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DIFFERENTIAL EFFECT OF CULTURE EPIMASTIGOTES AND BLOOD-FORM TRYPOMASTIGOTES ON NORMAL MOUSE SPLENOCYTE RESPONSIVENESS TO MITOGENS

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Blood form trypomastigotes of the Y strain of T. cruzi, *produced a strong inhibition of the blastogenic response to T and B cell mitogens, of the C3H/He, C57BL/6 and BALB/cJ strains of mice, while culture epimastigotes of the Y strain kept in a medium that allows parasite growth at 26º, 30º, 34º and 37ºC produced a strong stimulatory effect that was even higher than the effect of the mitogens alone. Both the inhibitory or the stimulatory effects were dose-dependent. The stimulatory effect of epimastigotes was also temperature-dependent producing increased stimulation indexes as the temperature of parasite cultures was raised. Metabolically active, living parasites seemed to be necessary for an improved lymphocyte stimulation suggesting a potential role of secreted metabolites as polyclonal activators of mouse lymphocytes.*

Key words: culture epimastigotes – blood-form trypomastigotes – mitogens – splenocyte

Only in the last few years have studies comparing the culture and blood forms of *Trypanosoma cruzi* been reported. Interesting differences have been found regarding antigenic glycoprotein markers of relevant immunological importance, both between culture- and blood-forms as well as between clones of epimastigotes or trypomastigotes separately (Araujo & Tighe, 1984; Astolfi-Filho, De Sá & Gander, 1984; Bongertz & Dvorak, 1983; Dvorak, 1984; Kirchhoff et al., 1984; Lanar & Manning, 1984; Plata, García-Pons & Eisen, 1984; Snary, 1983; Snary et al., 1981; Villalta & Kierszenbaum, 1983; Okanla, Stumpf & Dusanic, 1982). Also, an extraordinary effort has been put in the study and use of culture-forms of *T. cruzi*, treated in every imaginable way, as potential vaccines. The results obtained have been however only approximations to totally effective vaccination procedures.

Only a few reports though, have appeared, in which the potential role of trypomastigotes as immunoprotective agents have been evaluated and these reports are strongly suggestive that this later form of *T. cruzi* could be very valuable in the production of efficient vaccines (Culbertson & Kolodny, 1938; Basombrió, 1981; Krettli & Brener, 1982; Zweerink et al., 1984).

Therefore the object of this study was to assess the influence of both forms of trypanosomes on the cellular response of *in vitro* blastogenic transformation of normal mouse lymphocytes and to evaluate the effect of the culture conditions on this type of response, measured by a change in the temperature of culture of the parasites.

MATERIALS AND METHODS

Blood-form *T. cruzi*: Groups of 30 C3H/He, C57BL/6 and BALB/cJ mice were inoculated with 50,000 blood-form trypomastigotes of the Y strain of *T. cruzi* from blood of syngenic donors (Silva & Nussenzweig, 1953). After 10 days, they were bled by cardiac puncture and the blood collected into 0.4 ml of 3.8% (w/v) sodium citrate. The pooled blood was centrifuged at 910g for 5 min., the erythrocyte sediment was discarded and the supernatants containing the parasites saved. Equal volumes of RPMI-1640 were added to each plasma sample and the mixture incubated in 50ml centrifuge tubes at 37ºC, 5% CO_2, overnight, to decant remaining red and white cells. Trypanosomes remaining in suspension were carefully removed with a Pasteur pipet avoiding the resuspension of the cell-sediment formed by gravity. After centrifuging at 1200g for 15 min. and two washes with RPMI-1640, the parasites were finally resuspended in RPMI-1640 at adequate concentrations so as to add the desired number of trypomastigotes in 50 μl/well (see Table I). The parasite pellet was formed by 100% trypomastigotes with no contamination with blood cell elements as seen under the microscope.

Culture-form *T. cruzi* **and supernatants**: Epimastigote culture-forms of the Y strain of *T. cruzi* were maintained in a synthetic medium supplemented with 5% (v/v) fetal bovine serum, which permits parasite growth at 26º, 30º, 34º and 37ºC (O'Daly, Rodríguez & Garlin, 1985;

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submitted for publication). Parasites harvested during the exponential phase of growth were washed twice with RPMI-1640 and resuspended so as to add 0.5, 1 and 2 x 10^5 culture epimastigotes in 50 μl of RPMI-1640 per well.

Also, parasites grown at 26°, 30°, 34° and 37°C were resuspended in separate Falcon Flasks each containing 7.5ml of 5% FBS-RPMI-1640 with either 30, 15 or 7.5 x 10^6 cells, in order to maintain the same proportion of live parasites used in the blastogenic assay (0.5, 1 and 2 x 10^5 parasites/50 μl). After incubation for 48h at 37°C, 5% CO_2 in a humid chamber, the trypanosomes were removed by centrifugation at 1200g for 15 min. The supernatants were filtered through Millipore 0.22 μm filters into sterile tubes and used later on as 50 μl/well of each supernatant in the blastogenic assay.

Blastogenic assay: The spleens (3 per experiment) of C3H/He and C57BL/6 mice were teased apart in RPMI-1640 and the number of nucleated cells determined after staining with crystal violet. The suspensions were adjusted to 2 x 10^6 cells/ml in RPMI-1640 supplemented with 3% heat inactivated FBS and 100 μl aliquots placed in each well of flat microtiter plates. *T. cruzi* blood- and culture-forms as well as culture supernatants were added in 50 μl to triplicate cultures for each parasite concentration to be tested and incubated for 18h. at 37°C, 5% CO_2. Afterwards, optimal concentrations of mitogens: Concanavalin A, 1 μg/ml (Con A), Phytohemagglutinin, 0.25 μl/ml (PHA); Lypopolysaccharide, 20 μg/ml (LPS) and Dextran Sulphate, 10 μg/ml (DS) in 14% FBS-RPMI-1640 were added in 50 μl aliquots to complete a final volume of 200 μl/well. Each well contained 2 x 10^5 normal spleen cells, the trypanosomes or supernatant equivalents at the different proportions, FBS at 5% (v/v) and the mitogens at the optimal concentrations. The cultures were incubated for 48 additional hours and after that time 0.2 μCi/well of tritiated thymidine (Methyl-[^{3}H]-Thymidine, 2.5 Ci/mmol) was added in 10 μl aliquots. 18 hours later the cells were harvested on glass fiber filter paper with an automated cell harvester (MASH II) and the paper circles were placed in minivials containing Aquasol (NEN). Cultures containing trypanosomes at the different concentrations but no splenocytes were also set under the same conditions, as controls for incorporation of parasites *per se*.

Results are expressed as a stimulation index (SI) calculated as:

$$\frac{\text{cpm [spleen cells + parasites (trypomastigotes or epimastigotes)] } - \text{ cpm parasites}}{\text{cpm spleen cells}}$$

This formula was applied both for cultures with mitogens and without mitogens. Blood form-trypomastigotes when cultured alone, did not show thymidine incorporation. Each value represents the mean of the triplicate cultures, of one of two similar experiments, for each strain of mice used. Supernatants from culture epimastigotes obtained after incubating the equivalent number of parasites at each temperature were added to the spleen cell cultures in 50 μl aliquots and the stimulation indexes were calculated as: $\frac{\text{cpm (spleen cells + supernatants)}}{\text{cpm spleen cells}}$ in the presence or absence of mitogens respectively. Standard deviations were below 5-10% of the mean in all cases and are not shown in the table for the sake of simplicity.

RESULTS

As shown in Table I, blood-form trypomastigotes of *T. cruzi* could inhibit normal lymphocyte responsiveness at ratios of 1:10 and 1:40 parasite : splenocytes for C57BL/6, and to a lower extent at ratios of 1:10-1:20 for BALB/cJ, while C3H/He mice showed a completely blocked responsiveness at 1:1 or 1:2 ratios and no significant blockage at 1:400 parasite : splenocytes ratio. This dose dependency was seen in the presence or absence of mitogens.

Tables II and III show one of two experiments with similar results for the C3H/He and C57BL/6 strains of mice respectively used in the blastogenic assays with living culture epimastigotes grown at different temperatures. An important observation to be mentioned is the mitogenic effect of epimastigotes *per se* (in the absence of mitogens) for both C3H/He and C57BL/6 splenocytes. This effect was less intense for C3H/He mice (Table II) since stimulation indexes were 2.42, 6.25, 9.12, 12.43 at 1:2 parasite : splenocyte as the temperature increased and: 1.64, 1.94, 1.83, 2.33 at 1:8 ratios. For C57BL/6 mice the indexes were 15.25. 24.98, 30.57, 36.46, at 1:2 ratios and: 2.99, 7.84, 7.32, 8.80 at 1:8 ratios (Table III). This polyclonal effect of living epimastigotes was as strong as the Con A effect and much stronger than the effect of PHA, LPS or DS alone on the normal splenocyte responsiveness.

With the exception of the cultures containing Con A together with epimastigotes grown at 26°, 30° and 34°C, the presence of the living epimastigotes in the splenocyte cultures with mitogens, produced a significative additional stimulatory effect over the effect of T- or B-mitogens alone, as observed in the total cpm values.

TABLE I

Effect of blood-form trypomastigotes on the blastogenic response of normal mouse spleen cells

Strain	Trypomast.	Medium		Con A		PHA		LPS		DS	
	x10³/well	cpm	(SI)	cpm	(SI)	cpm	(SI)	cpm	(SI)	cpm	(SI)
C3H/He	200	0	(0.00)	0	(0.00)	0	(0.00)	0	(0.00)	0	(0.00)
	100	0	(0.00)	0	(0.00)	0	(0.00)	9	(0.00)	0	(0.00)
	0.5	692	(0.92)	9140	(0.99)	7214	(1.01)	3916	(1.00)	1446	(0.98)
	–	752	–	9193	–	7109	–	3919	–	1476	–
C57BL/6	20	394	(0.24)	123	(0.00)	40	(0.00)	1350	(0.09)	667	(0.30)
	10	1136	(0.69)	11530	(0.41)	4838	(0.27)	8680	(0.57)	1740	(0.80)
	5	1436	(0.87)	24561	(0.87)	15295	(0.86)	11876	(0.78)	2071	(0.95)
	–	1651	–	28137	–	17766	–	15266	–	2187	–
BALB/cJ	20	897	(0.61)	55189	(0.86)	24242	(0.78)	9741	(0.59)	3640	(0.75)
	10	1301	(0.89)	61536	(0.96)	26531	(0.85)	14387	(0.87)	4298	(0.90)
	–	1461	–	64349	–	31110	–	16597	–	4797	–

TABLE II

Effect of culture epimastigotes grown at 26°, 30°, 34°, and 37°C on the blastogenic response of normal C3H/He spleen cells.

T (°C)	Sp. C.	Epimast.	Medium		Con A		PHA		LPS		DS	
		x10³/well	cpm	(SI)	cpm	(SI)	cpm	(SI)	cpm	(SI)	cpm	(SI)
26	+	100	3662	(2.42)	11356	(0.66)	9770	(2.85)	7984	(1.15)	12332	(3.39)
	+	25	2419	(1.64)	5783	(0.33)	3362	(0.98)	3586	(0.65)	6186	(1.72)
	–	100	639	–	345	–	758	–	2697	–	669	–
	–	25	371	–	292	–	263	–	586	–	283	–
30	+	100	13110	(6.25)	12654	(0.61)	22206	(4.76)	20038	(2.76)	19487	(3.74)
	+	25	3105	(1.94)	5803	(0.33)	9424	(2.75)	5213	(0.97)	8612	(2.26)
	-	100	5298	–	2378	–	7155	–	7357	–	6624	–
	–	25	679	–	244	–	728	–	765	–	841	–
34	+	100	16482	(9.12)	16153	(0.91)	22362	(6.06)	19322	(3.10)	18500	(4.00)
	+	25	2891	(1.83)	9635	(0.57)	8266	(2.51)	6871	(1.37)	9473	(2.62)
	–	100	5082	–	1034	–	4200	–	5087	–	4756	–
	–	25	604	–	148	–	329	–	562	–	482	–
37	+	100	17770	(12.43)	41088	(2.38)	39865	(11.94)	23603	(4.64)	19247	(4.92)
	+	25	3369	(2.33)	16477	(0.97)	11036	(3.34)	7388	(1.51)	8512	(2.34)
	–	100	2234	–	1311	–	2110	–	2262	–	2340	–
	–	25	452	–	269	–	475	–	433	–	456	–
Controls	+	–	1250	–	16683	–	3162	–	4598	–	3436	–

In the case of the cultures with Con A and parasites there was an apparent inhibitory effect of the parasites grown at 26°, 30° or 34°C that diminished gradually as the temperature increased. This inhibition was not observed when epimastigotes were grown at 37°C, and cultured at a ratio of 1:8, while at a ratio of 1:2 it was stimulatory.

It should be noted than an increase in the temperature of epimastigote culture conditions from 26° to 37°C in the presence or absence of mitogens, leads to an increase in the stimulation indexes of normal C3H/He or C57BL/6 splenocytes. The temperature of 37°C being that at which maximum stimulations are reached (Table II and III).

TABLE III

Effect of culture epimastigotes grown at 26°, 30°, 34°, and 37°C on the blastogenic response of normal C57BL/6 spleen cells

T (°C)	Sp. C.	Epimastig.	Medium		Con A		PHA		LPS		DS	
		x10³/well	cpm	(SI)	cpm	(SI)	cpm	(SI)	cpm	(SI)	cpm	(SI)
26	+	100	8211	(15.25)	14165	(1.03)	17086	(2.68)	14530	(2.95)	9804	(5.81)
	+	25	1740	(2.99)	5863	(0.42)	6906	(1.09)	8487	(1.92)	3805	(2.28)
	–	100	337		162		630		2155		555	
	–	25	198		101		235		393		172	
30	+	100	16543	(24.98)	17828	(1.15)	20721	(2.30)	20075	(3.25)	14717	(5.76)
	+	25	4823	(7.84)	9917	(0.71)	12020	(1.83)	12851	(2.92)	8000	(4.65)
	–	100	3652		2150		6508		6431		5547	
	–	25	779		214		765		610		604	
34	+	100	19468	(30.57)	14848	(1.03)	19262	(2.43)	21368	(3.47)	14599	(6.44)
	+	25	4347	(7.32)	11614	(0.84)	11382	(1.79)	13591	(3.09)	4528	(2.62)
	–	100	3696		815		4312		6841		4339	
	–	25	565		128		384		641		352	
37	+	100	20206	(36.46)	23887	(1.68)	20438	(2.95)	19641	(4.10)	13916	(7.30)
	+	25	4958	(8.80)	13585	(0.98)	11857	(1.86)	10680	(2.43)	5080	(2.94)
	–	100	1393		970		2300		2462		2294	
	–	25	417		229		413		484		407	
Controls	+	–	516		13649		6150		4195		1592	

TABLE IV

Effect of supernatants from culture epimastigotes grown at 26°, 30°, 34° and 37°C on the blastogenic response of normal C57BL/6 spleen cells

Sup. of T.c.- T (°C)	Medium		Con A		PHA		LPS		DS	
	cpm	(SI)	cpm	(SI)	cpm	(SI)	cpm	(SI)	cpm	(SI)
S100 – 26°	1244	(2.34)	20800	(2.19)	6803	(2.09)	3629	(3.15)	3409	(3.13)
S25 – 26°	606	(1.14)	14051	(1.48)	5371	(1.65)	2016	(1.75)	1949	(1.79)
S100 – 30°	1136	(2.14)	21840	(2.30)	7984	(2.45)	5965	(4.57)	4193	(3.85)
S25 – 30°	523	(0.98)	16382	(1.73)	5190	(1.60)	2408	(2.09)	2058	(1.89)
S100 – 34°	1415	(2.66)	21020	(2.21)	7833	(2.41)	6451	(5.60)	3692	(3.39)
S25 – 34°	496	(0.93)	17088	(1.80)	4728	(1.45)	1843	(1.60)	1797	(1.65)
S100 – 37°	1126	(2.12)	28659	(3.02)	9627	(2.96)	9078	(7.88)	4084	(3.75)
S25 – 37°	484	(0.91)	18632	(1.96)	4889	(1.50)	2788	(2.42)	2200	(2.02)
Controls	531	–	9494	–	3255	–	1152		1089	–

Table IV shows that supernatants obtained from epimastigote cultures grown at the four temperatures, mimicked the stimulatory effect of living parasites, even though this effect was much less intense, mainly for the cultures with no mitogens added. The stimulatory effect of supernatants was nonetheless dose-dependent and in this case the correlation between temperature rise and increased stimulatory effectiveness was only clearly seen in the cultures supplementary treated with LPS. Under this experimental condition, the cultures with Con A and epimastigote supernatants, did not show an inhibitory but a stimulatory effect on normal splenocyte responsiveness at any of the parasite-equivalent concentrations tested. Similar results were seen when using C3H/He spleen cells under the same experimental conditions (data not shown).

DISCUSSION

A major finding reported in this paper is the striking difference between the blood-trypomastigotes and culture-epimastigotes of the Y strain of *T. cruzi* regarding their effect on the blastogenic response to T and B-cell mitogens of normal mouse splenocytes. Trypomastigotes proved to be strongly inhibitory of this type of response in ratios of parasite : splenocyte of up to 1:40 for the C57BL/6 strain of mice and also inhibitory, even though with less intensity, in ratios of up to 1:20 for the BALB/c strain of mice. This effect was shown regardless of the presence or not of any of the mitogens used and was apparently dose dependent. This confirms the results of Maleckar & Kierszenbaum (1983, 1984) and Corsini & Costa (1981) who found inhibition of blasto-

genic responsiveness of lymphocytes by living trypomastigotes or their extracts respectively, even though Ramos, Schadtler-Siwon & Ortiz-Ortiz (1979) did not find any trypomastigote effect *"in vitro"*. On the contrary, culture-form epimastigotes of the same strain, kept in culture for more than 20 passages in a medium that allows parasite growth at any temperature between 26° and 37°C (O'Daly, Rodríguez & Garlin, 1985) produced an extraordinary mitogenic effect that was in the same range or higher than the effect of Con A, and much stronger than the effect of PHA, LPS or DS, for both C57BL/6 and C3H/He mice. This stimulatory effect of culture epimastigotes was dependent on the dose of parasites used to stimulate the splenocytes and on the temperature at which parasites were grown, previously to the addition to the spleen cell cultures in the blastogenic assay. The higher the dose of parasites, the stronger the stimulation of thymidine incorporation obtained. Also, the higher the temperature of parasite culturing the stronger the incorporation seen. These results were similar for both strains of mice, only that C57BL/6 splenocytes seemed to be more prone to be stimulated by both mitogens and parasites than C3H/He.

When spleen cells were cultured with parasites and mitogens together, putting aside the results for the Con A stimulated cultures (see below), there seemed to be an additive effect on lymphocyte blastogenesis at the higher parasite concentration producing total cpm values that were, for C3H/He, equal or higher than the sum of the effect of parasites plus the ones of mitogens alone, while for the lower parasite concentration the additive effect seemed to be synergistic. For C57BL/6 the values were at the higher concentration, lower or equal to the summation of both the cpm for cultures stimulated by parasites or mitogens alone and again at the lower parasite density the effect was apparently synergistic. This suggests that living epimastigotes continuously secrete antigens that stimulate lymphocytes to proliferate, adding this effect to the polyclonal response of the mitogens.

The apparent inhibition reported for the cultures both with epimastigotes grown at 26°, 30° and 34°C and Con A could be interpreted as a partial temperature-dependent binding of Con A by the parasites, rather than as an inhibitory effect of the parasites *per se*, because: 1) the "inhibition" does not occur when parasites are grown at 37°C; 2) it does not occur with supernatants from parasite cultures in the presence of Con A, which mimic the stimulatory effect of the living parasites (see Table IV), and 3) considering the net cpm incorporated by the cultures with mixed parasites and mitogens as the result of the additive effect of both types of polyclonal activators, the diminution of incorporated cpm due to Con A absorption by parasites (hence changing the optimal concentration of the lectin) could be compensated by the polyclonal effect of the living epimastigotes. This would explain why there seems to be an apparently stronger inhibitory effect at lower parasite concentrations as there is less polyclonal stimulation by the lesser number of epimastigotes under probably equivalent suboptimal concentrations of Con A due to parasite binding. The fact that the parasite supernatants mimic the effect of living epimastigotes in a dose dependent fashion but to a much lower extent, support this idea. There being no inhibition in this case for the cultures containing Con A or any of the other mitogens. On the other hand, the lower blastogenic potency of parasite supernatants suggest that it is important that the parasites be alive to continuously stimulate normal splenocytes to proliferate, probably by active secretion of soluble molecules into the culture media.

Partial absorption of mitogens by trypomastigotes should also be taken into account to explain the inhibitory effect seen in Table I. The fact however that there are differential results under the same conditions (mitogen and parasite concentrations) for the various strains of mice used (which have similar dose-responses to mitogens) together with the fact that neither PHA, LPS or DS seem to be bound by trypanosomes in culture, as would be the case for Con A, make us think that we are observing a real inhibitory effect of trypomastigotes rather than a mitogen absorption on parasites.

Another interesting result reported here is the temperature dependency of lymphocyte stimulation produced by culture-epimastigotes. We have previously reported (O'Daly, Serrano & Rodríguez, 1983) protease activities of trypanosoma extracts grown at different temperatures (26° to 37°C) which showed a significant drop of more than 50% of the proteolytic activity when the temperature was increased from 26° to 37°C. This suggests that the secreted substances active as polyclonal activators could be less degraded when produced at 37°C, thus inducing a much better polyclonal effect on lymphocytes. Another possibility could be that there is an increase in the secretion of a particular substance with polyclonal activity or that different substances are produced when the temperature is increased up to 37°C.

In recent years some authors have found important differences between culture forms and virulent blood forms regarding the immunological relevance of particular immunogenic anti-

genic determinants (Araujo & Tighe, 1984; Astolfi-Filho et al., 1984; Lanar & Manning, 1984; Villalta & Kierszembaum 1983).

Finally it should be pointed out that despite the use in many laboratories of culture-form trypanosomes, treated in different ways the total absence of parasitemia in immunized mice has never been found (Brener, 1980; Teixeira, 1979; O'Daly & Azócar, 1984). The use of blood-form trypomastigotes as effective antigens in protecting mice from a challenge with virulent parasites has also been suggested (Culbertson & Kolodny, 1938; Basombrió, 1981; Krettli & Brener, 1982; Zweerink et al., 1984).

Efforts are under way in our laboratory to assess the potential protective capacity of the trypomastigote-form of *T. cruzi*. These efforts are mainly focused on the study and isolation of antigens that could be used as vaccine by the hybridoma technique.

RESUMO

Tripomastigotas de sangue da cepa Y de *T. cruzi* mostraram uma forte inibição da resposta de transformação blástica a mitógenos de células T e B, nas estirpes C3H/He, C57BL/6 e BALB/cJ de camundongos, enquanto epimastigotas de cultura da cepa Y mantidos em meio que permite o crescimento dos parasitas a 26º, 30º, 34º e 37ºC mostraram um forte efeito estimulante, que foi inclusive maior que o efeito dos mitógenos isolados. Os efeitos de inibição e de estimulação foram dependentes da dose. O efeito estimulante dos epimastigotas também foi dependente da temperatura, encontrando-se maiores índices de estimulação à medida que a temperatura da cultura dos parasitas foi aumentada. Parasitas vivos, metabolicamente ativos, parecem ser necessários para a obtenção de uma maior estimulação dos linfócitos, o que sugere um papel potencial dos metabólitos segregados como ativadores policlonais dos linfócitos dos camundongos.

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REFERENCES

ARAUJO, F. & TIGHE, L., 1984. Antigens of *Trypanosoma cruzi:* evidence that the 90kd protective glycoprotein antigen is expressed in blood-form trypomastigotes and may not be functional in dead epimastigotes. *J. Parasitol., 70* (1) :185-187.

ASTOLFI FILHO, S.; DE SÁ, M. & GANDER, E., 1984. Protein synthesis in purified trypo- and epimastigote forms of *Trypanosoma cruzi. Acta Tropica* (Basel) *41* (2) :109-115.

BASOMBRIO, M., 1981. Resistance to re-infection by *Trypanosoma cruzi* in chagasic mice. *Medicina* (Buenos Aires) *41* Suppl., :230-232.

BONGERTZ, V. & DVORAK, J., 1983. *Trypanosoma cruzi:* antigenic analysis of cloned stocks. *Am. J. Trop. Med. Hyg., 32* (4) :716-722.

BRENER, Z., 1980. Immunity to *Trypanosoma cruzi. Adv. in Parasitol., 18* :247-292.

CORSINI, A. & COSTA, M., 1981. Immunosuppression in mice infected with *Trypanosoma cruzi* (Chagas, 1909). II – Trypomastigote crude extract (TCE) suppress the humoral immune response in mice. *Rev. Inst. Med. Trop. São Paulo, 23* (3) :122-126.

CULBERTSON, I. & KOLODNI, M., 1938. Acquired immunity in rats against *Trypanosoma cruzi. J. Parasitol., 24* :83-90.

DVORAK, J., 1984. The natural heterogeneity of *Trypanosoma cruzi:* biological and medical implications. *J. of Cell. Biochem., 24* (4) :357-371.

KIRCHHOFF, L.; ENGEL, J.; DVORAK, J. & SHER, A., 1984. Strains and clones of *Trypanosoma cruzi* differ in their expression of a surface antigen and identified by a monoclonal antibody. *Mol. Biochem. Parasitol., 11* :81-89.

KRETTLI, A. & BRENER, Z., 1982. Resistance against *Trypanosoma cruzi* associated to anti-living trypomastigote antibodies. *J. Immunol., 128* (5) :2009-2012.

LANAR, D. & MANNING, J., 1984. Major surface proteins and antigens on the different *in vivo* and *in vitro* forms of *Trypanosoma cruzi. Mol. Biochem. Parasitol., 11* :119-131.

MALECKAR, J. & KIERSZENBAUM, F., 1983. Inhibition of mitogen-induced proliferation of mouse T and B lymphocytes by bloodstream forms of *Trypanosoma cruzi. J. Immunol., 130* (2) :908-911.

MALECKAR, J. & KIERSZENBAUM, F., 1984. Suppression of mouse lymphocyte response to mitogens *in vitro* by *Trypanosoma cruzi. Int. J. Parasitol., 14* (1) :45-52.

O'DALY, J. & AZOCAR, J., 1984. Immunological alterations in Chagas' disease. In: Critical Reviews in Tropical Medicine. Vol. 2 pp. 189-208. Plenum Publishing Corporation, New York.

O'DALY, J.; RODRIGUEZ, M. & GARLIN, G., 1985. Growth requirements of *Trypanosoma cruzi* at different temperature in fetal bovine serum or peptide supplemented media (submitted for publication).

O'DALY, J.; SERRANO, L. & RODRIGUEZ, M., 1983. Free aminoacid pool and porteolytic enzymes in *Trypanosoma cruzi* cultured *in vitro*. *Int. J. Parasitol., 13* (5) :433-440.

OKANLA, E.; STUMPF, J. & DUSANIC, D., 1982. Crossed-Immunoelectrophoretic analysis of *Trypanosoma cruzi* epimastigote, metacyclic and bloodstream forms. *J. Parasitol., 68* (4) :538-546.

PLATA, F.; GARCIA PONS, F. & EISEN, H., 1984. Antigenic polymorphism of *Trypanosoma cruzi:* clonal analysis of trypomastigote surface antigens. *Eur. J. Immunol., 14* (5) :392-399.

RAMOS, C.; SCHADTLER-SIWON, I. & ORTIZ-ORTIZ, L., 1979. Suppressor cells present in the spleens of *Trypanosoma cruzi* infected mice. *J. Immunol., 122* :1243-1247.

SILVA, L. & NUSSENZWEIG, V., 1953. Sobre uma cepa de *Trypanosoma cruzi* altamente virulenta para o camundongo branco. *Folia Clin. Biol.,* (São Paulo) *20* :191-208.

SNARY, D., 1983. Cell surface glycoproteins of *Trypanosoma cruzi:* protective immunity in mice and antibody levels in human chagasic sera. *Trans. R. Soc. Trop. Med. Hyg., 77* (1) :126-129.

SNARY, D.; FERGUSON, M.; SCOTT, M. & ALLEN, A., 1981. Cell surface antigens of *Trypanosoma cruzi:* use of monoclonal antibodies to identify and isolate an epimastigote specific glycoprotein. *Mol. Biochem. Parasitol., 3* (6) :343-356.

TEIXEIRA, A., 1979. Chagas' disease: trends in immunological research and prospects for immunoprophylaxis. *Bull. W.H.O., 57* :697-710.

VILLALTA, F. & KIERSZENBAUM, F., 1983. Cross-reactivity of vector-borne metacyclic forms of *Trypanosoma cruzi* with mammalian and culture stages. *J. Protozool., 30* (2) :329-331.

ZWEERINK, H.; WESTON, H.; ANDERSEN, O.; GARBER, S. & HAYES, E., 1984. Immunity against infection with *Trypanosoma cruzi* in mice correlates with presence of antibodies against three trypomastigote polypeptides. *Infect. Immun., 46* (3) :826-830.