MAJOR ARTICLE

Genotypes of the Mannan-Binding Lectin Gene and Susceptibility to Visceral Leishmaniasis and Clinical Complications

Diego Peres Alonso,[1] Afonso Flávio B. Ferreira,[3] Paulo Eduardo M. Ribolla,[1] Isabel K. F. de Miranda Santos,[2] Maria do Socorro Pires e Cruz,[3] Fernando Aécio de Carvalho,[4] Antonio Roberto R. Abatepaulo,[2] Dorcas Lamounier Costa,[3] Guilherme L. Werneck,[5] Teresinha J. C. Farias,[3] Maria José S. Soares,[4] and Carlos Henrique N. Costa[3]

[1]Department of Parasitology, Institute for Biology and Biomedicine, São Paulo State University, Botucatu, and [2]Department of Biochemistry and Immunology, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, São Paulo, [3]Laboratory for Research in Leishmaniasis, Natan Portella Institute for Tropical Diseases, and [4]Department of Parasitology and Microbiology, Federal University of Piauí, Teresina, Piauí, and [5]Institute of Social Medicine, State University of Rio de Janeiro, Rio de Janeiro, Brazil

Background. Visceral leishmaniasis (VL) is almost always lethal if not treated, but most infections with the causative agents are clinically silent. Mannan-binding lectin (MBL), an opsonin, is a candidate molecule for modifying progression to VL because it may enhance infection with intracellular pathogens. Mutations in the *MBL2* gene decrease levels of MBL and may protect against development of VL. This case-control study examines genotypes of *MBL2* and levels of MBL in individuals presenting with different outcomes of infection with *Leishmania chagasi.*

Methods. Genotypes for *MBL2* and levels of serum MBL were determined in uninfected control subjects ($n = 76$) and in individuals presenting with asymptomatic infection ($n = 90$) or VL ($n = 69$).

Results. Genotypes resulting in high levels of MBL were more frequent (odds ratio [OR], 2.5 [95% confidence interval {CI}, 1.3–5.0]; $P = .006$) among individuals with VL than among those with asymptomatic infections and were even more frequent (OR, 3.97 [95% CI, 1.10–14.38]; $P = .043$) among cases of VL presenting with clinical complications than among those with uneventful courses. Serum levels of MBL were higher ($P = .011$) in individuals with VL than in asymptomatic infections.

Conclusions. Genotypes of the *MBL2* gene predict the risk for developing VL and clinical complications in infections with *L. chagasi.*

Visceral leishmaniasis (VL), or "kala-azar," is endemic in >60 countries [1]. The majority of the 500,000 new cases that occur every year are from India, Nepal, Bangladesh, Sudan, and Brazil. VL is reemerging in the Mediterranean Basin, where most cases occur in individuals who are immunosuppressed by HIV. VL is caused by intracellular pathogens of the *Leishmania* species transmitted by sand flies; in Brazil, *L. chagasi* is the causative agent. Patients have high burdens of parasites in their spleen and liver; clinical manifestations are fever, anemia, wasting, splenomegaly, and hepatomegaly. Like in other infectious diseases, a persistent question has been why only a small proportion of infected individuals develop disease. VL is almost always lethal if not treated and even with treatment has a mortality rate of 10% [2]. Most infections remain cryptic unless immunological suppression occurs [3]. Young age and malnutrition are important risk factors for VL [2, 3], but other host susceptibility factors remain unknown.

Genetic analysis of families presenting multiple cases of VL in northeastern Brazil suggests that there is a

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Reprints or correspondence: Dr. Carlos Costa, Instituto de Doenças Tropicais Natan Portella, Rua Arthur de Vasconcelos 151-Sul, Teresina Piauí 64.001-450, Brazil (chncosta@gmail.com).

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single major gene determining the outcome of infection [4]. The information available on genetic susceptibility or resistance to VL is summarized as follows: an analysis of 15 polymorphic markers located within class II/III regions of HLA did not show any association between alleles and VL [5]. However, the tumor necrosis factor (TNF)–1 allele of the TNF-α gene, located in the class III region of HLA, has been shown to be associated with development of a positive skin test to *L. chagasi* [6]; a study conducted in Sudan showed linkage of polymorphisms across the SLC11A1 (Nramp) gene to cases of VL [7]. More than one single major gene can affect susceptibility to VL, because parasites that cause high mortality before reproductive age can select for independent mutations in different populations of hosts. This occurs in malaria, in which mutations in different genes independently confer protection against severe disease [8].

Mannan-binding lectin (MBL), a serum lectin and a component of innate immunity [9], is a candidate molecule that merits examination in the context of VL because it may promote progression of infection with *L. chagasi* to disease because of its possible enhancing effect on infections with intracellular pathogens [10]. MBL binds to glycosylated structures not found in hosts but present on many pathogens, including parasites of the genus *Leishmania* [11]. It acts as an "ante-antibody" and, in the case of bacterial infections, confers protection before the establishment of adaptive immune responses [9]. MBL is coded by the *MBL2* gene located on chromosome 10. Three mutations at exon 1 result in substitutions of amino acids from a collagenous region of the polypeptide chain [9, 12]. The corresponding alleles (B, C, and D) are collectively called O [9] because their physiological effect is similar: they hinder assembly of subunits into functional trimeric structures, render them vulnerable to degradation [13], and diminish levels of MBL in the serum; the wild-type allele is called A [9]. Mutations in the promoter, occurring at −550 bp (alleles H and L) and at −221 bp (alleles X and Y), affect transcription rates and further influence baseline levels [9, 12]. Low levels of MBL cause a common immunodeficiency [14], and studies show that variant alleles are associated with various infectious diseases and are useful prognostic markers [15].

In spite of the apparent deleterious physiological effects of low levels of MBL, the mutations that cause them are frequent and arose independently in populations from different geographical regions [9, 16]. This indicates that there may be a selective advantage for carriers of mutations. MBL seems to have dual and opposing roles that would explain the advantage

Table 1. Distribution of genotypes for the *MBL2* gene in Brazilians exposed to *Leishmania chagasi* and presenting with asymptomatic infection or visceral leishmaniasis (VL).

Group	Genotypes, no.		Odds ratio (95% CI)	P[c]
	High[a]	Intermediate/low[b]		
VL[1,2,3,4,5,6,7,8,9]	35	25		
Infected, asymptomatic				
All[1,10]	32	58	[1]2.5 ([1]1.3–5.0)	[1]**.006**
Neighbors[2]	19	34	[2]2.5 ([2]1.2–5.4)	[2]**.028**
Random[3]	13	24	[3]2.6 ([3]1.1–6.0)	[3]**.028**
Uninfected				
All[4,10]	37	51	[4]1.9 ([4]1.0–3.8)	[4].053
			[10]0.7 ([10]0.3–1.4)	[10].255
Neighbors[5]	16	28	[5]2.5 ([5]1.1–5.5)	[5]**.028**
Random[6]	21	23	[6]1.5 ([6]0.7–3.4)	[6].285
Control subjects[d]				
All[9]	95	136	[9]2.0 ([9]1.1–3.5)	[9]**.018**
Neighbors[7,11]	48	77	[7]2.2 ([7]1.2–4.2)	[7]**.017**
Random[8,11]	47	59	[8]1.8 ([8]0.9–3.3)	[8].084
			[11]0.8 ([11]0.5–1.3)	[11].361

NOTE. Odds ratios and χ^2 tests are performed with groups that bear the same nos. in superscript; the respective odds ratio, 95% confidence interval (CI), and P value for each comparison has the same no. in superscript. Bold indicates statistical significance.

[a] Genotypes predicted to result in high levels of mannan-binding lectin (MBL) (YA/YA and YA/XA).

[b] Genotypes predicted to result in intermediate or low levels of MBL (YA/YO, XA/XA, XA/YO, YO/YO, XO/YO, XO/XO, A/O, and O/O).

[c] χ^2 test.

[d] Includes individuals whose infection status is undetermined but who are healthy.

Table 2. Distribution of genotypes for the *MBL2* gene in individuals with visceral leishmaniasis presenting with or without clinical complications.

Group	Genotypes, no.		Odds ratio (95% CI)	P[c]
	High[a]	Intermediate/low[b]		
Without complications [1,2]	17	18		
With complications, not severely ill[1]	15	4	[1]3.97 ([1]1.10–14.38)	[1]**.043**
Severely ill	3	3	1.1 (0.1–9.0)	.98

NOTE. Odds ratios and χ^2 tests are performed with groups that bear the same nos. in superscript; the respective odds ratio, 95% confidence interval (CI), and P value for each comparison has the same no. in superscript. Bold indicates statistical significance.

[a] Genotypes predicted to result in high levels of mannan-binding lectin (MBL) (YA/YA and YA/XA).

[b] Genotypes predicted to result in intermediate or low levels of MBL (YA/YO, XA/XA, XA/YO, YO/YO, XO/YO, XO/XO, A/O, and O/O).

[c] Fisher's exact test.

for the wide range seen in the levels of this collectin [10]: whereas low concentrations and/or mutations in the promoter region or exon 1 have been associated with recurrent or severe infections caused by extracellular bacteria [14, 17] and *Plasmodium falciparum* [18], high concentrations may enhance targeting of intracellular organisms to host phagocytes, the milieu preferred by these pathogens [19, 20]. Garred et al. propose that mutations resulting in lower levels of MBL are maintained by heterosis, whereby heterozygotes may have an advantage over homozygotes [10]. We addressed this hypothesis by examining the association between clinical outcome of infection with *L. chagasi* and MBL as a quantitative trait and its genotypes for exon 1 and its promoter.

SUBJECTS AND METHODS

Study design and subjects. Individuals in this case-control study are from Teresina, Piauí, Brazil, where urban epidemics of VL have occurred since 1980 [21] and where there is no transmission of Chagas disease or cutaneous leishmaniasis. Informed consent from all participants and institutional approval were obtained for this study. Frequencies of wild-type and variant alleles and the levels of MBL were determined in unrelated individuals presenting with VL ($n = 69$); in unrelated, healthy individuals who had been infected with *L. chagasi* as determined by a positive Montenegro (leishmanin) delayed hypersensitivity (DTH) skin test and/or the presence of specific antibodies in an indirect immunofluorescent serological test (hereafter referred to as "infected, asymptomatic subjects"; $n = 90$); and in unrelated, healthy individuals who had no evidence of present or past infection with *L. chagasi* as determined by both a negative skin test and the absence of specific antibodies ($n = 76$). Ages of individuals ranged between 6 months and 73 years (mean ± SE, 22.7 ± 18.1 years). All groups of the study population represent an admixture of white, African, and Amerindian populations, estimated, respectively, at 21%, 31%, and 48% [22]. There is no statistically significant difference ($P = .361$) in the distribution of genotypes for high or for intermediary to low levels of MBL between randomly selected individuals and neighbors of individuals with VL (table 1, comparison 11), reflecting the similar ethnic composition of these study groups. Healthy infected asymptomatic or healthy uninfected individuals originated from 2 groups: persons living in the same neighborhoods as patients with VL ($n = 125$) and individuals randomly selected from the population ($n = 106$). This distinction takes into account the following confounding factors: first, the population we studied presents the third highest frequency of the B allele (B, 25.25%; C, 6.18%; D, 2.60%; O, 34.03%; A, 65.97%; values refer collectively to all individuals participating in the study) described to date [9], the other populations also being from South America and composed of Amerindians [9, 16], which in Brazil is a socioeconomically underprivileged population. Individuals of Amerindian extraction are, therefore, to be found more frequently in the poor neighborhoods where VL is more prevalent [23]. Another bias could be caused by a greater exposure to infected sand flies incurred by neighbors of individuals with VL [24]. Diagnosis of VL was confirmed by identification of parasites in the bone marrow or by positive serologic tests in patients presenting with fever, anemia, hepatosplenomegaly, and weight loss; patients presenting with VL were assigned to 1 of 3 categories: category 1 comprises patients who did not present complications, patients in category 2 required additional therapy (blood products or antibiotics), and patients in category 3 were severely ill and considered to be at immediate risk of death.

Quantification of MBL and genotyping of the* MBL2 *gene. Serum concentrations of MBL were measured in a double-antibody immune assay (clone Hyb-131-01; Staten Serum Institute); after an initial screening for mutations with DNA sequencing, single-nucleotide polymorphisms (SNPs) for exon 1 and promoter were typed with a SNaPshot Multiplex kit (Applied Biosystems) using specific primers for each SNP.

Statistical analysis. Statistical analysis was performed us-

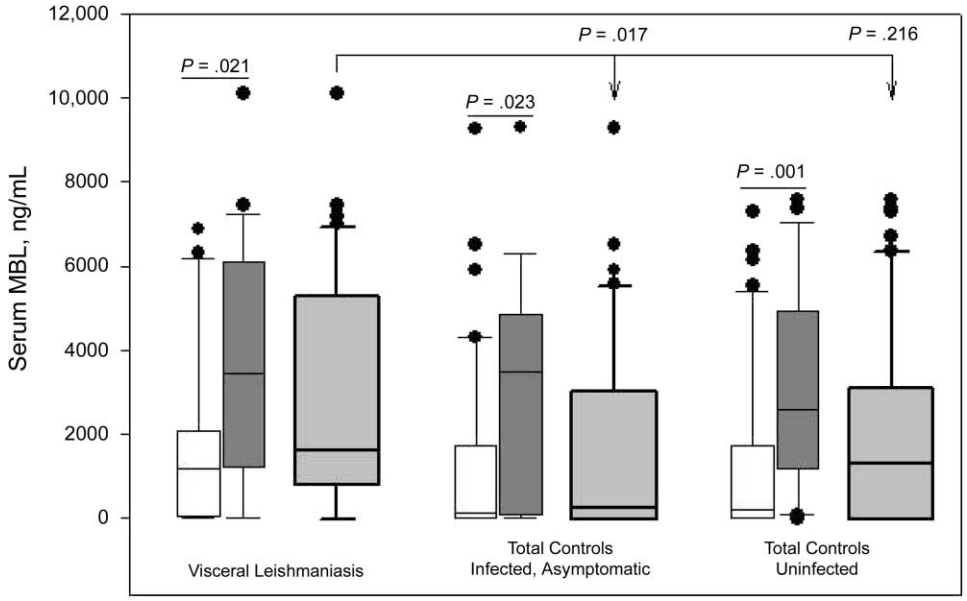

Figure 1. Box-whisker plots of concentrations of serum mannan-binding lectin (MBL) observed in different genotypes and clinical-epidemiological categories of *Leishmania chagasi*–infected individuals. White bars indicate bearers of genotypes resulting in intermediary to low levels of MBL, dark gray bars indicate bearers of genotypes resulting in high levels of MBL, and light gray bars indicate all genotypes combined. Levels of MBL are correlated with clinical outcome on exposure to *L. chagasi* and are significantly lower ($P = .017$, Mann-Whitney rank sum test) in infected, asymptomatic individuals than in those with a history of VL. The median concentrations of MBL were as follows: in patients with VL ($n = 42$), 2829 ng/mL (784 and 5278 ng/mL, for, respectively, the 25th and 75th percentiles); in infected, asymptomatic control subjects who are neighbors of individuals with VL or in the randomly selected individuals ($n = 56$), 1672 ng/mL (0 and 3010 ng/mL, for, respectively, the 25th and 75th percentiles); and in uninfected control subjects ($n = 66$), 2111 ng/mL (0 and 3110 ng/mL, for, respectively, the 25th and 75th percentiles).

ing SigmaStat for Windows (version 2.03; SPSS) and Stata (version 9; StataCorp). We used the χ^2 test to analyze differences in the proportion of genotypes of the *MBL2* gene within the case and 2 control groups and their association with the outcomes of infection with *L. chagasi*. We used Fisher's exact test to analyze differences in the proportion of genotypes within the different clinical categories of VL. We used the Mann-Whitney rank sum test to verify whether there was an association between clinical outcome of infection with *L. chagasi* and MBL as a quantitative trait and multiple logistic regression to examine whether levels of MBL are associated with the chance of developing VL.

RESULTS

***Genotypes of the* MBL2 *gene and outcome of infection with* L. chagasi.** The genotypes resulting in high levels of MBL were significantly more frequent among individuals with VL than among infected, asymptomatic individuals (neighbors plus randomly selected individuals; $P = .006$); than among infected, asymptomatic neighbors ($P = .018$); than among infected, asymptomatic randomly selected individuals ($P = .028$); than among neighbors regardless of their status of infection ($P = .011$); and than among healthy individuals in general, regardless of their status of infection ($P = .018$) (table 1). Individuals with a history of VL and genotypes resulting in high levels of MBL were almost 4 times more likely to have also developed category 2 clinical complications than those whose genotypes result in intermediate or low levels of this opsonin (odds ratio, 3.97 [confidence interval, 1.20–14.38]; $P = .043$, Fisher's exact test) (table 2). Very severe bacterial infections were treated in 5 of the 6 patients in category 3. Interestingly, 3 of these had genotypes resulting in intermediate or low levels of MBL, which may explain their unusual susceptibility. Genotypes resulting in lower levels of MBL were significantly more frequent ($P = .018$) among infected, asymptomatic individuals with anti–*L. chagasi* antibodies and a negative DTH test than among those with a positive DTH test (data not shown).

***Levels of MBL and outcome of infection with* L. chagasi.** We next examined the association between clinical outcome of infection with *L. chagasi* and MBL as a quantitative trait. The results displayed in figure 1 show that phenotypes for MBL are associated with the outcome of infection with *L. chagasi:* levels were significantly lower ($P = .017$, Mann-Whitney rank sum test) in infected, asymptomatic individuals than in individuals with a history of VL; levels of MBL were also lower in uninfected individuals, but the difference was not significant ($P = .216$). Levels of MBL were also significantly lower in infected, asymptomatic neighbors of individuals with VL and in infected,

asymptomatic, randomly selected individuals (respectively, $P = .039$ and $P = .038$, Mann-Whitney rank sum test), compared with those with a history of VL (data not shown). There was no discrepancy between genotypes and the respective expected values for levels of MBL: in all clinical-epidemiological categories, levels were always significantly lower among bearers of genotypes that cause low to intermediary levels of the opsonin (figure 1). There was no association between levels of MBL and sex or age of individuals and among the different clinical categories of VL. Multiple logistic regression analysis showed that levels of MBL >500 ng/mL are directly and significantly associated with the chance of developing VL ($P = .007$).

DISCUSSION

The hypothesis whereby MBL enhances infections with intracellular pathogens states that so-called insufficient levels of this opsonin will result in fewer parasites entering the phagocytes, their preferred milieu, thus maintaining levels of infection within the capacities of an effective immune response. This hypothesis was addressed by us in a previous study [20]: we found that the phenotypes for MBL (baseline levels) were directly and significantly correlated with the probability of developing VL. Homozygotes for wild-type alleles were more frequent among patients with a history of VL and variant alleles were more frequent in infected, asymptomatic individuals, but the association was not significant. This discrepancy is explained by the fact that phenotypes for MBL depend on the set of alleles not only at exon 1 but also at the promoter, which was not genotyped in that study, and/or by a type II error due to small sample size.

In the present study, we expanded the numbers of individuals evaluated in the case and control groups; included 2 distinct sets of asymptomatic, infected control subjects; and genotyped the promoter haplotypes as well as exon 1 from the *MBL2* gene. We confirm that serum levels of MBL are directly and significantly associated with the chance of developing VL and that there is a threshold effect of this opsonin at >500 ng/mL. We now show that genotypes resulting in high levels of MBL are significantly more frequent among individuals presenting with VL than among infected, asymptomatic individuals and are even more frequent among patients who developed clinical complications. To our knowledge, this is the first definite description of an association between a gene and the outcome of VL. Genotypes of the *MBL2* gene, therefore, might be useful for estimating the risk of developing VL and, once patients develop VL, the risk of developing severe anemia and other complications, warranting a prospective study to this effect. Conversely, our results indicate that low levels of MBL may protect against progression to disease on infection with *L. chagasi.* A practical test for genotyping patients and individuals living in areas of high transmission of the parasite would be a useful adjunct to clinical care and epidemiological interventions.

Interestingly, 3 of the 5 patients with bacterial infections at immediate risk of death had genotypes for intermediate to low levels of MBL, which are associated with susceptibility to sepsis [25]. VL may have developed in these patients because of risk factors other than high levels of MBL, and, once wasting had developed, low levels of MBL increased their risk for coinfections and sepsis. Patients with complications but who were not very severely ill presented the highest frequency of genotypes for high levels of MBL and required treatment for anemia and/or bleeding. MBL-opsonized *L. chagasi* enhances production of TNF-α and interleukin (IL)–6 by human macrophages and monocytes in a dose-dependent manner [20], and these cytokines are elevated in VL [26, 27]. TNF-α causes anemia in infections by inhibiting production of erythropoietin [28] and, through activation of macrophages, by promoting erythrophagocytosis [29]; hypoferremia of infection is caused by IL-6 and hepcidin [30]. It was also interesting to observe that genotypes resulting in lower levels of MBL were significantly more frequent among infected, asymptomatic individuals with anti–*L. chagasi* specific antibodies and a negative DTH test than among those with a positive DTH test. Other work reports that high antibody responses occur in resistant subjects and are not predictive of disease [31].

The mechanism whereby MBL promotes progression of infections with *L. chagasi* to disease must be elucidated. Glycosylated antigens are captured by multiple types of lectin pattern-recognition receptors that transduce qualitatively different signals. Targeting of *L. chagasi* to different types of antigen-presenting cells may depend on the combination of the levels of MBL with the various lectin receptors, resulting in distinct patterns of cellular activation and, consequently, of effector mechanisms [32–34]. MBL can regulate the availability of pathogen-derived polymannose structures for binding to receptors for induction of T cells [35] and for affecting the function of monocytes [20, 36] and dendritic cells [32]. Our results support the concept that MBL is a "double-edged sword" and that intermediary levels of MBL may be the most desirable phenotype for innate protection against a range of pathogens.

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