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Proliferation and bystander suppression induced by membrane and flagellar antigens of *Trypanosoma cruzi*.

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Key words: Chagas' disease, membrane, flagella, regulation, cellular immunity

Abstract. We have studied, *in vitro,* proliferation induced by flagella (FE) and membrane (ME) antigenic fractions of T. cruzi epimastigotes, as well as their regulatory effect on the proliferative response to PPD (Protein Purified Derivative). Crude flagella as well as bands from Western blots of flagella and membrane of epimastigotes were tested. Crude flagella elicited higher proliferation in mononuclear cells from patients with Chagasic cardiomyopathy (CDM) than in 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 FE, bands between 150 and 24.3 kDa (B3 to B18 with the exception of B4 and B13) induced higher CPM (Counts Per Minute) in CDM. In INF only bands B7 (87.3 to 80.1 kDa), 9 (69.8 to 64.6 kDa) and 13 (45.4 to 41.5 kDa) had high CPM. ME bands also elicited higher proliferation in CDM. However, only 5 out of 14 bands gave CPM higher than 1000 in CDM and none in INF. The mean down regulation (DR) of most bands was similar in both groups. But the frequency of relevant DR elicited by FE was significantly higher in CDM. In contrast the frequency of up regulation (UR) was higher in INF. Bands 13 and 14 of ME did not induce DR in most INF. The discordance between the frequency of relevant DR in CDM and INF was more evident with ME than with FE. The frequency of (UR) was 50% or higher with all ME bands in INF, but, lower than 12% in CDM. The higher UR in INF and of DR in CDM, suggest the presence of some balance or interaction in INF that is lost in CDM. In ME there might be antigens that could be relevant for the immunoprofilaxis of Chagas' disease. The difference in the clinical status of the two groups seems to be associated with the recognition of different groups of antigens together with variations in the nature of the regulation of the response of mononuclear cells to these antigens.

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Proliferación y supresión circunstancial inducida por antígenos de flagelo y membrana de *Trypanosoma cruzi.*
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Palabras clave: Enfermedad de Chagas, membrana, flagelo, regulación, inmunidad celular.

Resumen. Hemos estudiado, *in vitro,* la respuesta proliferativa de células mononucleares a antígenos de Flagelo (FE) y Membrana (ME) de epimastigotes de T. cruzi, así como su efecto regulador en la respuesta proliferativa a PPD (Protein Purified derivative). Fue evaluada tanto la respuesta a Flagelo crudo como a las bandas de Western blots de FE y ME. El flagelo crudo indujo una proliferación de las células mononucleares más intensa en los pacientes con cardiomiopatía (CDM) que en los pacientes sin evidencia de patología cardiaca (INF). Los antígenos fraccionados causaron una menor respuesta proliferativa, tanto en intensidad como en frecuencia, que los antígenos crudos. Las bandas de FE, entre 150 y 24,3 kDa (B3 a B18 con la excepción de B4 y B13), indujeron CPM mas altas en CDM. En INF solo las bandas B7 (87,3 a 80,1 kDa), 9 (69,8 a 64,6 kDa) y 13 (45,4 a 41,5 kDa) causaron CPM (Cuentas Por Minuto) altas. Las bandas de ME también indujeron una proliferación mayor en CDM. Sin embargo, solo 5 de 14 bandas tuvieron CPM promedio mayores de 1000 en INF. La mayor parte de las bandas causaron una baja regulación (BR) que fue similar en ambos grupos. Sin embargo, la frecuencia de BR relevante producida por FE fue significativamente mayor en CDM. Por el contrario la sobre regulación (SB) fue mayor en INF. Las bandas 13 y 14 de ME no indujeron BR en la mayor parte de los INF. La discordancia entre la frecuencia de BR relevante en CDM e INF fue mas evidente con ME que con FE. La frecuencia de SB fue 50% o mas con todas las bandas de ME en INF, pero menor que 12% en los CDM. El predominio de SB en INF y de BR en CDM sugiere la presencia, en los INF, de algún tipo de equilibrio o interacción que esta ausente en los CDM. En ME pudieran estar presente antígenos relevantes para la inmunoprofilaxis de la enfermedad de Chagas. En conjunto nuestros resultados sugieren que las diferencias en los cuadros clínicos pudieran tener relación con la respuesta observada a los diferentes antígenos tanto a nivel de regulación como de proliferación.

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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, a 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 studies (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 (7-12) as well as in Chagas' disease patients (7, 13).

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 (14). Furthermore, it has been reported that the frequency of natural regulatory cells (CD4+CD25+), in peripheral blood, is higher in INF than in CDM (15, 16).

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 in several antigens (17, 18). Miller *et al.* (19) 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 (13). On the other hand, Rowland *et al.* (20), 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 antigen induces pathology, while a flagellar antigen elicits protective immunity (21-23). 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 response elicited by different parasite antigens.

In a previous publication (24) we have demonstrated that the proliferative response of mononuclear cells from patients with Chagas' disease to bands of soluble, epimastigotes and trypomastigotes, antigens was poor with an important down regulation (DR) that was more frequent and intense with soluble antigens of trypomastigotes. The epimastigotes antigen used corresponds to the cell sap antigen reported as pathogenic and no protective. Because Ruiz *et al.* (23) have reported that the fraction of flagella induce an important protection we decided to study this fraction and the antigens of membrane of epimastigotes to see if they elicited a better response in mononuclear cells of patients. Surprisingly, Bands of FE induced a response compatible with our previous results with soluble antigens. However, with ME bands few patients with no evidence of cardiac pathology (INF) had a relevant DR and all ME bands gave a significant up regulation (UR) in more than 50 percent of the INF evaluated.

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 36 patients (23 males, 13 females), with positive serology to *T. cruzi* antigens, agreed to participate in this study. Patients were classified into two groups according to previous criteria (25). The 17 patients assigned to group I (INF) were seropositive for *T. cruzi* with no evidence of heart disease (mean age 47 years; range 32 to 63 years). The 19 patients assigned to group II, Chagasic cardiomyopathy, (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 52 years; range 28 to 72 years).

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 (25); 20 μL of this suspension were added to each culture well.

PPD (Protein Purified Derivative) (Statens Serum Institut, Denmark). Protein purified derivative (10 μg/mL), 20 μL/well.

Flagella (FE). The flagella subcellular fraction of epimastigotes of *Trypanosoma cruzi,* strain Y, were prepared as described by Piras *et al.* (26). Briefly, parasites were adjusted to a concentration of 3×10^8/mL in a solution of 0.32 M sucrose, 1% albumin, 0.1mM CaCl2. Sonicated with a Branson ultrasonicator (65 W for 30 seconds) using a microtip probe. After two cycles of sonication the quality of the preparation was evaluated by observation with a phase contrast microscope. Flagella were purified by differential centrifugation in a discontinuous gradient of sucrose. The purified flagella were evaluated with a phase contrast microscope and, if found to be adequate, used to prepare the flagella antigen.

Membrane (ME). The proteins from the membranes of epimastigotes were obtained by extraction with Triton X-114 as described by Bordier *et al.* (27).

Preparation of antigens adsorbed to nitrocellulose particles

Proteins in antigen samples were resolved by SDS-PAGE using a discontinuous buffer system (28). Either FE or ME (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. They were run under reducing and denaturalizing conditions, and transferred to nitrocellulose membranes (NC) by the method of Towbin *et al.* (29). 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 (24). 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 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 concentration of 2 × 10^5 cells/well. 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

TABLE I
RANGE OF MOLECULAR WEIGHTS OF WESTERN BLOTS BANDS

Bands	Flagella		Membrane	
	Upper	Lower	Upper	Lower
1	219.17	192.50	207.73	149.99
2	192.50	150.00	149.99	109.09
3	150.00	118.28	109.09	91.70
4	118.28	103.89	91.70	80.69
5	103.89	99.23	80.69	71.87
6	99.23	87.33	71.87	65.10
7	87.33	80.14	65.10	53.67
8	80.14	69.83	53.67	47.23
9	69.83	64.60	47.23	39.51
10	64.60	59.41	39.51	30.67
11	59.14	53.98	30.67	26.03
12	53.98	45.37	26.03	21.93
13	45.37	41.47	21.93	18.08
14	41.47	37.84	18.08	14.91
15	37.84	33.23		
16	33.23	29.95		
17	29.95	27.66		
18	27.66	24.32		

nitrocellulose particles from Western blots and soluble PPD were used at the same concentrations as for the cell proliferation assay.

The percentage of DR or UR was calculated as follows:

$$\text{Regulation} = \left(\frac{CPM(PPD + T.\ cruzi)}{CPM\ PPD + CPM\ T.\ cruzi} \times 100 \right) - 100$$

Regulation was evaluated only in patients with a significant proliferation to PPD (FE: INF 14, CDM 13; ME: INF 13, CDM 17).

Data analysis: criteria used to define relevant 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 relevant. Only patients with a relevant 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 relevant up or down regulation to the band.

– Frequency of relevant up or down-regulation: percentage of patients with relevant regulation to each band.
– The frequency of responses to crude antigens was evaluated using a Chi square test.
– The paired Wilcoxon signed-rank test was used to compare: percentage of patients with positive proliferation, mean values of down-regulation or up-regulation.

RESULTS

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 Flagella (FE) adsorbed to NC. The ME antigen was not evaluated because the presence of detergent minimize its adsorption to NC.

The proliferative response induced by antigen A was higher in CDM than in INF (Table II) without reaching the level of significance (p= 0.06). The CPM induced by FE were lower than with A but the tendency was similar, higher in CDM than in INF. The frequency of relevant proliferation with FE was similar in both groups.

Regulation. As reported (24) any value equal or lower than –10 was considered a relevant DR, and any value equal or higher than 10 a relevant UR.

The intensity of the DR of the response to PPD, induced by FE was similar in both experimental groups (Table III). The UR by FE was higher in CDM than in INF, but this difference was not statistically significant.

Analysis of the response to bands of western blots of FE or ME

Proliferation. In order to identify the antigens that stimulate and/or regulate the mononuclear cell response we studied the response of mononuclear cells to antigens of FE and ME semi purified by electrophoresis. The protein pattern is more complex in FE (18 bands) than in ME (14 bands) (Table I).

The analysis of patients with relevant proliferation to FE bands (Fig. 1B) shows that bands between 150 and 24.3 kDa (B3 to B18 with the exception of B4 and B13) gave high CPM (mean relevant CPM higher

TABLE II
MEAN PROLIFERATION INDUCED BY CRUDE ANTIGENS

Groups	Antigens	
	A	FE
CDM		
CPM	21738	4353
SEM	5253	1232
N	14	15
INF		
CPM	14416	2377
SEM	5898	612
n	18	19

SEM: Standard Error of the mean. FE: Flagella epimastigotes.

TABLE III
REGULATION INDUCED BY FLAGELLA

Regulation	CDM	INF
DR	–41.5	–50.5
%DR	75.0	64.3
	(9/12)	(9/14)
UR	108.7	72.9
%UR	25.0	35.7
	(3/12)	(5/14)

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.

than 1000) in CDM. In INF high CPM were observed only with bands 7 (87.3 to 80.1 kDa), 9 (69.8 to 64.6 kDa) and 13 (45.4 to 41.5 kDa). With ME (Fig 1A), the pattern observed was similar; in CDM the CPM were higher than in INF. and bands 3 and 4 (109.1 to 80.7 kDa), 8 and 9 (53.7 to 47.2 kDa)and 12 (26 to 21.9 kDa) had higher CPM in CDM. However, the number of bands with mean CPM greater that 1000 was lower (5 of 14) than with FE (14 of 18). Furthermore none of the ME bands induced a high proliferation in INF.

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 FE and ME bands (Fig. 1) 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 relevant 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 IV) was significantly higher in CDM (FE $p < 0.0001$; ME $p < 0.0001$). Bands 2, 3, 5, 6, 10, 12, 16, 17 and 18 of FE induced a relevant proliferation in more than 40% of the CDM. On the other hand in INF only band 2 elicited a relevant proliferation in more than 40% of the patients. The proliferation induced by

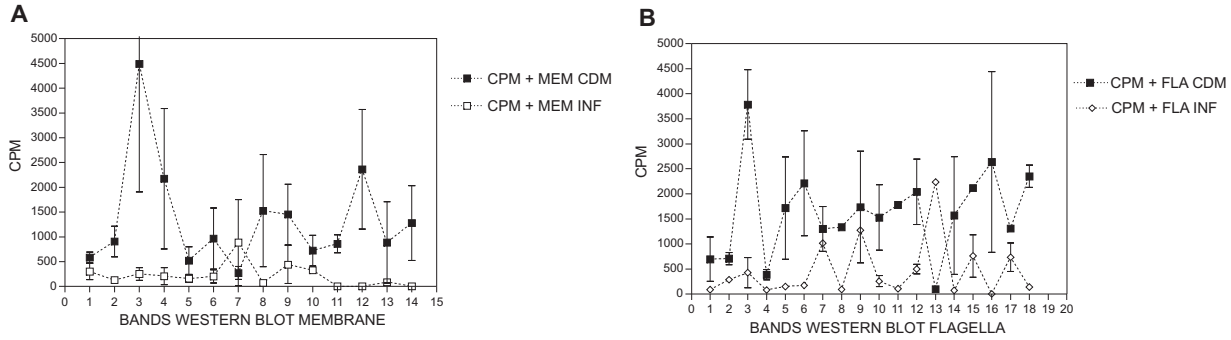

Fig 1. Average proliferation (CPM) induced by bands of ME (A) and FE (B) epimastigotes. Only CPM from patients with significant proliferation to the western blot bands were included. The data shown are means +/- standard error.

TABLE IV
PERCENTAGE OF PATIENTS WITH RELEVANT PROLIFERATION INDUCED BY WESTERN BLOT BANDS OF FLAGELLA AND MEMBRANE

Groups	FE Bands																		
	n	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
CDM	17	35	53	59	35	53	41	35	29	29	47	24	41	35	35	24	41	53	47
INF	19	11	42	21	5	16	21	16	21	5	32	11	16	5	11	16	0	37	21
Groups	ME Bands																		
	N	1	2	3	4	5	6	7	8	9	10	11	12	13	14				
CDM	17	12	18	53	35	24	24	18	41	41	35	53	24	12	12				
INF	15	20	7	20	20	27	40	13	13	27	7	0	0	20	0				

FE: Flagella epimastigotes. p 0.0001 (CDM vs INF). ME: Membrane epimastigotes. p 0.0001 (CDM vs INF).

Bands of ME was of lower in intensity, as well as in frequency of patients with relevant proliferation. Only bands 3, 8, 9 and 11 induced a relevant proliferation in more than 40% of the CDM and none in INF.

In summary the response of CDM to bands of FE or ME was more intense and frequent than in INF. Furthermore, bands of FE elicited a more intense response than bands of ME. Consequently the discrepancies observed were not only due to the difference in composition of FE and ME but also to the characteristic of the immune response of the patients in each group.

Regulation. As shown in Fig. 2, with most bands, of FE or ME the mean DR was similar in both groups. But, the frequency of relevant DR, induced by bands of FE (Fig. 2B) was higher in CDM and this difference was significant ($p = 0.0057$). In contrast with the former observation, the frequency of relevant UR elicited by bands of FE was significantly higher (Fig 2B) in INF, in intensity ($p = 0.0001$) as well as in frequency ($p = 0.028$). The low frequency of UR and the high frequency of DR suggest that the proliferation observed in CDM with bands of FE principally might be due to proliferation of regulatory cells.

Surprisingly, with ME the intensity of DR, in INF, oscillated more than with FE (Fig 2 A) and bands 13 and 14 (21.9 to 14.9 kDa) did not induce an important down regulation in most INF. The frequency of relevant DR was clearly higher in CDM for all bands. It was of interest that the discordance between the DR in CDM and INF was more evident with ME than with FE. This difference was highly significant ($p < 0.0001$) between CDM and INF (Table V).In agreement with the low frequency of DR, in INF, with bands of ME, the frequency of relevant UR, of all ME bands, was higher than 50 per cent. Most bands of ME induced UR only in a few CDM. The difference between CDM and INF was highly significant ($p < 0.0001$) and indicate a clear difference in the response to ME antigens.

The observation that the UR with ME bands was higher in INF and bands that induce DR in most of CDM, in INF elicited UR in more than 50% of the INF suggest, in INF, the presence of a balance, between up and down-regulation that is lost or diminished in CDM.

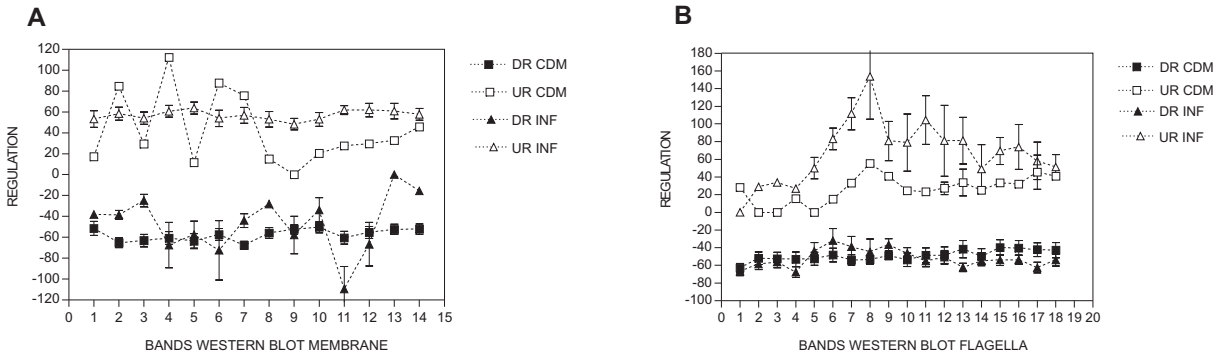

Fig 2. Frequency of patients with relevant down or up regulation with ME or FE. Mean down-regulation and up-regulation observed with Western blot bands of ME (A) and FE (B) of epimastigotes. Only patients with significant proliferation to PPD and significant down-regulation (DR) or up-regulation (UR) values were included. The data shown are means +/- standard error. Standard error of UR is included only when the number of patients with a significant UR allowed its estimation.

TABLE V
FREQUENCY OF RELEVANT DOWN AND UP REGULATION INDUCED BY WESTERN BLOT BANDS OF MEMBRANE AND FLAGELLA OF EPIMASTIGOTES OF *T. cruzi*

	Group	n	FE																	
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
DR	CDM	13	92	92	85	92	92	85	85	92	92	77	92	92	85	92	92	85	92	92
	INF	14	100	93	93	93	43	71	71	64	71	71	71	71	57	71	79	79	64	71
UR	CDM	13	8	0	0	15	0	23	8	8	8	8	15	15	15	15	8	8	15	8
	INF	14	0	7	7	7	43	29	29	36	29	21	21	14	29	14	14	14	29	29
	Group	n	ME																	
			1	2	3	4	5	6	7	8	9	10	11	12	13	14				
DR	CDM	17	82	82	88	88	88	88	82	82	88	82	88	76	76	82				
	INF	13	23	23	23	15	31	31	23	8	23	31	23	31	0	8				
UR	CDM	17	12	6	6	6	12	6	6	6	0	12	6	6	12	6				
	INF	13	62	62	54	85	62	62	62	92	77	62	54	62	77	85				

FE: Flagella epimastigotes: Frequency of DR p 0.0057 (CDM vs INF). Frequency of UR p 0.028 (CDM vs INF).
ME: Membrane epimastigotes: Frequency of DR p 0.0001 (CDM vs INF). Frequency of UR p 0.0001 (CDM vs INF).

DISCUSSION

In Chagas' disease the host parasite interaction is particularly complex. It has been reported that in the acute phase as well as in the chronic, the parasite induce regulation of the immune response. Previously, we have shown that *T. cruzi* specifically regulates the immune response (13) and that there are differences in the proliferation induced in CDM and INF (5). On the basis of these observations we have postulated that the development of heart pathology will depend on the dynamic equilibrium between immunopathology and control of the parasite (30). In a previous paper we have evaluated the regulation and proliferation induced by soluble antigens of epimastigotes or trypomastigotes (24). Most bands of either epimastigotes or trypomastigotes gave a low proliferation in CPM as well in frequency of patients with a relevant proliferation. None of the CDM showed UR with trypomastigotes bands but, several of them gave UR in more than 30 percent of the INF. Our previous data suggests that the cells proliferating might be regulatory cells more than effectors cells.

To increase our understanding of the role of different antigens in the genesis of the immune response to *T. cruzi* we have evaluated flagella, which have been reported as protector in experimental animals, and membrane antigens. FE bands gave a higher proliferative response in a larger percentage of CDM than of INF. This was surprising; if FE is protector, one would expect a better response in INF. Furthermore, most FE bands induced DR in more than 90 percent of CDM, suggesting that the proliferation might be due to regulatory cells more than effectors cells.

With ME these observation were more evident. There is a clear difference between the proliferative response of CDM and INF. As shown in Fig 1, the proliferation elicited by ME bands in INF was relevant in less than 20 percent of INF. However, all bands

gave a significant UR in more than 50 percent of INF. In our opinion the UR observed in INF it is not reflected as a proliferative response to ME bands because it is inhibited specifically by regulatory cells. This interpretation is in agreement with recent reports (15, 16) of a higher frequency of natural regulatory cells CD4+CD25+ in INF. CD4+CD25+ regulatory cells have been associated with regulation of immunopathology (31).

In conclusion the difference of the data obtained in INF and CDM suggests that the response to these antigens varies according to the clinical status of patients. The high frequency of UR observed with ME, in INF, implies the presence of antigens that might be relevant for the immunoprofilaxis of this disease.

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