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Proliferation and bystander suppression induced by antigens of *Trypanosoma cruzi*. Evaluation with a modification of the T cell blot technique.

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Key words: Chagas' disease, antigens, regulation, cellular immunity.

Abstract. We have studied *in vitro* proliferation induced by soluble antigenic fractions of *T. cruzi* epimastigotes and trypomastigotes, as well as their regulatory effect on the proliferative response to PPD. Both crude extracts of the parasite as well as bands from Western blots of soluble epimastigotes and trypomastigotes antigens were tested. Crude extracts elicited higher proliferation in mononuclear cells from patients with chagasic cardiomyopathy (CDM) than in those from patients with no evidence of cardiac pathology (INF). Fractionated antigens induced a lower proliferative response, in intensity as well as in frequency, than the crude extracts. With the soluble antigenic fractions of epimastigotes, cells from CDM patients gave higher responses to low molecular weight (MW) bands (17 to 30 kDa), and from INF patients, to bands of intermediate MW (31 to 62 kDa); this pattern was inverted with soluble antigenic fractions of trypomastigotes. The two crude preparations induced either up-regulation or down-regulation of the PPD response in variable numbers of patients from both groups. With fractionated antigens, down-regulation intensity was stronger in patients without evidence of heart disease, but frequency was greater in patients with Chagasic cardiomyopathy (CDM). Six bands of western-blot of soluble trypomastigote antigens (B1, 4-7, and 9) induced significant down-regulation in 100% of CDM patients. The up-regulation elicited by most bands of the antigenic fractions was significantly higher, and more frequent in patients without heart pathology. Most bands of soluble trypomastigote antigens (10/15; 66.6%) did not induce up-regulation in patients with cardiomyopathy. These data suggest that differences in the clinical status of the two groups may reflect the recognition of different groups of antigens together with variations in the nature of the regulatory response.

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Proliferación y supresión circunstancial inducida por antígenos de *Tripanosoma cruzi*. Evaluación con una modificación de la técnica de T cell blot.

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Palabras clave: Enfermedad de Chagas, antígenos, regulación, inmunidad celular.

Resumen. Se estudió tanto la respuesta proliferativa inducida por antígenos solubles de epimastigotes y tripomastigotes de *T. cruzi,* como su efecto regulador en la respuesta proliferativa a PPD. Se evaluaron tanto los extractos crudos del parásito como las bandas de "Western blots" de antígenos solubles de epimastigotes y tripomastigotes. La proliferación de las células mononucleares, en respuesta a los extractos crudos, fue mas intensa en los pacientes con cardiomiopatía chagásica (CDM) que en los pacientes sin evidencia de patología cardiaca (INF). La respuesta a los antígenos fraccionados fue menor, en frecuencia e intensidad, a la observada con los antígenos crudos. Las bandas de bajo peso molecular (17 a 30 kDa) de los antígenos solubles de epimastigotes dieron una respuesta mas alta en pacientes CDM y las de peso molecular intermedio (31 a 62 kDa). Este patrón se invirtió con las bandas de antígenos solubles de tripomastigotes. Ambos extractos solubles indujeron sobre regulación o baja regulación de la respuesta a PPD en un número variable de pacientes en ambos grupos. Con los antígenos semi purificados la intensidad de la baja regulación fue mas intensa en pacientes sin evidencia de enfermedad cardiaca, sin embargo la frecuencia fue mayor en los pacientes con cardiomiopatía chagásica (CDM). Seis bandas del Western blot de antígenos solubles de tripomastigotes (B1, 4-7, y 9) se asociaron con una baja regulación en 100% de los pacientes CDM. La sobre regulación observada con la mayoría de las bandas de la fracciones crudas fue significativamente mas alta, y mas frecuente en pacientes con patología cardíaca. La mayoría de las bandas de antígeno soluble de tripomastigotes (10/15; 66,6%) no indujeron sobre regulación en pacientes con cardiomiopatía. Estos datos sugieren que la diferencia en la clínica de los dos grupos pueden deberse a que los pacientes de cada grupo reconocen diferentes antígenos conjuntamente con variaciones en la regulación asociada con esto.

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INTRODUCTION

Chagas' disease is a parasitic zoonosis, endemic in Central and South America, caused by the protozoan parasite *Trypanosoma cruzi*. According to the World Health Organization (1), there are 18 million people infected with *T. cruzi* in Latin America. Thirty percent of these have, or will develop, chronic cardiomyopathy that will limit their productive capacity and require medical care.

Since *T. cruzi* is an intracellular parasite, the cell-mediated immune response plays a central role in its control. This aspect has been evaluated in numerous stud-

ies (2-7), all of which have shown *in vitro* proliferation of mononuclear cells stimulated with *T. cruzi* antigens. The existence of suppression of the immune response has been reported in experimental models (8-13) as well as in Chagas' disease patients (7, 14).

Recently, a proline racemase of the parasite has been described whose activity seems to be essential for B-cell mitogenicity and the induction of the suppression observed during the acute phase of the experimental infection of mice (15).

Antigens recognized by the immune system play an important role in determining the characteristics of the host response. Antigenic determinants that selectively activate *in vivo* T cells with either helper or suppressor activity have been shown with several antigens (16,17). Miller *et al.* (18) reported that cells from rats made tolerant by oral administration of antigen, down-regulate the response to a different antigen, to which the animal is immune, when stimulated with both antigens. This effect was termed "bystander suppression". Using an *in vitro* assay, we have demonstrated that *T. Cruzi* antigens induce bystander suppression of the proliferative response to PPD or tetanus toxoid (14). On the other hand, Rowland *et al.* (19), have shown that resistance or susceptibility of mice to *T. cruzi* infection is associated with the response to different antigens. Other studies have shown that immunization with a soluble sub cellular epimastigote fraction induces pathology, while a flagellar fraction induces protective immunity (20-22). These studies suggest that in Chagas' disease, the outcome of the host-parasite interaction depends on the balance established between the effector and regulatory immune responses elicited by the different parasite antigens.

To obtain a better understanding of these complex interactions, it is fundamental to study purified antigens rather than crude extracts of the parasite. The T-cell blot technique, described by Aboud Zeid *et al.* (23), is a powerful method for evaluating cell-mediated immunity to semi-purified antigens. However, it has not been widely used because of the variability observed among the responses produced by different batches of antigen. Antigens have to be trapped and internalized by antigen presenting cells (APC), processed and incorporated in MHC to activate T cells. To be phagocytosed efficiently the NC particles can not be too large. Furthermore, to minimize the random difference in phagocytosis, the size of NC particles should have a low dispersion. We have developed a modification of this technique that minimizes the variability of the size of NC particles and of the proliferative response of mononuclear cells, observed with different batches of NC particles. Combining this methodology with the evaluation of the bystander suppression of the proliferative response to PPD, we have compared the proliferation and regulation induced by trypomastigote, i.e. the infective form of *Trypanosoma cruzi*, and epimastigote antigens. Most trypomastigote and epimastigote antigens provoked negative regulation of the PPD induced proliferation.

MATERIALS AND METHODS

Patient groups

Patients were selected from the Cardiology Clinic for Chagas' disease at the Vargas Hospital in Caracas, Venezuela. A total of 38 patients (24 males, 14 females), with positive serology to *T. cruzi* antigens, agreed to participate in this study. Patients were classified into two groups according to previous criteria (23). The 19 patients assigned to group I (INF) were seropositive for *T. cruzi* with no evidence of heart disease (mean age 46 years; range 34 to 66

years). The 19 patients assigned to group II (CDM) presented electrocardiographic abnormalities characteristic of either complete right bundle branch block plus anterior or posterior hemiblock of the left bundle, or complete atrioventricular block with wide QRS complex (mean age 50.8 years; range 25 to 68 years).

Evaluation of different procedures to produce NC particles Preparation of NC particles as described by Abou Zeid *et al.* (23)

Pieces of 63 mm^2 of nitrocellulose membranes were adsorbed with 75 μL of PPD (10 g/mL), left to dry at room temperature for sixty minutes, and placed in serological tubes. To dissolve the NC one mL of DMSO was added to each tube and the mixture incubated for sixty minutes. At the end of this period one mL of carbonate/bicarbonate buffer (0.05M; pH 8.5) was added drop wise to each tube, while vigorously vortexing, to promote formation of NC particles. The NC particle suspension was centrifuged at 300 G for ten minutes. The supernatant was discarded and the NC particles were suspended in PBS. This procedure was repeated three times. After the last centrifugation the NC particles were suspended in five mL RPMI 1640 (Grand island Biological Company, USA) supplemented with gentamicin (50μg/mL), aliquoted and stored at –20°C.

Modification of the methodology described by Abou Zeid *et al.* (23)

To obtain smaller and more uniform NC particles we did not deliver the carbonate/bicarbonate buffer drop wise but as a continuous flow, directly into the DMSO and NC solution, using a peristaltic pump (400 μL/minute). The rest of the method was as described by Abou Zeid.

Estimation of the NC particle size

Twenty-five μL of each NC particle suspension were placed on a microscopic slide and the size of 400 particles measured using a calibrated ocular.

Evaluation of the reproducibility of the stimulation of mononuclear cells induced by PPD adsorbed NC particles

To evaluate the reproducibility of the proliferative response, four different batches of *PPD* adsorbed NC particles, either as described by Abou Zeid *et al.* (23), or as modified by us, were used to stimulate mononuclear cells from a healthy donor.

Antigens. Antigen A

Y strain epimastigotes were grown in 199 medium supplemented with 2% fetal calf serum. The parasites were harvested by centrifugation at 400 g for 20 minutes and washed twice with PBS. After the last wash, they were adjusted to 30×10^6/mL and autoclaved for 10 min (24); 20 μL of this suspension were added to each culture well.

Soluble parasite antigens

Soluble epimastigote (SE) and trypomastigote (ST) antigens were prepared as described by Segura *et al.* [20]. The only difference was that parasites were disrupted by pressure-depressure using a French pressure cell instead of a Sorvall-Ribi disintegrator. To prepare these crude fractions for mononuclear cell stimulation, 50 g of the crude antigen were adsorbed to 63.3 mm^2 of NC and processed as described for the Western blots bands.

PPD (Statens Serum Institut, Denmark)

Protein purified derivative (10 μg/mL) 20 μL/well.

Antigens semi purified by electrophoresis

Proteins in antigen samples were resolved by SDS-PAGE using a discontinuos buffer system (25). Either SE or ST (3 mg per gel) were applied to 10% acrylamide gels using a blank comb with a single reference well for molecular weight markers (Sigma Chemical Co. USA) for the determination of molecular mass. The soluble antigens were run under reducing and denaturalizing conditions, and transferred to (NC) membranes by the method of Towbin *et al. (*26). The Western blots were stained with Ponceau red (Sigma Chemical Co. USA) to identify the protein bands. The stained and unstained bands were marked, excised, cut into 63.3 mm^2 pieces and processed as described. After washing with PBS, the resulting NC particles were suspended in 5 mL RPMI 1640; 100 μL of this suspension were added to each culture well. The molecular weight (mw) range of the bands is shown in Table I.

Cell proliferation assays

A standard microtest procedure was employed. Briefly, mononuclear cells were separated from heparinized blood by

TABLE I
RANGE OF MOLECULAR WEIGHTS OF SE AND ST WESTERN BLOTS BANDS

Bands NC	Molecular weight			
	SE		ST	
	Upper	Lower	Upper	Lower
1	132	112	207	165
2	112	96	165	127
3	96	76	127	96
4	76	62	96	85
5	62	51	85	72
6	51	47	72	59
7	47	45	59	53
8	45	41	53	44
9	41	38	44	39
10	38	36	39	30
11	36	33	30	25
12	33	30	25	19
13	30	29	19	16
14	29	27	16	13
15	27	25	13	10
16	25	22		
17	22	20		
18	20	18		
19	18	17		
20	17	15		

centrifugation over Ficoll-Hypaque, washed three times, and cultured in triplicate for crude antigens and in quadruplicate for semi purified antigens, in plastic microtiter plates, at a 2×10^5 cells/well concentration. The medium used was RPMI-1640 (Grand Island Biological Company) supplemented with 50 μg/mL gentamycin and 10% autologous serum. After seven days of exposure to the antigen(s), 0.5 μCi of [^{3}H] thymidine was added to each well 18 hours before harvesting onto glass fiber strips and counting by liquid scintillation. As control, particles of nitrocellulose (NC) without protein, obtained from the same blot as the bands, were added to 10 wells with mononuclear cells.

Evaluation of regulation induced by *T. cruzi* antigen

To evaluate the regulation elicited by parasite antigens, mixtures of antigens or nitrocellulose particles from Western blots and soluble PPD were used at the same concentrations as for the cell proliferation assay.

The percentage of down-regulation (DR) or up-regulation (UR) was calculated as follows:

$$\text{Regulation} = \frac{\text{CPM (PPD} \quad \textit{T.cruzi} \text{ antigen)}}{\text{CPM PPD} \quad \text{CPM } \textit{T.cruzi} \text{ antigen}} \quad 100 \quad 100$$

Regulation was evaluated only in patients with a significant proliferation to PPD (SE: INF 13; CDM 9. ST: INF 13; CDM 13).

Data analysis. Criteria used to define proliferation or regulation induced by NC adsorbed antigen as relevant

Proliferation: Any band that gave a CPM greater than the mean plus two standard deviations of the CPM induced by NC alone.

Regulation: any regulation equal to or smaller than -10 (Down-regulation), or equal to or greater than 10 (Up-regulation) was considered significant. Only patients with a significant proliferation to PPD were included in this analysis.

Statistical analysis

The following parameters were used to compare the regulation induced by bands of the sub cellular fractions:

Mean regulation: Average of significant up or down regulation to the band.

Frequency of relevant up or down-regulation: percentage of patients with significant regulation to each band.

The frequency of responses to crude antigens was evaluated using a Chi square test.

To compare down-regulation or up-regulation mean values, the relevant proliferation (CPM) means induced by each band, and the percentage of patients with positive proliferation or suppression, we used the paired Wilcoxon signed-rank test.

The response of individual bands was compared using a non-parametric test (Mann-Whitney).

RESULTS

Evaluation of NC particles

The T-cell blot technique (23) was received with great interest when published. However, due to the variability of the data obtained it has not been widely used. Most NC particles obtained using the original methodology have a diameter larger than 2.4 μm (Table II). The sizes of particles affect their sedimentation and the interaction with antigen presenting cells. Therefore, the quality and the amount of antigen present in each aliquot, dispensed to stimulate mononuclear cells, may vary.

These considerations suggested that the variability of this technique could be minimized if we could produce smaller and

TABLE II
EVALUATION OF NC PARTICLES PRODUCED BY THE TECHNIQUE OF ABOUD ZEID AND AS MODIFIED BY AUTHORS

Technique	Diameter of NC particles		
	< 2.4 μm	2.4-5.0 μm	> 5.0 μm
Aboud Zeid (n = 9)			
Mean	9.0%	50.6%	36.1%
Standard Deviation	6.3	30.8	31.1
Modification (n = 64)			
Mean	92.0%	6.2%	1.8%
Standard Deviation	4.9	3.8	1.9

n = sample size.

NC batch	Proliferation induced by NC adsorbed with PPD and particulated		
	Control	I	II
1	220	400	800
2	150	300	1000
3	180	250	840
4	270	760	900

Control: NC particles without antigen. I: NC particles produced as described by Abou Zeid *et al.* (23).
II: NC particles produced by the modified technique.

more homogeneous NC particles. With this in mind, and after trying different procedures, we found that NC particles were smaller and more homogeneous when carbonate buffer was delivered as a continuous flow, instead of drop wise. With this simple modification, most NC particles produced had a diameter of less than 2.4 μm (Table II). When different batches of NC particles were evaluated (Table II), it was obvious that the NC particles, prepared as described by Abou Zeid *et al.* (23), induced a highly variable proliferative response of mononuclear cells (mean CPM 427; SD 230.3). On the contrary, with our modification, the NC particles induced a more intense response with low variability (mean CPM 885; SD 86.9).

Analysis of the response of patients to crude antigens

Proliferation. The proliferative response to crude antigens was evaluated with antigen A, the standard *T. cruzi* antigen used in our laboratory, and soluble subcellular fractions of epimastigotes and trypomastigotes adsorbed to NC. The proliferative response induced by antigen A was significantly higher in CDM than in INF (Table III). The CPM induced by the sub cellular fractions (SE and ST) were similar and, as with antigen A, higher in CDM than in INF. Nevertheless, in most cases they were lower than the proliferation induced by antigen A in the same patient. The intensity of the proliferation induced by an antigen is directly related to its concentration and immunogeneicity.

TABLE III
MEAN PROLIFERATION INDUCED BY *Trypanosoma cruzi* CRUDE ANTIGENS

Groups	Antigens		
	A	SE	ST
CDM			
CPM	22754*	4183+	5568!
SEM	5875	1596	1326
n	15	13	17
INF			
CPM	11377	2945	2825
SEM	5316	1283	929
n	18	15	18

SEM = Standard Error of the mean. * P = 0,0053 When compared with CPM INF. + P = 0,0197 When compared with CPM INF. ! P = 0,0394 When compared with CPM INF.

The amount of antigen A was selected based on previous experiments, where optimal proliferation, in most patients, was observed at this concentration. However, the soluble antigens of epimastigotes and trypomastigotes were used adsorbed to NC and the concentration of protein in each band was different. Furthermore, the stimulation induced by an antigen depends on its immunogenicity and not on a given concentration of proteins. To normalize the data and to be able to compare the proliferation induced by each band, the NC particles used to evaluate each experimental group were:

1. Produced from the same Western blot.
2. The gel was loaded with the higher concentration of protein compatible with an adequate separation of bands.

If the antigen was evenly adsorbed to NC, the amount of protein added to each well was 1 g; a low concentration for a crude antigen. Thus, it is obvious that antigens adsorbed to NC might have induced suboptimal stimulation of mononuclear cells. Therefore, it does not seem adequate to compare neither the CPM nor the stimulation index obtained with antigen A with the ones obtained with antigens adsorbed to NC. Accordingly, we compared the frequency of patients with relevant proliferation for antigen A, with the frequency of patients with CPM greater than the mean value of the CPM induced by NC alone plus two standard deviations of the mean.

As shown in Table IV, the frequency of relevant proliferation induced by crude antigens was lower in INF. This difference only was statistically significant when SE was used as antigen (p = 0.018). With ST the frequency of patients with significant proliferation was lower than with A, but it was not statistically significant (p = 0.1642).

Regulation

We have demonstrated that *T. cruzi* antigens elicit a bystander regulation of the proliferative response to PPD of mononuclear cells obtained from Chagasic patients. Our previous study (14) showed that 71% of INF patients had a down-regulation lower than -10 when their mononuclear cells were stimulated simultaneously with PPD and tetanus toxoid. Based on this find-

ing we defined as a significant down-regulation any value equal or lower than -10, and as a significant up-regulation any value equal or higher than 10.

Since the evaluation is based on the PPD generated proliferative response, only PPD positive patients were included.

The intensity of the down-regulation of the response to PPD, induced by SE as well as ST was higher in CDM although not significantly different (Table V). It could be presumed that the diminished proliferation is the consequence of sequestration of PPD in the NC. If this argument is correct, all antigens adsorbed to NC should induce down regulation. However, with soluble antigen of epimastigotes only 44 percent of CDM had down-regulation while 44 percent had up-regulation. Similar data were obtained with trypomastigote antigens.

The frequency of patients with SE induced down-regulation was higher in INF than in CDM patients. On the other hand, the percentage of patients with down-regulation with ST was similar in both experimental groups.

An important aspect of the assay is that it reveals, besides the down-regulation, the presence of up-regulation or synergism. With SE, the values of up-regula-

TABLE IV
PERCENTAGE OF PATIENTS WITH A SIGNIFICANT PROLIFERATION TO CRUDE ANTIGENS

Antigens	Experimental Groups	
	CDM	INF
A	91.7 (11/12)	75.0 (12/16)
SE	83.3 (10/12)	33.3 (5/15)
ST	76.5 (13/17)	50.0 (9/18)

TABLE V
REGULATION INDUCED BY SUBCELLULAR FRACTIONS

Regulation	SE		ST	
	CDM	INF	CDM	INF
DR	–66.0	–48.2	–66.6	–55.3
%DR	44.4 (4/9)	84.6 (11/13)	81.3 (13/16)	76.9 (10/13)
UR	40.4	21.0	151.4	86.2
%UR	44.4 (4/9)	15.4 (2/16)	6.3 (1/16)	23.1 (3/13)

DR: Average down-regulation of patients with significant down-regulation. UR: Average up-regulation of patients with significant up-regulation. % DR: percentage of patients with a significant down-regulation. % UR: percentage of patients with a significant up-regulation.

tion were similar in both experimental groups. The intensity of the up-regulation in INF was higher with ST than with SE. Furthermore, with ST only one CDM had a relevant up-regulation, while three INF had a significant up-regulation. Because the sample size of patients with up-regulation was small, no reliable statistics could be performed. However, the data obtained with crude antigens from the infective stage of the parasite suggest presence of considerable differences in the regulation of the immune response to the infective form of *Trypanosoma cruzi*.

Analysis of the response to semi purified antigens

Proliferation. In order to identify the antigens that stimulate and/or regulate the mononuclear cell response we studied the response of mononuclear cells to soluble antigen semi purified by electrophoresis. The protein pattern is more complex in SE than in ST. In SE 20 bands were identified, and 15 in ST (Table I).

The analysis of patients with significant proliferation to SE, bands (Fig. 1A) shows that bands between 29.8 and 61.6 kDa (B5 to B12 with the exception of B9)

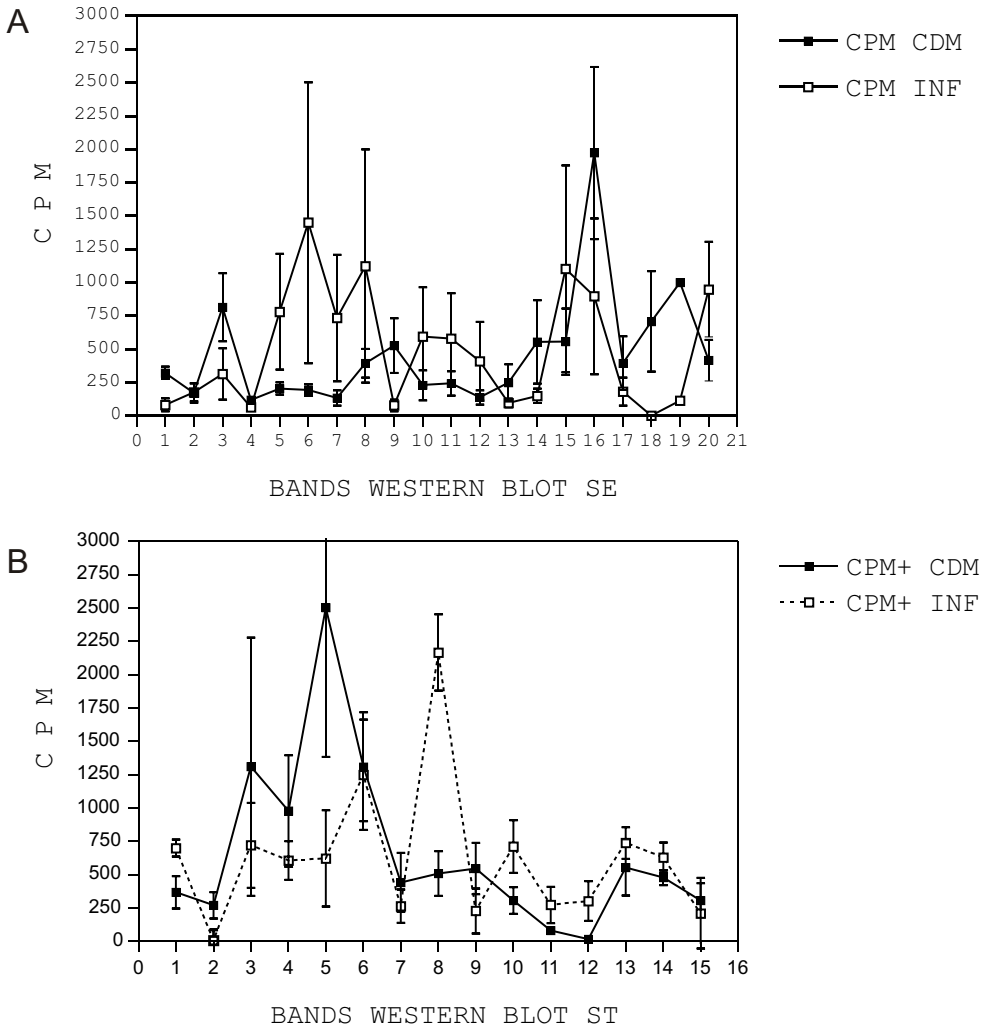

Fig. 1. Average proliferation (CPM) induced by bands of the soluble subcellular fractions of epimastigotes (SE) (A) and trypomastigotes (ST) (B). Only CPM from patients with significant proliferation to the western blot bands were included. The data shown are means +/– standard error.

gave higher CPM in INF, and bands between 17.2 and 29.8 kDa (B13 to B19) in CDM. With ST (Fig 1B), the pattern observed with SE was reversed; bands between 59 and 127 kDa (B3 to B6) were more active in CDM. These observations suggest that besides the complexity of the protein pattern, these sub cellular fractions have fundamental differences in their antigenic composition.

The frequency of positive responses to most SE and ST bands (Table VI) was low. Consequently, due to the sample size, any inference concerning the intensity of the positive response to each band will have a large margin of error. However, comparing the frequency of significant proliferation elicited by each band, we can assess if one experimental group responds more to one or both sub cellular fractions. The frequency of relevant proliferation (Table VI) was significantly higher in CDM (SE $p = 0.0158$; ST $p = 0.0002$). Bands 3, 5 and 6 of ST, that gave significant proliferation in an important percentage of CDM, also had high CPM (Fig. 1b), suggesting that they might be of importance in the host-parasite relationship.

These results confirm that the immunogeneicity of soluble antigens of *T. cruzi* is low. However, several of the bands induced proliferation with a frequency not much lower to the one observed with crude soluble antigens (Table VI).

Regulation. As shown in Fig. 2, the down-regulation mean of most bands, either of SE or of ST, was higher in INF (SE $p = 0.0021$; ST $p = 0.0028$). However, the frequency of down-regulation elicited by bands of both sub cellular fractions was higher in CDM than in INF (Table VII). This difference was statistically significant for ST ($p = 0.0009$) and SE ($p = 0.014$).

An interesting aspect was that more than 80% CDM had significant suppression with most ST bands. Six of them (B1, B4 to B7, and B9) induced down-regulation in 100% CDM. But, in a contrasting manner, band 3 induced relevant proliferation in 41% of CDM (mean CPM 1250), band 4 in 24% (mean CPM 1000), band 5 in 59% (mean CPM 2500) and band 6 in 29% (mean CPM 1250), suggesting that the proliferating mononuclear cells might be the ones that will modulate negatively the immune response

The up-regulation of the PPD proliferative response was more intense in INF with both sub cellular fractions (SE $p = 0.026$; ST $p = 0.0052$). As shown in Fig. 2, the up-regulation induced by SE bands was higher than the up-regulation elicited by ST bands.

The frequency of patients with relevant up-regulation (Table VII) was significantly higher in INF for SE ($p = 0.0075$) and ST ($p = 0.0015$). The difference of up-regulation frequency in INF and CDM was more pronounced with ST bands (Fig 2B). Bands 6, 7, 10, 13 and 15 of SE (Fig 2A) and bands 1, 3 to 10, and 15 of ST (Table VII) did not induce relevant up-regulation in any of the CDM studied. In INF only bands 2 and 4 of ST did not induce relevant up-regulation.

The observation that the UR with most bands was higher in INF and bands that induce DR in one hundred percent of CDM (bands 5 to 10 and 15) in INF induce UR in more than 31% of the INF (5-31%; 6-31%; 7-38%; 8-31%; 9-38%; 10-31% and 15-23%) suggest the presence of some interaction or balance, between up and down-regulation, in INF that is lost in CDM.

DISCUSSION

Our modification of the technique described by Aboud Zeid *et al.* had two fundamental effects: the NC particles generated had a size consistently similar to microorganisms (2.4 μm), and induced a more reproducible proliferation. In our opinion this is due to the fact that the size of NC parti-

TABLE VI

PERCENTAGE OF PATIENTS WITH RELEVANT PROLIFERATION INDUCED BY CRUDE SE OR ST ANTIGENS AND THEIR WESTERN BLOT BANDS

Group	Crude SE	SE Bands																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
CDM	83	18	29	35	12	35	29	35	29	29	35	29	24	24	18	29	18	29	12	12	18
INF	33	11	44	28	17	22	22	28	17	11	17	17	17	22	17	11	11	22	0	33	17
Group	Crude ST	ST Bands																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15					
CDM	76	24	18	41	24	59	29	41	24	29	12	18	6	35	18	18					
INF	50	16	5	11	26	26	11	21	11	5	5	11	5	11	11	11					

SE: Soluble Epimastigote antigens. ST: Soluble Trypomastigote antigens.

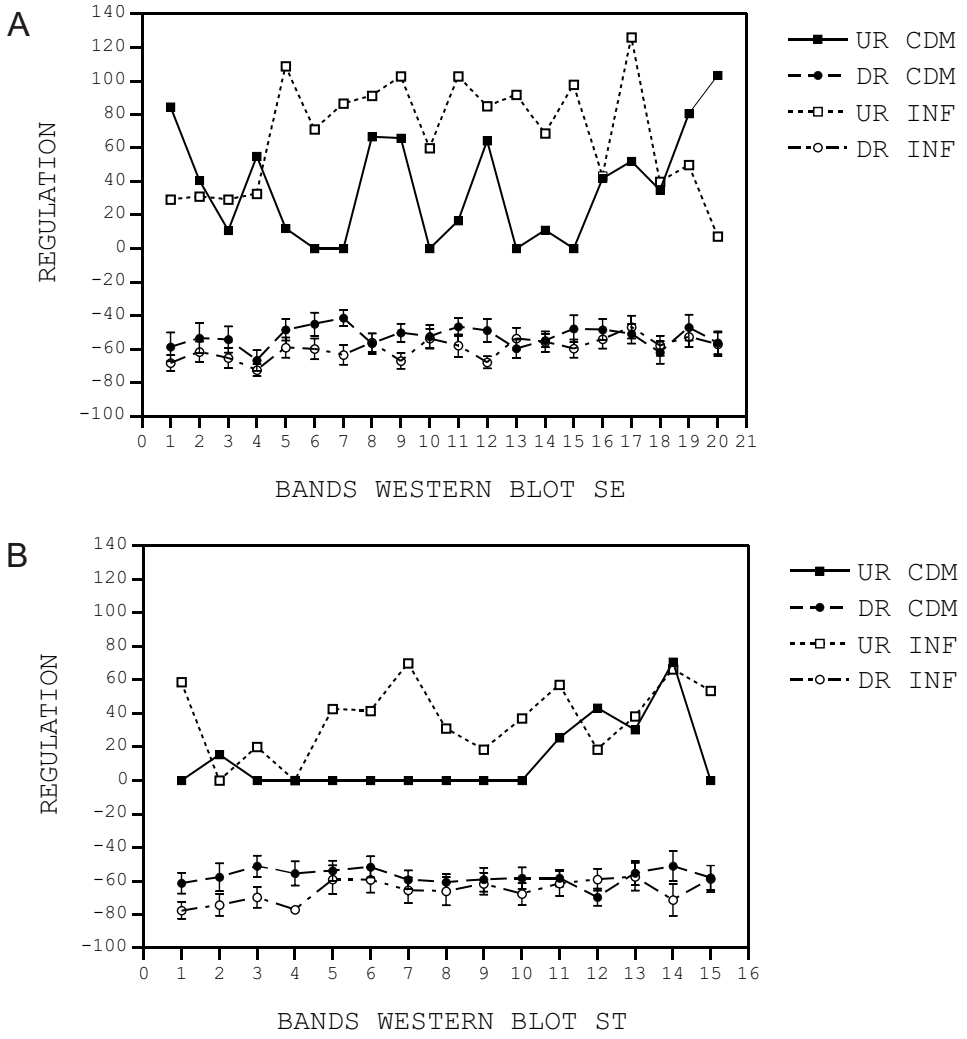

Fig. 2. Down-regulation and up-regulation observed with Western blot bands of the soluble subcellular epimastigote (SE) (A) and trypomastigote(ST) (B) fractions. Only patients with positive PPD proliferation and significant down-regulation (DR) or up-regulation (UR) values were included. The data shown are means +/– standard error. Standard error of UR is not included because, in most cases, the number of patients with a significant UR was small.

cles is more homogeneous and APC can internalize them easily. This should induce a more reproducible stimulation of lymphocytes as shown by our evaluation.

The proliferation elicited by soluble epimastigote antigens is less intense than the proliferation elicited by antigen A, and the proportion of patients with relevant proliferation is smaller in INF (33%) than in CDM (83.3%). These values are similar to those reported with a similar antigen (INF 27%; CDM 72.5%) by De Titto *et al.* (6), corroborating the validity of the results obtained with the antigens adsorbed to NC.

Using a similar technology Gazzinelli *et al.* (27) evaluated the proliferation induced by fragments of NC from Western blots of soluble epimastigote antigens. However, their approach was different from ours. First, there are technical differences with respect to the preparation of crude antigens and the processing of Western Blots.

TABLE VII

PERCENTAGE OF PATIENTS WITH RELEVAN TPROLIFERATION INDUCED BY CRUDE SE OR ST ANTIGENS AND THEIR WESTERN BLOT BANDS

	Group	n	SE	SE Bands																	
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
DR	CDM	10	44	80	80	80	70	90	90	70	70	80	80	80	70	90	80	80	80	100	70	80	60
	INF	14	85	93	86	71	93	64	64	50	50	64	71	71	57	64	71	64	71	64	64	64	79
UR	CDM	10	44	10	20	10	30	10	0	0	20	10	0	10	20	0	10	0	20	10	10	10	20
	INF	14	15	7	14	14	7	29	29	36	21	29	29	14	29	29	29	36	21	7	14	29	21
	Group	n	ST	ST Bands																			
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15					
DR	CDM	13	81	100	85	92	100	100	100	100	92	100	85	92	85	85	85	92					
	INF	13	77	85	77	92	92	69	62	54	54	62	62	62	92	77	46	62					
UR	CDM	13	6	0	8	0	0	0	0	0	0	0	0	8	8	15	8	0					
	INF	13	23	15	0	8	0	31	31	38	31	38	31	15	8	15	46	23					

SE: Soluble Epimastigote antigens. ST: Soluble Trypomastigote antigens.

Second, they did not evaluate the response to soluble trypomastigote antigens nor did they analyze the regulation induced by these antigens.

Contrary to our results these authors observe higher values of proliferation. This dissimilarity could be due to differences in the preparation and processing of antigens. On the other hand, they reported an important proliferation in the range of 43 to 57 kDa in both of their experimental groups (equivalent to our INF and CDM groups). Our data show that SE bands 3 to 6, enclosing the range from 43 to 57 kDa, also induced a important proliferation in CDM. In their study, two of 8 INF (24%) and one of 7 (14.3%) INF patients did not respond to any of the fractions evaluated. Our results also show that three of 18 (16.7%) INF, and two of 17 (11.8%) CDM did not respond to any of the SE bands. However, the frequency of patients that did not respond to any of the ST bands was significantly higher ($p = 0.0096$) in INF (55.6%) than in CDM (5.9%). This observation, together with the difference in response of INF and CDM to the different bands, reveals a difference in the intensity and pattern of response of INF and CDM, to antigens of the circulating form of the parasite, not evident with antigens of the culture form of the parasite.

It is important to underline that the average proliferative response, as well as the frequency of response to trypomastigote antigens of INF was lower, which indicates an association between the intensity of the response to these antigens and the presence of pathology. In this context, it is relevant to note that ST band 5 (72 to 84 kDa) induced proliferation with high frequency (59%) in CDM.

Down-regulation may play an important role in the persistence of chronic infection and in the development or control of immunopathology. Several authors have reported suppression in experimental models (8, 10-13, 28, 29). Morato *et al.* (7) reported experimental findings in Chagas patients that suggest the presence of suppression. Kierazenbaum *et al.* (30) demonstrated the presence of molecules, spontaneously released by trypomastigotes (termed Trypanosomal Suppressive Factor) that inhibit lymphocyte proliferation. In accordance with this, our results show that most epimastigote or trypomastigote antigens induce significant down-regulation. SE and ST bands gave a higher average down regulation in INF, but the frequency of relevant down regulation was higher in CDM for both antigens. Only one SE band, B17, induced down-regulation in all CDM patients. With ST, B1, B4 to B7 and B9 induced down-regulation in 100% CDM. On the basis of these differences, we postulate that regulation of the cell-mediated immune response to the parasite might affect different effector functions, such as control of parasitemia or induction of immunopathology, in the different patient groups.

It is reasonable to assume that the presence of significant proliferation should be associated with low or no down-regulation. However, in agreement with our previous data (14) we did not find an inverse correlation between proliferation and down-regulation.

The presence in INF of higher frequency and intensity of up-regulation with trypomastigote antigens suggests that in INF the response to parasite antigens is more efficient.

On the other hand our data support the notion that antigens or elements that induce down regulation of the immune response coexist with antigens that elicit an effector immune response; being prevalent the down regulation. The above conclusion is supported by data obtained by Hansen *et al.* (31), showing that the crude flagellar epimastigote antigen, previously reported as inducing protection against experimen-

tal infection (22) can be separated by affinity chromatography into two fractions with opposite immunological properties. The non-retained material induced important splenocyte proliferation. In contrast, ligand bound material inhibited the proliferation elicited by Concavalin A, as well as the proliferation induced by the non-retained material.

It is important to emphasize the importance of our observations for the formulation of a possible vaccine for Chagas' disease. In order to be effective, it will be necessary to identify and purify antigens that preferentially induce a protective response and eliminate those that stimulate suppression. Unfortunately, our data indicate that most antigens present in crude *T. cruzi* antigenic preparations induce down-regulation, more frequently than proliferation.

In conclusion, we have shown that most SE and ST semi purified antigens induce *in vitro* a significant down-regulation of the immune response and little proliferation. Furthermore, the bands that induce higher proliferation or down-regulation are different in INF and CDM. The difference of the data obtained in INF and CDM patients suggests that the response to these antigens varies according to the clinical status of Chagas' disease patients.

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