〔原著論文〕 HLA-B35 and MICA-A5 synergistically enhanced the susceptibility to Chagas heart disease

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Introduction

Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi* (*T. cruzi*), is a major public health problem in Central and South America (1). An epidemiological study in an endemic area, Guatemala, showed that, on average, 5-10% of the individuals were seropositive and considered to be persistently infected with *T. cruzi* (2). Five to 30 years after primary infection, about 30% of the seropositive individuals develop heart disease which is characterized by electrocardiographic alterations, heart failure, or sudden death, and has been suggested to be one of the major causes of death in this endemic area. The other 70% of seropositive individuals remain asymptomatic. In Guatemala, there are no chronic patient with digestive disorders such as megacolon, although this has been reported in other populations (1).

We are currently unable to predict whether exposed individuals will eliminate the organism or develop chronic Chagas disease. The natural course of infection in humans remains unclear, although there is little

epidemiological information such as incidence, cure rate, force of infection and death rate. In an experimental mouse model system, previous studies have elegantly showed that CD8⁺ cytotoxic T cells are essential for elimination of the parasite (3, 4, 5). Recently, Widel et al. showed that specific CD8⁺ T cells were also active in human Chagas patients in Guatemala (6). Autoimmune mechanisms have been reported to play an important role in the development of chronic Chagas heart disease, both in an experimental mouse model and in humans (7, 8, 9, 10). However, the pathogenic process by which chronic Chagas disease develops is still controversial (11).

Because immune response has been suggested to play a role both in the control of susceptibility to *T. cruzi* infection and in the development of chronic disease, genetic predisposition of the host may influence the outcome of the infection (12, 13). To determine the host genetic factors that influence susceptibility to Chagas heart disease, we typed cardiac Chagas patients for polymorphisms of the closely linked genes HLA-B, MICA and the TNF- α promoter region, and identified

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HLA-B35 and MICA-A5 as two major markers of the susceptibility.

Materials and Methods

Subjects. Typing for HLA-B, the TM region of MICA and TNF promoter region polymorphisms was performed on 33 unrelated, seropositive Chagas patients (11 men and 22 women, mean age 60.8 years) with heart problem, including 29 with typical electrocardiograph abnormalities such as ventricular conduction defects, atrioventricular block and atrial fibrillation described by Kawabata et al.(14) and Maguire et al. (15), and 4 with hypertrophy of heart in their chest X rays but without any ECG abnormalities. They were outpatients at the Department of Cardiology, Zacapa National Hospital, Zacapa, Guatemala. 138 seronegative healthy controls (46 men and 92 women, mean age 38.1 years) were also typed. The subjects were mestisos, all from Zacapa or surrounding areas, and the geographical distribution of

their residences was similar between patient and control groups. The serological diagnosis of Chagas disease was performed by indirect haemagglutination test (1) or immuno-double diffusion test (1) and in all cases by enzyme-linked immunosorbent assay for anti-*T. cruzi* IgG₁ (1).

DNA extraction. Genomic DNA was extracted from peripheral blood using a kit (DNA Extractor WB, Wako Pure Chemicals, Osaka, Japan) as previously described (16).

HLA-B typing. HLA-B typing by sequence specific oligonucleotide probe (SSOP) method was carried out following the protocol described by Yoshida et al. (17). Exon 2 of the HLA-B gene was amplified by polymerase chain reaction (PCR). Labeled SSO probes were hybridized with amplified DNA on nylon membrane and detected by the chemiluminescent method (17).

MICA typing. We analyzed the TM region

Table 1.

(a) The frequencies of HLA-B in the patients with Chagas disease and healthy controls.

ECBa	Chagas patients n=33 (%)	Healthy controls n=138 (%)	P	Pc	OR (95%CI)
B0703	3 (9.1)	1 (0.7)			
B0801	11 (33.3)	28 (20.3)			
B13	0 (0)	1 (0.7)			
B15	4 (12.1)	0 (0)			
B1524	0 (0)	4 (2.9)			
B1801/1802	0 (0)	5 (3.6)			
B27	0 (0)	3 (2.2)			
B2708	1 (3.0)	0 (0)			
B35	25 (75.8)	50 (36.2)	<0.0001	<0.003	5.50 (2.31-13.11)
B3801/1523	0 (0)	3 (2.2)			
B39	2 (6.1)	2 (1.4)			
B3902	0 (0)	4 (2.9)			
B40	5 (15.2)	45 (32.6)	0.0476	1.333	0.37 (0.13-1.02)
B41	0 (0)	1 (0.7)			
B44	1 (3.0)	9 (6.5)			
B4701	0 (0)	3 (2.2)			
B48	1 (3.0)	3 (2.2)			
B4901	1 (3.0)	15 (10.9)			
B4902	0 (0)	1 (0.7)			
B51	1 (3.0)	12 (8.7)			
B5201	4 (12.1)	3 (2.2)			
B55	0 (0)	13 (9.4)			
B57	2 (6.1)	15 (10.9)			
B58	1 (3.0)	5 (3.6)			
B5901	0 (0)	4 (2.9)			
B62	0 (0)	5 (3.6)			
B7301	2 (6.1)	0 (0)			
B7801	2 (6.1)	10 (7.2)			

a: Epitope combination of HLA-B

polymorphism of the MICA gene using the PCR-single-strand conformation polymorphism (SSCP) method (18). PCR amplification was performed by the method described by Mizuki et al. (19). Briefly, a total volume of 30 µl of amplification reaction mixture contained 100 ng of template DNA, 10 mM Tris-HCl (pH8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.25 U Ampli Taq DNA polymerase (Perkin Elmer, NJ, USA), 0.2 mM dNTPs, 5 pM of 5' primer (MICA5F; 5'-CCTTTTTTTCAGGGAAAGTGC-3') and 3' primer (MICA5R; 5'-CCTTACCATCTCCAGAAACTGC-3'). PCR was performed under the following conditions: denaturing at 95 °C for 9 min, followed by 38 cycles of denaturing at 94 °C for 15 sec, annealing at 56 °C for 15 sec, extension at 72 °C for 45 sec, and a final extension at 60 °C for 20 min. Heat-denatured PCR products with 2 volumes of formamide dye (88% formamide, 10mM EDTA, 0.01% bromphenol blue, 0.01% xylene cyanol) were loaded on a 14cm X 14cm X 1mm polyacrylamide gel (7% acrylamide, 0.4XTBE, 10% glycerol) and run at constant voltage (10 volt / cm) under a constant temperature of 26 °C. DNA fragments were visualized by silver staining (Silver Stain kit II, Wako Pure Chemicals, Osaka, Japan). We compared SSCP patterns of the samples with those of the homozygous B cell lines (BM92, OLGA, BM14, HHKB, RML, AMAL) whose MICA types have been well established (19).

TNF promoter region polymorphism. Polymorphisms of the TNF-α promoter region were analyzed by the SSCP method described by Higuchi et al. (20). The 1051 bp sequence of the TNF-α gene at position -1236 to -186 was amplified by PCR method with 5' primer (5'-

GGGGACACACAAGCATCAAGG-3') and 3' primer (5'-GCTGTCTGCTTGTGTGTGTGTGTCTGGG-3') under the following conditions: denaturing at 95 °C for 3 min and extension at 72 °C for 5 min, followed by 35 cycles of denaturing at 94 °C for 1 min, annealing at 60 °C for 2 min, extension at 72 °C for 3 min, and a final extension at 72 °C for 10 min. The PCR product was blotted on nylon membrane and hybridized with 10 different probes to identify the following dimorphic sites, -238G/A, -308G/A, -857C/T, -863C/A, -1031T/C, as described (20).

Statistical analyses. We compared the allele frequencies of HLA-B, TM region of MICA and TNF-α promoter region between Chagas patients and seronegative healthy controls by the chi-squared test (21). The obtained P-value was corrected by the Bonferroni correction (22), multiplication by the number of the alleles tested (Pc). The observed difference was considered statistically significant when Pc was less than 0.05. Odds ratio (OR) and 95% confidence interval (CI) were estimated to analyze the risk for the disease (23). The method of Mittal (24) was used for the linkage analysis among HLA-B, MICA and TNF-α promoter alleles. When the t-value was greater than 2.0, linkage disequilibrium (LD) was considered to be present.

Results

HLA-B typing. Twenty-eight epitope combination groups of HLA-B (ECB) were detected in the subjects. 75.8% of the patients were typed as HLA-B35, compared to only 36.2% of the controls. The frequency of HLA-B35 was significantly increased in the patients ($\chi^2 = 16.90$, $P_c < 0.003$, $OR = 5.50$, $95\%CI = 2.31 -$

Table 1.

(b) Frequencies of the MICA alleles in patients with Chagas disease and healthy controls.

MICA _b	Chagas patients n=32 (%)	Healthy controls n=134 (%)	P	P _c	OR (95%CI)
A4	5 (15.6)	22 (15.9)	0.240	1.200	1.61 (0.72-3.57)
A5	13 (40.6)	40 (29.9)			
A5.1	5 (15.6)	29 (21.0)			
A6	6 (18.8)	48 (35.8)	0.064	0.320	0.41 (0.16-1.07)
A9	24 (75.0)	96 (71.6)			

b: MICA; MHC class I chain related gene A. MICA alleles are determined by using the microsatellites polymorphism in the MICA TM region (exon 5). Terminology of the alleles are derived from Mizuki et al. (19).

13.11) (Table 1a).

MICA typing. Five alleles of MICA previously reported in TM region (19) were typed in Guatemalan subjects (Table 1b). The frequency of MICA-A5 was increased in the patients compared with controls. However, the differences was not significant ($X^2 = 1.38$, $P = 0.240$, $P_c = 1.200$) (Table 1b).

Haplotype analysis. There were three HLA-B-MICA combinations with positive LD, HLA-B35-MICA-A9, HLA-B55-MICA-A5.1 and HLA-B4901-MICA-A6. T-values were calculated using Mittal's method (24) to be 5.61, 2.79 and 2.26, respectively (Table 2). HLA-

Table 2.

Linkage disequilibrium between ECB and MICA alleles

MICA	HLA-B		
	35	55	4901
MICA-A5	-	-	-
MICA-A5.1	-	2.79a	-
MICA-A6	-		2.26
MICA-A9	5.61	-	-

a: t-value was calculated by the method of Mittal (24). A t-value of more than 2.0 indicates a positive linkage disequilibrium. The indication of negative (-) means the t-value was less than 2.0.

B35-MICA-A9 haplotype was significantly increased in the patients ($X^2 = 10.51$, $P = 0.0012$, $OR = 3.65$, $95\%CI = 1.62 - 8.23$) (data not shown). However, those associations may be due to the strong association between HLA-B35 and the disease, because MICA-A9 itself did not show any significant association as noted above. Although both HLA-B35 and MICA-A5 were increased in the seropositive patients, there was no significant LD between these two alleles.

To analyze the effects of HLA-B35 and MICA-A5 on the susceptibility, we classified the subjects into four groups according to their HLA-B and MICA markers: both HLA-B35 and MICA-A5 negative (B35⁻ / A5⁻), HLA-B35 positive and MICA-A5 negative (B35⁺ / A5⁻), HLA-B35 negative and MICA-A5 positive (B35⁻ / A5⁺), and both HLA-B35 and MICA-A5 positive individuals (B35⁺ / A5⁺) (Table 3). The patients were

observed at a greater rate in group B35⁺ / A5⁺ than in group B35⁺ / A5⁻ ($X^2 = 3.86$, $P = 0.0495$, $OR = 2.86$, $95\%CI = 0.98 - 8.31$), while there was no difference between group B35⁻ / A5⁺ and group B35⁻ / A5⁻ ($X^2 = 0.01$, $P = 0.920$, $OR = 1.08$, $95\%CI = 0.24 - 4.84$). Therefore, the effects of MICA-A5 on susceptibility to infection with *T. cruzi* appeared only in the presence of HLA-B35. As shown in Table 3, increased risk for the individuals who had both HLA-B35 and MICA-A5 ($OR_{B35/A5-1} = 9.80$) was much higher than the sum of the increased risks ((OR_{B35-1}) + (OR_{A5-1}) = 2.86) for either HLA-B35 alone or MICA-A5 alone positive individuals, showing that the effects of HLA-B35 and MICA-A5 on the susceptibility were synergistic.

TNF- α promoter typing. Located within 300 kb on the centromeric side of the MICA and HLA-B genes, are genes for two other potentially important regulators of protective immunity, TNF- α and b. To clarify whether the susceptibility effect of HLA-B35 and MICA-A5 was due to the HLA-B and MICA genes themselves or to reflection of LD between these markers with the polymorphisms in the TNF region, TNF- α promoter polymorphisms were analyzed in the Guatemalan subjects. There was, however, no association between dimorphisms at any of the five positions (-238, -308, -857, -863, -1031) in TNF- α promoter region polymorphism and seropositivity (data not shown).

Discussion

Using knockout mice, Tarleton et al. have shown that

Table 3.

Odd's ratio of each combination of HLA-B35 and MICA-A5

HLA-B35	MICA-A5	Chagas patients	Healthy controls	ORa
		n=32	n=134	
-	-	5	54	
-	+	3	30	1.08
+	-	14	40	3.78
+	+	10	10	10.80

a: OR was calculated against individuals negative both HLA-B35 and MICA-A5

both CD4⁺ and CD8⁺ T cells stimulated by HLA-class II and class I, respectively, were essential in the protective immunity against *T. cruzi* infection (3). CD8⁺ T cells isolated from mice chronically infected with *T. cruzi* expressed MHC restricted cytolytic activity against infected macrophages (4). Anti-CD8 antibody treatment increased susceptibility to *T. cruzi* in the resistant C57BL/6J mice (5). These studies suggest that the CD8⁺ T cell subset is important in immunity against *T. cruzi* in mice. In the present study, we found an association between HLA-B35 and Chagas heart disease, suggesting that CD8⁺ T cells restricted by MHC class I molecules may play an important role in immunity to *T. cruzi*, not only in mice, but also in humans. Although we did not check HLA-A type in the subjects, Indeed, Wizek et al. recently reported that HLA-A2 binding antigenic peptides stimulated the patients' CTL (6).

After the identification of HLA-B35 as a marker of susceptibility, we analyzed the neighboring HLA class I-like gene, MICA, and found another significant susceptible combination of HLA-B35 and MICA-A5, which was not derived from LD between these two markers. The effect of these was not additive, but synergistic, as shown in Table 3. MICA is similar in structure to mouse MHC class Ib (TL region), which can be recognized by $\gamma\delta$ T cells (25, 26, 27). Groh et al. demonstrated that MICA can be expressed in TAP-negative cells, suggesting that its expression is independent of peptide binding (28). They also reported that $\gamma\delta$ T cells bearing V δ 1 TCR from intestinal epithelium were stimulated by MICA and MICB (29). V δ 1 $\gamma\delta$ T cells represent 70 to 90% of the $\gamma\delta$ T cells in the intestinal epithelium that maintain the integrity of the gut epithelium by eliminating infected or damaged epithelial cells. These activated T cells may possibly be recruited to other damaged tissues where MICA is positive.

Because the effects of HLA-B35 and MICA-A5 on the susceptibility were synergistic, susceptibility is likely determined by an interaction between activated T cells stimulated by the MICA molecule and by the MHC class I, although there is always the possibility that other closely linked susceptibility genes may be involved. With regards to HLA-B35, susceptibility associations have also been observed with subacute thyroiditis (30) and AIDS (31, 32). The common mechanisms

underlining these associations should be an important subject of analysis.

Because TNF genes are located close to the MICA gene, the MICA-A5 effect might have been observed if there were any LD with some susceptibility allele of the TNF- α promoter region, which controls the transcriptional level of TNF- α (33). However, there was no association between TNF- α promoter type and susceptibility to Chagas disease, and no LD between TNF- α promoter and MICA type polymorphisms, indicating that the effect on the susceptibility and MICA-A5 was not due to LD between the TNF- α promoter and MICA.

The pathogenesis of Chagas heart disease in the chronic phase remains unknown. It has been suggested that some autoimmune mechanisms may result in damage to the heart (7, 8, 9, 10). Cunha-Neto et al. demonstrated that chronic Chagas patients with heart disease had *T. cruzi* -cardiac myosin cross reactive autoantibody in their sera (9). Santos et al. reported that anti-CD4 monoclonal antibodies abrogated the rejection of syngeneic newborn heart grafts in chronically infected mice, and that CD4⁺ T cells from chronically infected mice showed a proliferative response to syngeneic myocardium antigens (34). These reports suggest that autoimmunity is involved in the pathogenesis of Chagas heart disease, and that CD4⁺ T cells play an important role. However, there is no report that clearly described the association between HLA-class II antigens and Chagas heart disease. In this study, we also typed for their HLA-DR, DQ and DP and found no significant association (data not shown). Llop et al. reported that HLA-B40-Cw3 haplotype was significantly decreased in cardiac Chagas patients compared with non-cardiac patients, indicating this haplotype associates with resistance (12). In the present study, we also observed a decreased frequency of HLA-B40 in the cardiac Chagas patients (15.2%) than in the controls (32.6%), although it was not statistically significant after Bonferroni correction ($P=0.0476$, $P_c=1.333$) (Table 1a). But if we consider the previous study (12) as the first finding of resistant HLA-B40, it may not necessary to correct our results by Bonferroni and we may claim that the frequency of HLA-B40 decreased in Chagas heart disease. The observations that HLA-class I rather than class II associated with Chagas heart disease suggest that the deterioration of the disease is partially caused by

insufficient elimination of the parasites due to decreased activity of immunity. In conclusion, we found that HLA-B35 and MICA-A5 increased the risk for developing Chagas heart disease, and that the effects of the two alleles on susceptibility were synergistic.

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Summary

To determine the host genetic factors that influence susceptibility to Chagas heart disease, we typed 33 unrelated, seropositive Chagas patients with cardiac disorders at the Department of Cardiology, Zacapa National Hospital, Zacapa, Guatemala, and 138 seronegative controls for polymorphisms of HLA-B, MHC class I chain-related gene A (MICA), and the Tumor Necrosis Factor- α (TNF- α) promoter region. The frequency of HLA-B35 was significantly increased in the cardiac Chagas patients ($P_c < 0.003$, OR = 5.50, 95%CI = 2.31-13.11). Although there was no association between MICA, TNF- α promoter region polymorphisms and the disease, the increased risk for the individuals who had both HLA-B35 and MICA-A5 (delta odds ratio; OR_{B35/A5} -1=9.80) was higher than the sum of the increased risks for either HLA-B35 (OR_{B35} -1=2.78) or MICA-A5 single positive (OR_{A5} -1=0.08) individuals, indicating that the effects of the two alleles on susceptibility were synergistic.

Key words: Chagas disease, *Trypanosoma cruzi*, HLA-B, MHC class I chain-related gene A (MICA), sequence specific oligonucleotide probe (SSOP)