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Beta-adrenergic effect of antibodies from chagasic patients and normal human lymphocytes on isolated rat atria

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SUMMARY

It was previously shown that fresh sera from chagasic patients that contained antibodies reacting with the plasma membrane of striated muscle and endothelial cells (EVI(+) serum) could act in co-operation with complement as a partial beta-agonist increasing the frequency of contraction of isolated rat atria. This activity was absent in EVI(−) chagasic serum or normal human serum and was lost upon heat-inactivation of EVI(+) serum. Also, IgG purified from EVI(+) serum was virtually devoid of activity. In this report we demonstrate that normal human lymphocytes can collaborate with EVI(+) IgG or heat-inactivated EVI(+) sera and induce both positive ino- and chronotropic effects on isolated rat atria. Depletion of phagocytic mononuclear cells from the effector cell population did not alter its activity, whereas blockade of the receptors for the Fc fragment of IgG with heat-aggregated IgG abrogated the effect. After fractionation of the T and non-T cell populations by sedimentation of E rosette forming cells the activity was present in the non-T cell fraction. The mechanism triggered involved a beta-adrenergic reaction that could be blocked by 10^{-7} M (−)-propanolol and not by inhibitors of prostaglandin synthesis (10^{-6} M indomethacin and $1{\cdot}8 \times 10^{-4}$ M acetyl salicylic acid) or an anti-histamine drug (10^{-6} M pyrilamine). Since positive EVI reactivity and myocardial lympho-mononuclear cell infiltrates are frequent in patients with chronic Chagas' cardiomyopathy, the possibility that they could interact influencing the rhythm and contractile activity of the heart should be taken into account.

INTRODUCTION

An antibody reacting with the plasma membrane of striated muscle and endothelial cells (EVI antibody) has been described in the sera of 96% of the patients with chronic Chagas' cardiomyopathy and in 40% of the asymptomatic individuals infected with *Trypanosoma cruzi* (Cossio *et al.*, 1974a, 1974b; Diez *et al.*, 1976). In previous work, it was also documented that the sera of chagasic patients with positive EVI serology (EVI(+)) became fixed to, and induced functional changes on, isolated rat atrial preparations (Sterin-Borda *et al.*, 1976). It was suggested that EVI(+), in the presence of the complement system increased the influx of calcium and raised the Ca^{2+} content in the tissue through activation of the beta-adrenoceptors (Gimeno *et al.*, 1979; Sterin-Borda *et al.*, 1981a, 1981b).

In this study we have investigated if EVI(+) exerted its effect directly or in co-operation with another effector system capable of collaborating with the antibodies bound to the heart. It will be shown that heat-inactivated EVI(+) sera (HI-EVI(+)) or purified EVI(+) IgG had virtually no

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ino- and chronotropic effects when tested at low concentration. Addition of normal lymphocytes bearing free receptors for the Fc fragment of IgG (Fcγ) on their surface in combination with EVI(+) antibodies resulted in a marked stimulation of the isometric developed tension (IDT) and frequency of concentrations (FC) of the spontaneously beating isolated atria.

MATERIALS AND METHODS

Sera. Sera were obtained from chronic Chagas' patients residing in metropolitan Buenos Aires and from normal non-infected individuals. Chagas' serology was studied by three standard serological reactions against *T. cruzi* (complement fixation, passive haemagglutination and immunofluorescence) at the Instituto de Diagnóstico e Investigación de la Enfermedad de Chagas (INDIECH), Buenos Aires. 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). EVI positive (EVI(+)), EVI negative (EVI−(−)) and normal human sera (NHS) were heat-inactivated at 56°C, 30 min, diluted 1/6 in phosphate-buffered saline (PBS) and stored at −20°C.

IgG purification. IgG was isolated from the pooled heat-inactivated sera of 10 EVI(+) patients with titres above 1/160 or from NHS by precipitation with 50% ammonium sulphate and chromatography on DEAE-cellulose (Bio-Rad, Richmond, California, USA) equilibrated with 0·01 M, pH 8 phosphate buffer. The eluted IgG fractions were concentrated to 8 mg/ml. IgG fractions showed only one line of precipitation corresponding to IgG with polyvalent antisera.

Heat-aggregated IgG (HA-IgG). Normal human IgG (Cohn FII, Sigma Chemical, St Louis, Missouri, USA) was dissolved at 10 mg/ml in PBS and heated for 20 min at 63°C.

Effector cells. Peripheral mononuclear leucocytes (ML) were obtained by Ficoll-Hypaque centrifugation of defibrinated normal human blood (Böyum, 1968). The interphase containing the ML was washed twice with PBS, once with RPMI 1640 tissue culture medium (GIBCO Laboratories, Grand Island, New York, USA) containing 50 μg/ml gentamycin (Schering Corporation, Essex Argentina, Buenos Aires, Argentina), 10 mM HEPES (GIBCO) and 5% heat-inactivated fetal calf serum (GIBCO) (RPMI-FCS). ML contained 90–98% mononuclear cells. To eliminate monocytes, ML ($10–20 \times 10^6$/ml) were incubated at 37°C for 30 min with an equal volume of carbonyl iron (Lymphocyte Separating Reagent, Technicon Instr Corp, Tarrytown, New York). The lymphocyte fraction (L) contained less than 1% monocytes as determined by peroxidase staining. L were washed with RPMI-FCS three times and suspended at 8×10^6/ml in the same medium. When subpopulations of lymphocytes were required, T and non-T cells were separated by rosette formation as described before (de Bracco, Isturiz & Manni, 1976).

To inhibit the activity of cells with receptors for Fcγ, 4×10^6 ML were incubated during 2 hr at 37°C with 3 ml PBS containing 1·5 mg/ml HA-IgG and used in the pharmacological assays. The same concentration of HA-IgG when tested alone had no effect on IDT and FC of rat atria. ML, L, T and non-T lymphocytes were incubated 16–18 hr at 37°C in a 5% CO_2 humidified atmosphere before the reactions. Viability was tested by exclusion of trypan blue and effector cells containing more than 80% viable cells were used.

Isolated rat atrial preparations. Male albino rats of the Wistar strain were sacrificed by decapitation. The atria were separated from the ventricles, carefully dissected, attached to a glass holder and immersed in a tissue chamber containing the different dilutions of sera and/or cells in modified Krebs-Ringer-Bicarbonate solution (KRB) (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 analysed in terms of (1) isometric developed tension (IDT) (mg) (2) frequency of contraction (FC) (number of contractile cycles per minute). The atria were allowed to function for 150 min before the reaction. Records were taken forthwith, and the values of these initial controls were considered as 100%. The magnitude of IDT and FC of beating atria immersed in KRB or KRB containing heat-inactivated normal or chagasic sera in the absence of effector cells were similar to the initial control values (IDT = 460–510 mg; FC: 127–135 beats/min). Concentration–response curves were done according to the method of Van Rossum (1963). The time interval between concentrations was

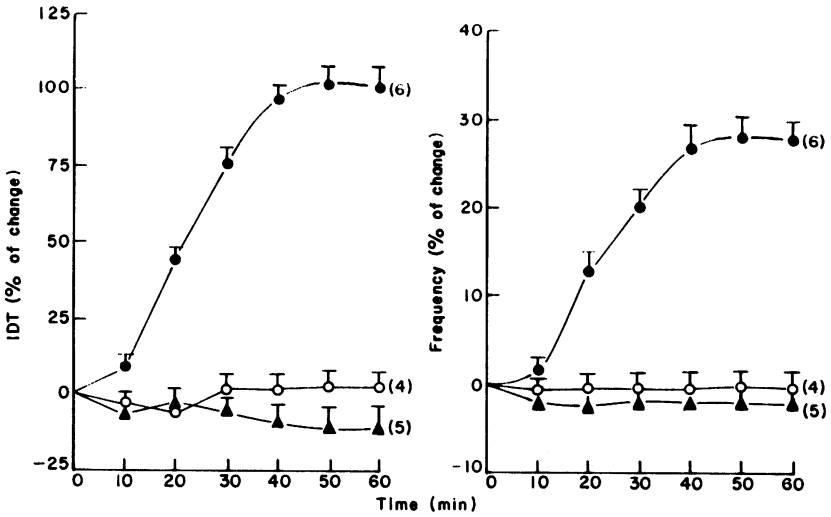

Fig. 1. Time course of the reaction of normal human lymphocytes and heat-inactivated chagasic sera with *in vitro* beating rat atria. Isolated rat atria were suspended in Krebs-Ringer-Bicarbonate solution (KRB) containing 4×10^5/ml normal human mononuclear leucocytes (ML) and 1/120 heat-inactivated EVI(+) (●——●) or EVI(−) (▲——▲) chagasic sera or heat-inactivated normal human sera (○——○). The number of sera of each class that was tested with ML from six different donors is indicated between parentheses. Changes in the isometric developed tension (IDT) or frequency (FC) were measured as described in Methods. The mean ± s.e.m. of IDT and FC at different times of reaction are shown in the graph.

that needed for every individual dose to produce a maximal effect sustained for at least 3 min. This period averaged 40 min (Fig. 1).

After the reaction, the tissue was frozen for immunofluorescence studies or fixed in formalin. Histological studies were performed by Dr J. Barcat of the Pathology Department, Instituto de Investigaciones Médicas.

Drugs. Freshly prepared solutions of the following drugs were used: (−)propanolol HCl 10^{-7} M (Ayerst Laboratory); pyrilamine 10^{-6} M and acetyl salicylic acid (ASA) $1{\cdot}8 \times 10^{-4}$ M (Sigma); indomethacin 10^{-6} M (Merck, Sharp and Dohme). All concentrations quoted in the text represent the final ones in the bath solution.

RESULTS

Effect of normal human mononuclear leucocytes and heat-inactivated Chagas' sera on the tension and frequency of spontaneously beating rat atria

Addition of normal human ML to isolated rat atria beating in KRB that contained HI-EVI(+) increased both the IDT and the FC (Fig. 1). This effect developed with time and was maximal after 40 min of reaction. In contrast, HI-EVI(−) or HI-NHS had no significant ino- or chronotropic effect in the presence of ML. IDT and FC increased to the same extent whether the donor of the EVI(+) serum had Chagas' cardiomyopathy or not.

The increment of IDT and FC of isolated rat atria was dependent on the concentration of effector cells (ML) and of HI-EVI(+) (Figs 2 & 3). In the absence of either reagent the FC and IDT of the isolated atria were similar to those of controls in KRB (Figs 2 & 3). Control and experimental preparations were indistinguishable by light microscopy.

The ability of the IgG fraction to induce changes in the IDT and FC in co-operation with the ML was assayed. The results shown in Fig. 4 demonstrate that the ino- and chronotropic effects of ML depended on the concentration of EVI(+) IgG present in the incubation mixture. In the absence of

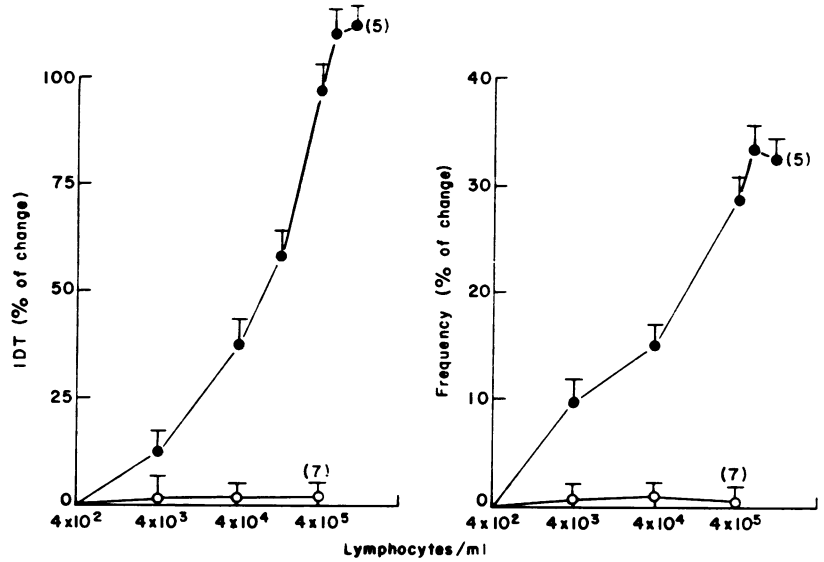

Fig. 2. Effect of different mononuclear leucocyte concentrations on the isometric developed tension and frequency of beating rat atria in the presence of EVI(+) chagasic sera. Different numbers of mononuclear leucocytes (ML) from seven individuals were reacted for 40 min with isolated rat atria suspended in Krebs-Ringer-Bicarbonate solution (KRB) (o——o) or KRB containing 1/120 dilution of heat-inactivated chagasic serum with an EVI titre of 1/640 (HI-EVI(+)) (●——●). Changes in the isometric developed tension (IDT) and frequency (FC) were measured as described in Methods. The mean ± s.e.m. of IDT and FC are shown in the graph and the number of experiments is indicated between parentheses.

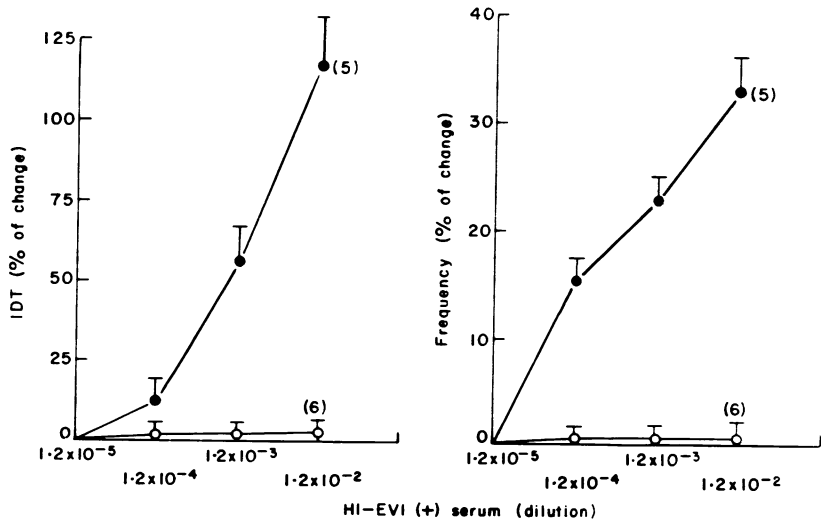

Fig. 3. Effect of different dilutions of heat-inactivated EVI(+) chagasic sera on the isometric developed tension and frequency of rat atria in the presence of normal human leucocytes. Dilutions of heat-inactivated EVI(+) sera from six different patients were reacted for 40 min with isolated rat atria suspended in KRB (o——o) or KRB containing 4×10^5/ml mononuclear leucocytes (●——●). Changes in the isometric developed tension (IDT) and frequency (FC) were measured as described in Methods. The number of experiments is indicated between parentheses.

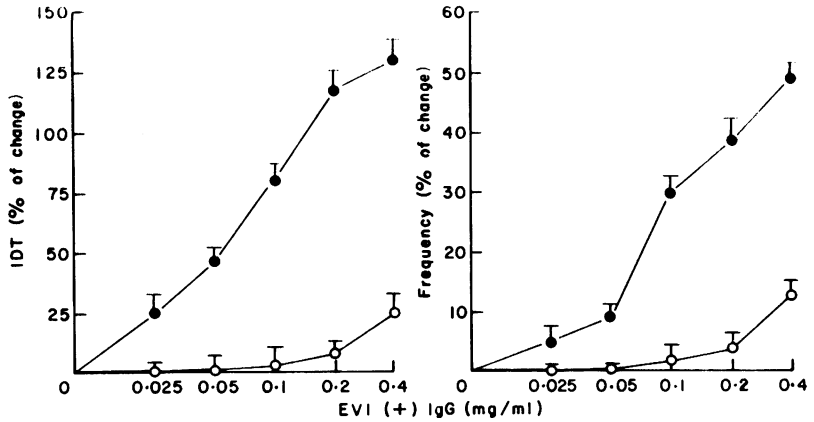

Fig. 4. Effect of different concentrations of EVI(+) IgG on the isometric developed tension and frequency of beating rat atria in the presence of normal leucocytes. Different concentrations of EVI(+) IgG were reacted for 40 min with isolated rat atria suspended in KRB (○——○) or KRB with 4×10^5/ml mononuclear leucocytes (●——●). Changes in IDT and FC were measured as described in Methods. The mean ± s.e.m. of five experiments is represented.

effector cells, EVI(+) IgG had weak ino- and chronotropic effects only at concentrations four-fold above those routinely utilized with whole HI-EVI(+) sera, whereas normal IgG was inactive.

Characterization of the effector cells

To determine which cells in the effector cell preparation were responsible for co-operating with EVI(+) antibodies to increase IDT and FC of the isolated atria, ML were depleted of phagocytic

Table 1. Characterization of the effector cells

Effector cells +HI-EVI(+)	Change (%)	
	IDT	FC
ML	+121·3	+30·1
ML+carbonyl iron	+117·5	+29·3
ML+HA-IgG	2·1	0
T	+ 1·2	0
non-T	+119·7	+31·2

Mononuclear leucocytes (ML) were treated with carbonyl iron or pre-incubated for 2 hr at 37°C with 1·5 mg/ml heat-aggregated IgG (HA-IgG). The T lymphocyte rich (T) and depleted (non-T) populations were prepared as described in Methods. T contained 69% E-RFC while non-T had 13% E-RFC. The percentage of change of IDT and FC was measured after 40 min incubation of the atria with 1/120 heat-inactivated EVI(+) Chagas' serum from a patient with 1/640 EVI titre and 4×10^5/ml effector cells. The IDT or FC were expressed as percentage of change calculated by comparison with the absolute values prior to the addition of the immunological reagents.

Table 2. Effect of different drugs on the isometric developed tension (IDT) and frequency of contraction (FC) of atria beating in control media or in suspending media containing lymphocytes and EVI(+) antibodies

	KRB			KRB + Lymphocytes + HI-EVI(+)		
Drug added	IDT*	FC*	*n*	IDT*	FC*	*n*
None	510±45	130±8	8	1,310±62	227±9	5
Propanolol 10^{-7} M	560±55	128±9	5	570±24	132±7	5
Pyrilamine 10^{-6} M	520±32	135±10	5	1,420±73	229±45	5
Indomethacin 10^{-6} M	490±37	125±7	6	1,298±42	224±8	6
Acetyl salicylic acid $1{\cdot}8 \times 10^{-4}$ M	480±25	132±9	4	1,325±64	218±12	4

* Mean values ± s.e.m. IDT = isometric developed tension expressed in mg; FC = frequency of contractions, expressed as number of beats/min.

cells. The resultant lymphocyte preparation (L) was as active as the original ML (Table 1). Blocking the receptors for Fcγ by pre-incubation with HA-IgG abolished the stimulatory activity (Table 1). The non-T population was active while the T lymphocyte fractions had no effect on the beating rat atria suspended in HI-EVI(+) (Table 1).

Effect of beta-adrenergic antagonists, anti-histamine drugs or inhibitors of prostaglandin synthesis
We investigated if the combined action of lymphocytes and EVI(+) antibodies triggered a beta-adrenergic mechanism. The results shown in Table 2 demonstrate that the stimulatory effects of lymphocytes plus HI-EVI(+) could be blocked by (−) propanolol while the anti-histamine agent or the inhibitors of prostaglandin synthesis had no effect. The drugs did not influence the activity of atria beating in KRB.

DISCUSSION

In the present study we explored the possibility that lymphocytes could be activated by the reaction with antibodies bound to the heart and stimulate the contractile activity of isolated rat atria. Our results demonstrate that normal human lymphocytes can co-operate with antibodies in chagasic sera increasing both the tension and frequency of *in vitro* beating rat atria (Fig. 1). Lymphocytes reacted in a dose-dependent manner and required the presence of HI-EVI(+) or purified EVI(+) IgG (Figs 2, 3 & 4). Fractionation studies indicated that the activity was exerted by non-T lymphocytes (Table 1) and that lymphocyte Fcγ receptors played a central role in the reaction. This was clearly demonstrated by the prevention of stimulation of rat atria by previous blockade of the lymphocyte's Fcγ receptors with HA-IgG (Table 1). The mechanisms whereby Fcγ receptor bearing lymphocytes react are not known. Cytolysis of EVI antibody coated cells in the atrial preparation by the K cells contained in the effector cell fraction (de Bracco *et al.*, 1976) could be a signal for the initiation of the stimulatory process. On the other hand, cross-linking of antibody molecules on the surface of atrial cells after binding to the lymphocyte Fcγ receptors could provide the necessary stimulus.

The immunological reagents acted as beta-adrenergic agonists and could be blocked by the corresponding antagonists and not by inhibitors of prostaglandin synthesis or anti-histamine drugs (Table 2). Thus, normal human Fcγ receptor bearing lymphocytes reacted in this experimental

model simulating the effector function of complement in the beta-adrenergic mechanism triggered by antibodies present in EVI(+) chagasic sera (Sterin-Borda *et al.*, 1976).

The fact that normal lymphocytes can co-operate with antibodies to induce changes in the pharmacological behaviour of living heart tissue may be relevant to the hypothesis that antibody- and cell-mediated immunopathogenic mechanisms could be involved in heart tissue damage during the chronic phase of Chagas' disease (Cossio *et al.*, 1980). However, our results indicate that antibodies that trigger a beta-adrenergic mechanism in collaboration with normal lymphocytes are present in the sera of both asymptomatic *T. cruzi* infected individuals and patients with chronic Chagas' cardiomyopathy. In addition, EVI(+) antibodies react very efficiently *in vitro* with xenogeneic tissue, but it is more difficult to obtain positive reactions with the EVI pattern using homologous tissue (Khoury & Fields, 1980; Cossio *et al.*, 1980). Therefore the reaction may only be of pathogenic relevance in the group of patients in which antibodies reacting with autologous tissue are present (Cossio *et al.*, 1980).

It is conceivable that similar mechanisms could be activated in other clinical conditions in which inflammatory cells and myocardium bound immunoglobulins co-exist: rheumatic fever (Kaplan *et al.*,) or heart transplant rejection (Rosen *et al.*, 1971).

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