oligomerized MBL protein.[28,39] When we analyzed the data taking into account MBL-sufficient versus insufficient halpotypes, the findings were not remarkable. In the same region of chromosome 10 are located several other candidate loci, namely *SDF1*, the ligand for chemokine receptor 4, *CXCR4*, which has a polymorphism that influences HIV progression.[41,42] It is also plausible that the association with the promoter polymorphism, X/Y, has unmasked a second gene, with which the X/Y variants are in linkage disequilibrium.

We analyzed class II HLA loci, namely, DQA, DQB, and DRB and did not identify an association with *W. bancrofti* infection nor outcomes within the infected group (data not shown). Our study represents one of several recent attempts to identify non-HLA associated genetic risk factors for susceptibility or resistance to parasitic infection, particularly in endemic areas. A recent report has shown that there could be genetic predisposition to microfilaremia and that predisposed subjects might be genetically unable to mount an efficient immune response against *Loa loa* antigens.[43,44] Genetic studies in severe clinical malaria using segregation analysis and linkage analysis for candidate chromosomal regions have identified specific loci in the human response to malarial infection.[45–47] In our study, we have identified two candidate genes, which could influence susceptibility to filarial infection. In a pilot analysis of the two informative loci, it appears that specific genotypes of *CHIT1* and *MBL2* (X/Y) could contribute to susceptibility to human filarial infection. Specifically, the presence of HH and absence of the XX genotype together were associated with increased susceptibility to infection (7 (11%) *vs* 30 (27%) and 59 (89%) *vs* 83 (73%); $P = 0.018$). Analysis of multiple candidate genes should be confirmed in subsequent studies.

The results suggest that small differences in the activity of one or both of these molecules, over time, could modulate host response and influence infection. Although speculative, our preliminary results identify an *in vivo* role for key molecules that merit further investigation. Before the results of our study can be applied to clinical decision making, confirmation of the findings are required in larger, prospectively recruited studies. Lastly, further genetic studies utilizing candidate genes derived from important host defense pathways, such as components of the Th1 and Th2 immune response, could be informative in identifying genetic markers and elucidating the biology of host response to filarial infection.

Materials and methods

Subjects

Samples for the study populations were collected between 1987 and 1993 year from South India, an area endemic with *W. bancrofti* infection as part of a prospective study.[3] The primary study population consisted of 180 individuals residing in South India. Study subjects were divided into three groups on the basis of strict parasitological evaluation: (i) normal and uninfected, (N, $n = 67$), (ii) microfilaria and antigen positive without signs or symptoms of clinical disease (MF+, $n = 63$) and (iii) patients with chronic lymphatic dysfunction/ elephantiasis (CP, $n = 50$). Antifilarial antibodies were measured in the three groups (eg N, MF+ and CP).[48] The geometric mean titer (μg/mL) with the range of measured values for each group were for N 36.8 (1.6–835), MF+ 137.6 (4.6–1862), and CP 67.1 (4.5–435.9). There was no difference in measured IgG antifilarial antibody levels between the separate groups (data not shown). In addition, occult filarial infection in the normal individuals was excluded by the absence of circulating filarial antigen using two separate tests (AMRAD-ICT (Sydney, Australia) and TropBio (Townsville, Australia)).[49,50] A separate collection of patients with the rare complication, tropical pulmonary eosinophilia (TPE, $n = 36$) was analyzed in the second stage of the study. These samples were collected under protocols approved by the Institutional Review Board of the National Institutes of Allergy and Infectious Diseases (USA) and the Tuberculosis Research Centre (Chennai, India).

Polymorphism analysis

Genomic DNA was obtained from peripheral blood by conventional methods. Polymorphism analysis was performed individually for each of six loci. Variants in five of the six loci have been previously reported. These included chitotriosidase (*CHIT1*),[26,27] myeloperoxidase (*MPO*),[24] Nramp-1 intron 4 (*NRAMP*),[25] p22-phox (*CYBA*),[22,23] and two linked polymorphic sites in exon 1 and the promoter of the mannose-binding lectin (*MBL2*).[28,29] We also analyzed two novel linked loci in the promoter of NADPH oxidase p67-phox (*NCF2*) (unpublished data). Each polymerase chain reaction (PCR) based assay was performed in duplicate. Primer pairs, annealing temperatures, and detection methods for each locus are shown in Table 3.

Statistical analysis

Distribution of genotypes for each candidate gene was compared by either the Fisher's exact test (f) or the chi-square statistic as appropriate using InStat® for Mac-

Table 3 Detection method for each genotype assay

Genes	*Primers*	*Annealing temperature*	*Detection method*
CHIT1 Exon 10	F: agc tat ctg aag cag aag R: gga gaa gcc ggc aaa gtc	55°C	Size differentiation of PCR product
MPO G-493A	F: cgg tat agg cac aca atg gtg ag R: gca atg gtt caa gcg att ctt c	58°C	Digest with *Aci*I
NRAMP Intron 4	F: tct ctg gct gaa ggc tct cc R: tgt gct atc agt tga gcc tc	60°C	Digest with *Apa*I
CYBA C242T	F: tgc ttg tgg gta aac caa ggc cgg tg R: aac act gag gta agt ggg ggt ggc tcc tgt	60°C	Digest with *Rsa*I
NCF2 G-153A G-149A	F: aca agg gtg tgg aaa tca ag R: gag cca ggg taa cct tgg S: cta ccc aaa ggc agg aaa gtc c	60°C	Cycle sequencing
MBL2 Exon 1	F: cct gag tat ggt ggc agc gtc tta ct R: cag gca gtt tcc tct gga agg S: act gtg acc tgt gag gat gcc caa aag	58°C	Cycle sequencing
–221 X/Y	F: cat gga gag aaa gag gaa gct cct R: tag gca cta tga tga gca gtg gg atc S: tcc cta agc taa cag gca taa		

Intosh Version 2.0 (Graphpad Software, SanDiego, CA, USA). The analysis of each locus is presented without correction for multiple statistical significance tests on the premise that each candidate gene was chosen because of prior *in vitro* data and association studies suggesting the biological significance of variant alleles. Because this is a pilot study, we interpret the findings as follows to guard against improper interpretation for specific candidate genes or genotypes; a *P*-value (two-tailed) between 0.05 and 0.10 indicates a weak association; a *P*-value between 0.01 and 0.05 indicates a strong relationship which may be worth exploring in subsequent or confirmatory studies; a *P*-value less than 0.01 indicates a strong association which is worthy of confirmation.[40]

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