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PERSISTENT INFECTIONS IN CHRONIC CHAGAS' DISEASE PATIENTS TREATED WITH ANTI-*Trypanosoma cruzi* NITRODERIVATIVES

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SUMMARY

We used a molecular method and demonstrated that treatment of the chronic human *Trypanosoma cruzi* infections with nitroderivatives did not lead to parasitological cure. Seventeen treated and 17 untreated chronic Chagas' disease patients, with at least two out of three positive serologic assays for the infection, and 17 control subjects formed the study groups. PCR assays with nested sets of *T. cruzi* DNA primers monitored the efficacy of treatment. The amplification products were hybridized to their complementary internal sequences. Untreated and treated Chagas' disease patients yielded PCR amplification products with *T. cruzi* nuclear DNA primers. Competitive PCR was conducted to determine the quantity of parasites in the blood and revealed < 1 to 75 *T. cruzi*/ml in untreated (means 25.83 ± 26.32) and < 1 to 36 *T. cruzi*/ml in treated (means 6.45 ± 9.28) Chagas' disease patients. The difference between the means was not statistically significant. These findings reveal a need for precise definition of the role of treatment of chronic Chagas' disease patients with nitrofuran and nitroimidazole compounds.

KEYWORDS: *Trypanosoma cruzi;* Chagas' disease; Treatment; Nifurtimox; Benznidazole.

INTRODUCTION

Recent publications have described notable advances in experimental chemotherapy of the *T. cruzi* infections, and results obtained with specific therapeutic regimes have been considered satisfactory[6,27,28]. The sterol and phospholipid synthesis inhibitors, which have been synthesized as anti-fungal drugs, and produced 70 to 90% cure of the experimental murine infections, did not prove to be effective in the treatment of human Chagas' disease[28]. In addition, synthesis of a novel chemical class of 60 nitrobenzofurans designed for advantageous activity against the murine *T. cruzi* infections yielded a single compound 1.6 times as effective than the reference drug nifurtimox[13]. However, several authors have shown the nitrobenzofurans and related compounds bear high potential for mutagenesis, carcinogenesis, teratogenesis and sterility[4,18,21-23,25].

Itraconazol used as an anti-fungal drug for 20 years, produced parasitologic cure in 53% of chronic Chagas' disease patients (6mg/kg/day, for 60 days) whereas allopurinol (8.5 mg/kg/day) yielded parasitologic cure in 44% of treated Chagas' disease patients[2]. The treatment of acute *T. cruzi*-infected patients with benznidazole resulted in conversion of serologic assays, which was considered equivalent to parasitological cure in 60% to 20% of treated cases[1,15]. However, the dichotomy between responders and non-responders of the specific drug therapy needs to be explained, since treatment did not appear to influence the clinical features of the acute infection[12,15]. Broad discrepancies in percentage of parasitological cure of chronic infections have been reported but they were explained by sectional observations, conducted on different occasions after treatment, in which the patients were not protected against the reduvid insect-vector and, therefore, the possibility of superinfection can not be ruled out[8-11,14-16,29].

In the absence of new drugs, nitroimidazol and nitrofuran are the compounds used to treat human Chagas' disease[8,9,29]. Here we present results of a study of chronic Chagas patients treated with benznidazole (N-benzyl-2-nitro-1-imidazolacetamide) or nifurtimox (4-[5-nitro-3-methyl-furfurylideneanimo]-tetrahydro-4-H-1,4-thiazine-1-1-dioxide). This study conducted in an insect-vector transmission-free urban area shows persistence of the *T. cruzi* infection in each Chagas patient, regardless of treatment.

MATERIALS AND METHODS

Study population

This study was conducted at the Medical Offices of the Brasília Refuse Department, and at the Chagas' Disease Multidisciplinary Research Laboratory, University of Brasília. The study protocol was approved by the Institutional Ethical Committee in Human Research. The street-cleaners enrolled in the programme gave written consent to participate in the study.

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Serologic assays

Each street-cleaner was subjected to immunological assays to determine specific antibodies for the *T. cruzi* infection. Patients showing at least two out of three positive tests (ELISA, indirect immunofluorescence [IF] and indirect hemagglutination [IH][26] were considered *T. cruzi* infected[30]. The results of the serological study in this series were validated by ELISA, IF and IH assays run in parallel with a panel of sera from 94 Chagas' disease patients with parasitological demonstration of *T. cruzi*[24].

Xenodiagnosis

This test was performed in each patient with positive immunological tests for the infection, essentially as described[22].

Study groups

The street-cleaners enrolled in this programme were subjected to immunological assays and 18.5% of them showed serological evidence of *T. cruzi* infections[26]. A cohort of these Chagas patients were treated with anti-trypanosomal nitrofuran or nitroimidazole (10 mg/kg/day, per os), because they showed severe electrocardiographic alterations. Among treated Chagas patients we selected 17 who had completed full treatment for 30 days or above. These treated patients were matched by gender and age with 17 untreated Chagas patients and with 17 control, non-Chagas individuals. As a result, three study groups were formed comprising 51 male street-cleaners: *i*) treated; *ii*) untreated; *iii*) control, with serologic tests negative for the *T. cruzi* infection.

Polimerase chain reaction (PCR) and hybridization

DNA samples extracted from 10 ml of peripheral blood from each street-cleaner were analysed by PCR with specific primers for the constant regions of minicircles of kinetoplast DNA (kDNA) and to highly repetitive sequences of nuclear DNA (nDNA) of *T. cruzi*[3, 17, 19]. The kDNA primer set (S35, 5'-ATAATGTACGGG(T/G)GAGATGC-3' and S36, 5'-GGTTCGATTGGGGTTGGTG-3') annealing to the constant region of minicircles yields a 330 bp product and its catamer of 660 bp[19]. The nDNA primer set (PON1, 5'-TGGCTTGGAGGAGTTATTGT-3' and PON2, 5'-AGGAGTGACGGTTGATCAGT-3') amplifies a 250 bp fragment[17]. A DNA thermal cycler (MJ Research, Watertown, MA) was used for 30-32 cycles, as described[7]. Negative controls (water and P388D1-IL1 DNA) and positive control (*T. cruzi* DNA) were always included to detect DNA contamination and ensure that the PCR worked efficiently. The PCR amplification products were transferred by capillarity to a nylon membrane. Prehybridization and hybridization were performed as described[7]. For S35/36 products, the sequence was S67 5'-GGTTTTGGGAGGGG(CG)-(G/C)-(T/G)TC-3'[19]. For PON1/2 products, the sequence was PON3 5'-CCGGCCTGTGTCTGCGGC-3'[17]. These oligonucleotides were radiolabelled with [(α 32P]-dATP (3000 CimMol) using the polynucleotide kinase method following the manufacturer's recommendation (Life Technologies).

Competition assays

These assays were conducted in blood samples of 17 untreated and of 17 treated Chagas patients who had received full treatment with nitroderivatives for 30 days and above. DNA samples from all these serologically positive patients that yielded positive PCR amplification products of the size expected with specific kDNA and nDNA primers were used for quantification of the parasitemia, as described[7].

Statistical analysis

The results are presented as means and standard deviation. The Student *t* test was used for group analysis.

RESULTS

Regardless of chemotherapeutic regime used, all Chagas' disease patients showed at least two out of three positive tests (ELISA, IF and IH) for the *T. cruzi* infections. Furthermore, the quantitative serological assays showed antibody titers in the treated Chagas group to be as high as those for the untreated Chagas patients (data not shown). No subject in the control group showed positive serology. Furthermore, we took advantage of results of xenodiagnosis showing parasitological demonstration of *T. cruzi* infections in chronic Chagas patients and used blood samples from these patients for covalidation of data obtained with serological and PCR assays. Results of these experiments are shown in Table 1.

Each Chagas patient yielded PCR amplification products with specific primers that annealed to *T. cruzi* nDNA (Figure 1). All these Chagas patients had shown PCR amplification product that formed a predictable size band in agarose gels, when specific kDNA primers were

Table 1
Covalidation of serological and PCR assays in blood samples of seven Chagas' disease patients with *Trypanosoma cruzi* demonstration by xenodiagnosis

Patient[a] Number	Seropositivity[b]	PCR[c]		
		Nuclear DNA	Kinetoplast DNA	Quantitative
Untreated Chagas patients				
142	Pos	Pos	Pos	50
159	Pos	Pos	Pos	0.3
162	Pos	Pos	Pos	0.5
282	Pos	Pos	Pos	50
335	Pos	Pos	Pos	50
Treated Chagas patients				
201	Pos	Pos	Pos	3×10^4
1250	Pos	Pos	Pos	5

[a] Eight Chagas patients had positive xenodiagnosis. One treated Chagas patient died before DNA tests were performed.
[b] Specific serum antibodies detected by immunofluorescence, hemagglutination and ELISA.
[c] PCR amplification products with kDNA and nDNA primers hybridized to specific internal sequences. The quantity of *T. cruzi* per ml of blood was determined by quantitative PCR, as described elsewhere[7]

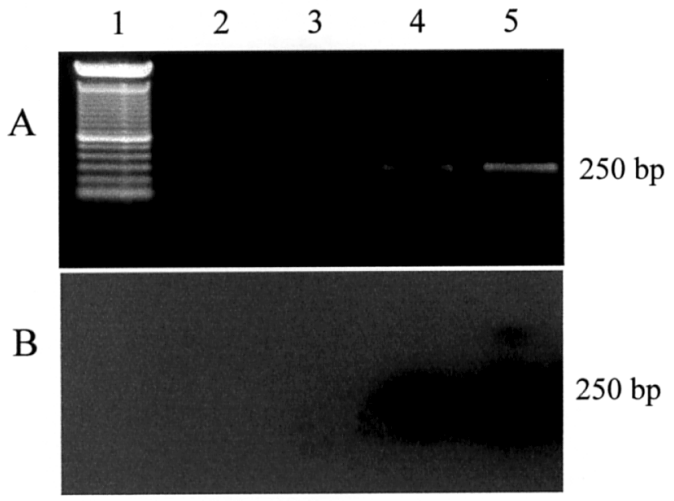

Fig. 1 - A: PCR amplification of sequences of nDNA from *Trypanosoma cruzi* with specific PON1/2 primers. Lane 1, 100 bp molecular weight marker; Lane 2, water (negative control); Lane 3, P388D1-IL1 DNA; Lane 4, Chagas' patient DNA; Lane 5, *T. cruzi* DNA. **B**: Southern hybridization with an internal fragment of DNA complementary to the PCR amplification product. Note the 250 bp bands in lanes 4 and 5 (see methods).

used. This product was hybridized with its complementary internal sequence. In contrast, none of the 17 control subjects, showing negative serological assays, yielded PCR amplification products with specific kDNA and nDNA primers.

The quantitative PCR was used to detect parasitemias in the blood of 34 patients with chronic Chagas' disease (Table 2). Among 17 Chagas patients treated with nitroderivatives, 41% received the drug for 30 days and 59% for 60 days. Citrated blood was collected from these patients 10 years after treatment. The competitive PCR assays showed *T. cruzi* kDNA bands at various points of equivalency with the competitor DNA bands, regardless of whether the template DNA was originated from treated or untreated Chagas patients. The competitive PCR quantification of *T. cruzi* in treated and untreated Chagas patients is shown in Table 2 and Figure 2. Seventeen untreated Chagas patients showed bands whose intensity was compared to that of DNA from a known quantity of *T. cruzi* showing equivalency in a range of < 1-to-75 parasites/ml of blood. The average means of *T. cruzi*/ml in this group was 25.83 ± 26.32. In addition, 16 treated Chagas patients showed bands whose equivalency ranged from < 1-to-36 parasites/ml of blood. The average means in this group was 6.45 ± 9.28. The statistical analysis demonstrated that the differences of means of parasitemias between treated and untreated Chagas patients are not significant. One treated Chagas patient (nº 201) showed exquisitely high number of parasites in the blood and was not considered for statistical analysis.

DISCUSSION

The prescription of nitroderivatives to treat the *T. cruzi* infections of street-cleaners with clinical and serological evidence of Chagas' disease was made by physicians at the Medical Offices of the Brasília Refuse Department, Federal District of Brazil. The Federal District is insect-

Table 2
Competitive PCR quantification of *Trypanosoma cruzi* in the blood of treated and untreated chronic Chagas' disease patients*

Treated Chagas patients		Untreated Chagas patients	
Patient number	*T. cruzi*/ml	Patient number	*T. cruzi*/ml
154	0.5	139	0.4
178	0.8	162	0.56
213	0.8	133	0.7
135	1.05	148	0.8
245	1.6	270	0.2
195	2.0	123	0.3
132	2.5	159	0.3
137	3.0	116	0.8
173	4.0	113	30.0
177	4.0	211	40.0
185	4.0	288	40.0
1250	5.0	157	40.0
707	8.0	142	50.0
136	10.0	335	50.0
175	20.0	282	50.0
524†	36.0	144	60.0
201†	3 x 10^{4}‡	140	75.0
X ± SD	6.45 ± 9.28	X ± SD	25.83 ± 26.32

* The assays were performed as described in the legend of Figure 2.
† These patients underwent two series of nitroderivatives, each for 60 days.
‡ Treated patient 201 with an absurbly high parasitemia was excluded from the group means.

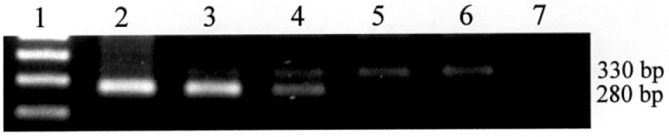

Fig. 2 - Quantitation of *T. cruzi* DNA in a treated Chagas patient by competitive PCR. Lane 1, molecular weight standard; Lanes 2-to-6, 1/50 aliquots of DNA extracted from a treated Chagas patient and various quantities of competitor DNA: Lane 2, 150 fg; Lane 3, 15 fg; Lane 4, 1.5 fg; Lane 5, 0.15 fg; Lane 6, 0 fg. Lane 7, water (negative control). These competitive PCR assay consisted of mixing an unknown amount of template DNA (*T. cruzi* minicircle DNA) with serial dilutions of a known quantity of competitor DNA (280 bp kDNA sequence obtained by cloning in the p7Blue vector). The competitor fragment that binds to the kDNA primers S35/36 yielded amplification product of a smaller size than that of the *T. cruzi* template DNA (280 vs 330 bp). The point of equivalency between the 330 and the 280 bp bands was given by tye tube showing equimolar concentration of template and of competitor DNAs in the 1.5% agarose gel. The quantity of *T. cruzi* was then calculated by the concentration of competitor DNA, assuming there are 10,000 minicircles/parasite and, therefore, 15 fg of 330 bp sequence/parasite[7]. This Figure illustrates the point of equivalency at 1.5 fg (Lane 4), which is approximately the amount of DNA in 0.1 parasite.

vector transmission-free of the *T. cruzi* infections and, therefore the possibility of superinfections was ruled out in this study.

Records in the files of all individuals in the study groups have shown that nitrofuran nifurtimox (10 mg/kg/day) was prescribed for 62% of Chagas patients. Nitroimidazole benznidazole (10 mg/kg/day) was prescribed to treat 38% of the remaining chagasic patients. In this series we report only those cases of chronic Chagas' disease undergoing full treatment for at least 30 days and showing PCR amplifications with specific kDNA and nDNA primers. In order to determine the efficacy of nitroderivative chemotherapy of the *T. cruzi* infections therefore, we used a quantitative PCR assay for detecting subclinical parasitemias in treated and untreated chronic Chagas patients. In this regard, previously we had run serological and quantitative PCR testings of blood samples from patients with positive xenodiagnosis, which showed consistently positive results. Essentially, the methods used were covalidated by these results.

Here we report that phenotypic and genotypic markers of the *T. cruzi* infections, which were used to evalutate the efficacy of nitroderivative therapy, did not shown significant differences in groups of treated and untreated Chagas' disease patients. In the first category, high titers of specific antibodies were observed in treated as well as in untreated Chagas patients. The lack of alteration of the serologic profile suggests that the specific chemotherapy did not affect the humoral immune responses to the parasitic antigens (data not shown). The discrepancy between these observations and those reported in the literature[1,9,11,12,15,29] might be explained by the fact that we used a highly sensitive molecular method to detect very low levels of parasitemias in treated and untreated chronic Chagas' disease patients. The persistence of *T. cruzi* shown in treated Chagas patients in this series may not be considered as resulting of reinfections, because our patients lived in an insect-vector transmission-free area.

Genotypic markers of *T. cruzi* infections were present in the treated as well as in the untreated chronic Chagas' disease patients in this series. We obtained PCR amplification products from Chagas' disease patients template DNA with nested sets of primers of nuclear DNA and kinetoplast DNA of *T. cruzi*, regardless of the patient belonging to the treated or the untreated group. In view of the observation that sequences of minicircles of *T. cruzi* may integrate in the genome of the host cell, we suggested that the nuclear DNA is the only genotypic marker that confers evidence of the living *T. cruzi* infection in treated and in untreated Chagas' disease patients[20]. In this regard, we used template DNA from both groups of Chagas' disease patients who had shown PCR amplification products with the primer set PON1/2, in order to quantify the parasitemia by competitive PCR[7,17].

The tests of competitive PCR were run with template DNA from 34 Chagas patients, treated and untreated, which have shown the 330 bp band amplified with the S35/36 kDNA primers. This criteria secured optimal amplification reactions and allowed comparison between the 280 bp band (amplification of the competitor DNA) and the 330 bp (amplification of the sequence of the minicircle kDNA of *T. cruzi*). The quantification of the minicircle DNA was determined at the tube of equivalency with known concentration of the competitor DNA. Considering that each parasite contains 15 fg of the 330 bp minicircle sequence, the quantity of parasite in each ml of blood was determined[7]. To our knowledge, this study shows, for the first time with a molecular method, that the treatment of chronic Chagas' disease patients with nitrofuran and nitroimidazole compounds do not lead to the parasitological cure of the *T. cruzi* infections.

The demonstration of persisting phenotypic and genotypic markers of the living *T. cruzi* infections in patients treated with anti-trypanosomal drugs indicates these drugs have limited efficacy. The results discussed here show that assessment of cure after treatment of acute and chronic Chagas' disease requires a highly sensitive molecular method for detecting subclinical parasitemias. Moreover, treatment of chronic Chagas' disease remains controversial[10], therefore requiring further research evaluation of benefits resulting from nitroderivative therapy[5]. In this regard, new drugs without undesirable side effects are needed, and that hopefuly promoting a true parasitological cure of the infection would halt the progression of the chronic disease.

RESUMO

Persistência das infecções em pacientes chagásicos crônicos tratados com nitroderivados anti-*Trypanosoma cruzi*

Usamos um método molecular e demonstramos que o tratamento de infecções crônicas pelo *Trypanosoma cruzi* com nitroderivados, que se mostraram eficientes em diminuir parasitemias nas infecções agudas, não produziu cura parasitológica. Trinta e quatro chagásicos crônicos, com pelo menos dois entre três testes sorológicos positivos para a infecção, e 17 controles com testes sorológicos negativos formaram os grupos de estudo. Os testes de PCR com primers de DNA de *T. cruzi* monitoraram a eficácia do tratamento. Foram obtidos produtos de PCR a partir de DNA de pacientes chagásicos tratados e não-tratados, com primers de DNA nuclear de *T. cruzi*. Os produtos amplificados hibridizaram com suas sequências internas e complementares. A técnica de PCR competitiva foi usada para quantificar o número de parasitos no sangue e revelou < 1 a 75 *T. cruzi*/ml em chagásicos não-tratados (média 25,83 ± 26,32) e < 1 a 36 *T. cruzi*/ml em chagásicos tratados (média 6,45 ± 9,28). A diferença entre as médias não foi estatisticamente significativa. O resultado mostra que o tratamento da doença de Chagas crônica com drogas nitroderivadas é insatisfatório.

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