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A circulating IgG in Chagas' disease which binds to β-adrenoceptors of myocardium and modulates their activity

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SUMMARY

It has been shown that sera from chagasic patients with positive EVI serology could act in co-operation with complement or normal human lymphocytes as a partial β-adrenoceptor agonist increasing the contractile tension and frequency of isolated rat atria, as occurs with IgG purified from chagasic serum. In this paper we demonstrate that IgG present in chagasic patients sera could bind to the β-adrenoceptors of the heart and stimulate contractile activity of myocardium. The positive inotropic and chronotropic effect could be blocked by the specific β_1-adrenoceptor antagonist but not by the β_2-adrenoceptor antagonist. Chagasic IgG inhibited the binding of (−) ^{3}H-DHA to β-adrenoceptors of purified rat myocardial membranes behaving as non-competitive inhibitors. The reactivity of chagasic serum or IgG with β_1-adrenoceptor was lost after absorptions with turkey red blood cells. In contrast, guinea-pig red blood cells were unable to remove the β_1 reactivity of chagasic serum or chagasic IgG. This supports the specificity of β_1-adrenoceptors of the chagasic IgG and the independence of β_1-adrenoceptor reactivity in relation to the EVI system. Clinical specificity of the β_1-adrenoceptor reactivity seems rather high in Chagas' disease since it was lacking in 14 individuals with other cardiopathies, such as ischaemic and rheumatic heart disease, even after heart surgery.

Keywords anti-adrenoceptor IgG Chagas' disease myocardium activity dihydroalprenolol binding

INTRODUCTION

Evidence accumulated over the last decade, concerning humans and experimental models, suggests an immunopathological mechanism may be involved in the pathogenesis of chronic Chagas' heart disease (Cossio *et al.*, 1980).

We have already reported that EVI chagasic sera stimulates the *in vitro* isolated rat atrial preparation, increasing tension and frequency. Data from those experiments pointed to an interaction between a serum factor with β-adrenoceptors. IgG from the reactive sera, carrying the EVI specificity, were active in this model and we suggested that this specificity was responsible for the stimulatory effect (Sterin-Borda *et al.*, 1976, 1981a, 1981b, 1982; Gimeno *et al.*, 1979).

In a recent report, adequate proof was presented supporting EVI specificity directed toward laminin, a contention not involving direct β-adrenoceptor reactivity (Szarfman *et al.*, 1982). In addition, recent studies strongly suggest that the EVI antibody is heterophil in nature (Khoury *et al.*, 1983), unable to participate through a direct mechanism in the pathogenesis of chronic Chagas' heart disease.

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In the present study, evidence is presented showing that IgG from chagasic sera reacts with β-adrenoceptors and this reactivity is independent of the EVI (laminin) system.

MATERIALS AND METHODS

Serum selection: chagasic patients and controls. Sera were obtained from 15 asymptomatic *Trypanosome cruzi* infected individuals residing at the time in metropolitan Buenos Aires and from three 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 was assayed by indirect immunofluorescence with fluorescein labelled rabbit F $(ab')_2$ anti-human IgG (Cappel Laboratories, Cochranville, Pennsylvania, USA) (Sterin-Borda *et al.*, 1976). Chronic Chagas' heart disease patients were not included in this study to avoid interference with medications. Besides congestive heart failure cases were also excluded as non-specific alterations influencing the immunopharmacology studies, might be included.

As controls, 15 serum samples from patients with other heart diseases were included. Diagnosis of these cases were: ischaemic heart disease (eight); rheumatic heart disease (four); aortic regurgitation due to Marfan syndrome (one); interatrial septal defect (one). Out of these cases, 10 serum samples were studied pre-heart surgery . Five of these samples presented circulating heart reactive antibodies as detected by indirect immunofluorescence with the well known pattern described for these diseases.

Purification of human IgG. IgG was isolated from the sera of seven EVI positive chagasic patients and three normal human sera by precipitation with 40% ammonium sulphate and chromatography with DEAE-cellulose (Bio-Rad, Richmond, California, USA) balanced with 0·005 M, pH 8 phosphate buffer. The eluted IgG fractions were concentrated and dialysed against PBS. IgG fractions showed one line of precipitation, corresponding to IgG with polyvalent antisera. Final IgG concentration was determined by radial immunodiffusion assay.

Absorption procedures. Guinea-pig and turkey red blood cells (GP-RBC or T-RBC) 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 for 1 h at 37°C and 1 h 4°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 GP-RBC had totally removed the EVI reactivity, and the absorption with T-RBC had not modified the EVI pattern, or even the sera titres.

These different absorptions with GP- and T-RBC were performed due to the fact that GP-RBC are very rich in EVI antigen (Cossio *et al.*, 1974; Khoury *et al.*, 1983), and lack β_1-adrenoceptors while T-RBC are very rich in the latter (Schreiber *et al.*, 1980). In addition, pilot absorption studies were able to demonstrate that T-RBC totally failed to absorb the EVI pattern.

The chagasic serum samples were divided in two groups: eight cases were used for the whole serum studies and seven cases for the studies with purified IgG.

IgG referred to in Results as absorbed with GP-RBC and T-RBC was obtained from absorbed sera and further controlled by indirect immunofluorescence over myocardium and skeletal muscle.

Isolated rat atrial preparations. Male albino rats of the Wistar strain were sacrificed by decapitation. The atria were separated from ventricles, carefully dissected, attached to a glass holder and immersed in a tissue chamber containing the various dilutions of sera and/or IgG in modified Krebs–Ringer–bicarbonate (KRB) solution (Sterin-Borda *et al.*, 1976). A constant resting tension of 750 mg was applied to the atria and the activity of spontaneously beating atria was anlaysed in terms of tension (T) (mg) and frequency of contractions (FC) (number of contractile cycles per minute). The atria were allowed to function for 150 min before the reaction. Records were then taken forthwith and the values of these initial controls were considered as 100%. The given serum or the IgG was studied both with and without fresh guinea-pig serum as a complement system. The values of T and FC of beating atria immersed in KRB or KRB containing heat-inactivated normal or chagasic sera in the absence of complement were similar to the initial

control values (T = 450–510 mg; FC = 127–135 beats/min). Concentration–response curves were carried out by the method of Van Rossum (1963). The time interval between concentrations was chosen to produce a maximal effect sustained for at least 3 min for every individual dose. This period averaged 20 min.

Preparation of purified membranes. Cardiac membranes for identification of β-adrenoceptors were prepared essentially as described by Limas & Limas (1978). Briefly, left ventricular tissues 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 was homogenized with Polytron PT-20 at a setting of 3 for 15 s, twice. The homogenates were filtered through four layers of gauze and spun at 700*g* for 15 min. The supernatant was centrifuged 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 $MgCl_2$.

Membrane suspensions (3–5 mg/ml protein) were pre-incubated with $2{\cdot}8 \times 10^{-7}$ M dilutions of normal and chagasic IgG (with or without absorptions with GP- or T-RBC) for 1 h at 30°C in 50 mM Tris-HCl buffer (pH 7·4), 10 mM $MgCl_2$. 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 Company, sp. act. 51·4 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 8·0), 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 GF/C glass fibre filtres; the filtres 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 guinea pig or turkey erythrocytes, was used as control.

Drugs. Freshly prepared solutions of the following drugs were used: butoxamine (Burroughs Welcome Co); practolol (Ayerst Laboratory) and *p*-oxy-prenolol (Ayerst Laboratory). All concentrations quoted in the text represent the final values in the bath solution.

Statistical analysis. The Student's *t*-test for unpaired values was used to determine the levels of significance. Differences between means were considered significant if $P \leqslant 0{\cdot}05$.

RESULTS

Effects of chagasic sera on isolated rat atria and the modification after absorption with GP- and T-RBC

As seen in Table 1, the addition of EVI (+) chagasic sera to the rat atrial preparation, resulted in a positive inotropic and chronotropic effect. These results are consistent with reported studies (Sterin-Borda *et al.*, 1976, 1982). When EVI (+) chagasic sera were previously absorbed with GP-RBC both effects were enhanced, whereas when absorbed with T-RBC the positive inotropic and chronotropic effect was abolished, and even a depression could be observed.

Three normal human sera (NHS) treated likewise to chagasic sera failed to show this effect as reported previously (Sterin-Borda *et al.*, 1976, 1982); as also did samples from patients with other heart diseases (Table 1).

A typical dose–response curve for different concentrations of chagasic sera absorbed with GP-RBC on the inotropic and chronotropic effect of rat atria were studied. It can be observed that the biological effect was dependent upon the concentration of serum. These biological effects were also assayed after pre-incubation of the rat atria with different concentrations of practolol; at concentrations of 10^{-8}–10^{-7} M, this drug completely inhibited the stimulatory effect (Fig. 1). In addition, *p*-oxy-prenolol acted similarly (data not shown).

Studies with purified IgG

To ascertain that IgG rather than other serum proteins were responsible for the observed biological

Table 1. Comparative action between chagasic sera, normal human sera and sera from non-chagasic cardiomyopathies

Sera		Controls* T	Controls* FC	Non-absorbed T†	Non-absorbed FC†	Absorbed GP-RBC T‡	Absorbed GP-RBC FC‡	Absorbed T-RBC T§	Absorbed T-RBC FC§
Chagasic	(a)	420±14·6	173±4·2	562±22·4	197±8·3	652±30·2	222±12·9	366±21·9	151±3·9
	(b)	360–480	160–190	500–680	190–240	550–770	190–280	300–460	140–170
		(*n*=8)		(*n*=8)		(*n*=8)		(*n*=8)	
Normal	(a)	432±15·2	171±4·0	422±13·2	168±3·9	445±7·3	172±6·2	437±13·2	170±3·9
		(*n*=3)		(*n*=3)		(*n*=3)		(*n*=3)	
Cardiomyopathies Non chagasic	(a)	421±10·2	169±5·1	426±12·3	170±4·3	—	—	—	—
		(*n*=15)		(*n*=15)					

* Pre-addition of serum. Tension (T) and frequency of contractions (FC) are expressed in mg and as number of beats per min, respectively. (a) mean values ± s.e. (b) Range.

Between T or FC Controls and T† $p < 0{\cdot}001$; FC† $< 0{\cdot}02$; T‡ $< 0{\cdot}001$; FC‡ $< 0{\cdot}01$; T‡ $< 0{\cdot}05$ and FC§ $< 0{\cdot}01$.

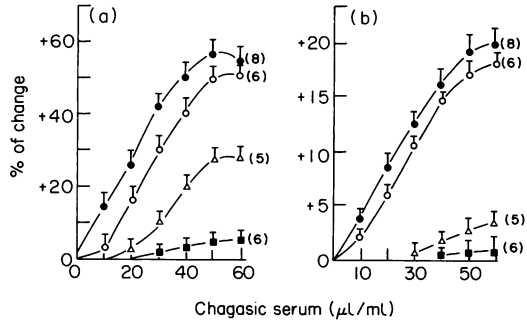

Fig. 1. Dose–response curves of chagasic sera absorbed with GP-RBC. Dilutions of sera from eight different patients were treated for 20 min with isolated rat atria suspended in KRB (●——●) or in KRB containing practolol 10^{-7} M (■——■); 10^{-8} M (Δ——Δ) and 10^{-9} M (○——○). Changes in (a) tension and (b) frequency are expressed as a percentage of variation from initial controls. Points represents mean values. Vertical bars ± s.e. The number of experiments is given in parenthesis.

effect. IgG from normal (N) or chagasic (Ch) sera was obtained. In both studied without any treatment (N-IgG; Ch-IgG) and after absorption with GP-RBC (N-IgG-GP; Ch-IgG-GP) or T-RBC (N-IgG-T; Ch-IgG-T), the IgG was tested for EVI reactivity before and after absorption in order to ascertain that this specificity was completely removed by GP-RBC but unaffected by T-RBC when present.

Normal IgG, whether unabsorbed or absorbed with GP-RBC or T-RBC had no effect either with or without complement. The employment of Ch-IgG unabsorbed or absorbed with GP-RBC increased both tension and frequency of rat atria. It should be noted that both tension and frequency were significantly greater with Ch-IgG-GP than with Ch-IgG. The above mentioned biological effect was dependent upon the concentration of IgG (Fig. 2). In contrast when the same IgG was absorbed with T-RBC the positive inotropic and chronotropic effects were not observed.

Practolol, *p*-oxy-prenolol and butoxamine were used as β_1 and β_2 adrenoceptor blockers (Levy, 1966; Wasserman & Levy, 1972; Dunlop & Shanks, 1968) to determine whether the atria stimulation by Ch-IgG-GP was triggered through a β_1 or β_2 adrenergic mechanism. As shown in Fig. 3, *p*-oxy-prenolol was an effective blocker at concentrations at which butoxamine had no action (10^{-7} M). None of the drugs had any action *per se* (Table 2). Furthermore, practolol exhibited the same effect as *p*-oxy-prenolol (data not shown).

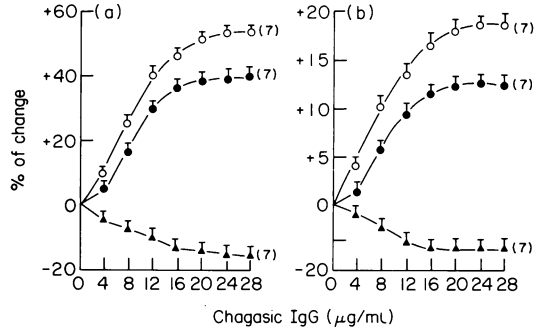

Fig. 2. Effects of different concentrations of chagasic IgG before (●——●) or after absorption with GP-RBC (○——○) or T-RBC (▲——▲). Different concentrations of chagasic IgG were treated for 20 min with isolated rat atria. Means values ± s.e. from seven different patients is represented. Other details as for Fig. 1. (a) Tension change and (b) frequency change.

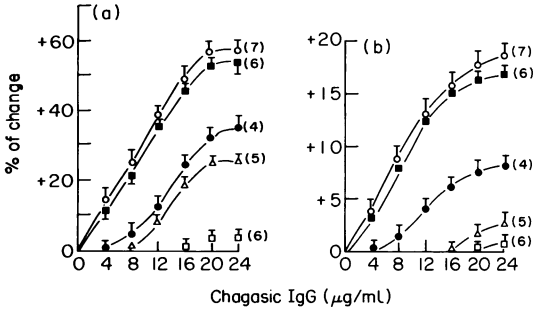

Fig. 3. Dose–response curves of chagasic IgG absorbed with GP-RBC. Different concentrations of chagasic IgG were treated for 20 min with isolated rat atria preparation suspended in KRB (○——○) or in KRB containing butoxamine 10^{-7} M (■——■) or *p*-oxy-prenolol 10^{-9} M (●——●), 10^{-8} M (△——△) and 10^{-7} M (□——□). Other details as for Fig. 1. (a) Tension change and (b) frequency change.

Table 2. Effect of adrenoceptor blocker drugs on the tension and frequency of isolated rat atria

Drug added	Tension*	Frequency†	*n*
None	432 ± 5·2	170 ± 5·2	6
Practolol (10^{-7} M)	425 ± 7·3	168 ± 4·3	6
p-oxy-prenolol (10^{-7} M)	412 ± 14·5	175 ± 8·3	6
Butoxamine (10^{-7} M)	425 ± 13·2	166 ± 3·2	6

Mean values ± s.e. *Tension expressed in mg. †Frequency of contractions expressed as number of beats/min.

Competition binding assay

As in Fig. 4, there was a dose-dependent inhibition of (−) ^{3}H-DHA binding to cardiac membranes when they were pre-incubated with different concentrations of Ch-IgG or GP-Ch-IgG. On the other hand, pre-incubation of membranes with T-Ch-IgG lacked any inhibitory effect.

As shown in Fig. 5a, GP-Ch-IgG competed with (−) ^{3}H-DHA for β-adrenoceptors from purified cardiac membranes. In agreement with Limas & Limas (1978), in our case binding of (−) ^{3}H-DHA occurred to a single class of non-co-operative binding site (90–120 fmol/mg of protein) with an equilibrium dissociation constant (K_d) of 10·5 ± 0·4 nM.

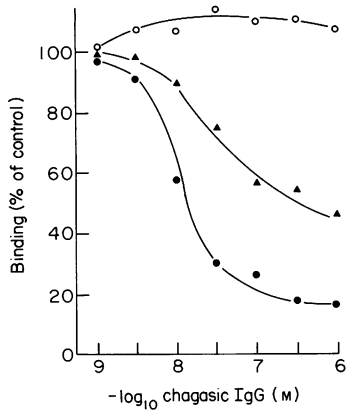

Fig. 4. Inhibition of binding of (−) ^{3}H-DHA by increasing concentrations of non-absorbed chagasic IgG (▲——▲); absorbed with GP-RBC (●——●) and absorbed with T-RBC (○——○). Membranes were pre-incubated with different concentrations of the immunoglobulins at 30°C for 60 min and then with 2·5 nM of (−) ^{3}H-DHA at 37°C for 15 min. Control binding of 100% refers to the radioactivity bound to normal IgG non-absorbed or absorbed with GP-RBC or T-RBC. Mean of five independent chagasic patients are plotted.

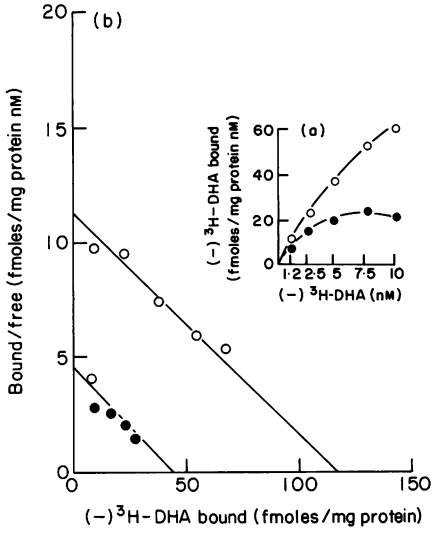

Fig. 5. (A) Inhibition of binding (−) ^{3}H-DHA to β-adrenoceptors of cardiac membranes by chagasic IgG absorbed with GP-RBC. Cardiac membranes were pre-incubated for 60 min at 30°C with 5×10^{-7} M normal IgG (○——○) or 5×10^{-7} M chagasic IgG (●——●) absorbed with GP-RBC and then were assayed for (−) ^{3}H-HDA binding. Values shown are the mean of four experiments. (b) Scatchard plots of the saturation binding data from (a). The lines for normal IgG and chagasic IgG were determined by linear regression analysis. Pre-incubation of the membrane with normal IgG at 5×10^{-7} M (○——○) yielded a total amount of binding sites; B_{max} of 118 fmol/mg of protein and a K_d of 10·6 nM. For the pre-incubation with chagasic IgG at 5×10^7 M (●——●), B_{max} 44 fmol/mg of protein and K_d 10·3 nM.

Pre-incubation of membranes with normal IgG absorbed with GP-RBC affected neither the K_d (10·6 ± 0·5 nM) nor the available number of binding sites (118 ± 9 fmol/mg of protein); whereas pre-incubation with GP-Ch-IgG led to inhibition. Scatchard plots (Fig. 5b) indicate that inhibition is essentially due to a decrease in the number of binding sites (44 ± 2 fmol/mg of protein) with no significant change in the K_d (10·3 ± 0·3 nM).

DISCUSSION

The present results demonstrate the existence of circulating IgG in chagasic patients independent of the EVI system which reacted with the β_1-adrenoceptor of the heart. This is based on the following observations: (a) chagasic sera and chagasic IgG stimulate the tension and the frequency of contraction of the isolated rat atrial preparation, further confirming and extending previous findings (Sterin-Borda *et al.*, 1976, 1981a, 1981b). This action is highly enhanced by the presence of complement or lymphocytes (Sterin-Borda *et al.*, 1982); (b) these inotropic and chronotropic effects could be blocked by specific β_1-adrenoceptor antagonists but not by β_2-adrenoceptor antagonists; (c) chagasic IgG inhibited the binding of (−) ^{3}H-DHA to the β-adrenoceptors on purified rat myocardial membranes: the number of binding sites decreased as IgG concentration became higher whereas the K_d of the still available sites remained unchanged. Thus, it might be concluded that chagasic IgG behaved as a non-competitive inhibitor for the β_1-adrenoceptors.

The reactivity of chagasic IgG with β_1-adrenoceptors could be significantly diminished by absorptions with T-RBC, a cell rich in β_1-adrenoceptors but devoid of EVI antigen. This together with the inability to remove β_1 reactivity with GP-RBC which lacks β_1-adrenoceptors and with high concentrations of EVI antigen, supports two conclusions: (1) the specificity of β_1-adrenoceptors of the chagasic IgG and (2) the independence of the β_1-adrenoceptor reactivity in relation to the EVI system.

Clinical specificity of β_1-adrenoceptor reactivity seems rather high in comparison to American trypanosomiasis: absent in normal human sera (Sterin-Borda *et al.*, 1976, 1982), it was also lacking in 14 individuals with ischaemic and rheumatic heart disease even after heart surgery. In addition, no reactivity of human sera from patients with dilated cardiomyopathies has been found with liver membrane beta adrenoceptors (Komajda *et al.*, 1982).

On the present evidence it is hardly possible to define the pathogenic role of β_1-adrenoceptor reactivity of chagasic IgG. However, it is worthwhile mentioning several studies presenting physiological proof that the chagasic patient behaves as a 'natural β-blocker' responder (Caeiro & Palmero, 1980; Palmero, Caeiro & Sosa, 1979). It is tempting to speculate that these physiological findings might be explained by the reactivity herein described.

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