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Antibodies to β_1 and β_2 adrenoreceptors in Chagas' disease

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SUMMARY

Evidence accumulated over the last decade concerning human and experimental models suggests that an immunopathological mechanism may be involved in the pathogenesis of chronic Chagas' disease. In this paper we demonstrate the existence of two different circulating IgG in chagasic patients which bind with myocardial β_1 and spleen cell β_2 adrenoceptors, acting as non-competitive inhibitors. Both chagasic IgG against β_1 and β_2 adrenoceptor increased intracellular levels of cAMP that could be blocked by specific β_1 and β_2 adrenoceptor antagonists. The specificity for β_1 and β_2 adrenoceptors and the independence of other tissue reactive antibodies was demonstrated by IgG absorption with turkey red blood cell (TRBC), human lymphocytes (HL) or guinea pig red blood cells (GPRBC). The $F(ab')_2$ fraction acted similarly. This supports the specificity of β_1 and β_2 adrenoceptors to the chagasic IgG and the independence of the other tissue reactive antibodies, such as EVI system. The probable pathogenic role of both β_1 and β_2 adrenergic chagasic antibody is discussed.

Keywords anti-adrenoceptor β_1 and β_2 IgG Chagas' disease dihydroalprenolol binding cAMP stimulation

INTRODUCTION

Immunity has long been proposed as a mechanism for the pathogenisis of chronic Chagas' heart disease. The immune hypothesis was advanced when an antibody which reacted with endothelium, vascular and interstitial structures of muscle (EVI-Ab) was discovered in chagasic patients (Cossio *et al.*, 1974).

Studies in humans showed that EVI-Ab was linked to *T. cruzi* infection (Szarfman *et al.*, 1975) and muscle biopsies of individuals infected with *T. cruzi* showed immunoglobulin deposits with a similar distribution (Cossio *et al.*, 1977). Moreover, *T. cruzi* infected patient sera showed autologous reactivity with muscle structure (Cossio *et al.*, 1974; Szarfman *et al.*, 1975), that was abrogated by antibody-absorption with epimastigotes of different strains of *T. cruzi* and by African Trypanosomes (Szarfman *et al.*, 1974), suggesting the possibility of the existence of cross-reactions between parasite antigens and host tissues. In addition, it has been demonstrated that humans and monkeys infected with *T. cruzi* produce antibodies that react with extracellular structures in some areas where basement membranes are present, and that they are specific for laminin (Szarfman *et al.*, 1982). Despite these antibodies, we have already demonstrated the existence of circulating IgG in chagasic patients independent of the EVI-Ab which reacted with the β_1 adrenoceptor of the heart. This antibody is able to bind to β_1 adrenoceptors of myocardium and modulates their activity (Borda *et al.*, 1984; Sterin-Borda *et al.*, 1976, 1981a, 1981b, 1986). On the other hand, we have demonstrated that peripheral chagasic human lymphocytes are active upon contact with cardiac antigens, and they appeared to be in 'active state' before further manipulations (Bracco *et al.*, 1984, 1985).

In this paper, we describe the presence of IgG in the sera of chagasic patients able to interact with lymphocyte β_2 adrenoceptor, that behaves as a β-agonist and induces a modulatory action in the immune system. This antibody was compared with the well known β_1 anti-adrenergic cardiac antibody. Both antibodies are independent of each other and the EVI-Ab (laminin) system. The recognition of both β_1 cardiac and β_2 spleen cell adrenoceptors by the chagasic IgG induces the activation of the adenylate cyclase system. The binding upon β adrenoceptors and the enzymatic effect is exerted by the $F(ab)'_2$ fraction of the chagasic IgG.

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MATERIALS AND METHODS

Serum selection: chagasic patients and controls

Sera were obtained from 11 asymptomatic *T. cruzi* infected individuals and from five normal non-infected individuals. Chagas' serology was studied by three standard serological reactions against *T. cruzi* complement fixation, passive haemagglutination and immunofluorescence (Cossio *et al.*, 1974). EVI reactivity, as assayed by IFI (Sterin-Borda *et al.*, 1976), was

positive in all chagasic sera used. Chronic Chagas' heart disease patients were not included in this study to avoid interference with medications.

Purification of human IgG

IgG was isolated from the sera of chagasic and normal human sera by precipitation with 40% ammonium sulphate and chromatography with DEAE-cellulose (Bio-Rad, Richmond, CA, USA) balanced with 0·005 M 8 pH phosphate buffer. The eluted IgG fractions were concentrated to 10 mg/ml and dialysed against phosphate buffer solution (PBS). IgG fractions showed one line of precipitation corresponding to IgG with polyvalent antisera. Final IgG concentration was determined by radial immunodiffusion assay (Borda *et al.*, 1984).

F(ab)′$_2$ isolation

The isolation of F(ab)′$_2$ from normal and chagasic IgG was done as described by Hudson & Hay (1980). Briefly, normal and chagasic IgG were subjected to pepsin digestion, incubated overnight at 37°C, pH 4·5, centrifuged and the supernatant was adjusted to a pH 7·4 and dialysed overnight with PBS. The dialysate was applied to G-100 Sephadex column. It produced two significant peaks, the first one with pepsin activity and the second one was the F(ab)′$_2$.

Absorption procedures

Human lymphocytes (HL), guinea pig red blood cells (GPRBC) and turkey red blood cells (TRBC) were washed three times with PBS and incubated with heat inactivated normal and chagasic serum in a proportion of 1 ml serum/ 3 ml packed RBC or 20×10^6 HL/ml for 1 h at 37°C and 1 h at 40°C. After absorption, EVI positive chagasic sera were tested by indirect immunofluorescence on heart and skeletal muscle reactions (Cossio *et al.*, 1974) to determine that the absorption with GPRBC had totally removed the EVI reactivity, and that the absorption with TRBC or HL had not modified the EVI pattern or even the sera titres. These different absorptions with HL, GPRBC and TRBC were performed due to the fact that GPRBC are very rich in EVI antigen (Cossio *et al.*, 1974; Khoury *et al.*, 1983) and lack β_1-adrenoceptors, while TRBC are very rich in the latter (Schreiber *et al.*, 1980) and HL have a homogenous population of β_2-adrenoceptors (Williams *et al.*, 1976).

Preparation of purified membranes

Cardiac membranes for identification of β-adrenoceptors were prepared essentially as described by Limas & Limas (1978) and Borda *et al.*, (1984). Briefly, left ventricular tissue from eight rats was mixed in four volumes of cold buffer containing 0·25 M sucrose, 60 mM Tris-HCl (pH 7·4), 10 mM $MgCl_2$ and homogenized twice with Polytron PT-20 at a setting of 3 for 15 s. The homogenates were filtered through four layers of gauze and spun at 700 ***g*** for 15 min, 10,000 ***g*** for 15 min and at 40,000 ***g*** for 30 min. The pellet was resuspended in 2·5 ml of 50 mM Tris-HCl (pH 7·4), 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 Lab) 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. Cells were then centrifuged and the pellet resuspended in 1:4 volumes of 50 mM Tris-HCl (pH 8), 10 mM $MgCl_2$. The suspensions were homogenized twice with Polytron PT-20 at a setting of 3 for 15 s. The homogenates were filtered and spun at 1000 ***g*** for 15 min and the supernatants were centrifuged at 40,000 ***g*** for 30 min. The pellets were resuspended in 2 ml of 50 mM Tris-HCl (pH 7·4), 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 pre-incubated with diffrent dilutions of normal and chagasic IgG or F(ab)′$_2$ (with or without absorptions with HL, GPRBC or TRBC) for 30 min at 30°C in 50 mM Tris-HCl (pH 7·4), 10mM $MgCl_2$. Their membranes were washed twice by centrifugation.

For (-)-^{3}H-dihydroalprenolol binding, 100 μl of membrane suspension and different concentrations of (-)-^{3}H-dihydroalprenolol ((-)-^{3}H-DHA, New England Nuclear Co., s.p. 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), 10mM $MgCl_2$. At the end of the incubation period, 100 μl aliquots were placed into 2 ml of ice-cold buffer and immediately filtered through GF/c glass fibre filters. The filters were washed with 10 ml of cold buffer, dried, added to 10 ml of triton-toluene based scintillation fluid and counted. Non-specific binding was determined by filtering aliquots of membranes incubated in the presence of 10^{-5} M ± propranolol not exceeding 25% of the specific binding.

Results are expressed as fmol of (-)-^{3}H-DHA specificity bound per mg of protein. Normal human IgG before and after absorption with GPRBC, TRBC or HL was used as control.

Cyclic AMP assay

Rat heart and spleen cells were excised immediately *post mortem*, prepared as described previously (Pascual *et al.*, 1986); Perez Leiros & Borda 1986) and suspended in 3 ml of Krebs-Ringer-Bicarbonate (KRB) solution gassed with 5% CO_2 in O_2 at 30°C with normal IgG and F(ab)′$_2$ (5×10^{-7} M) chagasic IgG and F(ab)′$_2$ (5×10^{-7} M) or alone (controls) for different times (indicated in results) in order to find the best incubation time that gave maximum cAMP levels in our system. We also analysed the effect of different amounts of normal and chagasic IgG, and normal and chagasic F(ab)′$_2$ on cAMP levels obtained at best incubation time, as well as the action of a 15 min incubation with the β-adrenoceptor blocker and (-)-propranolol (1×10^{-7} M). IgG and F(ab)′$_2$ effects on cAMP levels were compared to those obtained at the best incubation time with the β-agonist isoproterenol (5×10^{-9}M). 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 (TCA) and centrifuged at 2,500 ***g*** for 15 min at 4°C. Proteins were determined by the method of Lowry *et al.*, (1951) after dissolving the pellet in 1 ml of 1 M NaOH in boiling water. The TCA 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 of 100 μl were taken for nucleotide determination using a radio immunoassay procedure by a cyclic AMP-[^{125}I]-RIA KIT (New England Nuclear).

Drugs

Freshly prepared solutions of L-propranolol (Ayerst Lab.) and

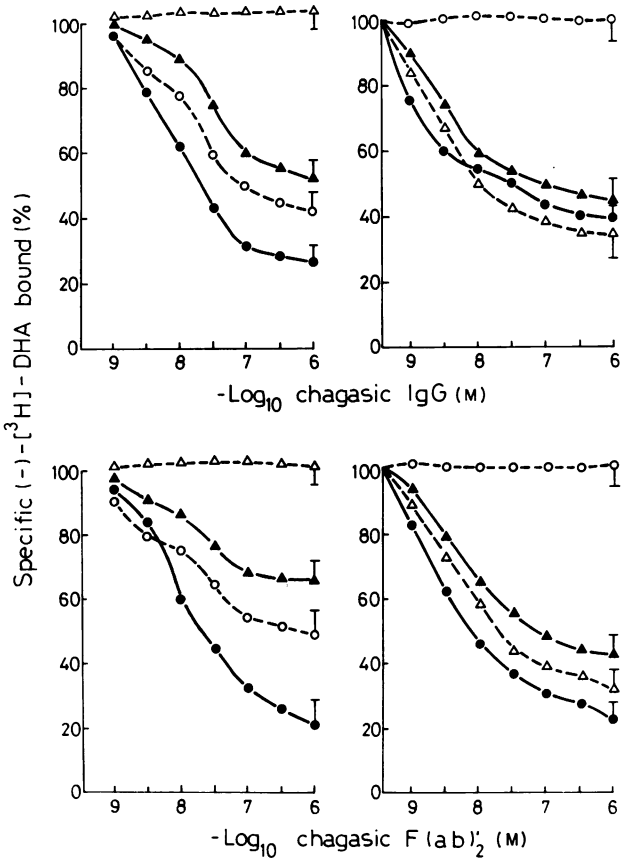

Fig. 1. Effect of chagasic IgG (upper panel) and the corresponding F(ab)'$_2$ (lower panel) on (-)-^{3}H-DHA binding to β adrenergic cardiac receptors (left) and spleen cell receptors (right). Tissues were incubated with increasing concentrations of non-absorbed (▲——▲); absorbed with GPRBC (●——●); absorbed with TRBC (△- - -△) and absorbed with HL (○- - -○). Chagasic IgG or the correspondent F(ab)'$_2$ were then assayed as described in Materials and Methods. Control binding of 100% refers to the radioactivity bound to normal IgG and F(ab)'$_2$ non-absorbed or absorbed with GPRBC, TRBC or HL. Mean of six independent chagasic patients are plotted.

isoproterenol (Sigma Chemical Co) were used. All concentrations quoted in the text represent the final values in the bath solution.

Statistical analysis

Student's *t*-test for unpaired values was used to determine the levels of significance. Differences between means were considered significant if $P \leq 0.05$.

RESULTS

Competition binding assay

Figure 1 shows a concentration-dependent inhibition of (-)-(^{3}H)-DHA binding to β cardiac (Panel a) and β spleen cells (panel b) adrenoceptors by chagasic IgG (upper) and the corresponding F(ab)'$_2$ (lower). It can be seen that chagasic IgG, either non-absorbed or GPRBC-absorbed, were able to inhibit specific binding in cardiac and spleen cell membranes. When membranes were pre-incubated with different concentrations of chagasic IgG absorbed with TRBC there was no inhibitory effect on cardiac membrane, whereas it persisted upon spleen cell membranes. By contrast, when membranes were pre-incubated with different concentrations of chagasic IgG absorbed with HL, the inhibitory effect upon spleen cells was abolished, but that on cardiac membrane was unaffected (Fig. 1 upper panel). F(ab)'$_2$ treated chagasic IgG behaves as the corresponding IgG (Fig. 1 lower panel). Normal IgG or normal F(ab)'$_2$ used at the same concentration as indicated for chagasic IgG, whether un-absorbed or absorbed with GPRBC, TRBC or HL had no effect.

Table 1. Chagasic IgG inhibit (-)-^{3}H-DHA binding on β adrenergic cardiac and spleen cell receptors

Assay performed with IgG	(-)-^{3}H-DHA binding			
	Heart		Spleen cells	
	K_d	B_{max}	K_d	B_{max}
none	3·4±0·3	210±8	1·70±0·12	25·2±2·5
normal	3·6±0·4	206±9	1·64±0·18	24·5±2·0
chagasic non-absorbed	4·1±0·3	101±6*	1·75±0·11	11·3±1·2*
chagasic absorbed GPRBC	4·6±0·6	64±3*	1·68±0·13	12·0±1·4*
chagasic absorbed TRBC	3·5±0·2	215±15	1·77±0·12	11·7±1·4*
chagasic absorbed HL	3·9±0·5	87±7*	1·82±0·20	23·9±1·7

Spleen cells or cardiac membranes were pre-incubated for 30 min at 30°C with 5×10^{-7} M normal or chagasic IgG, with or without absorptions and incubated with (-)-^{3}H-DHA as described in Materials and Methods. B_{max} and K_d values were calculated from linear regression analysis. K_d is expressed in nM and B_{max} in fmol/mg protein. Statistical analysis was performed following Sagripanti *et al.* (1984).
* Different significantly from normal IgG, $P < 0.001$.

In saturation studies (Table 1) non-competitive interaction could be established even in cardiac or in spleen cell membranes. The binding of (-)-^{3}H-DHA occurred to a single class of non-cooperative binding site (B_{max}) of cardiac membrane (200±8 mol/mg protein) and B_{max} of spleen cell membrane (24·2±2·1 mol/mg protein), with an equilibrium dissociation constant (K_d) of cardiac membrane (3·5±0·4 nM) and spleen cell membranes (1·65±0·1 nM) The membranes of spleen cells pre-treated with normal IgG un-absorberd or absorbed with GPRBC, TRBC or HL affected neither the K_d (1·68±0·1 nM) nor the available number of binding sites (25·4±2·8 fmol/mg protein). This was also true for samples from cardiac membranes (K_d: 3·8±0·7 nM; B_{max}: 218±10 fmol/mg protein).

As shown in Table 1, both cardiac and spleen cell membranes pre-incubated with chagasic IgG non-absorbed or absorbed with GPRBC led to inhibition essentially due to a decrease in the number of binding sites, with no significant change in the K_d. When chagasic IgG were previously absorbed with TRBC the inhibitory effect upon cardiac membranes was abolished, while on spleen cell membranes it was unaffected. By contrast, when chagasic IgG were absorbed with HL, they lacked the inhibitory effect on spleen cell membranes, while on cardiac membrane this was not altered.

Chagasic IgG action on intracellular cyclic AMP levels

In order to evaluate one intracellular signal trigger by the

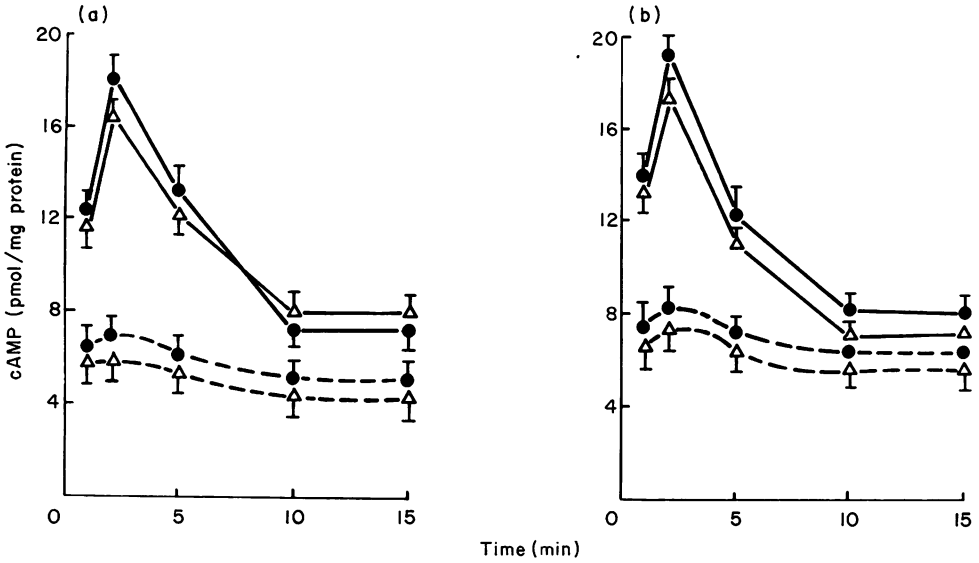

Fig. 2. Time course of cAMP effect of chagasic IgG (●——●) and F(ab)′$_2$ (△——△) on cardiac (a) and spleen cell (b) homogenates. Values are means ± s.e.m. of five separate experiments performed in duplicate. Pre-incubation of tissues with 10^{-7} M propranolol prevents the chagasic IgG (●- - -●) and F(ab)′$_2$ (△- - -△) cAMP increase.

Table 2. Responsiveness of cAMP levels of heart and spleen cells

Additions	cAMP (pmol/mg protein)	
	Heart	Spleen cells
none	6·3 ± 0·1	8·5 ± 0·2
isoproterenol	19·1 ± 0·4*	20·1 ± 0·5*
chagasic IgG non-absorbed	18·4 ± 0·3*	19·8 ± 0·3*
chagasic IgG absorbed TRBC	6·5 ± 0·2	18·8 ± 0·2*
chagasic IgG absorbed HL	18·7 ± 0·4*	8·7 ± 0·3
chagasic IgG absorbed GPRBC	19·0 ± 0·5*	21·3 ± 0·5*
normal IgG	6·7 ± 0·3*	9·1 ± 0·4
normal F(ab)′$_2$	6·9 ± 0·4	8·7 ± 0·3

Values are means ± s.e.m. of five separate experiments performed in duplicate. Values were measured in tissue homogenates after 2 min of reaction with 5×10^{-7} M normal or chagasic IgG or with 5×10^{-9} M isoproterenol.

*$P < 0{\cdot}001$ between none vs. isoproterenol or chagasic IgG.

specific recognition between chagasic IgG and β adrenergic receptors, the action of chagasic IgG and the corresponding F(ab)′$_2$ on intracellular cAMP levels in cardiac and spleen cells was explored. First we measured cAMP levels in cardiac homogenates and spleen cells alone or pre-incubated with propranolol (10^{-7} M) and then exposed to 5×10^{-7} M chagasic IgG and F(ab)′$_2$ for different exposition times.

As shown in Fig. 2 there was a significant increase of cAMP intracellular levels, with a peak at 2 minutes, then these levels decreased when the exposition time to chagasic IgG or F(ab)′$_2$ was longer, The cAMP effect of the chagasic antibody was blunted by propranolol (10^{-7} M) (Fig. 2.), confirming that the stimulatory effects of chagasic IgG and the F(ab)′$_2$ were mediated predominantly by a β-adrenergic mechanism.

The increment induced by a 2 min contact with chagasic IgG (5×10^{-7} M) was similar to that observed with 5×10^{-9} M isoproterenol in both cardiac and spleen celll preparations (Table 2). When chagasic IgG was absorbed with GPRBC, the stimulatory effect persisted in both tissue preparations, but when the IgG was absorbed with TRBC the effect was abolished on the heart but was not affected on spleen cells. By contrast, when the chagasic IgG was absorbed with HL the stimulatory effect upon cAMP spleen cell preparations was prevented, while the effect on cardiac cAMP was not modified. Normal IgG or normal F(ab)′$_2$ samples treated as chagasic did not increase the intracellular levels of cAMP above basal values.

It is important to note that butoxamine (1×10^{-6} M) inhibited chagasic IgG increase in spleen cell cAMP levels (9·1 ± 0·4 pmol/mg protein) while practolol (1×10^{-6} M) blunted it on myocardium (7·2 ± 0·2 pmol/mg protein).

DISCUSSION

The present results demonstrate the existence of two different circulating IgG in chagasic patients which reacted with β_1 or β_2 adrenoceptor-rich tissues. This is based on the observation that chagasic IgG inhibited the binding of (-)-^{3}H-DHA to the β_1 adrenoceptors on purified myocardial and β_2 adrenoceptors of spleen cell membranes, by acting as a non-competitive inhibitor, and that chagasic IgG from the same patients increased cAMP levels on both cardiac and spleen cell preparations. This stimulatory action could be blocked by specific β_1 and β_2 adrenoceptor antagonist. Furthermore, the binding and the response on the adenylate cyclase activity of chagasic IgG with myocardial β_1 adrenoceptors could be significantly diminished by absorption with TRBC, a cell rich in β_1 adrenoceptors, whereas they still persist when the IgG were absorbed with HL, a cell rich in β_2 adrenoceptors.

By contrast, the binding and the biological reactivity of chagasic IgG with spleen cell adrenoceptors could be abolished by absorption with HL, but was unaffected when the same IgG

were absorbed with TRBC. This together with the inability to remove β adrenergic reactivity on spleen cells and myocardium with GPRBC, which lacks β adrenoceptors, and with high concentrations of EVI antigen supports two conclusions. Firstly the specificity of the chagasic IgG to cardiac β_1 and spleen cell β_2 adrenoceptors and secondly the independence of the β adrenoceptor specificity of the IgG in relation to the EVI system. Thus, the specific antibodies for the β_1 and β_2 adrenoceptors are independent of each other and the other tissue reactive antibodies, such as the EVI system. It is well known that myocardium is rich in β_1 adrenergic receptors while in spleen cells β_2 adrenoceptors are predominant (Lands *et al.*, 1967; Minneman, Hedberg & Molinoff, 1979; Borda, Perez Leiros & Sterin-Borda, 1986; Perez Leiros & Borda, 1986).

The specificity of β_1 and β_2 adrenoceptor reactivity is assessed by the fact that F(ab)'$_2$ of the chagasic IgG is responsible for the binding of β adrenoceptors and the stimulation of the adenylate cyclase activity. Normal IgG and the corresponding F(ab)'$_2$ were unaffected in the systems studied.

On the present evidence it is hardly possible to define the pathogenic role of β adrenoceptor reactivity of chagasic IgG. However, it is tempting to speculate that these findings may explain the fact that the chagasic patient behaves as a natural β-blocked responder (Palmero, Caeiro & Iosa, 1979). Furthermore, we have already described that chagasic IgG which bind to β_1 adrenoceptors of myocardium interact with the membrane bound receptor-cyclase complex, inducing the stimulation of adenylate cyclase activity (Borda *et al.*, 1984; Sterin-Borda *et al.*, 1986), with a stimulation of Ca^{2+}-ATPase and inhibition of $Na^+ + K^+$-ATPase activity (Pascual *et al.*, 1986; Pascual, Borda & Sterin-Borda, 1987). The myocardial enzymatic effects of the chagasic IgG were prevented after β adrenoceptor blockade. These findings may involve β_1 adrenoceptor recognition by chagasic IgG in the genesis of cardiac damage observed in Chagas' heart disease.

In relation to the pathogenic role of β_2 adrenergic chagasic antibody, it is possible to speculate that the interaction of the IgG with lymphocytic β_2 adrenoceptors is an early step of activation represented by the increase in cAMP levels,. This phenomenon is of particular interest because peripheral blood lymphocytes of chagasic patients appear to be in an 'active state'. They do not require further activation of mitogen lectins to be active, and react in a similar way to mitogenic lectin-activated normal lymphocytes (Sterin-Borda, *et al.*, 1983; Bracco *et al.*, 1984, 1985, 1986).

We propose that the specific recognition of F(ab)'$_2$ chagasic IgG upon β_2 adrenoceptors of lymphocytes could induce, behaving as a β agonist, an immunosuppressor action on lymphocyte β_2 adrenoceptor-rich cells, modulating the immune system.

The data, taken together, indicate that chronic *T. cruzi* infection generates reactive antibodies against myocardial β_1 and lymphocytic β_2 adrenoceptors. Whether these immune phenomena are produced by the existence of cross-reacting epitopes between hosts and parasites or whether these phenomena are the consequence of tissue damage induced by the parasite infection still remains an open question.

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