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Detection and Significance of *Trypanosoma cruzi* Persistence in Inflamed Gingival Foci in Chagas Disease

Néstor Añez[1, *], Gladys Crisante[1], Sonia Araujo[2], Marinest Añez[3], Agustina Rojas[1], Henry Parada[4]

[1]Center for Parasitological Research "J.F.Torrealba", Faculty of Sciences, University of Los Andes, Merida, 5101, Venezuela
[2]Laboratory of Experimental Parasitology, Department of Biology, Faculty of Sciences, University of Los Andes, Merida, 5101, Venezuela
[3]Faculty of Dentistry, University of Los Andes, Merida, Venezuela
[4]General Hospital "Luis Razetti" Cardiological Unit, Barinas, Venezuela

Email address
nanes@ula.ve (N. Añez)

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Abstract
Sixty unquestionable chronic chagasic patients (CCP) bearing different degrees of gingival inflammation were selected for the study. The periodontal inflammatory processes, as including criterion, were clinically diagnosed as mild, moderate and severe in 56%, 37% and 7% of the selected individuals, respectively. Gum biopsies were taken from the included patients and processed combining immunohistopathological and molecular (PCR) methods in order to assess the persistence of *Trypanosoma cruzi*-tissue forms in long-term CCP. In 24 of the 60 CCP (40%) *T. cruzi* persistence was confirmed. This included the presence of the parasite itself detected in 18.3% of the samples processed by Giemsa stain, and the observation of *T. cruzi* antigenic deposits in 28.3% and 23.3% samples when peroxidase-anti-peroxidase technique and immunofluorescence test, were used respectively. In addition, the PCR-based method revealed persistence of a portion of the *T. cruzi* genome in 20% of the study samples. Interestingly, 87.5% of the CCP had been treated with Benznidazole when they suffered the acute phase of the disease, which suggests an unsatisfactory effect of the drug to eliminate *T. cruzi*-tissue forms. The biological significance of *T. cruzi* persistence in the human host is discussed and, its role on the host-parasite relationships is considered.

1. Introduction

Chagas disease is a systemic infection caused by *Trypanosoma cruzi,* a Kinetoplastida protozoon of the family Trypanosomatidae, afflicting nearly 10 million people and making more than 90 million individuals living in rural areas of 21 countries in Latin America (LA) under risk. These figures define Chagas disease as the major health problem and the largest parasitic disease burden of the American continent, which has been estimated to be more than 7 billion dollars per year (1,2). As a consequence of constant migration of infected people from LA endemic regions, Chagas disease has spread to non-endemic countries generating potential risks in USA, Western Europe, Oceania and Asia (3,4,5).

The parasite is naturally transmitted to humans by hematophagous insects, namely the Reduviidae triatomine bug, after depositing infected feces during blood feeding. This vectorial transmission is the most recognized method of infection in rural areas where Chagas disease is endemic. However, *T.cruzi* is also transmitted orally by ingestion of contaminated food (6,7,8), by transfusion of blood from infected donors (9) congenitally/transplacentary from infected mother to the offspring (5,10), by transplant of infected/contaminated organs (11), and by contamination while manipulating contaminated material or patients with Chagas disease infection (12).

Clinically, Chagas disease presents with a primary acute phase, with blood circulating parasites, characterized by variable signs and symptoms ranging from mild to severe; although asymptomatic cases are not rare (13). This initial phase is followed by a chronic one, after acute symptoms subside by the effect of chemotherapy against the circulating parasites or due to the host natural immune response (14,15). In any case, the drug and/or the host immune response are able to keep the disease in check, limiting parasitic proliferation. However, they are unable to eliminate the entire population, allowing the tissue form of the parasite to persist for longer periods during the chronic phase (15,16).The persistence of *T.cruzi* tissue form in chronic chagasic patients (CCP) has been demonstrated by detecting the parasite itself or part of its genome following endomyocardial biopsies, processed by immunohistochemical and PCR-based techniques (15). This finding in treated and/or untreated long-term CCP has been correlated with the presence of myocarditis and its progression, incriminating it as being responsible for clinical relapse when immunosuppression occurs (15,17). Nevertheless, considering the paninfective behavior of *T.cruzi*, a parasite capable of widely invade any organ of the mammal host, the persistence of the parasite in tissue other than the heart must be a concern. Interestingly, in a recent report *T.cruzi* persistence was confirmed in biopsies taken from CCP´s gingival inflammatory foci as revealed by PCR (18).This finding and the fact that recently it has been recognized that oral infection may affect the course and pathogenesis of several systemic diseases including cardiovascular affection, among others (19), stimulate us to assess the relationship between both conditions in CCP. Considering the above, in the present work we investigated the role of oral inflammatory foci in the persistence of *T.cruzi*-tissue form in long-term CCP following observations from combined immunohistochemical and PCR evidence. The current study has several clinical implications. On one hand, from a scientific viewpoint, they may add significance to the persistence of *T.cruzi* tissue form and help elucidate the currently controversial pathogenesis in CCP and on the other they may also make dentists aware of the risks they are exposed to, when treating gingival inflammation in patients from Chagas disease endemic areas.

2. Patients and Methods

2.1. Patients

Sixty unquestionable chronic chagasic patients (CCP) were evaluated during the present study. They were previously diagnosed clinically, parasitological, and serologically during the acute phase of Chagas disease. All patients were regularly re-evaluated every 6-12 months at the cardiologic unit of the "Luis Razetti" General Hospital in Barinas, Venezuela, to monitor their progress and clinical condition with time. To confirm their condition as CCP, a 10 ml blood sample was taken and processed for serology using a direct agglutination test (DAT), an indirect immunofluorescence antibody test (IFAT), for both polyvalent and specific IgM/IgG subtypes, and an enzyme-linked immunosorbent assay (ELISA), as described previously (7,13,15,18,20). To select patients for the study group, a cohort of 72 people with Chagas disease were examined to determine whether they fulfilled the established inclusion criterion of having gingivitis. The condition of the patient's oral cavity according to the degree of gingival inflammation was established by a dentist. Most patients received treatment with benznidazole when suffering the acute phase of Chagas disease.

2.2. Sample Collection and Processing

Once the two inclusion criteria were confirmed for every patient, i.e. demonstration of Chagas disease chronic infection and evidence of gum inflammation, a small gum biopsy of $3mm^3$ was taken from them. The tissue was divided into two parts: one was immediately frozen to be processed for PCR assay and the other was preserved in 10% formaldehyde to be used for immunohistochemical observations. The PCR assay to detect the presence of specific *T.cruzi*-DNA in the inflamed gum was performed using primers S35 (5'-AAA TAA TGT ACG GGT GAG ATG CAT GA-3') and S36 (5'-GGG TTC GAT TGG GGT TGG TGT-3') following protocols previously established (21). The immunohistopathological evaluation of the gum sample was carried out by Giemsa staining (GS) of histological sections of 7 μm in thickness to look for tissue parasites (*T.cruzi* amastigote-forms) as well as inflammatory signs. Two other immunohistochemical techniques included an indirect immunofluorescence test (IIFT) and peroxidase anti-peroxidase (PAP) assays to specifically identify *T.cruzi* antigenic deposits in the study tissue. Details for the immunohistochemical techniques have been previously reported (15, 18).

2.3. Ethical Considerations

A written consent including the agreement prior information (API forms) from all patients who entered the protocol was obtained prior sample collection. This study was approved by the ethical committee of the "Luis Razetti" General Hospital, Ministry of Health, Barinas, Venezuela and

by the Research Council of Universidad de Los Andes, Merida, Venezuela, in order to comply with the criteria established by the Biomedical Committee of The National Research Council of Venezuela.

3. Results

The serological results obtained from each of the blood samples taken from individuals to detect the presence and level of specific anti-*T.cruzi* antibodies, confirmed that most of them were seropositive as they showed levels of IgG similar to those characteristic to CCPs. These results, and the clinical cardiologic evaluation previously performed by electrocardiography and echocardiography, assured us that patients fulfilled one of the inclusion criteria. The second inclusion criterion was confirmed after the clinical evaluation of the patient's gum. The observations revealed different degrees of inflammation in the 60 patients in the study, 34 (56%) of them had mildly inflamed gums, 22 (37%) had moderate inflammation, and 4 (7%) of the patients had severe periodontal inflammation (Table 1). The inflammatory process was confirmed histologically in sections of gum stained with Giemsa stain (Fig. 1). In addition, the use of immunohistopathological (GS, PAP, IIFT) and molecular (PCR) techniques, revealed the detection of *T.cruzi*-tissue forms persistent in inflamed gums of CCP. Indeed, the presence of *T.cruzi* itself was revealed in histological sections of inflamed gum stained with Giemsa stain in which disperse scanty amastigote forms were detected in gingival fiber of the processed tissue (Fig. 2). Similarly, when immunohistochemical PAP and IIFT methods were applied specific *T.cruzi* antigenic deposits were observed in 28.3% and 23.3% of the processed samples, respectively (Fig. 3). Moreover, the use of the PCR assay allowed the detection of a portion of *T.cruzi* genome in the gingival samples of CCPs. This was revealed by the amplification of a band of 330bp from the kDNA minicircle variable region of the parasite (Fig. 4). The frequency of *T.cruzi*-persistence at different degrees of gingival inflammatory processes revealed 16 (26.7%), 7 (11.6%) and 1 (1.7%) CCPs with mild, moderate and severe gum inflammation, which represents proportions of 1:0.4:0.06, respectively (Table 1). The analysis of the results obtained from 4 different tests used during the study revealed *T.cruzi* tissue persistence, irrespective of the degree of periodontal inflammatory process found in CCPs. However, immunohistochemical tests (PAP and IIFT) showed slightly higher positive results (70.8% and 58.3%) than PCR (50%) and Giemsa staining (45.8%).Regarding the concordance among tests, two samples from the 24 CCPs showing mild gum inflammation revealed positive results with all the 4 techniques, while coincidences among 3 and 2 tests were observed in 6 and 12 samples, respectively. Details of the obtained results for each of the positive samples are given in Table 2.

Table 1. *Detection of degree of periodontal inflammatory process and Trypanosoma cruzi-persistence in selected chronic chagasic patients*

Number (%) of CCP with gingival inflammation		Detection of *T.cruzi*-persistence in inflamed gingiva using immunohistochemical and molecular tests				
		GIEMSA STAIN	PAP	IIFT	PCR	TOTAL *T.cruzi*-persistence
MILD	34 (56)	7 (11.6)	9 (15)	9 (15)	8 (13.3)	16 (26.7)
MODERATE	22 (37)	3 (5)	7 (11.6)	5 (8.3)	3 (5.0)	7 (11.6)
SEVERE	4 (7)	1 (1.7)	1 (1.7)	0 (0.0)	1 (1.7)	1 (1.7)
TOTAL	60 (100)	11 (18.3)	17 (28.3)	14 (23.3)	12 (20.0)	24 (40.0)

Table 2. *Characteristic of chronic chagasic patients showing Trypanosoma cruzi persistence at periodontal inflammatory foci detected by various methods*

Number	Code	Age (year)	Gender	Serology IgG	Degree of inflammation	Detection of *T. cruzi*-persistence using immunohistochemical and molecular tests			
						Giemsa	PAP	IIFT	PCR
1	24-95	25	F	-	Mild	+	+	+	+
5	20-96-1	18	F	-	Mild	+	+	+	+
6	02-11	27	F	1:32	Mild	-	-	-	+
23	23-96	53	M	1:256	Mild	-	+	+	-
28	12-94	31	F	1:128	Mild	+	-	+	-
29	SC1	31	F	1:64	Mild	-	+	+	-
30	SC2	15	F	1:128	Mild	+	+	+	-
32	22-96	73	M	1:64	Mild	-	-	-	+
36	03-90	27	M	1:32	Mild	-	-	+	+
37*	SC3	42	M	1:64	Mild	-	+	-	+
45*	IM-00	23	F	1:64	Mild	-	+	-	+
52	SC4	30	M	1:256	Mild	+	-	+	-
56	SC5	28	F	1:128	Mild	+	+	-	-
57*	SC6	32	F	1:512	Mild	-	+	+	-
59	SC7	39	M	1:128	Mild	+	-	-	-
60	239610	39	M	1:128	Mild	-	-	-	+
17	06-04	27	M	1:256	Moderate	-	+	+	-
21	01-90	50	M	1:256	Moderate	-	+	+	-

Number	Code	Age (year)	Gender	Serology IgG	Degree of inflammation	Detection of *T. cruzi*-persistence using immunohistochemical and molecular tests			
						Giemsa	PAP	IIFT	PCR
35	SC8	39	F	1:64	Moderate	-	+	+	+
39	06-10	28	F	-	Moderate	+	+	-	+
42	11-07	57	F	1:128	Moderate	+	+	-	+
53	07-94	33	M	1:64	Moderate	-	+	+	-
58	26-96	37	F	1:512	Moderate	+	+	+	-
13	08-95	39	M	1:128	Severe	+	+	-	+
Total (%)						11 (45.8)	17 (70.8)	14 (58.3)	12 (50.0)

*Untreated patients

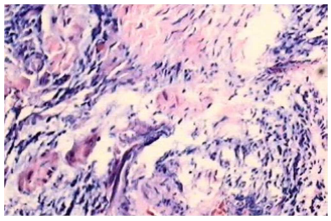


Figure 1. *Sectioned sample of the inflamed gum of a chronic chagasic patient (Giemsa stain. 200X)*

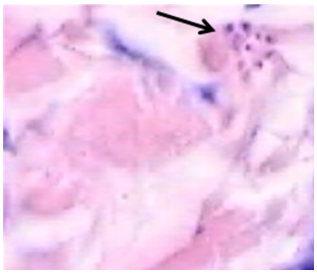
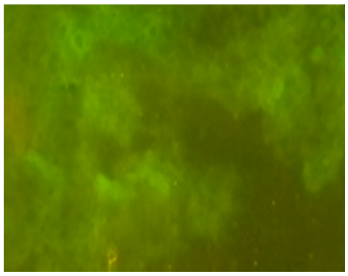


Figure 2. *Detection of persistence of T.cruzi-tissue forms in the inflamed gum of CCP. Arrow shows a scanty number of amastigote forms in gingival fiber. Giemsa stain 1000X*



A

B

Figure 3. *Trypanosoma cruzi antigenic deposits detected in the inflamed gum of a chronic chagasic patient detected: A. by indirect immunofluorescence test (IIFT) and B. by peroxidase anti-peroxidase test (PAP). Magnification 1000X*

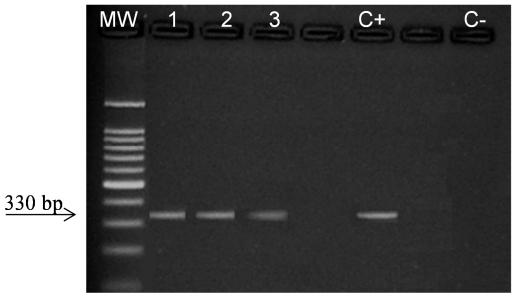


Figure 4. *PCR-assay in selected samples from the inflamed gum of chronic chagasic patients (Lanes 1-3). C+: Trypanosoma cruzi DNA as positive control. C-: Negative control. MW: Molecular weight ladder*

4. Discussion

It has been widely recognized that in Chagas disease the activation of a potent immune response and/or the etiological treatment are able to limit proliferation of *T. cruzi* in the human host. However, none of the two options are able to produce eradication of the infection, which consequently provokes inflammatory processes responsible for the development of symptomatic chronic forms of the disease in its more recognizable presentation: cardiomyopathy, and digestive mega-colon and mega-esophagus (14, 22). There is not yet a reliable biomarker to demonstrate conclusive cure of chronic Chagas disease, and more than 80% of treated patients in the acute phase may still harbor parasites according to classical diagnostic criteria (2, 23). This has been supported by molecular (PCR) and immunohistochemical techniques used to demonstrate the persistence of *T. cruzi* in endomyocardial biopsies in benznidazole-treated and untreated chagasic patients (15). In addition, the tissue persistence of *T. cruzi* in seropositive individuals diagnosed as CCPs has recently been demonstrated in biopsies from gingival inflammatory foci following PCR-based methods, suggesting the establishment and maintenance of the infection in organs other than the heart (18). This finding, together with the record of *T. cruzi* persistence in adipose tissue of CCPs may explain the role of these tissues as reservoirs of the parasite from which recrudescence of chagasic infection could occur during periods of immunosuppression (24).

In the present work we were able to confirm and complement the detection of persistence of *T. cruzi* tissue forms in samples of inflamed gums in 24 out of 60 (40%)

unquestionable CCPs combining immunohistopathological (GS, PAP, IIFT) and molecular (PCR) assays. The use of these methods made it possible to detect the way *T. cruzi* persists in the host tissue: i. as amastigote in histological sections stained with Giemsa stain in 45.8% (11/24) of the patients; ii. as specific *T. cruzi* antigenic deposits demonstrated by PAP and IIFT in 70.8% and 58.3% of the observed samples, respectively; and iii. as a portion of *T. cruzi* genome by PCR assay in 50% of the DNA samples from gingival inflammation of CCPs. These findings confirm that *T.cruzi* persistence is a phenomenon frequently occurring in tissues other than the heart, probably as a consequence of its biological behavior as a paninfective parasite able to invade any kind of cells including those from endodermic, mesodermic and ectodermic origins (25). Indeed, the fact that persistence of *T. cruzi* tissue forms were detected in 40% of the oral inflammatory foci in CCPs, suggest that this event seems to be more common in Chagas disease than previously believed. The present findings also suggest the need to investigate the detection of *T. cruzi* persistence in other inflamed areas such as the skin of chagasic patients, which is less invasive and more efficient method than the endomyocardial or gingival biopsies.

Another interesting aspect is the fact that from all 24 samples showing signs of parasite persistence only 12.5% belonged to untreated patients, while the remaining CCPs (87.5%) were treated with benznidazole during acute phase of Chagas disease. This demonstrates the unsatisfactory effect of benznidazole, and recommends the detection of *T. cruzi* in inflammatory periodontal foci for long-term follow up studies to evaluate the efficiency of anti-chagasic treatment in CCPs.

Parasitic persistence activates multiple immunopathogenic mechanisms, which are not completely understood (26). Our results suggest the necessity to consider etio-pathogenic roles of the persistent *T. cruzi* tissue forms for the development of immunopathologic mechanisms causing the chronic phase of Chagas disease, even those including its effects on the heart and digestive tract. Consequently, it is also necessary to design new therapeutic strategies capable of eliminating *T. cruzi* tissue forms.

The above analysis suggests that *T. cruzi* could be biologically provided to maintain a symbiotic relationship with the host to ensure a microhabitat for its own survival and long-term persistence. This behavior has significance from a biological viewpoint assuming that *T. cruzi* persistence is associated with chronic Chagas disease leading to parasite perpetuation in the host, supporting previous analysis (27) on the mechanisms of *T. cruzi* persistence.

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