

Studies of Membrane Fluidity and Heart Contractile Force in *Trypanosoma cruzi* Infected Mice

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In Chagas disease serious cardiac dysfunction can appear. We specifically studied the cardiac function by evaluating: ventricle contractile force and norepinephrine response, affinity and density of β -adrenergic receptors, dynamic properties of myocardial membranes, and electrocardiography. Albino swiss mice ($n = 250$) were infected with 55 trypomastigotes, Tulahuén strain and studied at 35, 75, and 180 days post-infection, that correspond to the acute, indeterminate, and chronic phase respectively.

Cardiac β -adrenergic receptors' affinity, myocardial contractility, and norepinephrine response progressively decreased from the acute to the chronic phase of the disease ($p < 0.01$). The density (expressed as fmol/mg.prot) of the receptors was similar to non-infected mice (71.96 ± 0.36) in both the acute (78.24 ± 1.67) and indeterminate phases (77.28 ± 0.91), but lower in the chronic disease (53.32 ± 0.71). Electrocardiographic abnormalities began in the acute phase and were found in 65% of the infected-mice during the indeterminate and chronic phases. Membrane contents of triglycerides, cholesterol, and anisotropy were similar in all groups. A quadratic correlation between the affinity to β -adrenergic receptors and cardiac contractile force was obtained. In conclusion the changes in cardiac β -adrenergic receptors suggests a correlation between the modified β -adrenergic receptors affinity and the cardiac contractile force.

Key words: Chagas disease - cardiomyopathy - contractile function - cardiac β -receptors

Chagas disease is caused by the parasite *Trypanosoma cruzi* and during the course of the disease characteristically develops cardiomyopathy and digestive complications (Tanowitz et al. 1992, Andrade 1999).

The mechanisms proposed to explain the cardiac damage are related to the persistence of *T. cruzi* at specific sites of the infected host and to the immune response induced by the infection. A complete understanding of the pathogenesis of the disease, however, needs to be elucidated (Brener & Gazzinelli 1988, Petri & Eissen 1989, Salomone et al. 2001).

Antibodies against different parasite components and other cardiac structures have been identified (Ferrari et al. 1995) and specific antibodies anti-cardiac β -adrenergic and muscarinic receptors (Borda & Sterin Borda 1996) seem to play an important role in the pathogenesis of the typical Chagas cardiopathy disorders: brady-arrhythmias and tachy-arrhythmias (Elizari 1999). Several evidences have shown that the neurohumoral catecholamine system plays a crucial role in cardiac diseases (Brodde 1996). Changes in β -adrenergic receptors such as down regulation, uncoupling from G-proteins and internalization or degradation of the receptors may contribute to the abnormalities of the contractile function (Chakraborti et al. 2000).

Additionally, the chronic activation of the sympathetic nervous system leads to a decrease of the function of adrenergic receptors in patients with chronic heart diseases. The number of β_1 -receptors is lower in dilated cardiopathies of any origin, and this fact is directly related to the severity of the disease (Brodde 1991, 1996, Bristow 1993, Harding et al. 1994).

Chagas cardiopathy has been described as a myoneurocardiopathy with inflammatory involvement, microcirculation alterations (Madoery & Madoery 1992) associated to modifications in catecholamine response and myocardial contractility (Paglini-Oliva et al. 1987, Enders et al. 1995, Peres Leiros et al. 1997, Sterin Borda et al. 1999). We have analyzed the dynamic properties of the myocardial membrane, cardiac electric function as well as density, and affinity of cardiac β -adrenergic receptors in TC-infected mice, and correlated them with the cardiac contractile force in different stages of experimental Chagas disease.

MATERIALS AND METHODS

Infection - We inoculated 250 mice intraperitoneally (Swiss albino males, weigh 30 ± 1 g each) with heparinized blood from infected mice with Tulahuén strains of *T. cruzi*. The number of parasites per milliliter of blood was counted in a Neubauer haemocytometer, so that each mouse was inoculated with blood containing 55 trypomastigotes. Tail-vein blood samples were collected from each infected mouse once a week from day 7 post-infection (PI) to follow the parasitemia level by using a Neubauer haemocytometer.

Mice were sacrificed by decapitation with previous Ketamina CIH (Parke Davis) 10 mg/kg anesthesia, and studied at 35, 75, and 180 days PI, corresponding to the acute,

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indeterminate, and chronic stages of the experimental infection, respectively. A group of non-infected mice were used as control. The investigation was performed according to the Guides for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH 1985).

Histopathological studies - The animals were killed by ether anesthesia and the hearts dissected. The organ was fixed in buffered (pH 7.0) 10% formalin and embedded in paraffin. The tissue was cross sliced from the apex to the auricles. The slices (5 μ m thick) were stained with the haematoxylin-eosin technique. A total of 50 slices from each group were analyzed. At least areas from each slice were examined with a $\times 40$ objective.

Membrane extraction - The extraction of membranes was performed from the right ventricle of the mice at 35, 75, and 180 days PI. A pool of two ventricles was homogenized in 10 volumes of ice cold homogenization buffer (250 mM Sucrose, 1 mM MgCl₂, and 20 mM TRIS-HCL, pH 7.4). Homogenates were centrifuged at 2000 \times g for 10 min. Pellets were homogenized again and centrifuged at 40,000 \times g for 30 min and then centrifuged two more times with KCl 0.6 M in homogenization buffer only. The final pellet was suspended in incubation buffer (mM composition: 125 MgCl₂; 1.5 EDTA; 75 TRIS-HCL; pH 7.65) in a volume of 1 ml/g of wet tissue.

Determination of lipid composition - To determine the triglycerides and cholesterol we used enzymatic methods (Abell et al. 1952, Mcgowan et al. 1983) with reagents from Weiner Lab. The quantification of triglycerides contained in the suspension samples of sarcoplasmic membranes was read in a spectrophotometer (Metrolab 1600 UV-Vis) at 505 nm and the concentration was obtained using a 2.26 mmol/l glycerol witness, equivalent to a 2 g/l triolein. To determine the triglycerides we used the following reagents: "buffer" Goods 50 mmol/l, chlorophenol 2 mmol/l, lipase lipoprotein ≥ 800 U/L, glycerol kinase ≥ 500 U/L, oxidase glycerol phosphate ≥ 1500 U/L, peroxidase ≥ 900 U/L and ATP 2 mmol/l, in a pH of 7.5.

The quantification of cholesterol contained in the suspension of sarcoplasmic membranes was read in a spectrophotometer (Metrolab 1600 UV-Vis) at 505 nm and the concentration was obtained using a 2 g/l cholesterol witness. To determine cholesterol we used a reagent formed with 4-AF 1.25 mmol/l, phenol 2.75 mmol/l, lipase ≥ 6000 U/L, cholesterol oxidase ≥ 60 U/L and peroxidase ≥ 400 U/L, in a pH 7.4 solution.

Fluorescence anisotropy measurement - Diphenyl hexatriene (DPH) fluorescence emission anisotropy (1,6-diphenyl 1,3,5-hexatriene) in the cardiac membrane suspension was determined using a SLM 4800 C spectrofluorometer controlled by a T format microprocessor. The r_{DPH} parameter showed the degree of impediment to the rotation of DPH molecules inserted in the membranes, providing a notion of the relative fluidity of these membranes. The term "membrane fluidity" was used to refer to the structural and dynamic properties that determined the order and relative movements of the lipids in the membrane. To determine the fluorescence, the suspension of cardiac membranes was diluted in 1000 μ l of incubation "buffer" (pH: 7.65), a 10 μ l DPH solution was added (dis-

solved in chloroform) at the rate of 1% of the final volume of the incubation solution, and after being shaken, was left to settle for an hour at 37°C. The sample was excited at 360 nm and the emission was measured at 420 nm. In all determinations, a 10 \times 10 \times 45 mm quartz cuvette was used. The cuvette was kept at a constant temperature. The DPH " r_{DPH} " anisotropy was calculated by the following equation: $r = [I_{VV} - (I_{HV} \div I_{HH}) I_{VH}] \div [I_{VV} + 2 (I_{HV} \div I_{HH}) I_{VH}]$. The letter "I" represents the intensity of fluorescence. The first and second sub indexes indicate the position (_V: vertical, _H: horizontal) of the excitation and emission polarizer, respectively.

β -adrenergic receptors - ³H/Dihydroalprenolol (³H/DHA, specific activity 3.515 $\times 10^{15}$ Bq/mol from NEN, US) was used as radioligand in β -adrenergic receptors binding assays. The experiments were carried out in triplicate with 100 μ l of membrane suspension (480 mg protein) and ³H/DHA (2.4-11.5 nM) and incubated at 37°C for 10 min in a final volume of 1 ml. The incubation process 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 to count radioactivity in Aquasol Universal LSC cocktail-NEN.

Specific binding was defined as the difference in radioactivity bound in the absence or presence of propranolol 1 μ M. Dissociation constant (Kd) and maximum ³H/DHA binding (Bmax) were determined by a saturation curve and Scatchard analysis using GraFit (Erithacus Software, Staines, UK).

Adrenoceptor functional studies - Non-infected and infected animals were sacrificed at 35, 75, and 180 days PI and right ventricles were dissected and placed in a glass chamber containing KRB - Ringer solution, saturated with 95% O₂ and 5% CO₂, glucose 11 mM, pH 7.4 and kept at 32°C. Control values for tension were recorded using a force transducer coupled to an ink writing oscillograph, as previously described (Paglini-Oliva et al. 1987).

Temperature and pH were kept constant throughout the experimental period. One end of the ventricle was hooked to a stimulating electrode and the other to a strain gauge (Statham, Universal Cell, model UC5). It was applied a resting tension of 5 mN (milliNewton).

Prior to the initiation of the experimental period, the tissue was allowed to equilibrate for 60 min, during the last 30 min of this period, the ventricle was stimulated with a 15 V current at a frequency of 30 pulses/minute and duration of 12 msec each. This stimulation pattern was maintained throughout the experimental period.

The effect of norepinephrine was studied by means of cumulative dose response curve; a progressively increasing dose of drug was added, producing a measurable effect before the addition of the next higher dose, until a maximum response was obtained. L-artenerol bitartrate (norepinephrine, Sigma Chemical Co.), 10⁻⁵ M was used.

Electrocardiography - Electrocardiograms were obtained with an electrocardiographic machine (Fukuda Denshi Model FD-16), under 10 mg/kg of Ketalar HCl (Parke Davis) anesthesia, in non-infected and infected groups at 35, 75, and 180 days PI. The electrocardiographic tracings were obtained with six standard leads (bipolar

leads D1, D2, D3 and unipolar leads aVR, aVL, aVF). The tracings were recorded at a paper speed of 50 mm/second and a calibration amplitude of 1 mV = 10 mm.

Statistical analyses - We used a linear model of variance analysis to analyze the results. After that, a comparison of all possible combinations of pairs of means was performed by the REGWQ multiple range test (Ryan-Einot-Gabriel-Welsch). A 0.05 significance level was established for all cases. To determine a possible correlation between the affinity (Kd) and density (Bmax) of cardiac β -receptors with the cardiac contractile force, a polynomic regression model was proposed, as follows: $Y_i = C_0 + C_1 X_{1j} + C_2 X_{2j}^2 + \dots + C_k X_{kj}^k + \epsilon_j$. "Y_i" were the observations of the response variable, "C_k" were the model parameters, "X_j^k" were the observations of the regressive variables and " ϵ_j " were aleatory errors with zero means and " σ^2 " variance. The regressive variables were "Kd" and "Bmax" and the response variable was the cardiac contractile force. Hypothesis test procedures of this model were performed by means of a generalized ANOVA for a simple linear regression.

RESULTS

Parasitemia and histopathological studies - The parasitemia from infected mice reached a value of 300 ± 50 parasites/ μ l by day 21st PI. Since day 50 until the end of the experiments no parasites were detected. Histological sections from hearts of *T. cruzi* infected mice 35 days PI showed mononuclear cell infiltrate and amastigotes nests, 75 days PI (indeterminate stage) hearts presented infiltrates and isolated fibrosis focus. In the chronic phase (180 days PI) fibber deorganization, necrosis, and fibrosis were observed.

Density and affinity of cardiac β -adrenergic receptors - Table I shows a progressive decrease in the affinity of cardiac β -adrenergic receptors (Kd) from the acute to the chronic stage of experimental disease when compared to the non-infected group ($p < 0.001$). Hearts from mice in the acute stage (35 days PI) presented a greater β -recep-

tor affinity than mice in the indeterminate (75 days PI) and chronic (180 days PI) phases ($p < 0.01$ and $p < 0.001$, respectively).

The density of cardiac β -adrenergic receptors (Bmax) is shown in Table I. Bmax were similar in mice from the acute and indeterminate stages, but higher than in non-infected mice ($p < 0.01$) and those with chronic disease ($p < 0.001$).

Measurements of lipid composition and determination as well as fluorescence anisotropy - The triglycerides and cholesterol contents obtained from the myocardium membrane of the infected groups resulted similar to non-infected ones, as shown in Table II.

The determinations of the emission anisotropies of the DPH fluorescence tested in suspensions of cardiac sarcoplasm membrane (Table II) were also similar in all the experimental groups.

Cardiac contractile force, and norepinephrine response - The cardiac contractile force measured as isometric developed tension (IDT) of right ventricles isolated from normal mice (n = 15) was 1.76 ± 0.14 mN. The addition of 10⁻⁵ M norepinephrine (NE) induced an increase of 2.10 ± 0.25 mN. This value as well as all drug effects was obtained as IDT, between basal IDT and the response reached with the drug used. The IDT value in ventricles from mice with acute Chagas disease (n = 15) was 2.84 ± 0.59 mN and significantly higher than the value measured in the control group ($p < 0.01$).

The IDT value found during the indeterminate stage (n = 15) was similar to the one observed in myocardium from non-infected mice, the addition of NE provoked atypical responses, in 35% of the ventricles studied, NE had no effect and in 50% and 15% induced a negative and positive inotropic effect, respectively. This IDT increment in the last group was significantly lower than in control tissues ($p < 0.01$). The basal IDT in ventricles isolated from mice in the chronic stage (n = 15) was lower than the one observed in the control group and the contractility was significantly lower when NE was added (0.84 ± 0.33 mN, $p < 0.01$).

TABLE I

Quantification of affinity (Kd) and density (Bmax) of cardiac β -adrenergic receptors during the evolution of the disease

Groups	n	Kd (nM)	Test	Bmax (fmol/mg.prot)	Test
Non-infected	15	3.610 ± 0.05	A	71.965 ± 0.36	A
Acute	15	5.632 ± 0.26	B	78.245 ± 1.67	B
Indeterminate	15	6.859 ± 0.20	C	77.282 ± 0.91	B
Chronic	15	11.208 ± 0.25	D	53.325 ± 0.71	C

Results are expressed as means ± ES; the means that have the same letter are not significantly different; REGWQ: multiple range tests

TABLE II

Triglyceride, cholesterol, and anisotropy values in animals infected or not with *Trypanosoma cruzi*

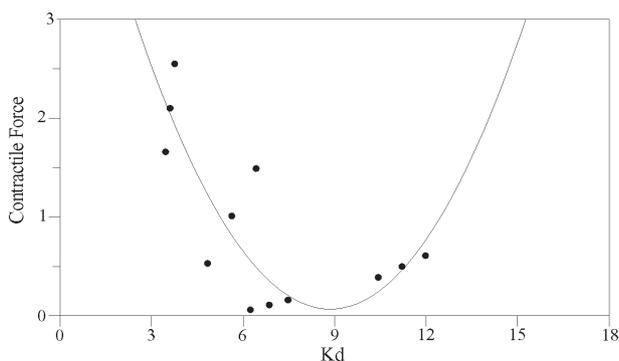
Groups	n	Triglyceride (g/l)	Cholesterol (mg%)	r (anisotropy)	Test
Non-infected	25	0.25 ± 0.01	40.46 ± 4.07	0.117 ± 0.008	A
Acute	25	0.26 ± 0.02	45.13 ± 2.42	0.136 ± 0.003	A
Indeterminate	25	0.18 ± 0.03	36.59 ± 3.03	0.123 ± 0.002	A
Chronic	25	0.20 ± 0.03	37.50 ± 4.10	0.126 ± 0.002	A

Results are expressed as means ± ES; they do not show a significant difference; REGWQ: multiple range tests

Correlation between affinity and density of β -adrenergic receptors (Kd and Bmax) and cardiac contractile force - A quadratic order model was obtained when myocardial contractile force and affinity were correlated, providing as a result, the coefficients corresponding to the function $Y = C_0 + C_1X + C_2X^2$. This fact explained the behavior of the "affinity" (Kd) as a regressive variable, when compared to myocardial contractile force as response variable. The coefficient values corresponding to the second-degree model were: $C_0 = +5.692 \pm 0.712$; $C_1 = -1.269 \pm 0.209$; $C_2 = +0.072 \pm 0.014$ ($p < 0.001$) (Figure).

A correlation between myocardial contractile force and density of β -adrenergic receptors could not be demonstrated.

Electrocardiography - Table III shows how the electrocardiographic findings of the infected mice worsened from the acute to the chronic (180 days PI) phases of the disease.



Correlation between the affinity (Kd) of cardiac β -adrenergic receptors and myocardial contractile force.

DISCUSSION

Cardiac dysfunction due to changes in the right ventricle contractility as well as β -agonist and antagonist responses were described in the course of experimental Chagas disease, (Paglini-Oliva et al. 1984, 1985, 1987) probably related to changes in cardiac β -adrenergic receptors. Borda and Borda (1996) have reported the existence of circulating IgG in chagasic patients and in experimental model of Chagas disease, which react with cardiac β_1 and β_2 adrenoceptors.

In our study, we confirmed that *T. cruzi* infected mice developed cardiac functional disorders such as: decreased contractility and modifications to norepinephrine response in right ventricles as well as electric conduction disturbances all along the acute, indeterminate and chronic stages of Chagas disease.

Cardiac hyporeactivity to neurotransmitters, a decrease in the contractile force, an increment in electrocardiographic changes and modifications in the affinity and density of β -adrenoceptors were typical findings of the acute period of *T. cruzi* infection (35 days PI). The level of the neurotransmitters become relevant during this acute stage, since it was demonstrated that in the hearts of rats the concentration of norepinephrine is reduced to non-detectable levels. It has been suggested that the depletion of this neurotransmitter produced by the destruction of the sympathetic cardiac innervation (Lohse et al. 1996) is the main cause of heart failure in the acute stage of the disease. Our results agree with this mechanism since we observed in the infected mice an increased number of binding sites and a decrease in the affinity values, demonstrating that the mechanism that regulates the affinity and density was maintained, although in different values compared to non-infected animals.

During the indeterminate stage (75 days PI) the right ventricle myocardium presented an atypical response to norepinephrine, such as negative inotropic effect or a complete lack of response to the neurotransmitter. Additionally, we were able to verify a decrease of the affinity when comparing the non-infected and acute groups. On the other hand, the number of β -adrenergic receptors was similar in mice in the acute stage, but higher than the number found in the non-infected group. Atrioventricular and intraventricular blocks were detected during this indeterminate stage, demonstrating that this stage of the disease is not silent and that these findings would probably determine the severity of the cardiopathy observed in the chronic stage. The results found on functional activity of β -adrenergic receptors are not enough to explain the absence of response to norepinephrine or the negative inotropic response described in myocardium of mice in the indeterminate stage. They might probably be explained after studying AMPc or other messengers that are currently under analysis in our laboratory.

The studies performed on myocardium contractility

TABLE III

Results of the electrocardiographic studies of 10 non-infected and 250 *Trypanosoma cruzi* infected mice

Electrocardiography	Non-infected	35 days post-infection n = 90	75 days post-infection n = 80	180 days post-infection n = 80
Mean (s.e.) pulse rate (beats/min)	446 (13.5)	468 (12.1)	575 (4.8)	526 (16.9)
Mean (s.e.) axes (grade)	66 (4.7)	51 (4.7)	44 (2.3)	55 (2.9)
PQ interval (s)	0.02	0.02-0.04	0.03-0.04	0.02-0.05
QRS interval (s)	0.02-0.03	0.02-0.03	0.02-0.04	0.02-0.06
% of mice showing abnormalities	2	12.5 ^a	66 ^b	60 ^b

a: $p < 0.01$ when compared with non-infected and $p < 0.001$ when compared with the acute (35 days post-infection) and indeterminate stage (75 days post-infection); *b*: $p < 0.001$ when compared with non-infected and acute groups

and pharmacological response during the chronic stage of Chagas disease showed a significant decrease in the contractile force, a marked hyporeactivity to norepinephrine and a significant increase of electrocardiographic abnormalities.

These results are related with findings in patients studied with specific functional tests, which have evidenced during the chronic phase of the disease alterations of the autonomous nervous system secondary to neurolysis (Madoery & Madoery 1992). This process can destroy up to 80% of heart nervous structures (Laucella et al. 1996). Similar results have been described by other authors in infected mice with a non-lethal strain of *T. cruzi* at nine weeks PI (Sterin Borda et al. 1999). Several non-excluding mechanisms have been proposed to explain the development of the chronic chagasic cardiomyopathy: the autonomous nervous system denervation (Caeiro 1994, Laucella et al. 1996), microvasculature disorders (Madoery & Madoery 1992), and immunological mechanisms (Tarleton 2001).

The microvascular and immunological theories, or the combination of both could explain the results of the marked decrease of affinity and density of the cardiac β -adrenergic receptors in the chronic stage. Besides, similar modifications have been described in dilated cardiomyopathies due to other etiologies (Bristow 1993), in which the reduction in the number of β -adrenergic receptors has been directly related to the severity of the disease (Brodde 1991, 1996, Harding et al. 1994).

The down regulation system of cardiac β -adrenergic receptors is a common finding of different diseases. The heart failure (Brodde 1996, Chakraborti et al. 2000) can be produced by an increment of catecholamine levels; however, these are not elevated in chronic chagasic patients (Iosa et al. 1989). It has been proposed that in chagasic patients, the down regulation may be induced by the specific circulating antibodies (Sterin Borda et al. 1999).

Having in mind that there is a cause-effect correlation between the function of β -adrenergic receptors and the cardiac contractile force, we suggest a linear model represented by a quadratic correlation between the affinity of β -adrenergic receptors and contractile force. This implies the dependence of the myocardial contractility on the affinity of the receptors, showing the minimum ability of contractile force after 75 days PI, with slightly recovery in the chronic stage of the disease.

These results may indicate that the changes in the number of β -adrenergic receptor binding sites would generate compensating mechanisms in the affinity of these receptors, with a correlation with the contractile force, that would be effective only until reaching the indeterminate stage of the chagasic cardiomyopathy. Notwithstanding, all the functional modifications observed in β -adrenergic receptors are neither followed by alterations in the membrane lipid composition nor in its fluidity, as it has been described in other cardiopathies (Villar et al. 1996).

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