

Lack of association between NRAMP1 gene polymorphisms and *Trypanosoma cruzi* infection

Key words:

Chagas' disease; NRAMP; polymorphism; *Trypanosoma cruzi*

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Abstract: Genetic analysis in mice and humans have established the key role of the human natural resistance-associated macrophage protein 1 (NRAMP1) in resistance to intracellular infections. In the present study we investigated whether four NRAMP1 polymorphisms (5'(GT)_n, -236 C→T, D543N, and 3'UTR deletion) were important in determining the susceptibility to *Trypanosoma cruzi* infections as well as in the development of chagasic cardiac disease. Genotyping for these variants was assessed in 83 seropositive (asymptomatic, *n*=51, cardiomyopathic, *n*=32) and 85 seronegative individuals from a Peruvian population where *T. cruzi* is endemic. No statistically significant differences either between patients and controls or between asymptomatic and cardiomyopathic individuals were observed with respect to NRAMP1 variants. Our data suggest that the NRAMP1 genetic polymorphism analysed do not play a major role in the pathogenesis of *T. cruzi* infection in this Peruvian sample.

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Trypanosoma cruzi is an intracellular parasite that causes Chagas' disease, a major health problem in Latin America (1). In endemic areas primary infections occurs mainly during childhood. A commonly mild acute phase is followed by a state of latent chronic infection characterised by lack of evidence of the disease other than positive serology. However, years or even decades post infection nearly one-third of infected individuals will eventually develop heart lesions (2). The reasons for the existence of uninfected individuals living in highly endemic areas and why only a proportion of patients suffer chronic heart disease remains unknown.

There is growing evidence that host genetic factors, together with environmental factors and pathogens strain differences, are major determinants of infectious disease prevalence and manifestations in humans (3). Indeed, it has been demonstrated that more than half of the variation in seropositivity to *T. cruzi* infections in



endemic areas may be attributable to genetic factors (4). In this context, HLA variation has been recently associated with differential resistance or susceptibility to *T. cruzi* infection (5–8). Since the resistance/susceptibility to most infectious diseases is clearly a polygenic trait, it is likely that other non-HLA genes, particularly those involved in the innate immune response, may also contribute to Chagas' disease genetic predisposition.

The human natural resistance-associated macrophage protein 1 (NRAMP1) represents a potential candidate for influencing the differential outcome of *T. cruzi* infection. This protein has been implicated in the early macrophage activation pathway leading to several effects on macrophage antimicrobial activity, including upregulation of inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF- α), interleukin 1 beta (IL-1 β) and major histocompatibility complex (MHC) class II expression, (for reviews, see refs. 9–11). Although the biochemical mechanism of action of NRAMP1 is presently unknown, it has been suggested that this protein controls the replication of intracellular pathogens by actively transporting divalent cations through the microbe-containing fagosome (9–12).

Genetic analysis in mice and humans have established the key role of NRAMP1 in resistance to mycobacteria and other intracellular pathogens (9–11). A single amino acid substitution in the mouse gene, *Nramp1*, was discovered to be responsible for resistance to intracellular infections (13). Furthermore, variants at human NRAMP1 gene have been found to be associated with susceptibility to tuberculosis, leprosy and HIV infection (14–16). Considering these previous disease association findings and the proposed role of NRAMP1 in macrophage activation and function, we evaluated the possible influence of NRAMP1 gene polymorphisms on susceptibility or resistance to *T. cruzi* infection and in the development of cardiac disease.

Material and methods

Participants

The study population ($n=164$) was recruited as part of a clinico-epidemiological study carried out in a rural settlement of Peru (South America), where *T. cruzi* infections is highly endemic. All participants were older than 15 years (range 15–74) and most of them (97%) recognised the presence of the local parasite vector, *Triatoma infestans*, around their dwellings. Thus, it is assumed that all the participants have experienced uniformly high levels of exposure to the vector. Clinical history, physical examination, and resting ECG were carried out.

Serological screening

Because of the low specificity of serological tests for Chagas' disease, it is generally recommended that a serum specimen tests positive in at least two different assays before it is accepted as positive. Therefore, sera were tested blindly using ELISA (Chagas' IgG ELISA; Gull Laboratories, Salt Lake City, UT, USA) and indirect immunofluorescence. When discordant results were obtained the indirect hemagglutination assay was used. Individuals were then divided into seropositive ($n=79$) and seronegative ($n=85$) groups. The mean age of the seropositive group was 35 (SD18) years, and that of the seronegative group 36 (SD15) years. Seropositive subjects with cardiac symptoms and/or ECG compatible with chagasic cardiomyopathy were ascribed to the cardiomyopathic group ($n=32$); the rest of seropositives made up the asymptomatic group ($n=47$).

NRAMP1 genotyping

DNA from all participants was purified by standard methods. In the present study we analysed four NRAMP1 gene polymorphisms: 5'(GT) n , -236 C \rightarrow T, D543N and 3'UTR. The 5'(GT) n is a dinucleotide microsatellite in the immediate 5' region of the gene and was typed as described (14). Four alleles have been identified in previous studies: allele 1 (t(gt) $_5$ ac(gt) $_5$ ac(gt) $_{11}$ g, allele 2 (t(gt) $_5$ ac(gt) $_5$ ac(gt) $_{10}$ g, allele 3 (t(gt) $_5$ ac(gt) $_5$ ac(gt) $_9$ g and allele 4 (t(gt) $_5$ ac(gt) $_8$ g. The promoter point mutation -236 C \rightarrow T (17) was analysed by a method developed in our laboratory and based on polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) with amplification-created restriction site (ACRS). A 165-bp fragment was amplified with sense primer: 5'-GTGTGGTCATGGGTATTGACATGAGTA-3' and antisense primer 5'-TGTTACCCATGGAGTGGACCTTTGTTC-3'. The mutated sense primer introduces a restriction site for the restriction enzyme *Rsa* I that recognises the wild type allele -236 C. The TGTG deletion in the 3'UTR of the gene (1729+55del4) and a non-conservative single base polymorphism at codon 543 that changes aspartic acid to asparagine (D543N), were detected by PCR-RFLP as previously described (18).

Statistical analysis

Genotype frequencies were determined by direct counting. Comparisons between seronegative healthy controls and total seropositive patients as well as between asymptomatics and cardiomyopathics were calculated using 2 \times 2 contingency tables and the chi-square test and Fisher's exact test when appropriate. Odds ratio (OR) were calculated by Woolf's method with 95% confidence interval or by Haldane's modification.

Genotype distribution of NRAMP1 polymorphism in chagasic patients and control subjects

Genotype	Controls n=85 (%)	Patients n=79 (%)	Asymptomatic n=47 (%)	Cardiac n=32 (%)
5' (GT)_n				
3/3	36 (42.4)	30 (38.0)	14 (29.7)	16 (50.0)
3/2	38 (44.7)	36 (45.6)	24 (51.1)	12 (37.5)
2/2	10 (11.8)	12 (15.2)	8 (17.2)	4 (12.5)
3/1	1 (1.2)	0	0	0
3/4	0	1 (1.3)	1 (2.1)	0
-236 C→T				
C/C	82 (96.5)	78 (98.7)	46 (97.9)	32 (100)
C/T	3 (3.5)	1 (1.3)	1 (2.1)	0
T/T	0	0	0	0
D543N				
G/G	60 (70.6)	54 (68.3)	33 (70.2)	21 (65.6)
G/A	22 (25.9)	25 (31.6)	14 (29.8)	11 (34.4)
A/A	3 (3.5)	0	0	0
3' UTR deletion				
+TG TG/+TG TG	60 (70.6)	54 (68.3)	33 (70.2)	21 (65.6)
-TG TG/+TG TG	22 (25.9)	25 (31.6)	14 (29.8)	11 (34.4)
-TG TG/-TG TG	3 (3.5)	0	0	0

Table 1

Results

To study the role of NRAMP1 in *T. cruzi* infection we selected four NRAMP1 polymorphisms with the potential to affect NRAMP1 expression, those located in regulatory sequences in either the promoter (the 5'(GT)_n and -236 C→T) or 3' untranslated regions (3' UTR), or function (nonconservative D543N amino acid substitution in the predicted cytoplasmic C terminus of the protein). The prevalence of NRAMP1 genotypes in chagasic patients with or without cardiomyopathy and in controls subjects is shown in Table 1. The study population was shown to be in the Hardy-Weinberg equilibrium. We found no significant differences in the distribution of these genetic variants among controls and total group of patients. In this Peruvian sample, the D543N and the 4-bp deletion in the 3'UTR appears to be in strong linkage disequilibrium, as has been previously reported in other populations with different genetic background (14–17). All the four alleles at (GT)_n microsatellite were observed in the Peruvian population; allele 1 and 4 were present at very low frequency. The promoter single base transition -236 C→T was uncommon (allelic frequency=0.012); none of the samples were

homozygotes for this mutation and only four individuals were heterozygotes (3 healthy controls and 1 asymptomatic patient), therefore, it was not possible to assess its influence of *T. cruzi* infection.

In order to investigate the possible influence of the NRAMP1 variants in the development of Chagas' disease cardiomyopathy, the genotype frequencies between seropositive patients with manifestation of the disease (asymptomatic) and those with chagasic cardiomyopathy were compared. We failed to detect statistically significant differences in the distribution of NRAMP1 allele or genotypes between asymptomatic and cardiac individuals. Interestingly, we noted an increased frequency, in the borderline of statistical significance, of individuals homozygous for the 5' (GT)_n allele 3 in cardiomyopathic compared with asymptomatics ($\chi^2=3.30$; $P=0.07$).

Discussion

It has been clearly demonstrated that macrophages are important effector cells for the control and killing of intracellular pathogens. However, macrophages may also serve as long-term host that facilitate the replication and survival of some parasites. *T. cruzi* is capable of infecting a variety of nucleated cells including the macrophage lineage. Infection by this parasite results in a wide spectrum of disease phenotypes, ranging from asymptomatic infections to the severe cardiac and/or gastrointestinal forms of Chagas' disease. Therefore, it is likely that a genetic defect at any key point in the macrophage activation pathway will contribute to Chagas' disease susceptibility/outcome. In this context, it has been suggested that NRAMP1 plays an important role in macrophage activation and function, and that this protein affects the intraphagosomal pathogen replication by modulating the divalent cations content in this organelle (9–12).

Although there is no doubt about the importance of mouse *Nramp1* in natural resistance to infection with certain intracellular pathogens (*Mycobacteria*, *Salmonella*, *Leishmania*), the results of genetic association studies between human NRAMP1 variants and infectious disease have been controversial. A number of studies have established an association between allelic variants of NRAMP1 gene and susceptibility to infectious disease (14–16), although no causal relationship between NRAMP1 alleles and risk of infection can be drawn from these association studies. However, other studies have failed to detect an association between NRAMP1 and susceptibility to infection (18–23). The reason for these discrepant results remains to be determined but the complex genetic model of most infectious diseases, involving more than one susceptibility locus, may in part account for these contradictory findings.

In the present study a clear association between the four NRAMP1 variants and *T. cruzi* infections in a Peruvian population have not been demonstrated, indicating that these polymorphisms may not have a major role in determining disease susceptibility and/or outcome. The molecular mechanisms controlling infection with *T. cruzi* might be different from those that have been previously shown to play an important role in other diseases caused by intracellular pathogens. In this sense, an interesting correlations has been observed between the effect of Nramp1 mutations in infection and the intracellular survival strategies adopted by different pathogens (10). Thus, the phagosome containing those intracellular pathogens affected by Nramp1 mutations, as *Mycobacteria*, *Salmonella* and *Leishmania*, acquire a late endosomal marker Lamp-1, which co-localizes with Nramp1, while *Listeria* or *Legionella*, not affected by Nramp1 mutations, do not acquire endosomal or lysosomal markers (10, 24, 25). Of note, epimastigotes forms of *T. cruzi* do not contain lysosomal markers as Lamp1 (26). Although further studies to elucidate the functional significance of these observations are warranted, it is tempting to speculate that the lack of association between NRAMP1 and *T. cruzi* susceptibility may be related to the presence of certain endosomal or lysosomal proteins with the capability to modify the intracellular behaviour of this protozoan parasite.

Genetic susceptibility to Chagas' disease is complex and heterogeneous and, as most infectious diseases, possibly involves a large number of polymorphic genes, perhaps each contributing with a

small incremental effect (3). On the other hand, the strength of association can depend on the degree of linkage disequilibrium of the examined polymorphisms with the putative causal genetic variation, and this event may vary greatly according to the history and genetic background of the particular population being tested (27). Interestingly, a very recent report has suggested that in mice, the contribution of Nramp1 to tuberculosis resistance may be confused by the presence of another closely linked locus, designated *sst1*, that has a stronger effect in the development of infection (28). An interesting finding observed here was the higher prevalence, in the borderline of statistical significance, of homozygosity or the 5' (GT)_n allele 3 found in cardiomyopathics compared with asymptomatic individuals. This is consistent with the hypothesis that chronic hyperactivation of macrophages associated with the 5' (GT)_n allele 3 is functionally linked to autoimmune disease susceptibility (29, 30) and, simultaneously, with the theory that the heart damage in chronic Chagas' disease is due to an autoimmune process (31). The possibility that a larger study will show a statistically significant association between allele 3 and susceptibility to Chagas' cardiomyopathy cannot be discounted.

To date, this is the first study dealing with the possible association between NRAMP1 genetic variants and Chagas' disease in an endemic population. Although we have not shown any evidence of linkage between *T. cruzi* infection and NRAMP1 polymorphisms in this data set, clearly it would be necessary to have greater number of individuals to complete exclude linkage.

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