Parasite Persistence Correlates with Disease Severity and Localization in Chronic Chagas’ Disease

L. Zhang* and R. L. Tarleton

The protozoan parasite Trypanosoma cruzi infects up to 20 million people in Latin America, and the resulting disease (Chagas’ disease) is a leading cause of heart disease and death in young adults in areas endemic for the parasite. The clinical symptoms of Chagas’ disease have been attributed to autoimmune reactivity to antigens shared by the parasite and host muscle or neuronal tissue. In the present study, in situ polymerase chain reaction analysis was used in murine models of Chagas’ disease to demonstrate an absolute correlation between the persistence of parasites and the presence of disease in muscle tissue. Clearance of parasites from tissues, presumably by immunologic mechanisms, correlated with the abatement of inflammatory responses and the resolution of disease. These data provide strong evidence for parasite persistence as a primary cause of Chagas’ disease and argue for efforts to eliminate T. cruzi from the host as a means for prevention and treatment of Chagas’ disease.

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before storage at −80°C. PCR was performed in an in situ PCR system (GeneAmp 1000; Perkin-Elmer, Foster City, CA) according to the manufacturer’s instructions. Primers specific for a conserved region of kDNA (forward primer, 5'-GGTTTCGATTGGGGTTGGTGAATATA-3'; reverse biotinylated primer, 5'-biotin- CCAAAATTGTAGCCTCCCTCCAAAAA-3') of T. cruzi were used for the amplification reactions, at 0.1 µM in PCR buffer (10 mM Tris-HCl/50 mM KCl, pH 8.3; Boehringer Mannheim, Indianapols) containing 1.5 mM Mg²⁺, 0.5 mM dNTP, and 25 U/mL Taq polymerase. The cycling conditions were 1 cycle of 95°C for 5 min, 60°C for 2 min, and 72°C for 2 min and 29 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 1 min. After completion of the PCR reaction, slides were washed 4 times in PBS for 20 min each, 3 times in PBS containing 0.05% Tween 20 for 10 min each, and 4 times in PBS for 15 min each, and then were incubated in avidin-peroxidase (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Sections were washed in PBS–Tween 20 and in PBS alone as above before detection with the peroxidase substrate 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis) and counter-staining with hematoxylin.

Heart transplantation and kDNA injection. T. cruzi kDNA was isolated by the protocol of Simpson and Berliner [17]. Transplantation of neonatal syngeneic hearts to adult mice was done as described elsewhere [13]. In brief, normal C57Bl/6J mice were anesthetized with rompun (3.5 mg/kg) and ketamine (35 mg/kg), and a small pocket was prepared on the dorsum of one ear. Syngeneic neonatal hearts of mice <5 days of age were removed and immediately inserted into the pocket. The transplanted hearts displayed mechanical (beating) and electrocardiographic activity 10–14 days after transplantation and at that point were injected with kDNA.

Results

To address the question of parasite persistence and its association with disease development, 2 murine models of Chagas’ disease were used. These vary with respect to the site and severity of tissue damage. Inflammatory disease is apparent in the heart and skeletal muscle in both models but is more severe in the heart of C3H/HeSnJ mice infected with the Sylvio X10/4 clone [15] and in the skeletal muscle of C57Bl/6J (B6) mice infected with the Brazil strain [16].

To monitor parasite persistence, we established in situ PCR protocols that allowed us to detect with very high sensitivity intact parasites or residual parasite kDNA. In situ PCR analysis of tissues from T. cruzi–infected mice revealed 2 patterns of amplification of kDNA. Infected host cells (pseudocysts) detected early in the acute phase of the infection showed an intense brown staining that localized specifically around intracellular parasites (figure 1A). The PCR amplification products were highly specific for T. cruzi–infected tissues, since no amplification products were detected in uninfected host cells or in tissues from uninfected mice (figure 1B). Tissues from chronically infected mice (>120 days after infection) were characterized by a diffuse inflammatory reaction in the absence of obvious intact pseudocysts [15, 16]. Amplification of T. cruzi kDNA by in situ PCR in these latter tissues resulted in a similarly diffuse pattern of amplification products that colocalized with the inflammatory infiltrates (figure 1C). To confirm that this amplification reaction also was specific for T. cruzi and not nonspecifically associated with sites of inflammation, complete Freund’s adjuvant was injected into muscle tissue to induce an inflammatory response. Although substantial inflammation was induced by this treatment, no amplification products were observed after in situ PCR analysis using kDNA primers (figure 1D). Control experiments in which Taq polymerase or at least one of the primers was omitted from the amplification reaction or in which primers specific for targets not present in T. cruzi were included in the reaction failed to yield any detectable amplification product in tissues from normal or acutely or chronically infected mice (data not shown). These results confirm that the in situ PCR technique specifically detects the presence of T. cruzi kDNA in tissue sections.

The PCR analysis of various tissues from infected mice confirms previous reports [15] of the difficulty in detection of tissue parasites during the very early stages of infection (3–7 days) when parasite burden presumably is very low (table 1). However, by postinfection day 14, kDNA and associated inflammation was detected in the heart, skeletal muscle, liver, kidney, and brain in both the C3H/Sylvio model and the B6/Brazil model, again consistent with previous reports of a wide tissue distribution of T. cruzi in acutely infected animals [15, 18]. In both models, the heart and skeletal muscle exhibited the strongest inflammation, which was associated with intense staining for kDNA, whereas only moderate inflammation and kDNA were found in the liver, kidney, brain, and spleen. In all cases, the intensity of kDNA staining was proportional to the degree of inflammation, and there was a close association between the presence of kDNA and inflammatory cell clusters.

In contrast to the wide tissue distribution of kDNA observed in mice during the acute stage of infection, detection of kDNA in chronically infected mice was much more restricted, with only the skeletal muscle and/or heart exhibiting a diffuse distribution of PCR products (table 1). In the B6/Brazil model, kDNA was detected in the heart (figure 2A) and skeletal muscle (figure 2B) at postinfection days 130–244 (table 1). However, in more chronically infected B6 mice (postinfection days 620–797), kDNA was detected only in skeletal muscle (figure 2D) but not in the heart (figure 2C). In contrast, C3H mice infected for >450 days with the Sylvio X10/4 clone exhibited evidence of kDNA only in the heart (figure 2E) but not in skeletal muscle (figure 2F).

In all tissues examined, the presence of kDNA was closely associated with the presence of inflammatory cells; in the absence of detectable kDNA, no inflammatory infiltrates were observed in either model at any point during infection (table 1, figure 2). In order to rule out the possibility that kDNA was inducing inflammation or that kDNA persists in tissues for long periods in the absence of parasites, 10⁶ parasite equivalents of T. cruzi kDNA was injected into heterotopic transplanted hearts
Figure 1. In situ detection of *Trypanosoma cruzi* kinetoplast DNA (kDNA) in tissues from B6 mice infected with *T. cruzi* Brazil strain. **A,** Heart tissue 26 days after infection. Note restriction of brown peroxidase product identifying kDNA polymerase chain reaction (PCR) product primarily to pseudocyst (*T. cruzi*-infected host cell containing multiple amastigotes). **B,** Normal heart tissue subjected to in situ PCR exhibiting no PCR products. **C,** Skeletal muscle tissue obtained 244 days after infection. Note colocalization of PCR products (brown precipitate) and inflammatory cells; pseudocysts are not readily apparent. **D,** Presence of inflammation but not kDNA PCR products in transplanted heart tissue from noninfected mouse injected 1 week earlier with 10 μL of Freund’s complete adjuvant (Sigma, St. Louis). (Original magnification ×200.)
in normal or chronically infected mice or into skeletal muscle of normal mice. No inflammation and only weak PCR amplification of kDNA was detectable in skeletal muscle and transplanted heart tissues 2 days after injection of kDNA (data not shown). At subsequent time points (postinjection days 3, 4, 5, 7, and 10), neither inflammation nor kDNA was observed in these tissues. Therefore, the kDNA amplified in the tissues of infected mice is likely derived from recently released or dead parasites and not from the long-term persistence of kDNA.

**Discussion**

Conflicting and controversial hypotheses have been proposed to explain the pathogenic mechanisms involved in the slow but progressive cardiomyopathy characteristic of Chagas’ disease. These hypotheses include, among others, the destruction of myocardial cells by parasitosis [19], damage of parasympathetic ganglia in target organs [20], microvascular abnormalities [21], and autoimmunity [2, 3, 22, 23]. The failure to detect substantial numbers of parasites in chagasic lesions and the local inflammatory response to apparently nonparasitized myocardial cells have been offered as support for an autoimmune etiology for Chagas’ disease [19]. Documentation of anti–self-immune responses and the existence of cross-reactive antigens between host heart and *T. cruzi* have further fueled the autoimmune hypothesis [22–26].

We [13] and others [27] previously used a syngeneic heart transplant model to attempt to assess the contribution of autoimmune responses to disease development in chronic *T. cruzi* infections. Although initial results with this model system suggested that chronically infected mice rejected neonatal syngeneic heart transplants in an autoimmune fashion [27], subsequent analysis of the system strongly implicated infection of the transplant by *T. cruzi* as the initiating and required factor in “rejection” [13]. In order to further characterize the role of local parasitic infection in disease development during *T. cruzi* infection, we used in situ localization of *T. cruzi* kDNA to define the dynamic relationship between the presence of parasites and disease development during the acute and chronic stages of the infection in mice. In 2 different models of chronic *T. cruzi* infection, parasite persistence and disease severity showed an absolute correlation. Furthermore, immune clearance of parasites from particular tissues resulted in the disappearance of inflammatory lesions and the resolution of disease in those tissues.

Previous studies using conventional histologic methods failed to show a correlation between parasite burden and disease progression and severity (reviewed in [1]). However, in general, those analyses focused on detection of pseudocysts containing relatively large numbers of intact parasites and thus would likely have missed host cells containing few parasites or parasite-infected cells destroyed by immune mechanisms. In contrast, the in situ PCR method used in this study is highly sensitive for detection of parasite DNA and can detect intact parasites and parasite kDNA that might be released after parasite destruction at the sites of inflammatory responses. With this technique, we obtained the strongest evidence to date linking the persistence of parasites and the presence of severe disease in specific host tissues in chronic *T. cruzi* infections.

The distinct patterns of PCR amplification products observed in tissues from mice with acute and chronic infections with *T. cruzi* are consistent with previously documented distribution patterns and tissue parasite loads at these points in infection [15, 28, 29]. Acutely infected mice exhibit well-defined pseudocysts containing intact parasites that are visible by both standard histologic techniques and in situ PCR. However, in concert with the development of an effective immune response, the level of parasites in the blood drops significantly and the presence of detectable pseudocysts likewise decreases. Mice that lack the ability to develop effective immune responses, such as the CD8+ T cells that can recognize parasite-infected host cells [30], exhibit extremely high numbers of pseudocysts and early death [31, 32]. Together, these observations suggest that the developing immune response provides the means for recognition and destruction of parasite-infected cells before they are fully formed pseudocysts and account for why pseudocysts are not prevalent in the chronic stage of the infection.

Nevertheless, despite the absence of clearly defined pseudocysts in inflamed tissues of chronically infected hosts, these

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>No.</th>
<th>Days after infection</th>
<th>Parasite strain</th>
<th>Heart</th>
<th>Skeletal muscle</th>
<th>Liver</th>
<th>Spleen</th>
<th>Brain</th>
<th>Kidney</th>
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<tr>
<td>C57B/6J</td>
<td>3</td>
<td>12–25</td>
<td>Brazil</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>3</td>
<td>18–26</td>
<td></td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td></td>
<td>3</td>
<td>130–244</td>
<td></td>
<td>+</td>
<td>+</td>
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<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>6</td>
<td>620–797</td>
<td></td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C3H/HeSnJ</td>
<td>2</td>
<td>3–7</td>
<td>Sylvo X10/4</td>
<td>–</td>
<td>–</td>
<td>ND</td>
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<td>+</td>
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<tr>
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<td>6</td>
<td>425–455</td>
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</table>

**NOTE.** Groups of mice infected for indicated periods were used as sources of tissues for in situ polymerase chain reaction (PCR) detection of parasite kDNA and for assessment of inflammatory response. + or – indicates presence or absence, respectively, of both *T. cruzi* kDNA and inflammatory cells in all tissue sections in all animals in respective group. At least 8 tissue sections/organ/animal were examined. As documented in figures 1 and 2, amount of kDNA PCR product and intensity of inflammation varies, in different strain combinations and in different tissues, and data in this table do not attempt to reflect this quantitative difference. ND, not determined.
Figure 2. Colocalization of *Trypanosoma cruzi* kinetoplast DNA (kDNA) and inflammatory infiltrates in heart (A, C, E) and skeletal muscle (B, D, F) of mice in chronic stage of infection. B6 mice infected for 130 days with *T. cruzi* Brazil strain exhibit kDNA (brown peroxidase product) and inflammatory cells in heart (A) and, at higher levels, in skeletal muscle (B). By 600 days after infection, parasite kDNA and inflammation is apparent only in skeletal muscle (D) and essentially is absent from heart (C). C3H/HeSnJ mice infected with Sylvio X10/4 clone of *T. cruzi* have opposite pattern with colocalization of parasite kDNA products and inflammatory cells in heart (E) 450 days after infection but not in skeletal muscle (F). (Original magnification ×200.)
tissues are not parasite free. Parasites or parasite products are detectable by a variety of sensitive techniques [4, 8–10, 12, 33], tissue parasite loads increase significantly on suppression of the immune system [34–39], and parasites can be relatively easily cultured from tissues of chronically infected hosts [40]. Instead of intact pseudocysts, in situ PCR analysis reveals a diffuse pattern of staining that may represent individual parasites or, more likely, the kDNA released by the destruction of parasites prematurely released from infected host cells. The specificity of the reaction products for \( T. cruzi \) kDNA is supported by the fact that such products are not present in inflammatory sites induced by agents other than \( T. cruzi \) (figure 1D). In addition, the diffuse nature of the amplification products in chronically infected tissue is unlikely to be due to technical issues such as the “smearing” of kDNA during tissue sectioning; such smearing clearly is not detected in sections containing pseudocysts, despite the high level of kDNA in such tissues (figure 1A). Since each parasite contains 40,000 copies of the template for the kDNA minicircle primers used in the in situ PCR reaction, kDNA released from only one or a few parasites might be sufficient to account for the amplification pattern observed in inflammatory sites. Thus, the pattern of amplification observed in tissues of chronically infected hosts is the likely product of parasite persistence and the developing, and at least partially, immune response.

Our data are consistent with an emerging body of work linking parasites or parasite products with the severity of disease in chronic \( T. cruzi \) infections [4–10, 41, 42]. Although many of these reports, especially those of human cases of Chagas’ disease, admittedly are anecdotal, as a whole they present a compelling case for localized infections with \( T. cruzi \) in the initiation of the disease process and for the persistence of parasites in the maintenance of disease-causing immune responses characteristic of chronic Chagas’ disease. Although it is not possible to completely rule out autoimmune responses in the Chagas’ disease process, it is clear that autoimmunity does not need to be invoked to account for disease development. The ability of syngeneic heart transplants to survive in chronically infected mice for more than a year without any signs of disease, despite the presence of active parasitization and inflammatory disease in the native heart [13], is further evidence for mechanisms other than autoimmunity in the development of chagasic cardiomyopathy. Thus, even if anti–self-immune responses are induced in these models of Chagas’ disease, these responses are insufficient to generate disease in the absence of local parasitic infection.

A second interesting revelation from this study relates to the tissue restriction of the persistent infection. It is well documented that \( T. cruzi \) infects a wide range of tissues but is restricted primarily to skeletal, heart, and gut muscle rather early in the infection [15]. In mouse models, we found that the distribution of \( T. cruzi \) can be further restricted much later in the infection because of selective clearance from certain tissues—namely, the heart in the B6/Brazil model and skeletal muscle in the C3H/Sylvio model. The mechanisms responsible for this selective clearance are not known but may include a variety of immune effectors and other host and parasite factors. The observation of parasite clearance from selected tissues also provides two additional insights into \( T. cruzi \) infection. First, it appears that systemic immune responses during chronic \( T. cruzi \) infections are sufficient to limit parasites to selected tissues, a conclusion that is supported by the extremely low parasitemia level in chronically infected hosts and by absence of infection of syngeneic tissue transplants in these animals [13]. Second, these results indicate that clearance of parasites can be achieved, although not in all tissues. Nevertheless, understanding how parasite clearance is accomplished in certain tissues may identify the factors and conditions required for obtaining clearance in all tissues. These observations suggest that prevention or therapeutic treatment of Chagas’ disease might be achieved with immuno- or chemotherapies that enhance parasite clearance and reduce parasite load and thus inhibit the progression of chronic disease.

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