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Experimental Parasitology 111 (2005) 80–86
Experimental Parasitology
www.elsevier.com/locate/yexpr

Trypanosoma cruzi: Chemotherapeutic effects of clomipramine in mice infected with an isolate obtained from an endemic area

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Received 25 August 2004; received in revised form 12 May 2005; accepted 13 May 2005
Available online 8 August 2005

Abstract

The susceptibility of *Trypanosoma cruzi* strains to nifurtimox and benznidazole has been investigated and resistant strains have been described. Some tricyclic drugs are lethal for trypomastigote and epimastigote forms of *T. cruzi* (Tulahuen strain) and prevent the disease in mice. We investigated whether clomipramine, a tricyclic antidepressant drug with anti-trypanothione reductase and anti-calmodulin effects, could be effective in treating Albino Swiss mice infected with trypomastigotes of a new *T. cruzi* isolate from a chronic patient from an endemic area of Argentina in two different treatment schedules. Both treatment schedules were effective in reducing electrocardiographic changes and preventing myocardial structural damage. The cardiac β-receptors low affinity was compensated for by an increment in their density. This probably maintained cardiac function since 70% of the mice survived for more than 2 years even though anti-cruzipain titers remained high. These results demonstrate that clomipramine, clinically used as a neuroleptic, could be a promising trypanocidal agent for the treatment of Chagas' disease.

Keywords: Chagas' disease treatment; *T. cruzi* strains; Clomipramine

1. Introduction

Chagas' disease is a parasitosis caused by several strains of the flagellate protozoan parasite *Trypanosoma cruzi*. Studies using cloned or uncloned populations of this parasite (Macedo and Pena, 1998), have demonstrated its heterogeneity and that generally the *T. cruzi* strains are composed of subpopulations with differences in their growth rate, histotropism, antigenicity, pathogenicity, and number of chromosomes (Machado and Ayala, 2001; Murta and Romanha, 1999).

The correlation between *T. cruzi* electrophoretic patterns and genetic background with the different clinical forms of Chagas' disease, as well as their differential susceptibility to drugs, have been extensively investigated (Andrade, 1999; Revollo et al., 1998). Strains resistant to nifurtimox and benznidazole, the only drugs available for treatment of this disease, have been described (Filardi and Brener, 1987; Magdesian et al., 2001; Murta et al., 1998; Murta and Romanha, 1998; Rodrígues Coura and de Castro, 2002).

It has also been proposed that the host's genetic background could be related to the development of clones with different susceptibility to the available drugs (Veloso et al., 2001).

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0014-4894/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2005.05.005

Our previous work has demonstrated that tricyclic drugs such as clomipramine and thioridazine are lethal for trypomastigotes and epimastigotes of *T. cruzi* (Tulahuen strain) (Barioglio et al., 1987; Paglini-Oliva et al., 1998) and also prevented the evolution to the chronic phase in mice infected with the same parasite strain (Lo Presti et al., 2004; Rivarola et al., 2001; Rivarola et al., 1999).

An isolate obtained from a chronic patient who lived in an endemic area of Argentina was demonstrated by its electrophoretic pattern (Montamat et al., 1996) to belong to zymodeme 12 from Argentina (Bustamante et al., 2003). It has been shown that patients infected with Z12 parasites have a higher risk of cardiac lesions (Montamat et al., 1996). This isolate was found to be susceptible to thioridazine, a phenothiazine, which inhibited the *T. cruzi* tryphanothine reductase through the peroxidase/H_2O_2/system (Gutierrez-Correa et al., 2001) modifying Chagas' disease evolution in mice (Lo Presti et al., 2004).

The lack of an effective treatment for Chagas' disease and the toxicity and side effects of the available drugs (Castro, 2000), encouraged us to investigate whether clomipramine, a tricyclic antidepressant drug with anti-calmodulin effect (Barioglio et al., 1987) shown to be effective in *T. cruzi* (Tulahuen strain) infected mice, would also be effective for the treatment of an infection produced by a new *T. cruzi* Z12 isolate.

2. Materials and methods

Drug. clomipramine: Sigma Chemical, St. Louis, MI, USA.

2.1. Experimental infection

Bloodstream trypomastigotes of *T. cruzi* were isolated by xenodiagnosis from a chronic patient from Santiago del Estero, an endemic area of Argentina. The isolate was designated as MHOM/AR/1999/SGO-Z12 (*T. cruzi*). It was maintained in mice and was used for isoenzymatic and biological characterization as previously described (Bustamante et al., 2003).

2.1.1. Infection

One hundred and twenty Albino Swiss male mice weighing 30 ± 1 g ($n = 140$) were inoculated with 500 trypomastigote forms of *T. cruzi*, SGO-Z12 isolate by intraperitoneal injection and 20 were used as uninfected controls. The parasite load used for infection was sufficient to reproduce the acute, indeterminate and chronic phases of the experimental disease. The number of parasites/ml of blood was determined using a Neubauer Haemocytometer and the blood (0.1 ml) was then diluted in physiological solution and 30% BSA (V fraction) to obtain the appropriate parasite concentration.

Parasitaemias were determined in a Neubauer haemocytometer using tail vein blood samples obtained twice a week, beginning 7 days post-infection (p.i.). The mice were divided into the following groups: **uninfected**: treated daily with isotonic saline intraperitoneally ($n = 20$); **infected**: infected with *T. cruzi* without treatment ($n = 40$); **Clo 5**: infected with *T. cruzi* and treated intraperitoneally with clomipramine 5 mg/kg/day for 30 days, the first dose 60 min post-infection (p.i.) ($n = 40$); **Clo 40**: infected with *T. cruzi* and treated intraperitoneally with clomipramine 40 mg/kg/day 60 min p.i. and 7 days p.i ($n = 40$).

The investigation was carried out according to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health, NIH Publication (No. 85-23, revised 1996).

2.1.2. Electrocardiographic study

Electrocardiograms were obtained with an electrocardiographic unit (Model FD-16 Fukuda Denshi) under ketamine hydrochloride (Parke Davis) anesthesia, 10 mg/kg before infection and 50, 90, and 135 days p.i. for the animals infected with *T. cruzi*, SGO-Z12 isolate (infected, treated with **Clo 5** or **Clo 40** groups). Electrocardiographic tracings were obtained with six standard leads (dipolar leads DI, DII, DIII, and unipolar leads aVR, aVL, and aVF), recording at 50 mm/s with amplitude set to give 1 mV/10 mm.

2.1.3. Histopathological studies

The hearts were removed from **uninfected, infected, Clo 5**, and **Clo 40** mice ($n = 10$ for each group at each time point) on days 50, 90, and 135 p.i.; fixed in buffered (pH 7.0) 10% formaldehyde, and embedded in paraffin. Each heart was cut horizontally into 5 μm sections from the apex to the auricles. The sections were stained with hematoxylin–eosin. A total of 50 slices from each group was analyzed and at least 30 areas from each slice were examined with a 40× objective.

2.1.4. Determination of cardiac β-adrenergic receptor binding

The density and affinity of β-receptors was performed in right and left ventricles obtained from **uninfected, infected, Clo 5**, and **Clo 40** groups. A pool of two ventricles was homogenized in 10 volumes of ice-cold homogenization buffer (250 mM sucrose, 1 mM $MgCl_2$, and 20 mM Tris–HCl, pH 7.4). Homogenates were centrifuged at 2000*g* for 10 min. Pellets were homogenized again and centrifuged at 40,000*g* for 20 min and twice with KCl 0.6 M in homogenization buffer only. The final pellet was suspended in incubation buffer (mM composition: 125 $MgCl_2$, 1.5 EDTA, and 75 Tris–Cl, pH 7.65) in a volume of 1 ml/g of wet tissue.

^{3}H/dihydroalprenolol (^{3}H/DHA, specific activity 3.515×1015 Bq/mol from NEN Life Science Products,

Boston, MA, USA) was used as radioligand in β-adrenergic receptors' binding assays. Experiments were performed in triplicate with 100 µl of membrane suspension (480 mg protein) and ^{3}H/DHA (2.4–11.5 nM) incubated at 37 °C for 10 min in a final volume of 1 ml. The incubation was concluded by adding 1 ml of cold incubation buffer to each tube and rapidly filtering the contents under reduced pressure through Whatman GF/B filters. The filters were dried and transferred to vials with scintillation liquid (Aquasol Universal, NEN) for counting.

Specific binding was defined as the difference in radioactivity bound in the absence or presence of 1 µM propranolol. Dissociation constant (K_d) and maximum ^{3}H/DHA binding (B_{max}) were determined by a saturation curve and Scatchard analysis using GraFit (Erithacus software, Staines, UK).

2.1.5. Serology

Blood samples were collected 50, 90, and 135 days post-infection, representing the acute, intermediate, and chronic phases of the experimental infection, respectively. These samples were then assayed for antibodies to "cruzipain" antigen (see below), by ELISA (Gea et al., 1992).

2.1.6. Trypanosoma cruzi antigen

Epimastigote forms of *T. cruzi* SGO Z12 isolate were grown at 26 °C in liver digest neutralized medium (Oxoid America, Columbia, USA) supplemented with 0.5% tryptose, 10% FCS, 200 mg/ml hemin, 100 U/ml streptomycin. Parasites were harvested at the exponential growth phase, centrifuged at 5000*g* for 10 min, and washed with PBS. One millimolar *N*-α-*p*-tosyl-L-lysine chloro methyl ketone (TLCK) was added as a cysteine protease inhibitor and the parasite pellet was lysed by eight cycles of freezing and thawing. The parasite homogenate was centrifuged at 105,000*g* and the supernatant was used for cruzipain purification. Cruzipain was purified by affinity chromatography as previously described (Labriola et al., 1993). The absence of enzyme activity was controlled in gels containing 0.1% gelatin (Sigma) incubated after the PAGE run at pH 5.7 and stained with Coomassie blue R250; the samples were neither reduced nor boiled.

2.1.7. Survival

The survival was monitored every day, at 13:00 h.

2.1.8. Statistical analysis

The data obtained were compared by analysis of variance for the electrocardiographic studies; for the β-adrenergic receptor studies a comparison of all possible combinations of pairs of means was performed by REGWQ multiple test (Ryan–Einot–Gabriel–Welsch). Student's *t* test was used for parasitaemias and χ^2 was used for categorical variables (histopathological and survival studies). The significance level was set at $p < 0.05$.

3. Results

3.1. Parasitaemias

Fig. 1 shows the evolution of the parasitaemia observed in infected mice with and without treatment. It can be seen that untreated mice had a peak of parasitaemia by day 35 post-inoculation, decreasing and becoming negative from day 45, whereas mice treated with **Clo 5** mg/kg daily for 30 days or with **Clo 40** mg/kg for only two doses showed significantly lower parasitaemias with values close to zero throughout the evolution of the infection.

3.2. Electrocardiography

The electrocardiographic studies (Table 1) showed that untreated, infected animals had increased electrocardiographic changes as the disease evolved. Treated animals had lower conduction disturbances from 90 days p.i. to the end of the study ($p < 0.01$).

3.3. Cardiac β-adrenergic receptor affinity and density

The results of the analysis of cardiac β-adrenergic receptors on myocardium membranes obtained from the four groups of mice in the acute, indeterminate and chronic phase are shown in Table 2. Cardiac β-adrenergic receptor affinity diminished and their density increased from the acute to the chronic phase in the infected untreated animals ($p < 0.01$).

Clo 5 and **Clo 40** treated animals showed a significantly lower affinity throughout the infection ($p < 0.01$), but Clo 40 showed an affinity value not different from non-infected animals in the chronic stage (135 days p.i.)

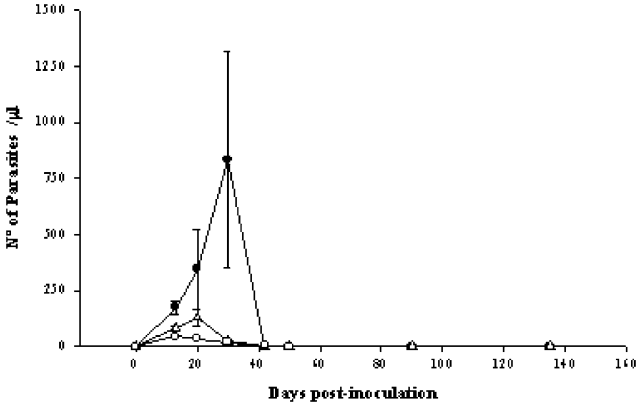

Fig. 1. Mean parasitaemia in SGO-Z12 infected and non-treated (●; $n = 40$), infected and treated with Clo 5 (○; $n = 40$), and infected and treated with Clo 40 (Δ; $n = 40$).

Table 1
Results of the electrocardiographic of 20 uninfected mice, and 120 mice infected with *T. cruzi*: 40 left untreated (INFEC), 40 treated with clomipramine 5 mg/kg daily for 30 days (Clo 5), and 40 treated with clomipramine 40 mg/kg at 1 h and 7 days after infection (Clo 40)

	Pulse rate (beats/min)	Axes (grade)	PQ interval (s)	QRS interval (s)	Mice showing abnormality (%)
Uninfected	624.8 (21)	49 (2.77)	0.015–0.03	0.015–0.03	6
55 d.p.i.					
Infected	501.8 (15.8)	44 (6)	0.01–0.03	0.02–0.04	18
Clo 5	483 (54.7)	32 (8.3)	0.01–0.03	0.01–0.01	11
Clo 40	464 (67)	34 (27)	0.01–0.02	0.01–0.01	17
90 d.p.i.					
Infected	509.7 (17.5)	48.3 (8.4)	0.02–0.04	0.02–0.04	36
Clo 5	560 (54.7)	36 (19)	0.01–0.02	0.01–0.01	20
Clo 40	560 (51.6)	57 (8.2)	0.01–0.02	0.01–0.01	10
135 d.p.i.					
Infected	467.7 (12)	38.2 (6.5)	0.015–0.03	0.015–0.07	55*
Clo 5	510 (22)	57 (10)	0.01–0.02	0.01–0.01	18*
Clo 40	517 (72)	48 (15.2)	0.01–0.02	0.01–0.01	16*

Numbers in parentheses are standard errors. d.p.i., days post-infection.
* $p < 0.01$.

Table 2
Results of β-receptor studies of 20 uninfected mice, and 120 mice infected with *T. cruzi*: 40 left untreated (INFEC), 40 treated with elomipramme 5 mg/kg daily for 30 days (Clo 5), and 40 treated with clomipramine 40 mg/kg at 1 h and 7 days after infection (Clo 40)

	K_d (nM)	B_{max} (fmol/mg protein)
Uninfected	3.610 (0.05)[a]	71.96 (0.36)[a]
55 d.p.i.		
Infected	5.71 (0.56)[b]	207.6 (8.1)[b]
Clo 5	9.05 (0.86)[c]	271.3 (10.1)[c]
Clo 40	6.06 (0.7)[b]	255.9 (10)[c]
90 d.p.i.		
Infected	6.28 (0.4)[b]	228 (6.02)[b]
Clo 5	8.10 (0.8)[c]	292.2 (11.2)[c]
Clo 40	5.32 (0.44)[b]	249.8 (6.6)[b]
135 d.p.i.		
Infected	7.32 (0.19)[b]	184.1 (2.1)[b]
Clo 5	7.74 (0.94)[b]	302.4 (13.6)[d]
Clo 40	4.9 (0.48)[a]	230.6 (6.98)[c]

Numbers in parentheses are standard errors. d.p.i., days post-infection. Within each row, values with different superscript differ significantly ($p < 0.01$).

(see Table 2). The density of cardiac β-receptors of both treated groups remained higher compared with uninfected animals, but their incremental increase was different; with **Clo 40** having lower values than **Clo 5** (see Table 2).

3.4. Serology

Titres of anti-cruzipain IgG were determined in the acute (50 days post-infection), indeterminate (90 days post-infection), and chronic (135 days post-infection) phase of the experimental infection. Titres in the treated and untreated infected mice were similar and significantly higher than those in the uninfected group ($p < 0.01$) on each sampling day (Fig. 2).

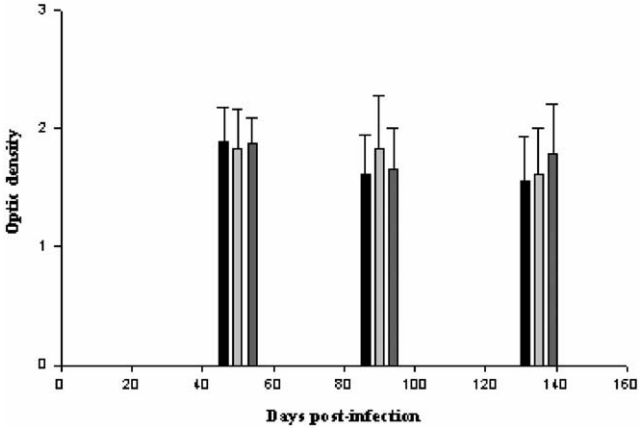

Fig. 2. Serology of *T. cruzi*-infected mice that were untreated (■), or treated with clomipramine 5 mg/kg daily for 30 days (▩) or with clomipramine 40 mg/kg at 1 h and 7 days post-infection (■).

3.5. Histopathology

The histopathologic studies of infected untreated hearts in the acute phase of the experimental infection showed extensive mononuclear infiltrates whereas treated groups had only a few isolated mononuclear infiltrates.

Hyalinization and lympho/monocyte infiltrates were observed 90 days p.i. in **untreated** mice; the **treated** groups had significantly less change than the untreated animals at the same stage ($p < 0.05$).

In the chronic stage, hearts of infected, **untreated** mice presented hyalinization and profuse necrotic areas, but the myocardium obtained from both the **treated** groups showed a cardiac structure no different from the **uninfected** animals (see Figs. 3A and B).

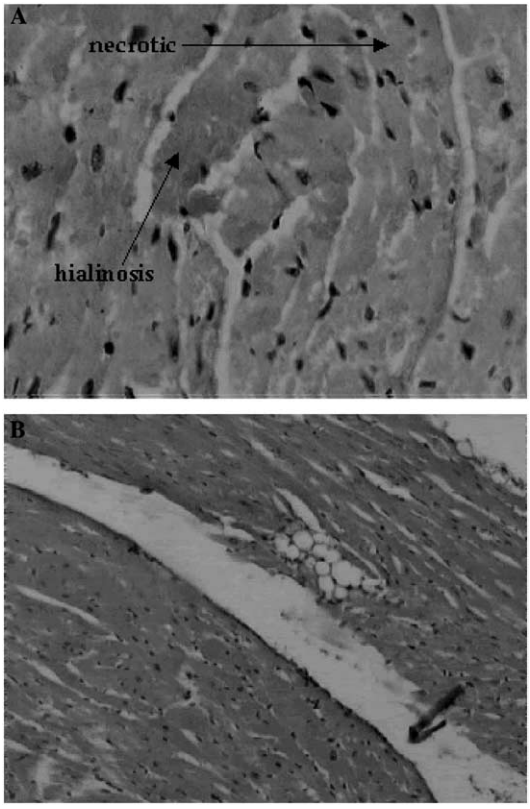

Fig. 3. (A) Myocardium from *T. cruzi* infected and untreated mice with necrotic and hialinosis (400×). (B) Hearts from *T. cruzi*-infected mice that were treated with clomipramine 40 mg/kg at 1 h and 7 days post-infection (p.i.), sacrificed 135 days p.i., showed no structural alterations (50×) (haematoxylin and eosin).

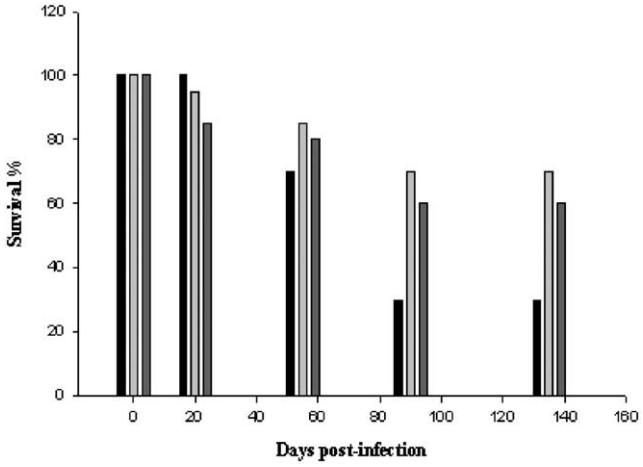

Fig. 4. Survival of *T. cruzi*-infected mice that were untreated (■), or treated with clomipramine 5 mg/kg daily for 30 days (■) or with clomipramine 40 mg/kg at 1 h and 7 days post-infection (■).

3.6. Survival

The survival of the treated animals at the end of the experiments (135 days) was 70% in the **Clo 5** group and 60% in **Clo 40** group. Two years later all these animals were alive. Only 30% of the non-treated animals were alive 135 days p.i. ($p < 0.01$) (Fig. 4).

4. Discussion

In most parasitic diseases, the parasite has evolved to evade the host immune system, so the host can control, in most cases, but not eliminate the parasite. Vaccine development against Chagas' disease faces great difficulties, and drugs therefore remain the only way to prevent or treat it (Hopkins Sibley and Hunt, 2003).

Chronic cardiac Chagas' disease represents the result of the interaction between the host and the parasite, causing different clinical features. The inflammatory response to the *T. cruzi* presence is probably responsible for progressive neuronal damage, microcirculatory changes, heart matrix deformation and heart failure (Higuchi et al., 2003). Our treated experimental model, which normally produces a disease similar to that described in humans in endemic areas, did not demonstrate this typical evolution; the acute phase did not evolve to the chronic one, perhaps because Clo treatment was effective in achieving the clearance of parasites, interrupting the positive cycle between the parasite presence and immunological response, inflammatory reaction and evolution to chagasic cardiopathy (Higuchi et al., 2003; Tarleton, 2003). Small mononuclear inflammatory infiltrates were observed in the animal hearts 55 days post-infection, but no structural changes were detected later that would be typical of the chronic stage. Similar results were obtained in *T. cruzi* (Tulahuen strain) infected mice treated with the same drug (Rivarola et al., 2001).

Some authors (Girones and Fresno, 2003) have proposed that a "trigger" is necessary for the initiation of pathology. The trigger signal (parasite or immunological response) would activate specific T cells to secrete inflammatory cytokines which produce the cardiac damage.

Simultaneously the cardiac damage would favor stimulatory molecules, whose presence would be necessary for the activation of the reactive T cells (Girones and Fresno, 2003). It is clear that a reduction of parasite concentration would produce a real benefit to the host.

The drug investigated here was lethal to the parasite, thus modifying the normal pathological processes, because treated mice showed a cardiac structure similar to non-infected animals and the electrocardiographic changes typical of chagasic disease were almost absent ($p < 0.01$). Similar results were previously described with different *T. cruzi* strains (Rivarola et al., 2001; Rivarola and Paglini-Oliva, 2002) and a different tricyclic drug (Lo Presti et al., 2004).

Chronic chagasic heart disease is a cardioneuropathy in which sympathetic and parasympathetic systems are affected (Borda et al., 1984). The β-adrenergic receptor-G protein-adenyl-cyclase system is the most powerful physiological mechanism to increase contractility (Brodde, 1996), so the state of β-adrenergic receptors gives us information about the inotropism of the heart.

Previous studies of cardiac receptor function, analyzing their affinity and density in hearts obtained from *T. cruzi* infected mice, have demonstrated that the changes are characteristic of each chagasic phase (Enders et al., 1995; Fernández et al., 1996). Others (Sterin-Borda et al., 1999) have related β-adrenergic changes to the presence of circulating antibodies characteristic of chagasic patients.

In *T. cruzi*, (Tulahuen strain) infected mice, the high titres of anti-cruzipain IgG did not return to normal even in treated animals (Rivarola et al., 2001; Rivarola and Paglini-Oliva, 2002; Rivarola et al., 1999). Here anti-cruzipain IgG titres in infected untreated rodents were 10 times higher than those obtained with Tulahuen strain infections and similar to previous work using SGOZ12 (Lo Presti et al., 2004).

Sterin-Borda et al. (1999) have proposed that chagasic antibodies would act as cardiac β-receptor agonists. With this in mind, one would expect a down regulation because of the high titres of antibodies acting as agonists. On the contrary, cardiac β-receptor density was significantly higher through the three phases of the *T. cruzi* infection in both infected untreated and treated mice. This abnormally high density could be a compensatory mechanism for diminished affinity. **Clo 5** treatment did not alter receptor affinity in the chronic phase but the receptor density was higher. This compensation could relate to the 70% survival observed in treated mice. The receptor affinity in the **Clo 40** group was similar to uninfected animals and their survival similar to the **Clo 5** treated mice.

Treated chagasic patients remain with positive serology for Chagas' disease for years (Segura et al., 1994, 1996). Our experimental model presents very high antibody titres that remain throughout the studies. This could be one of the reasons why β-receptor density and affinity do not return to normal values; presumably this high concentration of antibodies could be interacting with the receptors and more studies are needed to clarify this. The SGOZ12 isolate was susceptible to clomipramine presumably because it interacted with the parasite trypanothione reductase (Gutierrez-Correa et al., 2001) and calmodulin (Bondy, 1995), both being targets for the drug and interrupted the evolution of the infection to the chronic chagasic phase.

We have therefore reproduced the disease as described in humans and have shown that clomipramine, currently used as a neuroleptic, is a promising trypanocidal agent for the treatment of Chagas' disease, without any apparent toxic effects in mice at the doses used.

Acknowledgments

This work was supported by grants from the Agencia Córdoba Ciencia, Secretaría de Ciencia Técnica (SECYT) from Universidad Nacional de Córdoba and from Uiversidad Nacional de La Rioja and Banco Nación Foundation.

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