Defective Production of Interleukin 2 in Patients with Chagas' Disease. Purified IL-2 Augments *in Vitro* Response in Patients with Chagasic Cardiomyopathy

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The production of interleukin 2 (IL-2) by peripheral blood mononuclear cells, from patients with different clinical forms of Chagas' disease and healthy controls, was evaluated after stimulation with Trypanosoma cruzi *antigen, PPD and PHA. PHA induced higher production of IL-2 in infected patients than healthy controls. No diferences were found between infected groups. With PPD the trend was similar, the only difference was that asymptomatic infected patients (INF) showed higher levels of IL-2 production than patients with cardiomyopathy (CDM). With* T. cruzi *antigen, most patients showed little or no IL-2 production at 24 hr, a peak at 48 hr and an abrupt fall at 72 hr. A similar pattern of IL-2 production was observed in INF and CDM. To evaluate the physiologic relevance of the deficit in IL-2 production, we studied the effect of non-mitogenic concentratios of IL-2 in the proliferative response to specific antigens. The addition of IL-2 only enhanced the proliferative response of CDM patients. These observations suggest that patients suffering Chagas' disease, particularly CDM, have a significant reduction in the capacity to produce IL-2. These findings could be of importance in the pathogenesis of Chagas' disease.*

Key words: *Trypanosoma cruzi* - Chagas' disease - immunoparasitology - interleukin-2

Trypanosoma cruzi, a protozoan parasite belonging to the order Kinetoplastidae, is the causal agent of American trypanosomiasis or Chagas' disease. In Venezuela, as in many other Latin American countries, the chronic cardiac form is an important public health problem. The digestive tract pathology, associated with Chagas' disease, has not been reported in Venezuela.

Although humoral and cell-mediated immune responses to *T. cruzi* antigens can be demonstrated in Chagas' disease (Tschudi et al. 1972, Montufar et al. 1977, Mosca et al. 1985), the parasites persist in blood and tissues, albeit at low levels that require culture or xenodiagnosis for detection. During the chronic phase some individuals develop a progressive cardiomyopathy, and there has been much speculation that autosensitization to heart antigens participates in the pathogenic process. Despite this, the available information (Todd et al. 1983, Khoury et al. 1983, Mosca et al. 1985) does not necessarily support the presence of an autoimmune component in Chagas' disease. This uncertainty makes a better understanding of the host's immune response essential in the evaluation of the pathogenesis of Chagas' disease.

During the development of a cell-mediated immune response to antigen, or a proliferative response to mitogen, activated lymphocytes produce interleukin-2 (IL-2) that is essential for T cell proliferation and differentiation (Smith 1980), and appears to have an important role in the regulation of the immune response (Malkowsky & Medawar 1984, Asherson et al. 1985). Based on these observations and the role assigned to this molecule in experimental Chagas' disease (Harel-Bellan et al. 1983, 1985) and other parasitic diseases (Lelchuck et al. 1984, Watson et al. 1985), we have studied, using specific antigens and PHA, the *in vitro* production of IL-2, in patients with different clinical forms of Chagas' disease. The results showed a defective production of IL-2 in patients with Chagas' disease compared to healthy controls, this being more evident in patients with chagasic cardiomyopathy. The kinetics of IL-2 production was also different in patients with Chagas' disease. To evaluate the relevance of these results we studied the effect of adding IL-2 to mononuclear cells stimulated with *T. cruzi* antigens or PPD. Only CDM patients had a significant increase of the responses under these conditions.

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MATERIALS AND METHODS

Patient groups - The participants in this study were selected from patients of the Cardiology Clinic for Chagas' disease at the J.M. Vargas Hospital in Caracas. A total of 68 patients with positive complement fixation tests (CFT) and hemagglutination to *T. cruzi* agreed to participate in the study. Patients were divided into two groups as described in a previous study (Mosca 1980). The 34 patients assigned to the first group (21 men and 13 women; mean age 39 years; range 24 to 58) were seropositive but had no evidence of heart disease (asymptomatic infected patients: INF). The 34 patients assigned to the second group (22 men, 12 women; mean age 44 years; range 28 to 64) had electrocardiographic abnormalities characteristic of either: complete right bundle branch block plus left anterior or posterior fascicular block, or complete atrioventricular block with wide QRS complex (Chagas' disease cardiomyopathy group: CDM).

A total of 17 healthy volunteers, from the Municipal Blood Bank of the J.M. Vargas Hospital formed a third group (control: 11 males and 6 females; mean age 29 years; range 19 to 39). These subjects had negative CFT for *T. cruzi*, and, on an epidemiological basis, had probably experienced no contact with the parasite.

Antigens - Antigen A: epimastigotes of *T. cruzi* strain Y were grown in medium 199 supplemented with 2% fetal calf serum (Grand Island Biological Company, USA). They were separated and washed twice with Hank's solution by centrifugation (700 g, 20 min), adjusted to 30 x 10^6/ml in PBS, and autoclaved for 10 min at 120°C (protein concentration 500 µg/ml). This antigenic preparation was the same used in previous publications (Mosca 1980, Mosca et al. 1985). *Trypomastigotes (T)*: strain Y trypomastigotes, harvested from Vero cell supernatants, were purified using a discontinuous gradient of Percoll, washed twice with Hanks' solution, and adjusted at a final concentration of 1 x 10^6/ml. *PPD* (*Mycobacterium tuberculosis* Purified Protein Derivative): a commercial preparation from the Pasteur Institute (France) was adjusted at a final concentration of 10 µg/ml. *PHA*: a solution 1/100 (v/v) of Phytohemagglutinin P (Difco Laboratories, USA) was used.

In vitro *stimulation of IL-2 production* - The production of IL-2 by mononuclear cells was evaluated in 14 INF, 15 CDM patients and 17 healthy volunteers. A standard microtest procedure was employed. In which mononuclear cells were separated from heparinized blood by centrifugation over Ficoll-Hypaque, washed three times, and cultured in triplicate in 96 wells flat bottom plates at a concentration of 2 x 10^5 cells/well. The medium used was RPMI 1640 (Grand Island Biological Company, USA) supplemented with 10% autologous serum and 50 µg/ml gentamicin. Twenty microliters per well of the antigens or PHA, were used in triplicate. After 24, 48 and 72 hr of incubation with antigens or PHA, 100 µl of the culture supernatants were withdrawn and stored frozen until assayed.

Bioassay for IL-2 activity - The method described by Gillis et al. (1978) was used. Briefly, each sample was diluted two-fold in RPMI-1640 (100 µl/well in 96-well flat bottom plates). To each well 100 µl of a suspension of 5 x 10^4 CTLL cells/ml (an IL-2 dependent murine line of cytotoxic lymphocytes) was added, and the plates were incubated for 24 hr at 37°C in 5% CO_2. They were pulsed with 0.5 µCi of ^{3}H-thymidine for 6 hr harvested with a Skatron multiple cell culture harvester (Titertek Flow Laboratories, USA) and counted in an LKB liquid scintillation counter (LKB, Sweden). The uptake of ^{3}H-thymidine by CTLL in response to the tested samples was compared with the response to a standard IL-2 preparation by probit analysis. Results were expressed as units of IL-2/ml (U/ml).

Effect of IL-2 on response to T. cruzi *antigens* - This evaluation was carried out in 20 INF and 19 CDM patients. Mononuclear cells from venous blood, and T cells purified by passage through columns of nylon wool, were tested in triplicate, in 96 wells culture plates, as described. The cells were stimulated with antigen A or PPD alone or in combination with IL-2.

As it has been reported that IL-2 has a mitogenic activity on unstimulated cells (Lakhanpal et al. 1987), we used the concentration of IL-2 with the lowest mitogenic activity as established in healthy controls. IL-2 (BIOSOFT, France) was diluted 1:50 and 20µl of this, were added in triplicate, to each culture well. Mononuclear cells or T cells in the absence of antigens or IL-2 were the controls for the background proliferation of the cells. After six days of culture, the wells were pulsed for 18 hr with 0.5 µCi of ^{3}H-thymidine and processed as described previously.

To measure the changes induced by IL-2 in the proliferative response to antigens, we calculated a ratio of enhancement:

$$RE = \frac{\text{CPM (antigen + IL-2)}}{\text{CPM antigen + CPM IL-2}}$$

values of RE equal or greater than 1 were, arbitrarily, defined as a significant enhancement of the proliferative response to the antigen.

Statistical analysis - The statistical significance of differences in the production of IL-2 induced with antigens or PHA among groups were tested by the Wilcoxon Rank Sum Test.

The differences in the kinetics of response to antigens or PHA were evaluated by comparing the mean IL-2 values of INF and CDM using the Sign Test.

The ratios of enhancement induced by IL-2 in each subgroup were compared using Student's t test.

RESULTS

IL-2 production by mononuclear cells stimulated with T. cruzi *antigen* - Mononuclear cells incubated with antigen A induced detectable IL-2 production in 57.1% (8/14) of INF and 46.6% (7/15) of CDM patients. Similar percentages were observed with live trypomastigotes (T).

T. cruzi antigens, both A and T, induced higher production of IL-2 in INF (Table I). Due to the small sample size and the dispersion of values, the difference in means is not statistically significant. However, the evaluation of this tendency, using the Sign Test, shows a significant difference ($p \leq 0.01$).

IL-2 production by mononuclear cells stimulated with PPD - With PPD, 70.6% (12/17) of the controls (Ct), 40% (4/10) of the INF and 62.5% (10/16) of the CDM patients produced detectable levels of IL-2. Controls produced significantly more IL-2 ($p \leq 0.01$) at 24 and 72 hr than CDM. IL-2 production by mononuclear cells from INF patients was also appreciably lower than the controls (Table I), although, due to the low number of INF patients with detectable levels of IL-2, no meaningful statistical analysis was possible.

IL-2 production by mononuclear cells stimulated with PHA - The difference between controls and patients became more evident in mononuclear cells stimulated with PHA (Table I). Controls produced more IL-2 than CDM patients at 24, 48 and 72 hr ($p \leq 0.01$), and at 72 hr when compared with INF patients ($p \leq 0.05$). INF had significantly higher IL-2 values at 24 and 48 hr ($p \leq 0.05$), than CDM.

Kinetics of IL-2 production - In patients with Chagas' disease stimulation with *T. cruzi* antigens or PPD induced low levels of IL-2 at 24 hr, a marked rise at 48 hr, and an abrupt fall to zero or near-zero values at 72 hr (Figs 1, 2). With PHA the kinetics were similar, but less accentuated (Fig. 3).

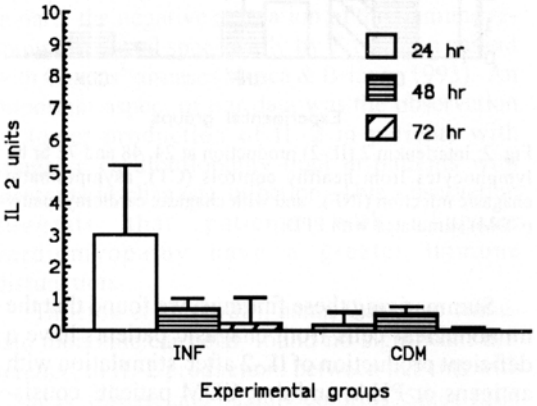

Fig. 1: interleukin 2 (IL-2) production at 24, 48 and 72 hr by lymphocytes from patients with asymptomatic chagasic infection (INF) and with chagasic cardiomyopathy (CDM) stimulated with antigen A.

Mononuclear cells from controls did not produce IL-2 when stimulated with *T. cruzi* antigens, but responded effectively to PPD. The latter pattern of response was, characterized by higher levels of IL-2 at 24 hr and no abrupt fall at 72 hr (Fig. 2).

TABLE I

Interleukin-2 production by lymphocytes, from patients with Chagas' disease, stimulated with *Trypanosoma cruzi* antigens, PPD an PHA

Antigens/hours		CT	INF	CDM[d]
A	24	0.00	3.01 ± 2.20	0.18 ± 0.40
	48	0.00	0.71 ± 0.30	0.53 ± 0.23
	72	0.00	0.24 ± 0.80	0.06 ± 0.09
T	24	0.00	3.20 ± 1.60	0.29 ± 0.21
	48	0.00	2.30 ± 1.60	0.65 ± 0.28
	72	0.00	0.22 ± 0.10	0.05 ± 0.02
PPD	24	2.67[a] ± 0.9	1.70	0.25 ± 0.18
	48	1.70[a] + 0.4	1.40 ± 0.78	0.77 ± 0.22
	72	1.78[a] + 0.45	0.03	0.10 ± 0.07
PHA	24	7.80[a] ± 2.80	2.80[c] ± 0.90	0.80 ± 0.30
	48	6.90[a] ± 2.60	3.30[c] ± 1.20	1.36 ± 0.16
	72	7.50[b] ± 1.40	1.10 ± 0.50	0.50 ± 0.80

Values are expressed in U/ml ± standard error

a: $p \leq 0.01$ when compared with CDM patients; *b*: $p \leq 0.05$ when compared with INF patients; *c*: $p \leq 0.05$ when compared with CDM patients; *d*: $p \leq 0.01$ when the tendency in IL-2 production of CDM is compared to INF using a sign test; CT = control healthy individuals; CDM = chagasic patients with cardiomyopathy; INF = chgasic patients without heart involvement

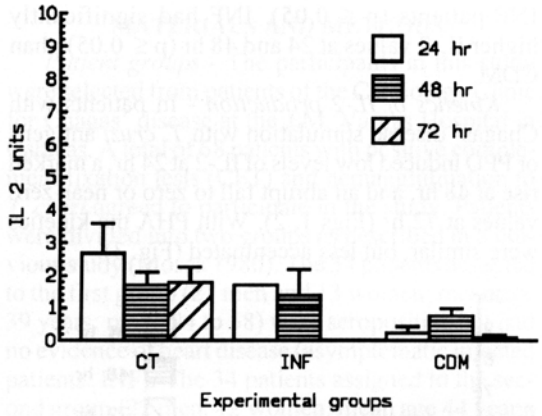

Fig. 2: interleukin 2 (IL-2) production at 24, 48 and 72 hr by lymphocytes from healthy controls (CT), asymptomatic chagasic infection (INF), and with chagasic cardiomyopathy (CDM) stimulated with PPD.

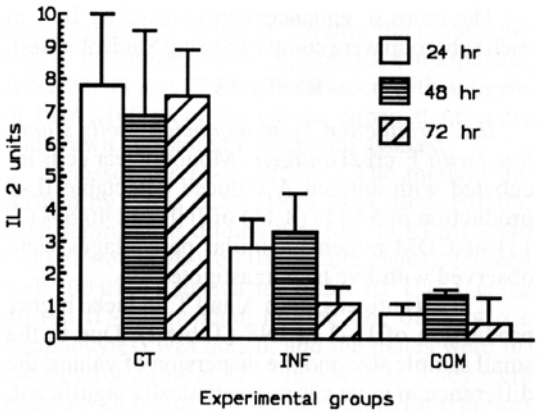

Fig. 3: interleukin 2 (IL-2) production at 24, 48 and 72 hr by lymphocytes from healthy controls (CT) chagasic infection (INF), and with chagasic cardiomyopathy (CDM) stimulated with PHA.

Summarizing these findings, we found that the mononuclear cells from chagasic patients have a deficient production of IL-2 after stimulation with antigens or PHA, and that CDM patients consistently produced lower levels of IL-2 than INF patients. In addition, the kinetic of IL-2 production is different in the control group.

Stimulation of mononuclear cells with antigens in the presence of IL-2 - Due to the central role played by IL-2 in the cell mediated immune response it was assumed that the effect of IL-2 should be more evident in patients with low proliferative response to *T. cruzi* antigens. Consequently patients, in each group, were classified in two subgroups according to their Stimulation Index (SI) to antigen A. Patients with SI less than 2.5 were considered non-responders (CDM-NR; INF-NR), and patients with SI equal or greater than 2.5 were considered responders (CDM-R; INF-R). Twenty percent (4/20) of INF and 63 % (12/19) of CDM were non responders. This difference in frequency was statistically significant ($p \leq 0.02$).

The ratios of enhancement (RE) for antigen A of CDM-NR and CDM-R patients (Table II) were higher than those of INF-R patients ($p<0.05$).

TABLE II

Effect of the addition of interleukin 2 (IL-2) on the, *in vitro*, proliferative response of mononuclear cells stimulated with *Trypanosoma cruzi* or PPD

				CPM			RE+	
Group	Control	A	PPD	IL-2	A+IL-2	PPD+IL-2	A	PPD
CDM-NR								
mean	232.82	273.25	4132.99	499.05	1218.00	245.00	2.6[a]	1.20[a]
SE	41.35	87.48	3436.33	172.73	631.77	86.55	0.37	0.24
n	11.00	11.00	11.00	11.00	11.00	11.00	11.00	11.00
CDM-R								
mean	1292.04	8474.25	13313.84	1868.38	9629.84	14324.16	1.73[a]	1.08[b]
SE	827.44	2818.95	3662.74	583.79	2433.02	3392.90	0.35	0.16
n	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00
INF-NR								
mean	256.00	394.15	3624.35	297.50	785.43	3781.43	2.28	1.38
SE	97.50	164.10	2730.00	105.46	380.14	2646.55	0.43	0.27
n	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
INF-R								
mean	355.69	6157.40	6520.00	892.88	5789.85	4509.39	0.98	0.71
SE	76.37	1219.50	1863.81	90.73	1243.74	1349.12	0.19	0.09
n	16.00	16.00	16.00	16.00	16.00	16.00	16.00	16.00

ND : not determined; *a*: $p \leq 0.02$ when compared with INFR; *b*: $p = 0.05$ when compared with INFR; RE+: ratio of enhancement; n: sample size; SE: standard error of the mean

As with antigen A, the RE for PPD of INF-R were lower than non-responder CDM-NR ($p \leq 0.02$) and CDM-R ($p \leq 0.05$).

Stimulation of T-cells with antigens in the presence of IL-2 - The results of adding IL-2 to T-cells are presented in Table III. Evidently the data are similar to those observed for whole mononuclear cells. The RE of CDM were significantly higher than those of INF ($p \leq 0.02$).

DISCUSSION

The interaction of the immune system with a pathogen induces a series of events. Among them, the interaction of antigen presenting cells with different subsets of T lymphocytes is of great importance. This interaction will induce the production of cytokines with a wide range of actions (Morimoto et al. 1986, Heeg et al. 1987, Meltzer & Nancy 1989, Hirohata & Lipsky 1989). A fundamental aspect of this complex process is the pattern of cytokines produced as well as the kinetics of their production (Spits et al. 1987, Serreze & Leiter 1988, Hodes 1989). Among the cytokines, IL-2 plays a central role in the expansion as well as in the regulation of immune response (Smith 1988). The difference in the capacity to give a good immune response to intracellular pathogens, observed in some strains of mice, has been shown to be directly correlated with the capacity of their mononuclear cells to produce IL-2 when stimulated with ConA (Lipoldova et al. 1992). The stimulation of T lymphocytes in the absence of IL-2 induces a specific anergy to the stimulating antigen. This state of specific anergy reverts, *in vitro*, if IL-2 is added when the mononuclear cells are stimulated (Essery et al. 1988, Proust et al. 1991). The experimental tolerance to alloantigen is abolished if IL-2 is administered when the animal is transplanted (Malkovsky & Medawa 1984, Essery et al. 1988, Dallman et al. 1991). Besides its importance during the genesis of the immune response, IL- 2 have shown, in clones and in lymphocytes subsets, to play an important role in the regulation of the immune response and the induction of different effector functions of T cells subsets (Otten & Germain 1991, Takahashi et al. 1991). All the foregoing suggests the physiological importance of the lower production of IL-2, induced by antigens or PHA, in patients with Chagas' disease. This deficit in the production of IL-2 must have physiological importance and could explain, in part, the negative regulation of the immune response, induced specifically by *T. cruzi*, in patient with Chagas' disease (Mosca & Briceño 1993). An important aspect of our data was the observation of lower production of IL-2 in patients with chagasic cardiomiopathy than in patients with asintomatic chagasic infection. This observation suggests that patients with chagasic cardiomyopathy have a greater immune disfunction.

Tarleton et al. (1988), using Con A to stimulate mononuclear cells, found no significant difference, in IL-2 production, between patients with Chagas' disease and healthy controls. Since PHA and Con A stimulate different subsets of lymphocytes (Stobo & Paul 1973), the difference in the mitogen used may explain this disagreement.

The observation of a lower production of IL-2 by mononuclear cells from patients with Chagas' disease, makes necessary to evaluate the possible relevancy of this observation. With this objective, we studied the effect of adding IL-2 to mononuclear cells stimulated with antigens. The addition of IL-2 induces a significant increase of the proliferative response only in CDM (Fig. 4). This observation reinforces the hypothesis that patients with chagasic cardiomyopathy have a greater disfunction of the immune response. On the other hand it seems that in INF the amount of IL 2 produced remain within the physiological range, since the addition of IL 2

TABLE III

Effect of the addition of interleukin 2 (IL-2) on the, *in vitro*, proliferative response of t cells stimulated with *Trypanosoma cruzi* or PPD

Experimental				CPM			RE	
group	Control	A	PPD	IL-2	A+IL-2	PPD+IL-2	A	PPD
CDM-NR								
mean	362.29	538.00	2489.14	3049.00	7635.00	13048.43	1.87[a]	3.13
SE	160.90	182.82	1040.21	941.16	2124.02	3586.85	0.33	3.81
n	7.00	7.00	7.00	7.00	7.00	7.00	7.00	7.00
INF								
mean	256.00	725.13	2285.75	1677.38	3561.75	3337.50	0.84	0.90
SE	94.95	379.70	518.94	335.76	1338.13	1309.11	0.17	0.16
n	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00

a: $p < 0.02$ when compared with INF; RE: ratio of enhancement; SE: standard error of the mean

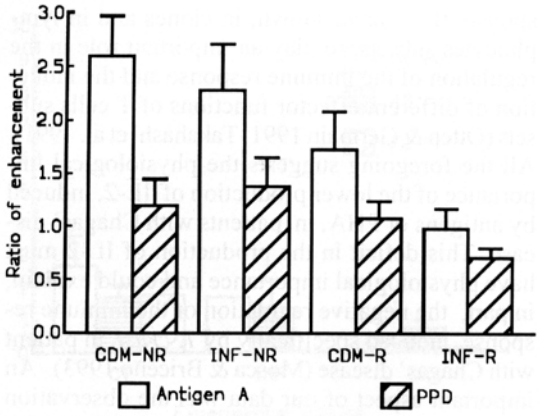

Fig. 4: ratio of enhancement (RE) induced by the addition of interleukin 2 (IL-2) to mononuclear cells, from patients and controls, stimulated with *Trypanosoma cruzi* antigens and PPD.

does not modify significantly the proliferative response to antigens.

Kierszenbaum et al. (1989) have reported that living trypomastigotes can induce, on T cells of healthy controls a down regulation of IL-2 receptors (IL-2R). Addition of exogenous IL-2 does not improve the proliferative response to mitogens. This information is apparently contradictory with our results obtained by the addition of exogenous IL-2. However, apart from differences in experimental conditions, an important consideration is the amount of trypomastigotes required to induce this down-regulation: at least one parasite per mononuclear cell, concentration that could only occur during the acute phase of Chagas' disease. Patients with chronic Chagas' disease, such as those studied, have subpatent parasitemia. Consequently, the findings of Kierszenbaum et al. (1989), might be relevant in acute Chagas' disease and ours for the chronic form of the disease.

Recent studies in experimental animals (Soong & Tarleton 1992), and in normal human T cells (Majunder & Kierszenbaum 1995) have shown that *T. cruzi* induces, in spleen cells as well as normal PBMN, a reduction in the levels of IL-2 mRNA. These experimental observations are in agreement with our experimental data showing a lower production of IL-2 in patients with Chagas' disease. Consequently, it is possible that antigens of *T. cruzi* induce a negative regulation of the effector response that could eliminate the parasite. The report of a higher frequency of positive xenodiagnosis in patients with chagasic cardiomyopathy (Maekelt 1973, Mosca et al. 1985) support this hypothesis. Furthermore, in a longitudinal study of the immune response of patients with Chagas' disease, we have shown that PBMN from patients with positive xenodiagnosis had a lower proliferative response to antigens of the parasite (Mosca et al. 1985).

It has become evident that IL-2 has effects on a wide range of cells. To confirm that IL-2 was acting on T cells, we evaluated the proliferative response of purified T lymphocytes, to antigens, in presence of IL-2. The results support the contention that the results obtained with mononuclear cells were mainly due to the response of T cells.

In summary, we have shown that PBMC from patients with Chagas' disease have a significant alteration in the kinetics and the amount of IL-2 produced when stimulated with PHA or antigens. This deficit was more intense, in CDM patients, when antigens of *T. cruzi* were used. At this point, the cause of the deficit in IL-2 production is not clear. Since IL-2 enhances the proliferative response to *T. cruzi* antigens, the deficit in IL-2 production might be part of the event that induces the specific suppression reported in patients with Chagas' disease (Mosca & Briceño 1993). An important point to be studied will be the role of different subsets of T cells in the genesis of this deficit.

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