Distribution of Antibodies against β-Adrenoceptors in the Course of Human *Trypanosoma cruzi* Infection (43244)

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Abstract. **We examined the possible role of altered humoral immunity in Chagas' disease by analyzing the effect of sera on the binding of radioligand to β-adrenoceptors during the course of human *Trypanosoma cruzi* infection. We described two circulating IgG which bind with myocardial β_1- and spleen cell β_2-adrenoceptor. Both chagasic IgG against β_1- and β_2-adrenoceptors increased intracellular levels of cAMP, which could be blocked by specific β_1- and β_2-adrenoceptor antagonists. The IgG against the β_1-adrenoceptor inhibited the action of norepinephrine on the contractility of atria. We also found differences in the distribution of β_1- and β_2-adrenoceptor antibodies in the course of infection. The anti-β_2-adrenoceptor IgG appears during the acute stage, peaks on the group with less than 10 years of infection, and then decreases. The prevalence of anti-β_1-adrenergic antibody is low in the acute stage, but it increases over time since infection, being higher in the group with more than 15 years of infection. The probable pathogenic role of both β_1- and β_2-adrenergic chagasic antibodies is discussed.**

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Altered immunity has been proposed as a mechanism for the pathogenesis of chronic Chagas' disease. Evidence for the participation of an immunologic mechanism is limited, but it includes the presence of antimyocardial antibodies (1–5), the presence of myocardial lymphomononuclear cell infiltration (6), abnormalities in circulating lymphocyte subpopulations (7–9), and an immunosuppressive state described in Chagas' disease (10–12) that could contribute to its chronic course.

In previous works, we have shown the presence of antibodies in the sera of patients with chronic Chagas' disease, with at least two different activities against neurotransmitter receptors. One specific antibody reacted with the β_1-adrenergic receptor of cardiac cells and it could be playing a role in the pathogenesis of myocarditis, since it causes alterations of the physiology, biochemistry, and pharmacology of the normal myocardium (3, 4, 13–16). We have described another antibody with reactivity against β_2-adrenoceptors of lymphocytes that behaving as a β-agonist, increases production of endogenous cAMP (17, 18). This phenomenon is of particular interest, since the specific recognition of lymphocyte membrane receptors by antibodies may result in modulation of the immune response in Chagas' disease.

In general, the infection with *Trypanosoma cruzi* occurs during early childhood (19). This acute phase of the disease has different manifestations than those observed in the chronic phase. Patients are asymptomatic or show few symptoms. The electrocardiogram alterations are nonspecific, and a complete recovery from signs and symptoms occurs within a short time (20). The manifestations of the chronic phase of the disease (characterized by severe involvement of the heart) arise around the third to fourth decade of life (21). Some scattered reports have demonstrated the presence of chronic Chagas' heart disease among children and adolescents (21–24). However, before the development of the chronic phase of the disease, alterations of autonomic nervous function without cardiovascular symptoms have been described (25).

The dysautonomia is characterized by a slow and progressive blockade of the β-adrenergic receptor in

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patients that are asymptomatic, with normal electrocardiograms and chest x-rays. Due to the presence of an adrenergic receptor blockade, patients show symptoms and signs that reflect pharmacological denervation (25, 26). In an attempt to elucidate the nature of this dysautonomia in patients with Chagas' disease, we characterized the changes in serum immunoglobulin G (IgG) anti-β-adrenergic receptor activity during the naturally long course of *T. cruzi* infection of patients from *T. cruzi*-endemic areas of northwest Argentina. The prevalence of β-adrenergic cardiac and lymphocyte antibodies in acute and asymptomatic patients was studied.

Materials and Methods

Test Sera. *T. cruzi*-infected patients from Salta, where *T. cruzi* infections are endemic, were studied. The sera of two groups of seropositive patients were characterized: Group 1, children (5–12 years old (n = 20)) with acute parasitemia and subcutaneous swelling around the eye; and Group 2, asymptomatic seropositive patients without association with electrocardiogram and chest x-ray changes. Group 2 is divided into two subsets according to the years of infection: 2A, less than 10 years of infection (6–13 years old (n = 30)), and 2B, more than 15 years of infection (18–20 years old (n = 44)). In all patients, serologic studies (passive hemagglutination, complement fixation, and immunofluorescence), chest x-rays, and electrocardiograms were performed. Acute *T. cruzi* infection was established by direct examination of the peripheral blood for free-swimming flagellates. The ages at the time of original infection of asymptomatic seropositive patients ranged from 1 to 2 years old. Control sera (Group 3) were from normal, noninfected individuals from Salta who were the same age as *T. cruzi*-infected patients (3A, 5–13 years old (n = 40); 3B, 18–20 years old (n = 40)). All sera samples were coded and frozen-stored at −20°C until used. Chronic Chagas' heart disease patients were not included in this study to avoid interference from medications.

Purification of Human IgG. IgG was isolated from the sera of chagasic and normal human sera by precipitation with 40% ammonium sulfate and chromatography with DEAE-cellulose (Bio-Rad Laboratories, Richmond, CA) balanced with 0.005 M (pH 8) phosphate buffer. The eluted IgG fractions were concentrated to 10 mg/ml and dialyzed against phosphate buffer solution. IgG fractions showed one line of precipitation corresponding to IgG with polyvalent antisera. Final IgG concentration was determined by radioimmunodiffusion assay (4).

Preparation of Purified Membranes and Binding Assay. Cardiac membranes for identification of β-adrenoceptors were prepared essentially as described by Borda *et al.* (4). Briefly, left ventricular tissue from 10 rats was mixed in 4 volumes of cold buffer containing 0.25 M sucrose, 50 mM Tris-HCl (pH 7.4), and 10 mM $MgCl_2$, and homogenized twice with a Polytron PT-20 at a setting of 3 for 15 sec. The homogenates were filtered through four layers of gauze and spun at 700g for 15 min, 10,000g for 15 min, and 40,000g for 30 min. The pellet was resuspended in 2.5 ml of 50 mM Tris-HCl (pH 7.4) and 10 mM $MgCl_2$. Spleen cell membranes were prepared from the spleens of 10 rats that were removed and homogenized in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) in a Teflon glass homogenizer. Cell suspensions were depleted of red blood cells by water lysis and then washed with 5% fetal calf serum-supplemented medium. The cells were then centrifuged and the pellet was resuspended in 1:4 volumes of 50 mM Tris-HCl (pH 8) and 10 mM $MgCl_2$. The suspensions were homogenized twice with a Polytron PT-20 at a setting of 3 for 15 sec. The homogenates were filtered and spun at 1000g for 15 min, and the supernatants were centrifuged at 40,000g for 30 min. The pellets were resuspended in 2 ml of 50 mM Tris-HCl (pH 7.4) and 10 mM $MgCl_2$.

Membrane suspensions (3–5 mg/ml protein for heart, and 0.5–1 mg/ml protein for spleen cell membranes) were preincubated with different dilutions of normal and chagasic serum or IgG for 30 min at 30°C in 50 mM Tris-HCl (pH 7.4) and 10 mM $MgCl_2$. The membranes were washed twice by centrifugation. For [^{3}H]dihydroalprenolol ([^{3}H]DHA) binding, 100 μl of membrane suspension and different concentrations of [^{3}H]DHA (New England Nuclear, Boston, MA; sp act 81.4 Ci/mmol) were incubated with shaking for 15 min at 37°C in a total volume of 150 μl of 50 mM Tris-HCl (pH 8) and 10 mM $MgCl_2$. At the end of the incubation period, 100-μl aliquots were placed into 2 ml of ice-cold buffer and immediately filtered through Whatman GF/C glass fiber filters. The filters were washed with 10 ml of cold buffer, dried, added to 10 ml of triton-toluene-based scintillation fluid, and counted. Nonspecific binding was determined by filtering aliquots of membranes incubated in the presence of 10^{-5} M propranolol not exceeding 25% of the specific binding. Control values are 37.5 ± 0.4 fmol/mg protein for cardiac membranes and 26.4 ± 0.2 fmol/mg protein for spleen cell membranes. Results are expressed as femtomoles and [^{3}H]DHA specifically bound per milligram of protein. Normal human IgG was used as a control.

Cyclic AMP Assay. Rat heart and spleen were excised immediately after death, prepared as described before (16, 27), and suspended in 3 ml of Krebs-Ringer bicarbonate solution gassed with 5% CO_2 in O_2 at 30°C with normal IgG (5×10^{-7} M) or chagasic IgG (5×10^{-7} M), or alone (controls) for 3 min. The action of a 15-min incubation with β-adrenoceptor blocker propranolol (1×10^{-7} M) upon chagasic IgG was also

studied. In all cases, total incubation time was 30 min in order to have similar experimental conditions. Tissues were then homogenized in 1 ml of 6% ice-cold trichloroacetic acid and centrifuged at 2500*g* for 15 min at 4°C. Proteins were determined after dissolving the pellet in 1 ml of 1 *M* NaOH in boiling water. The trichloroacetic acid supernatant fractions were extracted three times with 4 ml of water-saturated ethyl ether. The ether phase was discarded, and the aqueous phase was heated at 56°C to remove the ether and evaporated to dryness under a stream of nitrogen gas. Cyclic AMP in the residue was dissolved in 300 μl of 0.05 *M* sodium acetate buffer (pH 6.2). Aliquots (100 μl) were taken for nucleotide determination using a radioimmunoassay procedure by a cyclic AMP-[^{125}I]-radioimmunoassay kit (New England Nuclear).

Contractility Studies. Male rats weighing 200 g were decapitated and bled. Their chests were opened and their hearts were quickly excised. The atria were immediately dissected and mounted on a polygraph, as described previously (3). One end of each atrium was attached to a stationary glass holder, immersed in a tissue chamber filled with a modified Krebs-Ringer bicarbonate solution, and gassed with a mixture of 95% O_2 and 5% CO_2, composed as reported previously (3). The other end of each atrium was connected to a force transducer (Statham UC-3 Gold Cell). Throughout the experiments, the preparations were subjected to a constant resting tension of 750 mg by means of a micrometric device attached to the transducer, the output of which was amplified and recorded with a direct ink-writing oscillograph. The tissue bath solution was gassed with a mixture of 5% CO_2 in O_2 and kept at a constant temperature of 30°C and pH of 7.4. Inotropic effects were determined by recording the maximum rate of isometric force (dF/dt) developed above the externally applied resting tension. Control values (equal to 100%) refer to the spontaneous dF/dt before the addition of different IgG or norepinephrine. The absolute values of dF/dt at the end of equilibration ranged between 8.1 and 8.9 g/sec.

Drugs. Freshly prepared solutions of propranolol and norepinephrine (Sigma Chemical Co., St. Louis, MO) were used. All concentrations quoted in the text represent the final values in the bath solution.

Statistical Analysis. Patient groups were compared by chi-square test with Yates' correction. A pooled variance *t* test was done for all unpaired two-group comparisons. All statistical significances were justified at $\alpha = 0.05$.

Results

The age distribution of the inhibitory effect of 1/10 dilution of sera from three groups (acute, asymptomatic seropositive, and asymptomatic seronegative patients) on [^{3}H]DHA binding to cardiac or lymphocyte membranes is shown in Tables I and II. The positive or negative effects were defined as >10% or <10% inhibition of [^{3}H]DHA binding, respectively. Table analysis (2 × 2) shows that the inhibitory effect of sera on [^{3}H]DHA binding on lymphocyte β-adrenergic receptors is strongly associated with Salta asymptomatic children (6–13 years old), and that the sera interference on β-adrenergic radioligand of cardiac membranes is strongly associated with Salta asymptomatic young men (18–20 years old).

In the acute stage of the disease, the prevalence of the anti-β-adrenergic activity of the sera is higher on lymphocyte membranes than on cardiac membranes. In acute patients, anti-β-adrenergic activity of the sera was 26% on lymphocyte membranes, and only 11% on cardiac membranes.

The sera anti-β-adrenergic activity on lymphocyte membranes appeared in the acute stage, peaked in the group with less than 10 years of infection (Group 2A), and then decreased in the group with more than 15 years of infection (Group 2B). On the other hand, the sera anti-β-adrenergic activity on cardiac membranes was low during the acute stage of the *T. cruzi* infection, but it was present in the asymptomatic children (Group 2A) and increased proportionately to the time postinfection (Group 2B). An inverse correlation ($r = -0.52$, $P < 0.02$) between the inhibition of the [^{3}H]DHA binding on cardiac and lymphocyte membranes of sera from the two groups of the asymptomatic infected individuals is observed (Fig. 1). To ascertain that IgG, rather than another serum protein, is responsible for the observed competition binding assay, IgG from pooled sera belonging to Groups 2A and 2B that showed positivity only on lymphocyte membranes (Group 2A) or on cardiac membranes (Group 2B) were used. As control (Group 3), normal IgG was tested. Figure 2 shows a competitive dependent inhibition of [^{3}H]DHA binding to β-cardiac membranes (A) and β-spleen membranes (B) by IgG from Groups 2A and 2B. It can be seen that IgG from Group 2A was able to inhibit the specific binding of [^{3}H]DHA on spleen cell membranes, but failed to do so on cardiac membranes. By contrast, when membranes were incubated with different concentrations of chagasic IgG from Group 2B, there was no inhibitory effect on spleen cells, whereas an inhibitory effect did occur on cardiac membranes. Normal IgG used at the same concentration as indicated for chagasic IgG had no effect.

In order to evaluate a signal transduction trigger by the interaction of chagasic IgG with β-adrenoceptors, the action on intracellular levels of cAMP in cardiac and spleen cells was explored. As shown in Figure 3, there was a significant increase in cardiac cAMP production by a 2-min contact with IgG from Group 2B. By contrast, IgG from Group 2A was ineffective in the system. On the other hand, when spleen cells were

Table I. Distribution of the Inhibitory Effect of Sera from *Trypanosoma cruzi*-Infected Patients on the [^{3}H]DHA Binding to Rat Cardiac Membrane Related with the Evolution Periods of *T. cruzi* Infection[a]

Groups	Subset	Age	Antimyocardial antibody	
			Number positive/total	%
1 (acute)		5–12	2/18	11
2 (asymptomatic)	A	6–13	8/35	23
	B	18–20	20/44[b]	45
3 (control)	A	5–13	2/40	5
	B	18–20	1/40	2

[a] Cardiac membranes (3 mg/ml) were incubated for 30 min at 30°C with serum 1/10 dilution from acute parasitemia in children (Group 1), asymptomatic seropositive patients infected for less than 10 years (Group 2A), or patients infected for more than 15 years (Group 2B). Then, they were incubated with 3 n*M* [^{3}H]DHA, as described in Materials and Methods. Means ± SE of percentage of inhibition of [^{3}H]DHA binding were: Group 1, 19.8 ± 0.9; Group 2A, 24.3 ± 1.0; Group 2B, 34.4 ± 1.7 fmol/mg protein; and control Groups 3A and 3B, 2.2 ± 0.4 fmol/mg protein.

[b] Different significantly from other groups; $P < 0.05$.

Table II. Distribution of the Inhibitory Effect of Sera from *Trypanosoma cruzi*-Infected Patients on the [^{3}H]DHA Binding to Rat Spleen Cells Related with the Evolution Periods of *T. cruzi* Infection

Groups	Subset	Age	Antilymphocyte antibody	
			Number positive/total	%
1 (acute)		5–12	5/19	26
2 (asymptomatic)	A	6–13	22/29[b]	76
	B	18–20	8/44	18
3 (control)	A	5–13	1/40	2
	B	18–20	1/40	2

[a] Spleen cell membranes (1 mg/ml) were treated with 1.2 n*M* of [^{3}H]DHA. For details, see footnote to Table I. Mean ± SE of percentage of inhibition of [^{3}H]DHA binding were: Group 1, 24.8 ± 1.5; Group 2A, 35.6 ± 1.6; and Group 2B, 19.7 ± 0.8 fmol/mg protein. Control groups 3A and 3B gave 2.6 ± 0.5 fmol/mg protein.

[b] Different significantly from other groups; $P < 0.005$.

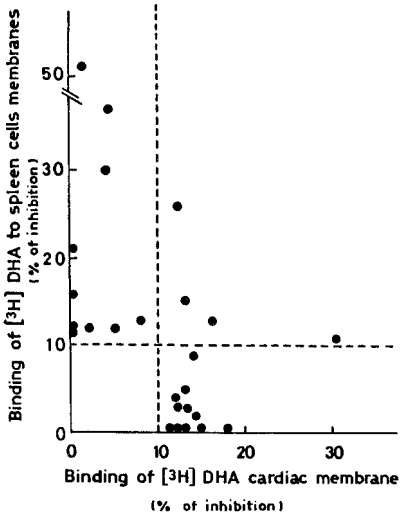

Figure 1. Inhibition of [^{3}H]DHA binding to β-adrenoceptors by chagasic sera: correlation between the inhibitory effect of each serum on cardiac and spleen cell membranes. The ability of 25 chagasic sera from asymptomatic individuals (Group 2A and 2B) to interact with cardiac and spleen membranes was plotted showing an inverse correlation ($r = -0.52$, $P < 0.02$). Positive interaction indicated with both cut-off lines indicates more than 10% of inhibition.

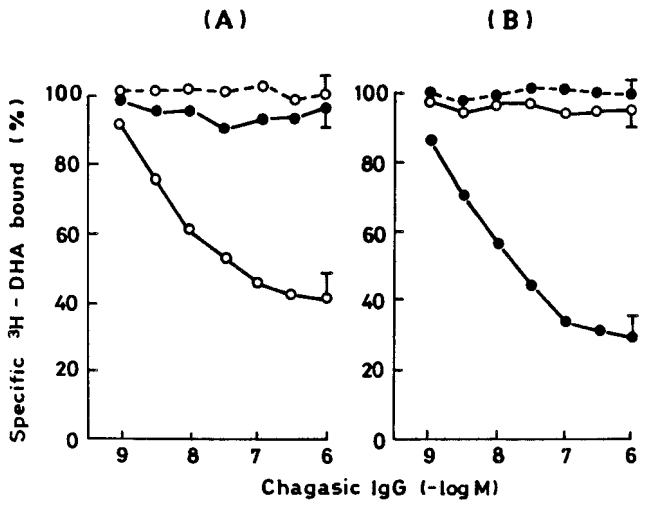

Figure 2. Effect of chagasic IgG on the [^{3}H]DHA binding to β-adrenergic spleen cell receptors (A) and cardiac receptors (B). Tissues were incubated with increasing concentrations of chagasic IgG from asymptomatic individuals in Group 2A (O—O), Group 2B (●—●), Group 3A (control; O- - -O), or Group 3B (control; ●- - -●). Control binding of 100% refers to the radioactivity bound to membrane alone. The mean of five experiments in each group is plotted.

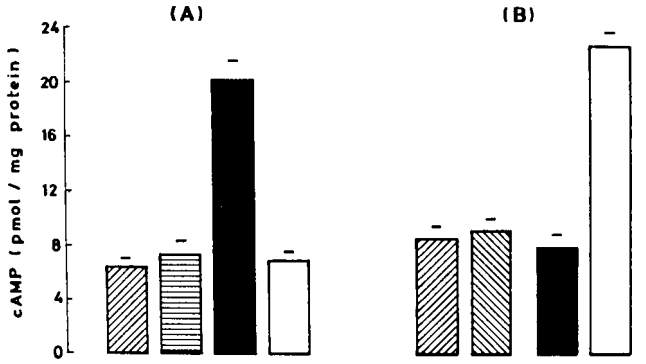

Figure 3. cAMP effect of chagasic and normal IgG on intracellular levels of cAMP by cardiac (A) and spleen cell (B) homogenates. Values were measured in tissue after 2 min of reaction without (▨) or with chagasic IgG from Group 2A (□) and Group 2B (■), and normal IgG from Group 3A (▧) and (▤). Values are mean ± SE of five experiments in each group.

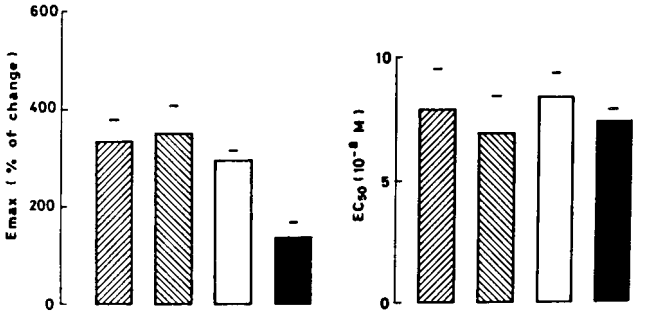

Figure 4. Inhibitory effect of chagasic IgG from Group 2B on dF/dt dose-response curve of exogenous norepinephrine. Atria were exposed to NE alone (▨) or in the presence of 1×10^{-8} *M* normal IgG (Group 3B + norepinephrine; ▧), chagasic IgG (Group 2A + norepinephrine; □), and chagasic IgG (Group 2B + norepinephrine; ■). Atria were incubated for 30 min with IgG, then the norepinephrine was added. Each concentration of norepinephrine reacted with atria for 2 min. The Lineweaver-Burke plots of the dose-response curves show a typical noncompetitive inhibition between both norepinephrine and chagasic IgG from Group 2B (decrease E_{max} without changes in ED_{50}). Zero refers to the basal values of dF/dt; they were 8.5 ± 0.4 g/sec. Values represent the mean ± SE for six experiments in each group.

exposed to IgG from Group 2A, an increased production of cAMP was observed, whereas IgG from Group 2B was unable to modify lymphocyte cAMP levels. Normal IgG increased neither the level of cAMP of the myocardium nor of the spleen cells above basal values. It is important to note that a β_1-adrenergic antagonist (practolol, 10^{-6} *M*) inhibited a chagasic IgG increase in the myocardium cAMP levels (7.5 ± 0.2 pmol/mg protein), whereas a β_2-adrenergic antagonist (butoxamine, 10^{-6} *M*) blunted a chagasic IgG increase in spleen cell cAMP levels (8.2 ± 0.5 pmol/mg protein). The absolute values of myocardial and spleen cell cAMP levels in the presence of chagasic IgG from the corresponding groups were 19.8 ± 0.3 and 20.2 ± 0.4 pmol/mg protein, respectively. To ascertain whether the biologic effect of the IgG from Group 2B and β-adrenergic agonist norepinephrine is mediated by the same receptor site, the action of norepinephrine on dF/dt of isolated atria in the presence of chagasic IgG was tested. IgG from Group 2B, unlike IgG from Group 2A or normal IgG (Group 3), inhibited the dose-response curve of norepinephrine in an uncompetitive fashion (decreasing the E_{max} without changes in the ED_{50} (Fig. 4)).

Discussion

Among the immunologic factors suspected of involvement in the pathophysiologic mechanism of the Chagas' disease, we can mention a certain component(s) of the IgG fraction in the sera of *T. cruzi*-infected individuals. Cell-mediated immunity and autoreactive antibodies to the antisarcolemmal and/or antimyofibrilar epitope of the heart have been described (1, 2, 6).

We have already reported the existence of two circulating IgG in Chagas' disease patients that react with β_1- or β_2-adrenoceptor-rich tissues (17, 18). Here we confirm this issue, based on the following observation: the sera and the IgG from *T. cruzi*-infected patients inhibit the binding of [^{3}H]DHA to the β-adrenoceptors on purified myocardial and spleen cell membranes, tissues rich in β_1- and β_2-adrenergic receptors, respectively (27, 28). Moreover, chagasic IgG increased cAMP levels in both cardiac and spleen cell preparations, and this stimulatory action could be blocked by specific β_1- and β_2-adrenoceptor antagonists.

The specificity of the antibodies for β_1- and β_2-adrenoceptors is independent of the other, as demonstrated by the fact that the same IgG that bound on lymphocyte β-adrenoreceptor stimulated the adenylate cyclase activity on this tissue, but was unable to do so on myocardial β_1-adrenoceptors. By contrast, the chagasic IgG that bound and stimulated cAMP on the myocardium was unaffected when the same IgG reacted with spleen cell preparations. Furthermore, only the IgG that recognized the β_1-adrenergic receptor of the myocardium could inhibit the dose-response curve of exogenous norepinehrine upon contractility of isolated atria. We also show differences in the distribution of β_1- and β_2-adrenergic receptor antibodies in the course of human *T. cruzi* infection. Thus, we observed that the sera or IgG that recognized lymphocyte β-adrenoceptors were strongly associated with asymptomatic individuals with less than 10 years of infection (Group 2A), whereas the sera or IgG that bound to myocardial β_1-adrenoceptors were strongly associated with individuals who had more than 15 years of infection (Group 2B). In the acute stage of the *T. cruzi* infection (Group 1), the prevalence of the β_2-adrenergic antibody was higher than that of the β_1-adrenergic antibody.

Analyzing the prevalence of anti-β_1- and anti-β_2-adrenergic antibodies in function of the time of infection, we conclude that the β_2-adrenergic antibody appears during the acute stage peaks on the group with

less than 10 years of infection and then decreases with time after infection. By contrast, the prevalence of β_1-adrenergic antibody in the early stage of the infection (acute stage) is very low, but it increases with time postinfection, being higher in the group with more than 15 years of infection than in the group with less than 10 years.

An inverse correlation was observed between the β_1- and β_2-adrenergic activity of sera from asymptomatic *T. cruzi*-infected patients (Group 2A and 2B), confirming that the β_1-adrenergic activity increases while the β_2-adrenergic activity decreases during the course of the *T. cruzi* infection. It is tempting to speculate on a pathogenic role of β-adrenergic reactivity of chagasic antibodies, that the interaction of the IgG with lymphocytic β_2-adrenoceptors, increasing cAMP production, induced immunosuppressor action on lymphocyte β_2-adrenergic-rich cells. We propose that if this specific recognition and modulation takes place *in vivo*, it could be the suppressor cell mechanism described in the early stage of Chagas' disease (29) which contributes to the chronic course of the disease.

Concerning the pathogenic role of β_1-adrenergic chagasic antibody, it is possible to speculate that the interaction of the IgG with β_1-adrenoceptors of the myocardium may explain the fact that the chronic chagasic patient behaves as a natural β-blocker responder (30). Koberle (31) has reported a neurogenic nature of the chagasic heart disease, demonstrating that it is caused by a poor regulation of the autonomic control of heart activity. Denervation of the sympathetic system of the heart was verified before the development of symptoms and signs of Chagas' cardiomyopathy (25, 32). The deposit of the antibody on β_1 cardiac adrenoceptors could induce a progressive blockade of myocardial adrenergic receptors with sympathetic denervation, already described in the course of Chagas cardiomyopathy (25).

The "switch" from IgG anti-β_2- to IgG anti-β_1-adrenergic receptor activity in the long course of the *T. cruzi* infection is difficult to explain. It is possible that, during acute infection, as a consequence of the immune response directed against the blood stream flagellates, the production of IgG with anti-β_2-adrenergic receptor activity is induced. This, in turn, triggers an immunosuppressor response (18), with a down-regulation in the production of this IgG with anti-β_2-adrenergic receptor activity. On the other hand, the invasion of the myocardium by the parasite induces myocardial injury (20), and cardiac tissue antigens could trigger the production of antibodies against cardiac β_1-adrenergic receptor. The immunosuppressor stage would then be temporary, inasmuch as it disappeared with the decrement of the production of the anti-β_2-adrenergic antibody. The production of the anti-β_1-adrenergic antibody, triggered by myocardial injury, would then appear.

The data, taken together, indicate that *T. cruzi* infection generates reactive antibodies against myocardial β_1- and lymphocytic β_2-adrenoceptors. The distribution of both β_2- and β_1-adrenoceptor antibodies in the course of the human *T. cruzi* infection appears to be related to the genesis of immunosuppressor and cardiac damage, respectively, observed during the course of the disease. More direct correlation of antibody titers and clinical progression of the disease is needed.

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