

Stimulatory effect of lymphocytes from Chagas' patients on spontaneously beating rat atria

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SUMMARY

The aim of this work was to study the effect of lymphocytes from individuals infected with *Trypanosoma cruzi* (Chagas' patients) on the contractile behaviour of living heart tissue. Chagas' lymphocytes (ChL) reacted with isolated rat atria preparations increasing the isometric development tension (IDT) and frequency of contractions (FC) in a dose-dependent manner. The maximal stimulatory effect was reached after 30–40 min of contact. In contrast, normal lymphocytes (NL) did not alter the basal IDT and FC values. β -adrenergic antagonists, anti-histamine agents and inhibitors of the synthesis and action of arachidonic acid (AA) products were used to study the mechanisms of the reaction. (–)-propranolol (10^{-7} M) and pyrilamine (10^{-6} M) had no effect ruling out the participation of β -adrenergic agonists or histamine. However, indomethacin (10^{-6} M) and acetylsalicylic acid (1.8×10^{-4} M) enhanced the effect of ChL. Inhibitors of the lipooxygenase pathway (5,8,11,14-eicosatetraenoic acid, 10^{-7} M; nordihydroguaiaretic acid, 10^{-5} M) and FPL55712, an antagonist of one of its terminal products: the slow reacting substance of anaphylaxis (SRS-A), abolished the reaction. Therefore, a fundamental role for SRS-A in the production of the stimulatory effect is postulated. Lymphocytes of the T cell lineage (E rosette forming cells, ERFC) are the effector cells involved in this reaction, whereas non-rosetting ChL depressed IDT. To ascertain if effector cells could be replaced by soluble factors, ChL were reacted with homogenates of rat atria and the cell free supernatants were added to beating rat atria. Positive ino- and chronotropic effects were obtained, indicating that soluble factors generated during the reaction can substitute for the intact effector cells. On the other hand if the effector cells were purified from Chagas' patients that had been treated 1 month to 6 years before the assay with trypanocidal drugs (3-methyl-4-(5'-nitrofurfurylidene-amino)-tetrahydro-4H-1, 4-tiazine-1, 1-dioxide, nifurtimox or *N*-benzyl-2-nitro-imidazolacetamide, benznidazole) only depressor effects were found. The depressor inotropic action of lymphocytes from treated patients (tr-ChL) was abolished with indomethacin and acetyl salicylic acid indicating that products of the cyclooxygenase pathway of AA were involved. While this work provides additional evidence for the hypothesis that lymphocytes from *T. cruzi* infected patients may react with heart tissue and alter its contractile behaviour, the results should not be extrapolated to the *in vivo* situation. However, it is important to take into account that whenever an infiltrate of lymphocytes is found in the cardiovascular system, it is possible that the production of AA metabolites occurs, and that these in turn, may alter the physiological behaviour of the tissue.

Keywords Chagas' disease lymphocytes SRS-A inotropic effects

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INTRODUCTION

The presence of mononuclear cell infiltrates is a consistent finding in the heart of Chagas' patients dying with chronic chagasic cardiomyopathy (Cossio *et al.*, 1980). The role of such cells in the genesis of the disease is a matter of controversy (Cossio *et al.*, 1980). We have recently demonstrated that antibodies present in the sera of Chagas' patients (EVI antibodies) added to beating rat atria together with normal Fc receptor bearing lymphocytes can stimulate the contractile behaviour of the isolated rat atria preparations (Sterin-Borda *et al.*, 1982). The mechanism(s) triggered by EVI antibodies plus normal lymphocytes (NL) involves β -adrenergic receptors (Sterin-Borda *et al.*, 1982). In the present study we have investigated if lymphocytes from chronic Chagas' patients (ChL) could alter the contractile activity of beating rat atria. We will show that ChL increase the isometric developed tension (IDT) and frequency of contractions (FC) of isolated rat atria *in vitro* and that this stimulatory effect can be prevented by blockade of the lipoxygenase pathways of arachidonic acid (AA) metabolism. In contrast, lymphocytes from Chagas' patients that underwent treatment with trypanocidal drugs (tr-ChL) inhibit the contractile activity of the rat atria.

MATERIALS AND METHODS

Patients. Patients with chronic Chagas' disease residing in a non-endemic area (Buenos Aires) for more than 1 year were referred by the Department of Cardiology of the Hospital Ramos Mejía, CEMIC or the Institute of Cardiology (Fundación H. Pombo de Rodriguez) of the National Academy of Medicine. On the basis of clinical, electrocardiographic, electrophysiological and pharmacological tests they were classified in two groups: (1) with chronic *Trypanosoma cruzi* infection and no signs of cardiomyopathy ($n=9$) and (2) with chronic Chagas' cardiomyopathy ($n=12$). An additional group of patients had completed a sequence of treatment with trypanocidal drugs 1 month to 6 years before the study. Four patients had been treated with nifurtimox (3-methyl-4-5'-nitrofurfurylidene-amino)-tetrahydro-4H-1,4-thiazine-1, 1-dioxide) and three patients received benznidazole (*N*-benzyl-2-nitro-1-imidazolacetamide).

Effector cells. Mononuclear leucocytes were obtained by Ficoll-Hypaque centrifugation of defibrinated blood (Böyum, 1968) from chagasic patients and normal donors. Monocytes and adherent cells were eliminated by treatment with carbonyl iron (Lymphocyte Separating Reagent, Technicon Inst. Corp. Tarrytown, New York, USA) or adherence (18 h) to plastic surfaces. The resulting lymphocyte preparation (L) contained less than 1% monocytes as determined by peroxidase staining. L were suspended at 8×10^6 /ml in RPMI 1640 tissue culture medium (GIBCO, Grand Island, New York) containing 5% heat-inactivated fetal calf serum (FCS) (GIBCO) and antibiotics (RPMI-FCS). When subpopulations of L were required, T lymphocyte rich (ERFC) and T depleted fractions (non-ERFC) were separated by formation of rosettes with sheep erythrocytes (E) as described before (de Bracco, Isturiz & Manni, 1976). L, ERFC, and non-ERFC were incubated 16–18 h at 37°C in a 5% CO₂ humidified atmosphere before the reactions. Viability was tested by trypan blue exclusion, and effector cells containing more than 80% viable cells were used.

Isolated rat atria 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 10 ml of the different types of effector cells diluted in a 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) and (2) the number of contractile cycles per min (FC). Records were taken after 60 min of equilibration and the values were taken as 100%. In the absence of effector cells IDT was: 460–510 mg and FC was 127–135 beats/min. Concentration–response curves were done according to the method of Van Rossum (1963). The time interval between each concentration of L was that needed to achieve a maximal effect sustained for at least 3 min.

Drugs. Freshly prepared solutions of the following drugs were used: (–)–propranolol HCl

(Ayerst Lab), 10^{-7} M; pyrilamine (Sigma), 10^{-6} M; indomethacin (Merck, Sharp & Dohme), 10^{-6} M; acetylsalicylic acid (Sigma), 1.8×10^{-4} M; nordihydroguaiaretic acid (Sigma) (NDGA), 10^{-5} M; 5,8,11,14 eicosatetraenoic acid (ETYA) (Hoffman LaRoche), 10^{-7} M; FPL 55712 (Fisons Ltd), 10^{-7} M; All concentrations quoted in the text represent the final ones in the bath solution and they did not affect the viability of L or the normal contractile behaviour of the rat atria during the reaction period (Sterin-Borda *et al.*, 1983).

Supernatants of L reacted with atria. Cell free supernatants were obtained by incubation of 5×10^6 ChL or normal lymphocytes (NL) with 0.5 ml of an homogenate of rat atria (RAH) for 90 min at 37°C followed by centrifugation of the reaction mixture 15 min at 800g. Control supernatants of RAH alone or ChL and NL alone were prepared in the same way. Supernatants were kept at -20°C .

RESULTS

Effect of ChL on the contractile activity of rat atria

ChL had a marked stimulatory effect on the tension and frequency of heart beats when compared to NL. This effect was apparent at concentrations above 4×10^4 ChL/ml (final concentration in the tissue chamber) (Fig. 1). When fractionated T lymphocyte rich cell preparations (Ch-ERFC) from Chagas' patients were used, stimulation was significantly higher. Moreover, Chagas' non-rosetting cells (Ch-non-ERFC) moderately depressed the IDT (Figs 1 & 2). Both the stimulatory effect of ChL, Ch-ERFC and the depressor action of Ch-non-ERFC developed gradually reaching a plateau at approximately 30–40 min (Fig. 2). Fig. 3 shows a representative trace of the effect of unfractionated ChL on the frequency and tension of beating rat atria.

Mechanisms of the reaction of ChL with beating rat atria

Several inhibitors acting on different pathways that could result in stimulation of frequency and tension of the rat auricles were used in order to determine the nature of the mechanisms triggered by

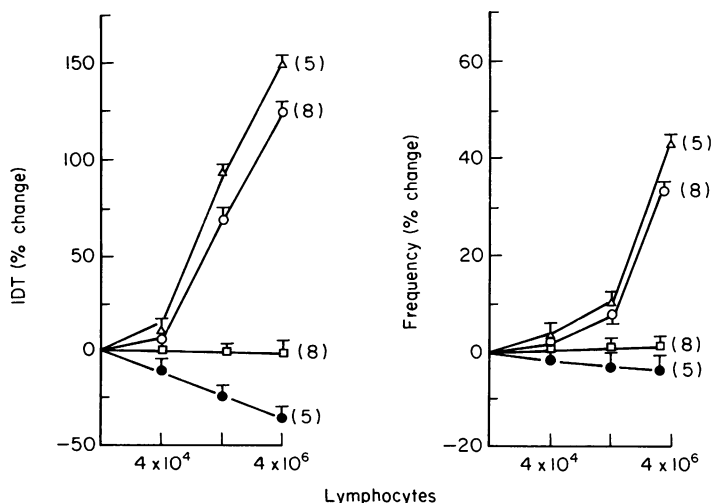


Fig. 1. Comparative effect of different concentrations of lymphocytes from Chagas' patients and normal donors on isolated rat atria. Half a millilitre of a 8×10^6 /ml suspension of unfractionated Chagas' lymphocytes, ChL (○—○); T lymphocyte enriched ChL, Ch-ERFC (△—△); T depleted ChL, Ch-non-ERFC (●—●) and normal lymphocytes, NL (□—□) were reacted with spontaneously beating rat atria immersed in 10 ml KRB as described in Materials and Methods. The time interval between each concentration of L was 20 min. Mean \pm s.e. percentage changes of isometric developed tension (IDT) and frequency (FC) are shown in the ordenates, and the number L added to the tissue chamber are shown in the abscissa. Figures in parentheses represent the number of different ChL, NL or ChL cell fractions tested.

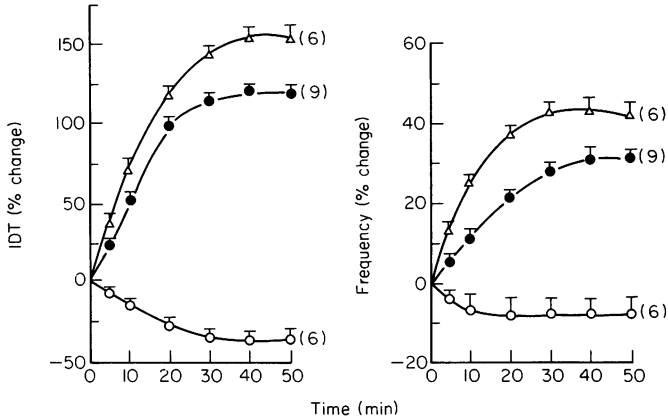


Fig. 2. Time course of the reaction of ChL with beating rat atria. Isolated rat atria were suspended in KRB in the presence of 4×10^5 /ml Ch-ERFC (Δ — Δ); Ch-non-ERFC (O—O) or unfractionated ChL (\bullet — \bullet). Percentage changes in IDT and frequency (Mean \pm s.e.) were calculated for each time as described in Materials and Methods. NL or NL cell fractions did not modify the basal values. The number of ChL or fractionated ChL tested are shown between parentheses.

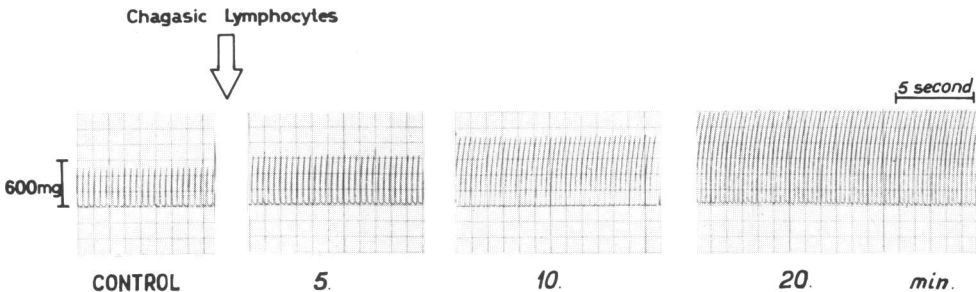


Fig. 3. Effect of the addition of Chagas' lymphocytes upon cardiac function. A representative trace of frequency (FC) and tension of contraction (IDT) of isolated rat atria before and after 5, 10 and 20 min of addition of ChL (4×10^5 /ml, final concentration in the organ bath).

ChL. Stimulation was not prevented by an anti-histamine drug (pyrilamine) or by a β -adrenergic antagonist ([—]—propranolol) (data not shown). On the basis of the results of previous work (Sterin-Borda *et al.*, 1983) with NL that had been activated with mitogens, we suspected that metabolites of AA could be involved in the reaction. Fig. 4 shows that, while inhibitors of the cyclo-oxygenase pathway of AA metabolism (indomethacin and acetylsalicylic acid) increased the IDT of rat atria above the levels produced by ChL, inhibitors of the lipoxygenase enzymes (NDGA and ETYA) abolished the stimulatory effect of ChL on FC and IDT. In addition, FPL 55712, an inhibitor of SRS-A abrogated the positive inotropic and chronotropic effects of ChL (Fig. 4).

Role of soluble factors in the reaction of ChL with isolated rat atria

To investigate if contact between ChL and the beating atria was necessary, we challenged the isolated beating atria with cell free supernatants from ChL that had been incubated previously with homogenates of atrial tissue (RAH). The results shown in Table 1 indicate that soluble factors present in the supernatants of the reaction between unfractionated ChL and rat atria could stimulate the contractility of beating rat atria, although to a lesser extent than intact cells.

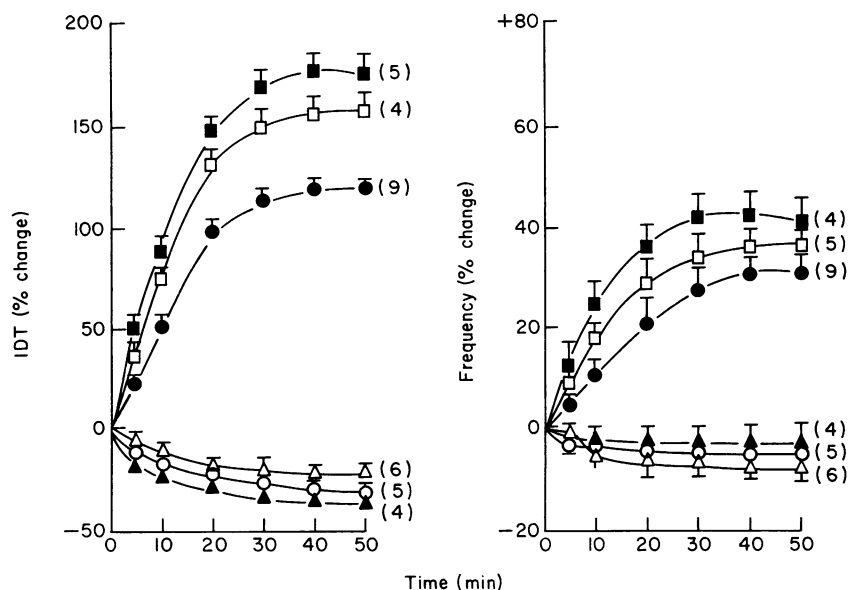


Fig. 4. Effect of inhibitors of the biosynthesis and action of metabolites of arachidonic acid on the stimulatory action of Chagas' lymphocytes. Four hundred thousand per millilitre of unfractionated ChL were reacted with beating rat atria in KRB (●—●) or in KRB containing either 10^{-6} M indomethacin (■—■); 1.8×10^{-4} M acetylsalicylic acid (□—□); 10^{-5} M nordihydroguaiaretic acid (Δ — Δ); 10^{-7} M FPL 55712 (○—○) or 10^{-6} M 5,8,11,14 eicosatetraynoic acid (\blacktriangle — \blacktriangle). Basal (100%) values of IDT and frequency were taken with each of the inhibitors or with KRB and the percentage changes (mean \pm s.e.) after addition of lymphocytes are shown in the graph. The number of ChL tested is shown in parentheses.

Table 1. Effect of supernatants from the reaction of Chagas' lymphocytes and homogenates of rat atria on spontaneously beating rat atria

Supernatants of	<i>n</i>	IDT (%)	FC (%)
ChL+RAH	7	+52.2 \pm 9.1	+11.0 \pm 2.0
NL+RAH	7	+4.2 \pm 2.8	+1.2 \pm 0.8

Five million ChL or NL were incubated at 37°C for 90 min with 0.5 ml RAH. After centrifugation, the cell free supernatants were added to spontaneously beating rat atria and the percentual changes in isometric developed tension (IDT%) or frequency (FC%) after 40 min of reaction were calculated as described in Materials and Methods. Supernatants of RAH, ChL or NL alone had no effect on the contractile activity of rat atria.

Effect of lymphocytes from patients treated with trypanocidal drugs

In contrast to the above mentioned results, when ChL were obtained from patients that had undergone trypanocidal treatment (tr-ChL) depression of the contractile activity was observed (Fig. 4) and the FC was unchanged. The depressor effect was abolished with inhibitors of the

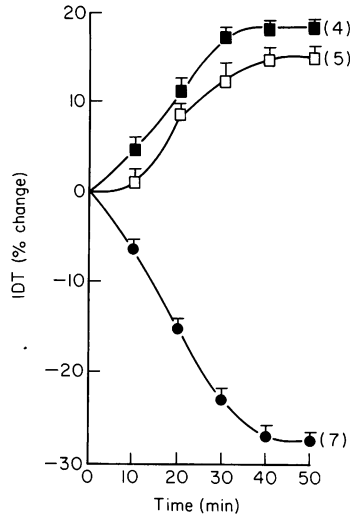


Fig. 5. Effect of lymphocytes from treated Chagas' patients on isolated rat atria. Four hundred thousand per millilitre of unfractionated lymphocytes from Chagas' patients that received treatment with trypanocidal drugs were reacted with isolated rat atria suspended in KRB (●—●); KRB containing 10^{-6} M indomethacin (■—■) or acetylsalicylic acid 1.8×10^{-4} M (□—□). Changes in the isometric developed tension (IDT) (Mean \pm s.e.) are shown. The number of experiments is shown in parentheses.

cyclo-oxygenase pathway (Fig. 4) and was present when either unfractionated tr-ChL or both the ERFC and the non-ERFC populations of tr-ChL were used (data not shown)

DISCUSSION

The results of this study demonstrate that lymphocytes from Chagas' patients can react *in vitro* with spontaneously beating atria increasing the contractile force and beating rate in a concentration-dependent manner (Fig. 1) while control normal lymphocytes are inactive. We have previously shown (Sterin-Borda *et al.*, 1983) that activation of NL with phytohaemagglutinin (PHA) induced a stimulatory reaction between the stimulated NL and the isolated rat atria. Since it has been shown that ChL recognize antigens in xenogeneic and homologous heart tissue (Cossio *et al.*, 1976; de la Vega, Damilano & Diez, 1975), we propose that ChL are activated upon contact with antigens in the atrial tissue and react in this experimental system in a similar way as PHA activated NL. Therefore, in contrast to NL, ChL do not require further activation with mitogens to produce stimulatory effects on the beating rat atria.

The fact that Ch-ERFC cell fraction was required for the positive ino- and chronotropic effects supports the role of cells from the T cell lineage in the reaction (Fig. 1 & 2). On the other hand the depressor action of Ch-non-ERFC is more difficult to interpret because this cell fraction is composed of many different cell types (B, null and K lymphocytes, low affinity ERFC, monocytes). In these experiments we could not detect differences between the activity of ChL from untreated patients with or without Chagas' cardiomyopathy, indicating that this assay is not a good correlate of the *in vivo* clinical situation.

The use of metabolic inhibitors of pharmacological antagonists provided evidence for the role of products of the lipoxygenase pathway of AA metabolism in the stimulatory effect of ChL (Fig. 2). The fact that FPL 55712, an antagonist of SRS-A (Burka & Paterson, 1981) was an effective blocker of the ChL's effect, strongly suggests that leukotrienes are the lipoxygenase metabolites involved in the reaction. It has been suggested that SRS-A is composed of leukotrienes C_4 and D_4 (Lewis *et al.*, 1980) and that these products of the lipoxygenase catalysed pathway are able to increase the tension on isolated guinea-pig atria (Terashita *et al.*, 1981).

The direct action of ChL on the rat atria is reminiscent of the effect of PHA activated NL (Sterin-Borda *et al.*, 1983) and differs from the co-operative reaction of NL and antibodies from Chagas' patients. The later reaction triggered a β -adrenergic mechanism (Sterin-Borda *et al.*, 1982).

The experiments with cell free supernatants from ChL reacted with atrial homogenates (Table 1) indicate that soluble factors are generated by contact of ChL with the heart tissue and that these factors can trigger positive ino- and chronotropic effects on beating atria. Therefore, close contact with whole ChL is not a requisite to produce these effects. However, we cannot know if the soluble stimulatory factor/s present in the supernatants are products of ChL or are derived from the atrial tissue after contact with the ChL.

The results of the experiments done with lymphocytes from Chagas' patients that had been treated with trypanocidal drugs are puzzling. Our group of patients received the trypanocidal drugs that are widely used in Argentina for the treatment of acute or chronically infected Chagas' patients. Therapy involves oral ingestion for 30–60 days of either nifurtimox or benznidazole. Treatment is thought to be effective for the disappearance of circulating parasites but the serological responses to *T. cruzi* persist (Cerisola, 1977). The loss of the ability of ChL to produce positive ino- and chronotropic effects is difficult to interpret. It has been reported that ChL from nifurtimox treated patients lose the ability to give certain cell-mediated responses to *T. cruzi* antigens (Lelchuk, Cardoni & Levis, 1977b) while other parameters of the non-specific cell-mediated immune response are preserved (Lelchuk, Cardoni & Fuks, 1977a). In this study we provide further evidence for the alteration of the lymphocyte reactivity in treated Chagas' patients. It is noteworthy that tr-ChL responded to confrontation with beating rat atria giving a depressor effect. This effect depended on the generation of products of the cyclo-oxygenase pathway of AA, as it could be blocked by treatment with indomethacin and aspirin (Fig. 4). The cyclo-oxygenase pathway is also active in the reaction of beating atria and L from Chagas' patients that did not receive trypanocidal drugs, because indomethacin and aspirin enhanced the inotropic positive effect of ChL (Fig. 4). These drugs acted probably by preventing the utilization of AA for the cyclo-oxygenase pathway, leaving more substrate to be converted into stimulatory lipoxygenase products. In addition, non-ERFC lymphocytes from untreated patients had a moderate depressor action (Figs 1 & 2). Elimination of this action would allow free expression of the Ch-ERFC stimulatory effect. Probably, the inhibitory effect of tr-ChL and Ch- non-ERFC from untreated patients depends on prostaglandins of the E series (PGE) because PGE is the only one that produces a negative inotropic action on isolated rat atria (Sterin-Borda *et al.*, 1980). The fact that tr-ChL depressed the IDT of beating rat atria suggests that they can indeed recognize the atrial tissue and react to it, although with the opposite effect to that of ChL from untreated patients. At the present time it is not possible to speculate on the reasons for this abnormal behaviour from individuals that have completed their treatment a long time before the assay and that do not show evidence of generalized immunosuppression.

Although the possibility that ChL can influence the contractile behaviour of the heart is interesting and would provide some evidence for the hypothetic role of autoimmune phenomena in the development of Chagas' cardiomyopathy, the results of these acute *in vitro* experiments cannot be extrapolated to the long standing *in vivo* situation. Furthermore, it is not known if ChL can react with autologous heart tissue and produce similar effects. In regard to the possible role that AA metabolites could play in the development of the cardiopathy, there are no reports pertaining the action of non-steroid anti-inflammatory drugs in Chagas' disease. Nevertheless, the fact that L can be activated by contact with heart tissue and can in turn trigger the production of metabolites of the lipoxygenase and cyclo-oxygenase pathways of AA with important biological activities on the cardiovascular system must be taken into account in cardiac diseases in which myocardial lymphocyte infiltrates are encountered.

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REFERENCES

- BÖYUM, A. (1968) Separation of leukocytes from blood and bone marrow. *Scand. J. lab. Invest.* **21**, Suppl 97, 77.
- DE BRACCO, M.M.E., ISTURIZ, M.A. & MANNI, J.A. (1976) Cell-mediated cytotoxicity. Characterization of the effector cells. *Immunology*, **30**, 325.
- BURKA, J.F. & PATERSON, A.M. (1981) The effect of SRS-A and histamine antagonists on antigen-induced contraction of guinea pig trachea. *Eur. J. Pharmacol.* **70**, 489.
- CERISOLA, J.A. (1977) Chemotherapy of Chagas' disease. *PAHO Scientific Publication*, **347**, 35.
- COSSIO, P.M., DAMILANO, G., DE LA VEGA, M.T., LAGUENS, R.P., CABEZA-MECKERT, P., DIEZ, C. & ARANA, R.M. (1976) *In vitro* inrecaction between lymphocytes of chagasic individuals and heart tissue. *Medicina (Buenos Aires)*, **36**, 287.
- COSSIO P.M., DIEZ C., LAGUENS R.P. & ARANA R.M. (1980) Immunopatología de la enfermedad de Chagas. Hechos y perspectivas. *Medicina (Buenos Aires)*, **40**, Suppl 1 222.
- DE LA VEGA, M.T., DAMILANO G. & DIEZ C. (1975) Leukocyte migration inhibition test with heart antigens in American trypanosomiasis. *J. Parasitol.* **62**, 129.
- LELCHUK, R., CARDONI, R.L. & FUKS, A.S. (1977a) Cell-mediated immunity in Chagas' disease. Alterations induced by treatment with a trypanocidal drug (Nifurtimox) *Clin. exp. Immunol.* **30**, 434.
- LELCHUK, R., CARDONI, R.L. & LEVIS, S. (1977b) Nifurtimox-induced alterations in the cell-mediated immune response to PPD in guinea-pigs. *Clin. exp. Immunol.* **30**, 469.
- LEWIS, R.A., AUSTEN, K.F., DRAZEN, J.M., CLARK, D.A., MARFAT, A. & COREY E.J. (1980) Slow reacting substance of anaphylaxis: identification of leukotrienes C₁ and D from human and rat sources. *Proc. Natl. Acad. Sci. USA.* **77**, 3710.
- STERIN-BORDA, L., BORDA, E., FINK, S. & DE BRACCO, M.M. DE E. (1983) Effect of phytohemagglutinin-stimulated human lymphocytes on isolated rat atria. Participation of lipoxigenase products of arachidonic acid metabolism. *Naunyn Schmied. Arch. Pharmacol.* **324**, 58.
- STERIN-BORDA, L., CANGA, L., PISSANI, A. & GIMENO, A.L. (1980) Inotropic effect of PGE₁ and PGE₂ on isolated rat atria. Influence of adrenergic mechanisms. *Prostaglandins*, **20**, 825.
- STERIN-BORDA, L., COSSIO, P.M., GIMENO, M.F., GIMENO, A.L., DIEZ, C., LAGUENS, R.P., CABEZA-MECKERT, P. & ARANA, R.M. (1976) Effect of chagasic sera on the rat isolated atrial preparation: immunological, morphological and functional aspects. *Cardiovasc. Res.* **10**, 613.
- STERIN-BORDA, L., FINK, S., DIEZ, C., COSSIO, P.M. & DE BRACCO, M.M. DE E. (1982) Beta-adrenergic effect of antibodies from chagasic patients and normal human lymphocytes on isolated rat atria. *Clin. exp. Immunol.* **50**, 534.
- TERASHITA, Z., FUKUI, H., HIRATA, M., TERAOKA, S., OHKAWA, S., NISHIKAWA, K. & KIKUCHI, S. (1981) Coronary vasoconstriction and PGL₂ release by leukotrienes in isolated guinea pig hearts. *Eur. J. Pharmacol.* **73**, 357.
- VAN ROSSUM, J.M. (1963) Cumulative dose-response curves. II. Technique for making of dose-response curves in isolated organs and the evaluation of drug parameters. *Arch. Int. Pharmacodyn. Ther.* **143**, 299.