RELATIONSHIP BETWEEN _TRYPANOSOMA CRUZI_ AND HUMAN CHAGASIC MEGAESOPHAGUS: BLOOD AND TISSUE PARASITISM

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Abstract. The persistence of _Trypanosoma cruzi_ in tissue and blood of 52 patients in the digestive form of chronic Chagas disease was studied. These patients had chagasic megaesophagus and underwent corrective surgery. Parasitologic (xenodiagnosis, hemoculture, or both), histopathologic (hematoxylin and eosin, and peroxidase-anti-peroxidase staining), and molecular (polymerase chain reaction [PCR] followed by slot-blot hybridization) tests were used in the analysis. The presence of _T. cruzi_, its genomic fragments, or its antigens could be detected in 98% (51 of 52) of the patients. The parasite was randomly identified in 76.9% of esophageal tissues and in 90.4% by PCR and in 73.1% by parasitologic methods from the blood. Fifty percent (26 of 52) of tissue samples had inflammation, 80.8% of which was associated with the parasite. _Trypanosoma cruzi_ was also identified unassociated with inflammatory alterations. Higher tissue parasitism and intense inflammatory processes were observed in esophageal tissue from patients with Grade IV megaesophagus. These data demonstrate that in the digestive form of Chagás disease, particularly in cases of megaesophagus, _T. cruzi_ is frequently found, both in blood and tissues and may contribute to the pathogenic mechanisms involved.

INTRODUCTION

Although Chagas disease was first described 9 decades ago, its pathogenesis and the mechanisms of parasite-host relationship are still not well understood. This infection has a variable clinical course: most of patients remain free of alterations in the indeterminate phase, but others can develop severe chronic disease, with cardiac or gastrointestinal involvement. The prevalence of digestive manifestations during the chronic phase of the disease varies considerably in different endemic areas. The digestive form seems to be absent from certain countries, such as Panama and Venezuela, whereas in central Brazil, 2–8.8% of patients have megaesophagus. The pathogenesis of chagasic megaesophagus may be related to host characteristics as well as to different strains of the parasite _Trypanosoma cruzi_. One of the most important factors that determines both symptoms and complications of this clinical form is the derenervation of the smooth muscle of the esophagus, causing several morphological and motor changes, according to which megaesophaghi are classified by Grades I–IV. Until a short time ago, microscopic techniques were unable to disclose parasites in the affected tissues, leading many authors to invoke autoimmune mechanisms as responsible for the cellular lesions. More recently, new, highly sensitive techniques such as immunohistochemistry and protocols based on the polymerase chain reaction (PCR) have consistently shown the presence of the parasites in diseased organs and their absence from unaffected tissues. This, together with an increase in efficiency in detection of _Trypanosoma cruzi_ in blood by serial hemocultures and by PCR, has provided convincing evidence that presence of parasites in tissues is associated with the pathogenic process. In the present study, chagasic megaesophagus was evaluated by means of parasitologic, clinical, and histopathologic approaches. Specific PCR, followed by hybridization as well as peroxidase-anti-peroxidase (PAP) and hematoxylin and eosin staining, were used to detect _T. cruzi_ in esophageal tissues from those patients. The blood parasitism was also identified through PCR, hemoculture, and xenodiagnosis and correlated with tissue parasitism.

MATERIALS AND METHODS

Patients. Blood and tissue parasitism was evaluated in 52 chagasic patients with megaesophagus who had esophagomyotomy (Heller surgical procedure) with antireflux valve construction at the Hospital Escola da Faculdade de Medicina do Triângulo Mineiro, Uberaba, in Minas Gerais, Brazil. The chagasic etiology of the megaesophagus was confirmed by 3 serologic tests: indirect immunofluorescence, indirect hemagglutination, and enzyme-linked immunosorbent assay. Clinical classification of patients was performed according to the results of esophagogram, electrocardiogram, barium enema, or number of days of constipation. The medical ethics committee of the Faculdade de Medicina do Triângulo Mineiro approved the project, and all procedures were carried out with the informed consent of patients. Experiments were double-blind, and patients were numbered chronologically in order of study entrance.

Esophageal tissue collection. During the megaesophagus surgery, a longitudinal strip of esophageal muscle (3–5 cm long by 1 cm wide) was excised from the lower portion of the esophagus near the gastric junction. After washing with isotonic saline, the tissue was cut into fragments of ~1 cm3 and frozen at –70°C. Upon thawing, the samples were divided into 2 groups: one was used fresh for DNA extraction and the other was fixed in absolute ethanol for histopathologic techniques. As a control, 3 esophageal samples were used that had been taken from patients who underwent surgery for idiopathic megaesophagus who were negative by serology, xenodiagnosis, and hemoculture for Chagas disease.

Extraction of tissue DNA. For PCR, tissue samples were minced, subjected to alkaline lysis with 50 mM NaOH, heated in boiling water for 10 min, neutralized with 130 mM...
Tris-HCl (pH 7.0), and used directly in PCR reactions after 10-fold dilution in double-distilled water.\textsuperscript{27}

**Histopathology and immunohistochemistry.** After-fixing in absolute ethanol, tissue fragments were routinely processed for paraffin embedding. The slides were made via 2 sequential cuts, one of which was stained with hematoxylin and eosin to evaluate the inflammatory process, and the other was used for the immunohistochemical PAP anti-*T. cruzi* parasite or antigen detection tests.\textsuperscript{23} The slides used for PAP were boiled in a microwave oven at maximum potential (700 W) for 18 min. Anti-*T. cruzi* serum obtained from rabbit was used as a primary antibody (diluted 1:1,000; 4°C for 22 hr), followed by swine anti-rabbit immunoglobulin G (Dako, Carpinteria, CA; diluted 1:100, 25°C for 60 min) as secondary antibody. The reaction localization was made by the use of PAP complex (Dako; diluted 1:100 for 60 min) followed by 0.048% diaminobenzidine (DAB) (Sigma-Aldrich, St. Louis, MO) as chromogen. Slides were counterstained with hematoxylin.

**Evaluation of parasitemia by use of hemoculture and xenodiagnosis.** To detect circulating parasites in the blood of patients, xenodiagnosis was performed at the same time as the collection of blood for culture and PCR. In general, this was done 3 days before the esophageal tissue fragment was collected, 3 days after, or both. Hemoculture was performed according to the methods described in the literature.\textsuperscript{19,26} Immediately after collection, 30 mL of the blood was centrifuged at 4°C to remove the plasma. The packed cells were washed by centrifugation at 4°C in liver infusion tryptose medium. The concentrate erythrocytes were resuspended in 30 mL of liver infusion tryptose and uniformly distributed in 6 test tubes. Cultures were maintained at 28°C and homogenized weekly. Microscopic examination was done on the 30th, 60th, and 90th days after culture in 10 μL aliquots of suspension.

Each xenodiagnosis was performed with 40 third-stage nymphs of *Triatoma infestans* that were placed on patients’ forearms for 30 min of feeding. Insects were individually examined for *T. cruzi* in the feces by abdominal compression, and the content was examined by light microscopy after staining. In general, this was done 3 days before the esophageal tissue fragment was collected, 3 days after, or both. Hemoculture was performed according to the methods described in the literature.\textsuperscript{19,26} Immediately after collection, 30 mL of the blood was centrifuged at 4°C to remove the plasma. The packed cells were washed by centrifugation at 4°C in liver infusion tryptose medium. The concentrate erythrocytes were resuspended in 30 mL of liver infusion tryptose and uniformly distributed in 6 test tubes. Cultures were maintained at 28°C and homogenized weekly. Microscopic examination was done on the 30th, 60th, and 90th days after culture in 10 μL aliquots of suspension.

**Blood collection for DNA extraction and PCR.** Blood (10 mL) was collected from chagasic patients for PCR reactions. The samples were immediately mixed with an equal volume of 6 M guanidine hydrochloride and 0.2 M ethylenediaminetetraacetic acid (EDTA) solution, stored at room temperature for 1 week, then stored at 4°C for 22 hr, followed by swine anti-rabbit immunoglobulin G (Dako, Carpinteria, CA; diluted 1:100, 25°C for 60 min) as secondary antibody. The reaction localization was made by the use of PAP complex (Dako; diluted 1:100 for 60 min) followed by 0.048% diaminobenzidine (DAB) (Sigma-Aldrich, St. Louis, MO) as chromogen. Slides were counterstained with hematoxylin.

**Polymerase chain reaction.** The protocol used to detect *T. cruzi* in blood and tissue samples was the same as previously described\textsuperscript{23} and consisted of specific PCR amplification of the 330-bp fragment corresponding to the 4 variable regions of *T. cruzi* kinetoplast minicircle DNA, followed by slot-blot hybridization. The PCR was carried out in a final volume of 20 μL containing 2 μL of blood or tissue DNA solution; 10 mM Tris-HCl (pH 9.0); 75 mM KCl; 3.5 mM MgCl\textsubscript{2}; 0.1% Triton X-100; 0.2 mM of dNTPs (Sigma-Aldrich); 20 pmol of the primers 121 5′-AAATAATGTACGGG(T/G)GAGATGCATGA-3′ and 122 5′-GTTTCGATTGGGGTTGGTGTAATATA-3′ (Operon Technology, Alameda, CA); and 1.0 U of *Tag* DNA polymerase enzyme (Promega, Madison, WI). The reaction mixture was overlaid with 30 μL of mineral oil and subjected to 35 cycles of amplification in a MJ Research (Watertown, MA) Programmable Thermal Controller (PTC-150). The temperature profile as follows: 95°C for 1 min of denaturation with a longer initial time of 5 min at 95°C, 65°C for 1 min for annealing of primer, and 72°C for 1 min for extension, with final incubation at 72°C for 10 min to extend the amplified primers. The PCR products were visualized in 6% polyacrylamide gel electrophoresis and silver stained.

**Slot-blot hybridization.** This technique was performed to confirm the *T. cruzi* specificity of the ampiclons observed in the polyacrylamide gels. In the hybridization reaction, the S-67 5′-TTGTTTTTGGGGGCGTCAAATTT-3′ probe recognized to the sequences in the conserved region of the *T. cruzi* minicircle.\textsuperscript{22} detecting 0.1 fg of DNA.\textsuperscript{23} The PCR products were denatured with 0.1 M NaOH and applied to Biodyne B nylon membranes (Life Technologies, Gibco BRL, Gaithersburg, MD) with a slot-blot apparatus (Hoeffer Scientific Instruments, San Francisco, CA). The membrane was prehybridized for 1 hr at 55°C and hybridized for 30 min with alkaline phosphatase-labeled oligonucleotide probe S-67 that was synthesized by Lifecodes (Stanford, CT). Detection was performed via chemiluminescence with Luminex Plus substrate (Life Technologies) and X-ray film exposure as previously described.\textsuperscript{22}

**Statistical analysis.** For comparing the degree of megasophagus with the levels of *T. cruzi* in tissue and blood and with inflammatory processes we used Epi Info version 6 (CDC, Atlanta, GA) in tests of simple proportions and the chi-square trend test. Results were considered significant at *P* < 0.05.

**RESULTS**

**Clinical and epidemiological characteristics.** The clinical classification of the patients revealed that 23.1% (12 of 52) presented the digestive form exclusively: 17.3% (9 of 52) had only megaesophagus and 5.8% (3 of 52) had megasphagus with megacolon. The cardiac form was encountered in 76.9% (40 of 52) of patients, in whom 48.1% (25 of 52) was associated with megaesophagus and 28.8% (15 of 52) with megaesophagus and megacolon (Table 1). The megaesophagus varied from Grade II to IV, with 30.8% (16 of 52) of patients presenting with Grade III and 34.6% (18 of 52) with Grades II and IV. The average age of subjects was 55.9 ± 11.7 years; 69% (36 of 52) were men and 31% (16 of 52) were women.

**Tissue parasitism.** The PCR to detect *T. cruzi* DNA was performed randomly (i.e., without previously determining inflammatory foci) in the esophageal tissue fragments collected from the 52 patients. The *T. cruzi* k-DNA 330-bp product was detected in 69.2% (36 of 52) of esophageal
samples (Table 2), of which 25% (9 of 36) were confirmed only after hybridization (Figure 1). The PAP test was performed on tissue fragments adjacent to those used for PCR analysis and revealed the presence of the parasite, its antigens, or both in 34.6% of the tissue samples (18 of 52). When we compared the results of both tests, we found positive correlations in 26.9% (14 patients); however, PAP was positive in 4 patients who tested negative via PCR. The use of both techniques together resulted in the detection of T. cruzi in 76.9% (40 of 52) of the tissue samples from patients with megaesophagus.

### Comparison of the inflammatory process with the presence of parasite identified by immunoperoxidase and PCR

An inflammatory process was observed in 50% (26 of 52) of the megaesophagus tissues. In 80.8% (21 of 26), we found an association with the presence of T. cruzi, its antigens, or genomic fragments detected by PAP and PCR. When inflammation-free tissues were evaluated, T. cruzi was
were amplified and where slight positive hybridization was observed. Patients with chagasic or idiopathic megaesophagus analysed by polymerase chain reaction (PCR).

Lanes 1–4 and 7–10, 92.5% (37 of 40) had positive blood PCR, and parasitism because of 40 patients who had tissue parasitism, direct relationship between positive tissue and positive blood of the amplified products. It was not possible to show a of which 83% (39 of 47) were detected before hybridization and negative hybridization. Lane 5, The PCR product from an idiopathic megaesophagus not amplified in polyacrylamide gel corresponding to Trypanosoma cruzi kinetoplast (k-DNA) amplified by PCR from chagasic megaesophagus tissue samples and respective slot blots with strong positive hybridization.

When the different degrees of megaesophagus were compared with parasitism and inflammatory processes, we found that Grade IV patients had more frequent manifestations of both (Table 2), and T. cruzi was detected in 94.4% (17 of 18) of the tissues in which moderate to intense inflammation was present (Table 1). However, the chi-square trend test did not reveal an association between increasing grades of megaesophagus and positive tissue parasitism detected randomly in 76.9% of the megaesophagus tissues and inflammatory process; ME = megaesophagus; PAP = peroxidase–anti-peroxidase; PCR = polymerase chain reaction; Xeno = xenodiagnosis. Chi-square significance at $P < 0.05$. Chi-square test is the comparison of simple proportions among grades of megaesophagus.

**Table 2**

Comparison of the grade of megaesophagus with tissue and blood parasitism and inflammatory processes

<table>
<thead>
<tr>
<th>ME</th>
<th>Number</th>
<th>PCR</th>
<th>PAP</th>
<th>PCR + PAP</th>
<th>Inflamm</th>
<th>Hemo/Xeno</th>
<th>PCR</th>
</tr>
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<tr>
<td>ME II</td>
<td>18</td>
<td>12</td>
<td>04</td>
<td>14</td>
<td>09</td>
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<td>12</td>
<td>17</td>
<td>13</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>Chi-square</td>
<td>6.10</td>
<td>12.85</td>
<td>6.97</td>
<td>11.81</td>
<td>0.04</td>
<td>3.10</td>
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<td>$P$</td>
<td>0.047</td>
<td>0.001</td>
<td>0.03</td>
<td>0.002</td>
<td>0.978</td>
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</tr>
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<td>36</td>
<td>18</td>
<td>40</td>
<td>26</td>
<td>38</td>
<td>47</td>
</tr>
</tbody>
</table>

* Hemo = hemoculture; Inflamm = inflammatory process; ME = megaesophagus; PAP = peroxidase–anti-peroxidase; PCR = polymerase chain reaction; Xeno = xenodiagnosis. Statistical significance at $P < 0.05$. Chi-square test is the comparison of simple proportions among grades of megaesophagus.

**FIGURE 1.** Representative 6% polyacrylamide gel (A) and slot blot hybridization (B) from the same DNA esophageal tissue samples from patients with chagasic or idiopathic megaesophagus analysed by polymerase chain reaction (PCR). Lanes 1–4 and 7–10, The 330-bp fragment corresponding to Trypanosoma cruzi kinetoplast (k-DNA) amplified by PCR from chagasic megaesophagus tissue samples and respective slot blots with strong positive hybridization. Lane 5, The PCR product from an idiopathic megaesophagus not amplified in polyacrylamide gel and negative hybridization. Lanes 6, 11, and 12, The PCR from chagasic esophageal samples in which little or no T. cruzi k-DNA products were amplified and where slight positive hybridization was observed.

The guiding principles of clinical microbiology for > 100 years have been Koch’s postulates. According to these guidelines, to demonstrate an etiology, the suspected agent must be present in affected individuals. In this study of 52 patients with chagasic megaesophagus, the presence of T. cruzi was found in 51 of them (98%) by PCR or culture proof. These data provide definitive evidence of long-term persistence of T. cruzi in esophageal tissue and blood from patients with chagasic megaesophagus and point to a key role of the parasite in unleashing the pathological processes in host. The parasite, its antigens, or its genomic fragments were detected randomly in 76.9% of the megaesophagus tissues and were associated with inflammatory processes in 80.8% of these. The patients also presented increased levels of parasitemia during the tissue collection period: T. cruzi was detected in 90.4% by PCR and in 73.1% by xenodiagnosis, hemoculture, or both, which could be interpreted as reflecting accentuated tissue or blood parasitism.

The use of more sensitive techniques and the focused search for the parasite in selected areas with inflammatory lesions revealed a relationship between persistence of T. cru-
and disease severity in certain organs, both for cardiopathy and for megaesophagus. This association was seen in esophageal tissue in a small study in which PCR identified T. cruzi in 4 patients with megaesophagus but not in 3 who had cardiopathy. In endomyocardial biopsies of chagasic patients and in hearts from autopsies of people with severe chagasic cardiopathy, the parasite was detected by PCR in 91.3% and 94.7%, respectively, of the tissue samples adjacent to the inflammatory foci. The PAP test also revealed parasite antigens associated with inflammatory foci in cardiopathy cases; however, no correlation was established between the quantity and intensity of the inflammatory process in tissue; severe cases had small amounts of antigen.

Despite its lower sensitivity (34.6%) compared with PCR (69.2%), the PAP test detected parasite antigens in 4 tissue samples that were negative by PCR. This discrepancy regarding PCR sensitivity may be explained by the parasite’s focal distribution in the chronic phase of infection, which leads to parasites being in only one specific tissue region. An exhaustive search of T. cruzi by PAP was performed in 8 chagasic patients with megaesophagi and in 8 without; the parasite was only found in 50% of those with megaesophagus.

Some patients appeared to present elevated tissue parasitism because 75% of the positive cases had amplified more than 10 fg of DNA, which was detected by PCR alone. With hybridization it was possible to identify cases of DNA concentrations of up to 0.1 fg, suggesting lower tissue parasitism. Although several patients had blood and tissue DNA samples with differences in the intensity of the amplified PCR product, there was no evidence that this could account for the number of parasites present. Positive results and variability of T. cruzi DNA concentrations in tissues may be related to the presence of the parasite genomic fragments inside host cells and inflammatory infiltrate cells. But the k-DNA amplified from infected tissues is probably from recently liberated or dead parasites because inoculation of T. cruzi k-DNA in tissue of normal mice disappears after 48 hr.

Inflammatory lesions have been reported in 57% of esophageal-gastric junction biopsies in cases of megaesophagus and can vary from case to case and within the same patient, depending on the point where the sample section is made. This finding agrees with our results in that 50% of the patients with megaesophagus presented with inflammatory alterations.

A correlation between the frequency and intensity of the inflammatory process in tissues and the presence of T. cruzi could be observed, especially in cases of advanced megaesophagus. However, the detection of T. cruzi not associated with inflammation may be explained by the presence of intact nests of parasites. Such parasite nests were indicative of tissue invasion so recent that the local inflammatory process has not yet developed, or then are related to some degree of host immunosuppression, which has been shown in severe gastrointestinal forms and suggested in cardiac forms of Chagas disease. The cellular immune response is an important factor in the control of T. cruzi in all stages of the disease, and an immune imbalance could result in an increase in tissue and blood parasitism, a fact that is consistent with observations in the present study among chagasic patients with megaesophagus.

The sensitivity of the parasitologic tests, xenodiagnosis, and hemoculture techniques could be related to the genetic characteristics of the T. cruzi strains, which vary according to geographic region. This same situation seems to occur with PCR performed in blood, where positive results in different chagasic populations and techniques fluctuate 45–100%. The simultaneous application of 2 parasitologic tests increases diagnostic sensitivity, but PCR is more sensitive in detecting circulating parasites. Our data indicate 74.5% agreement between positive PCR in blood and the presence of circulating parasites detected by indirect parasitologic tests, which is similar to findings in published data. However, it was not possible to establish a direct relationship between the presence of the parasite in analyzed tissues and the patients’ parasitemia.

As has previously suggested for the cardiac form, the parasite is probably playing an important role in the pathogenesis of esophageal lesions, particularly because of its continuous presence throughout the chronic phase. Trypanosoma cruzi, whether or not associated with lesions, is perhaps most responsible for the development of histopathologic changes because of on-going immune response activation. This can be demonstrated by the elevated percentage of T lymphocytes (CD4+ and CD8+) expressing Class II molecules of major histocompatibility complex (MHC) human leukocyte antigen D-related (HLA-DR) in the peripheral blood of patients with the gastrointestinal form of Chagas’ disease.

Acknowledgments: We thank Dr. Lúcia Maria da Cunha Galvão for help in PCR hybridization; Orlando Carlos Magno and Afonso da Costa Viana from the Departamento de Parasitologia, Universidade Federal de Minas Gerais; and Aparecida Correa, Helena Moraes, and Isabel Moraes from the Departamento de Ciências Biológicas, Faculdade de Medicina do Triângulo Mineiro (FMTM), for technical assistance.

Financial support: This study was supported by grants from the Financiadora de Estudos y Proyectos 41/96/0894/00 (FINEP/PRONEX); Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ); Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq); Fundação de Ensino e Pesquisa de Uberaba da Faculdade de Medicina do Triângulo Mineiro (FUNEP/FMTM); UNDP/World Bank/WHO—Special Programme for Research and Training in Tropical Disease (TDR); and a fellowship from the Coordenação de Aperfeiçoamento do Pessoal de Ensino Superior-Piano Institucional de Capacitação Docente (CAPES/PICD).

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