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Differential distribution of *Trypanosoma cruzi* clones in human chronic chagasic cardiopathic and non-cardiopathic individuals

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ABSTRACT

PCR and Southern blot hybridization were used to determine the distribution of *Trypanosoma cruzi* clones in 37 chronic chagasic cardiopathic and non-cardiopathic patients. Parasite DNA amplified from peripheral blood or dejections of *Triatoma infestans* fed on patient blood was hybridized with probes containing hypervariable minicircle nucleotide sequences capable of detecting three sublineages of *T. cruzi*. Probes Z-I and Z-IIb detect unique sequences in lineages TcI and TcIIb, respectively. Probe Z-hybrid detects sequences of lineages TcIId and TcIIe. *T. cruzi* clones of the Z-I sublineage were detected in 62.2% of *T. infestans* dejections and 5.4% of peripheral blood samples. Clones of Z-IIb and Z-hybrid sublineages had similar distribution in blood and dejection samples. Interestingly, clones of the Z-IIb sublineage were significantly lower in cardiopathic than in non-cardiopathic patients (23.5% *versus* 75%; $P = 0.0006$). Clones of the Z-hybrid sublineage were found in 29.4% of cardiopathic and 75% of non-cardiopathic patients, respectively ($P = 0.0051$). By contrast, clones of sublineage Z-I were similarly distributed in both groups of patients. The low frequency of Z-IIb and Z-hybrid sublineage clones detected in cardiopathic patients suggests that the immunological mechanisms involved in controlling and eliminating these *T. cruzi* parasites may be detrimental to the host, leading to the development of chagasic cardiomyopathy.

1. Introduction

Trypanosoma cruzi is the etiological agent of Chagas disease (Miles, 2003). The World Health Organization estimates that 11–18 million individuals are infected worldwide (WHO, 2002). Infection with *T. cruzi* is characterized by acute non-specific symptoms that in most patients are followed by an "indeterminate" phase defined by the appearance of *T. cruzi* specific antibodies in the blood stream and the absence of clinical signs and cardiac abnormalities. Between 10 and 30 years after infection, approximately 30–40% of "indeterminate" patients show recognizable signs and/or symptoms of a unique form of heart disease referred to as chagasic cardiomyopathy (CCM). Patients usually develop a variety of symptoms with several degrees of intensity, which has led to the establishment of a diagnostic classification for CCM (Rocha et al., 2003). Non-specific symptoms suggestive of heart misfunction include palpitations, dizziness and syncope. Clinical manifestations of CCM include congestive heart failure, brain, limb and lung thromboembolism, and ventricular fibrillation (Rassi et al., 2000). The mechanism(s) leading to the development of CCM are uncertain and several fundamental enigmas concerning this disease still remain unanswered. A considerable amount of information supports the hypothesis that an immune response – constantly triggered by either parasite persistence or host response to self-antigens (autoimmunity), or both – plays an important role in the development and/or propagation of the tissue lesions (Teixeira et al., 2002; Cunha-Neto et al., 2006). Parasite persistence implies that a few parasites constantly trigger immune responses that lead to chronic inflammation and cell death. The concept of autoimmunity hypothesizes that parasites have molecules that mimic human antigens. In an attempt to control *T. cruzi* infection, the host produces antibodies and T cells that subsequently recognize self-antigens and destroy myocardial tissue. Several lines of evidence indicate that a low level of parasites remains in the bloodstream and/or in the heart tissue (Añez et al., 1999; Zhang and Tarleton, 1999). A significant number of studies support the hypothesis of parasite persistence and its pathologic

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role in the development of chagasic cardiomyopathy (Añez et al., 1999; Zhang and Tarleton, 1999).

Phylogenetic studies have shown that this microorganism undergoes long-term clonal evolution and that it is divided into two main phylogenetic lineages, namely *T. cruzi* I and *T. cruzi* II (Tibayrenc et al., 1986; Tibayrenc, 1995; Souto et al., 1996; Zingales et al., 1999). According to some authors, *T. cruzi* II is further partitioned into five sublineages or "discrete typing units" called DTUs (Brisse et al., 2000; Tibayrenc, 2003). It is not known why different patients develop cardiac, digestive, cardiodigestive or indeterminate clinical forms of the disease. One hypothesis proposes that the genetic characteristics of infecting *T. cruzi* clones are the cause which produce these diverse clinical manifestations (Macedo and Pena, 1998; Andrade, 1999; Vago et al., 2000). Evidence showing that clones of *T. cruzi* isolated from cardiac tissue differ from those found in the esophagus support this idea. In order to obtain more insight about the pathogenesis of Chagas cardiopathy, here we report the distribution of *T. cruzi* clones in chronic chagasic cardiopathic and non-cardiopathic patients.

2. Materials and methods

2.1. Patients

Thirty-seven chronic chagasic patients with positive conventional serology (ELISA, IFAT) and circulating *T. cruzi* (PCR positive) were included in this study. The patients were from the IV, V and Metropolitan Regions of Chile, Chagas endemic areas, and they were treated with allopurinol (8.5 mg/(kg day for 60 days)) in 1992. Compliance with *ad hoc* protocols and regulations of the Ethics Committee of the Faculty of Medicine, University of Chile, were followed throughout the course of this investigation (Apt et al., 1998).

The titers of the patients studied by IFAT fluctuated between 1/60 (minimum positive) and 1/1280 (maximum obtained by us). In relation to ELISA, all patients had antibody levels on an optical density (OD) at 490 nm ranging between 1.596 and 2.026. The cut-off value was 0.2 OD, using epimastigotes as antigens as usual (Zulantay et al., 2004).

2.2. Cardiology

Each patient was subjected to a 12-lead electrocardiogram (EKG) examination, twice a year, during the 13-year follow-up. Therefore, each patient had at least 20 ECG at the time of study. The cardiac evaluation, in addition to the EKG, included a chest radiograph and, in some cases, an Eco-Doppler. The double-blind protocol recommended by the World Health Organization was followed (Arribada et al., 1986). The cardiologist analyzing the EKG profiles was unaware of the patient's infection status or any previous EKG results (Apt et al., 2003).

Each chagasic patient was classified as cardiopathic only after the altered EKG profile was maintained over a period of 10 years, together with the other analyses mentioned above.

2.3. XD test and triatomine samples

The xenodiagnostic test (XD) uses two cylindrical wooden boxes, each containing seven uninfected third-instar *T. infestans* nymphs, free of infection, maintained in our laboratory since 1950 (Schenone, 1999). The insects were allowed to feed for 20–30 min on the arm of each patient. Microscopic examination of insect dejections was performed 30, 60, and 90 days after feeding triatomines with peripheral blood from infected individuals. The sensitivity of this test was about 80% of the cases, as was previously published (Zulantay et al., 2007).

The fecal samples of all the triatomines obtained after 30, 60 and 90 days from each patient were pooled and used for PCR analysis. The material was placed in Diamond medium, incubated at 98 °C for 15 min and centrifuged at 4000 rpm for 3 min. The supernatant was loaded onto a P6 column to remove low molecular weight DNA polymerase inhibitors and the wash through was collected and frozen at −20 °C until used.

2.4. Identification of T. cruzi clones in peripheral blood and triatomine dejections

PCR was performed in triplicate using DNA extracted from peripheral blood samples (PCR-B) or triatomine dejections (PCR-D) following procedures previously described (Solari et al., 2001). Briefly, 800 μL of blood sample was used for nucleic acid extraction and the DNA suspended in 50 μL of sterile water. The reaction mix contained 10 pmol of forward primer 121 (5′-AAA TAA TGT ACG GG(T/G) GAG ATG CAT GA-3′), 10 pmol of reverse primer 122 (5′-GGT TCG ATT GGG GTT GGT GTA ATA TA-3′), 5 μL of amplification buffer (1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl pH 8.3), 0.2 mM of each dNTP, 1.5 U of Taq DNA polymerase (Promega) and 5 μL of template in a final volume of 50 μL. The amplification procedure included an initial denaturation at 94 °C for 4 min followed by 35 cycles (94 °C for 30 s, 64 °C for 30 s and 72 °C for 30 s). A final incubation at 72 °C for 10 min was included to ensure full elongation of the amplified fragments. A Techne TC-412 thermocycler and a DNA clean chamber (AirClean 600 PCR workstation) were used to prevent contamination. DNAs prepared from a confirmed infected individual or from a non-infected individual were included as positive and negative controls, respectively. A similar PCR protocol was used to process the triatomine samples. Amplification products were fractionated by electrophoresis in 2% agarose gels in 0.5× TBE buffer (44.5 mM Tris–HCl, pH 8.0; 44.5 mM boric acid; 1.1 mM EDTA), denatured, transferred onto nylon membranes and cross-linked by UV irradiation (Solari et al., 2001).

2.5. Cloning and nucleotide sequence determination of kDNA segments

DNA from *T. cruzi* clones Cutiacl1, CBBcl3 and SC43cl1 (kindly provided by Dr. Christian Barnabé) was amplified using primers 121 and 122 (Solari et al., 2001) and the PCR products were cloned into the pGEM-T easy vector (Promega). Recombinant plasmids derived from amplified material of clones Cutiacl1, CBBcl3 and SC43cl1 were designated pS120, p3d and pS122, respectively. The GeneBank accession numbers for each cloned minicircle segment are: FJ481519 (S120), FJ481521 (3d) and FJ481520 (S122). Plasmid DNA was prepared from each recombinant clone to determine the nucleotide sequence of the *T. cruzi* insert. BLAST programs and GenBank data were used in the nucleic acid sequence analyses. Multiple sequence alignments were performed with ClustalX (Jeanmougins et al., 1998) and BioEdit programs (Hall, 1999).

2.6. Preparation of minicircles probes and Southern blot analysis

Minicircle probes S120, S122 and 3d were obtained by PCR using plasmids pS120, pS122 and p3d as templates. Probe S120 was obtained with forward primer P-120-D (TTA TAA CCC AAC TTT ATC CGA AAT ATC) and reverse primer 122. Probe S122 was obtained with forward primer P-122-D (TAA CAA CAA TAC TAC ATT AAC AAC AAC) and reverse primer 122. The forward and reverse primers to synthesize probe 3d were primers 121 and 122. PCR reactions contained 50 ng of template, 0.2 mM of each dNTP, 0.2 μM of forward and reverse primers, 2.5 mM of $MgCl_2$ and 0.3 U of Taq DNA polymerase (Promega) in a final volume of 50 μL. Amplification conditions included 4 min incubation at 94 °C and 35 cycles (1 min