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IMMUNOSUPPRESSION IN MICE INFECTED WITH TRYPANOSOMA CRUZI (CHAGAS, 1909)

I — Evidences of polyclonal B cell activation in experimental infections mimicked by an extract prepared from circulating trypomastigotes

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SUMMARY

Balb/C mice injected i.v. with an extract (TCE) prepared from circulating trypomastigotes of **Trypanosoma cruzi** strain Y exhibited polyclonal B cell activation. An increase in PFC against SRBC and Fl-SRBC was detected in the spleen of TCE injected mice similarly to experimentaly infected mice. Bone marrow colony forming units were also increased in size when singeneic mice were given the extract together with the marrow cells. This phenomenon may be related to the humoral immunosuppression verified in experimental American trypanosomiasis.

INTRODUCTION

Experimental infections with **Trypanosoma cruzi** profoundly suppress humoral and cell-mediated immune responses in mice. This has been demonstrated in heavily infected animals usually after 6 days of infection [7,18,20].

The mechanisms of that immunosuppression are poorly understood and suppressor T cells have been involved with "in vivo" suppression to thymus dependent and independent antigens and lack of response of spleen cells to mitogens (PHA and LPS) "in vitro" [18].

Suppressor cells, T lymphocytes and macrophages, were demonstrated to participate in suppressive mechanisms raised by **T. brucei** infections in mice [8,15].

On the other hand, an exhaustion of B and T cell potential share with suppressor cells an important part in African trypanosomiasis suppression particularly after the second week of infection [2,8].

Moreover, a correlation between polyclonal activation of the immune system and immunodepression was described in **T. brucei** infection [14]. This phenomenon would be explained by a mitogen secreted by the parasites pushing B cells towards a pathway of proliferation and differentiation [13]. Evidences of this mitogen were obtained with lysates of African trypanosomes [4,12,17]. Consequently, both phenomenons i e. suppressor cells and clonal exhaustion possibly raised by a mitogen secreted by the parasites seem to operate in African trypanosomiasis to suppress immune responses.

In this paper we report the evidences of polyclonal B cell activation in **T. cruzi** infections and the mitogenic activity of an extract (TCE) obtained from circulating trypomastigotes.

This mitogen could account for the humoral suppression in infected mice as shown in the following paper.

MATERIAL AND METHODS

1. **Animals** — Balb/C and CBA female and male mice, bred and kept in our animals facilities were used with 6-8 weeks of age.

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2. **Trypanosome — Trypanosoma (Schizotrypanum) cruzi** strain Y, obtained from Dr. Zigman Brener (Belo Horizonte — Brazil) weekly passaged in Swiss-55 mice according to instructions [5] were used throughout.

3. **Trypanosome infections** — Balb/C mice were infected i.p. with 100 parasites obtained from Swiss infected mice at the peak of parasitaemia (7 days after i.p. infection with 10^5 parasites). Blood was harvested from the axillary plexus in MEM-1% FCS with 10 IU/ml of heparin and kept in ice bath. Parasites were counted in a Neubauer haemocytometer. Final dilutions were done in serum-free MEM.

4. **Parasitaemia** — Parasites in peripheral blood were counted as described by BRENER[5]. Mice were marked in the foodpad so parasitaemia could be followed-up in every single mouse.

5. **Separation of parasites from infected blood** — Five ml of infected blood were centrifuged at room temperature (240g) during 10 minutes in Falcon 50 ml conical plastic tubes (2070 Tube — Falcon, Oxnard, USA) then rested without disturbance 1 hr at room temperature. The plasma was then carefully removed and the pellet resuspended to the initial volume with Eagle — MEM 5%/FCS. The same procedure described before was repeated and the supernatant added to the plasma harvested before. Parasites were then washed (3x) in serum free medium and suspensions at the desired concentration were prepared and kept at -70°C. Yield was usually 50-60% with no visible (40x magnification) contamination with red blood cells.

6. **Trypomastigote crude extract (TCE)** — The extract was prepared by freezing-thawing (3x) the suspension of trypomastigotes containing different amounts of parasites in Eagle-MEM. The preparation was then centrifuged at 10.000g during 10 minutes at 4°C. The pellet was discarded and the supernatant called Trypomastigote Crude Extract (TCE) kept in 1 ml vials at-70°C. Microscopical examinations of the extract did not show any evidence of viable parasites.

7. **PFC assay** — Jerne PFC were assayed following instructions described by DRESSER[11].

8. **Inhibition of PFC against fluoresceinated — SRBC** — Fluorescein was attached to SRBC following instructions described by CALDERON et al.[6]. Inhibition experiments were performed adding 10 μl of a 5 mg/ml Fluorescein solution in Carbonate-Bicarbonate buffer to the cell suspension just before plaquing.

9. **Colony forming units (CFU) in the spleen** — CFU in the spleen were determined in CBA mice as described by TILL & MCCULLOGH [21]. Viable bone marrow cells ($3x10^4$) were injected i.v. into irradiated (750 rads — Co^{60} source) mice. The number of CFU was determined 8 days later.

RESULTS

1. **Parasitaemia in BALB/C mice infected with 100 parasites** — Mice infected i.p. with 100 parasites developed an increasing parasitaemia detected from the 7th day onwards peaking on day 10 after infection. Mice usually controlled the parasitaemia around day 12 keeping very low numbers of circulating parasites up to the 20th day after infection. Mortality is usually very high reaching 90% by day 20. Only few (less than 5%) mice survived longer than 30 days of infection. There was not any correlation between parasitaemia and mortality. Female and male mice had practically the same behaviour towards infection (Fig. 1).

2. **Spleen cell number during infection — T. cruzi** acute infections usually determine splenomegaly both in human beings and in experimental animals. To score the increase in spleen cells mice were killed several days after infection. Viable spleen cells increased significantly after the 3rd day peaking on day 7. This cell number remains high during all the 20 days of infection. Survivors usually had a decrease in viable cell numbers but the number was still 2x normal values (data not shown). Viability, curiously did not change significantly in infected mice (Fig. 2).

3. **Background PFC in the spleen of infected mice** — Since a suppression of humoral response against SRBC has been described [7,18], a mechanism underlying suppression was investigated in infected mice. An increase in background PFC was noticed early in infection peaking on day 3. Background PFC number was between normal range after 7 days of infection. Increased numbers of PFC were noticed when

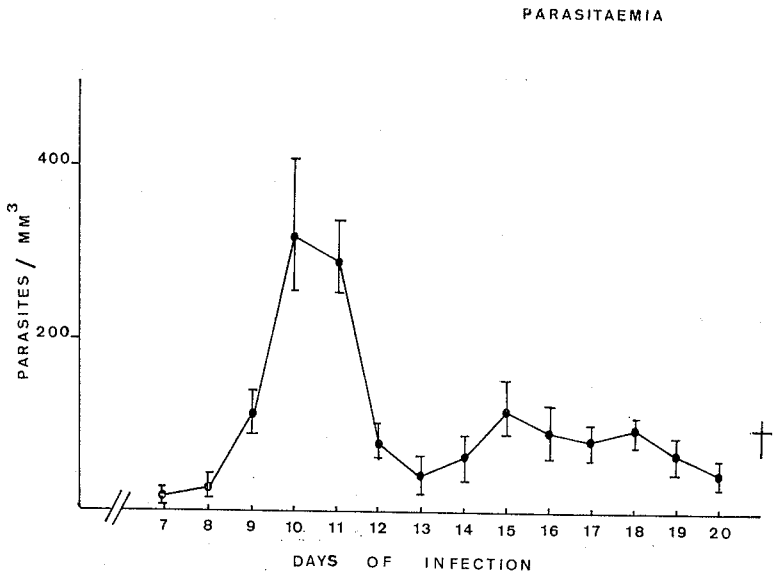

Fig. 1 — Parasitaemia in Balb/C mice infected with **Trypanosoma cruzi** strain Y. Mice infected i.p. with 100 parasites. Results express the geometric mean ÷ SD

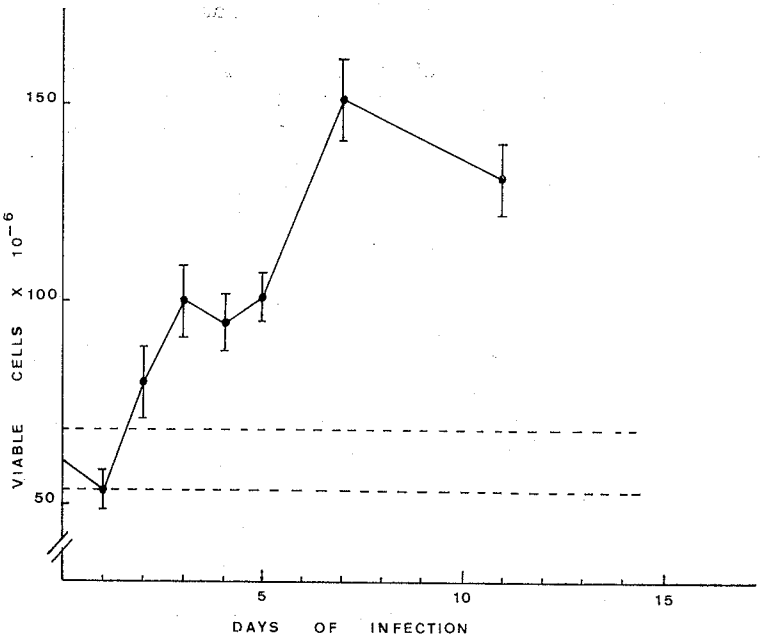

Fig. 2 — Viable cells in the spleen of **Trypanosoma cruzi** infected mice. Mice infected i.p. with 100 parasites. Viability assessed by trypan blue exclusion. Results express the arithmetic mean ± 1 SEM (n = 5). Dotted lines represent the normal values ± 2 SEM

expressed either as PFC/10^6 viable spleen cells or PFC/spleen (Fig. 3).

4. **Mitogenic activity of an extract prepared from circulating trypomastigotes** — Since an increase in background PFC in the spleens of infected mice would suggest the possibility of a mitogen secreted by the parasites pushing B cells clones towards proliferation and differentiation we examined the activity of an extract prepared by freezing-thawing trypomastigotes in different concentrations ($4x10^6$ to $1,0x10^6$ parasites/ml) when inoculated i.v. into normal mice. Results in Table I show a 20 to 30 times fold increase in PFC against SRBC in mice inoculated with 0.2 ml of the extract.

The kinectics of this mitogenic activity was studied in mice inoculated i.v. with 0.2 ml of a suspension containing $2x10^6$ parasites/ml. A

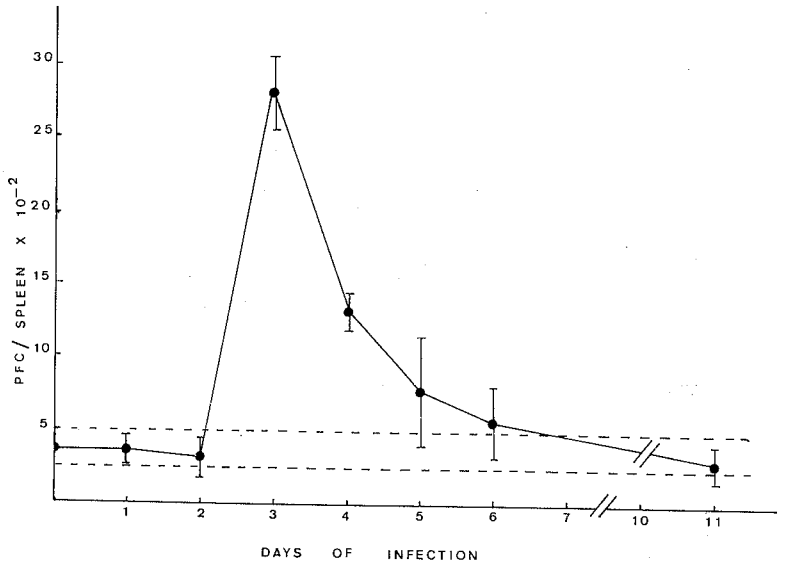

Fig. 3 — IgM PFC against SRBC in the spleen of mice infected with **Trypanosoma cruzi.** Mice infected i.p. with 100 parasites. Results express the arithmetic mean ± 1 SEM (n = 5). Dotted lines represent the normal values ± 2 SEM

significant increase in PFC against SRBC was obtained from the 2nd day onwards peaking on day 4. PFC were still high 6 days after inoculation (Fig. 4).

Since **T. cruzi** and SRBC might share common determinants and consequently PFC increase would indeed measure this cross-reaction we looked for the response to other determinants. Mice were inoculated i.v. with 0.2 ml of a suspension containing several amounts of trypomastigotes and the PFC against F1 — SRBC determined 3, 4 and 5 days afterwards. Table II presents the results expressed as PFC/spleen or PFC/10^6 viable cells on the 4th day after inoculation.

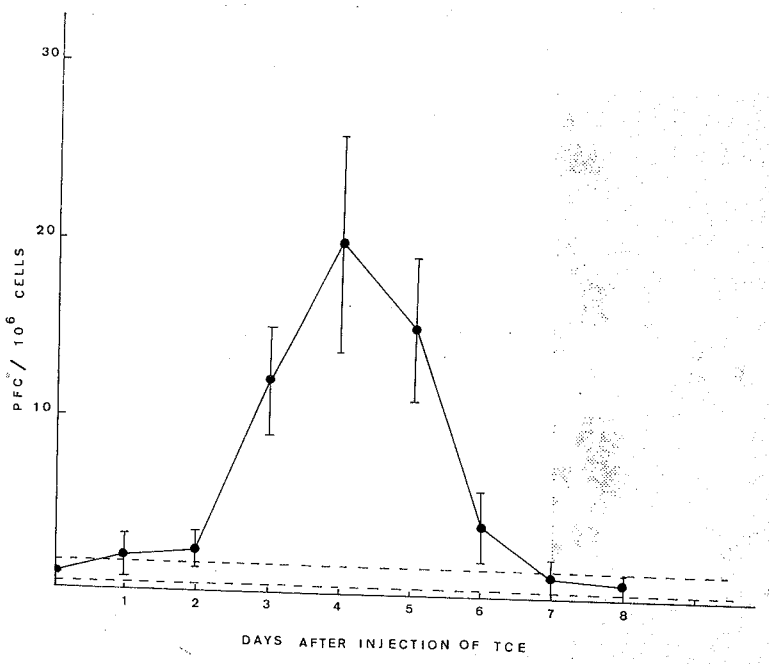

Fig. 4 — IgM PFC against SRBC in the spleen of mice injected with **Trypanosoma Crude Extract** (TCE). TCE prepared as described in **Material & Methods.** Results express the arithmetic mean ± 1 SEM (n = 5). Dotted lines represent the normal values ± 2 SEM

T A B L E I

PFC against SRBC in mice injected with Trypomastigote Crude Extract

Parasites/ml x 10^6	PFC/spleen	PFC/10^6 viable cells
4.0	2352 ± 112	30.6 ± 2.4
2.0	2320 ± 196	31.4 ± 3.0
1.0	1720 ± 242	21.7 ± 2.5
0	110 ± 17	1.1 ± 0.2

TCE prepared as described in **Material & Methods.** Mice injected with 0.2 ml of TCE. PFC assayed 4 days after inoculation. Arithmetic mean ± 1 SEM (n = 5)

T A B L E II

PFC against F1-SRBC in mice injected with Trypomastigote Crude Extract

Parasites/ml x 10^6	PFC/spleen	PFC/10^6 viable cells
2.0	1920 ± 102	25.9 ± 1.4
1.0	1112 ± 101	14.0 ± 0.7
0.5	856 ± 63	10.8 ± 0.8
0.25	1152 ± 147	11.8 ± 1.5
0.1	416 ± 59	5.4 ± 1.4
0	80 ± 14	1.9 ± 0.4

Mice injected i.v. with 0.2 ml of TCE. PFC assayed 4 days after inoculation. Arithmetic mean ± 1 SEM (n = 5)

An increase in PFC was noticed in a large range from 0.25 to 2.0x10^5 parasites/ml. Even 0.2 ml prepared from as little as 1x10^5 parasites/ml gave a positive result.

Inhibition experiments performed with free Fluorescein added to the spleen cell suspension immediatly before plaquing confirm the specificity of the reaction since free F1 inhibited 75% of the PFC (Table III).

T A B L E III

Inhibition of PFC against F1-SRBC with Fluorescein

Groups	PFC/spleen	PFC/10^6 viable cells
F1 — Treated	85 ± 22[a]	1.4 ± 0.5
Control	381 ± 37[b]	5.6 ± 1.0

a — b $p < 0.001$

Mice injected i.v. with 0.2 ml of TCE from 1x10^6 parasites/ml. Background PFC from uninjected controls (155 ± 23) discounted from PFC of experimental groups. Arithmetic mean ± 1 SEM (n = 5)

We concluded that a mitogen in TCE interferes with the proliferation and maturation of B cells pushing them towards antibody secretion.

5. **Colony forming units (CFU) in spleens of mice injected with TCE** — In order to verify if TCE would interfere with bone marrow CFU in spleen of animals injected with syngeneic cells, mice received either on day of cell transfer or during 3 days 0.2 ml of TCE prepared from a suspension containing 2x10^6 trypomastigotes.

There was not any difference in CFU number between injected and controls groups. On the other hand, the size of the colonies in TCE injected mice was considerably greater than controls, particularly in animals injected on the day of cell transfer (Fig. 5). This result implies that cells divided faster, consequently we

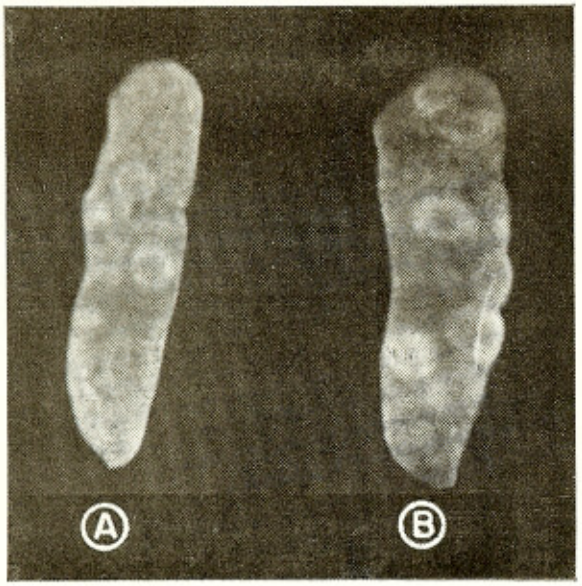

Fig. 5 — Colony Forming Units in the spleen of mice injected with Trypanosoma Crude Extract (TCE). A) **Normal controls.** B) **TCE injected mice.** Mice injected with TCE on the day of cell transfer.

have more cells in those colonies than in normal controls. This is a direct evidence of a mitogenic activity in TCE.

DISCUSSION

Balb/C mice are highly susceptible to **T. cruzi** strain Y. One hundred parasites injected i.p. were able to kill 95% of the animals in 20 days. Parasites were detected in peripheral blood 7 days after infection. During this prepatent period spleen cells increased 3 x in infected animals.

Background PFC against SRBC were also increased during this period. As many as 2.500 PFC/spleen were detected in 3 days infected mice which corresponds to a 30 fold increase. Background PFC returns to normal levels after 1 week of infection at the moment when parasites begin to be detected in peripheral blood. These data suggest that important alterations in spleen cell populations took place during the pre-patent period.

It has been demonstrated that Ig producing cells require several phases of proliferation and differentiation before they mature in to IgG producing and secreting cells [3,22]. The lymphocytes are driven to these pathways of proliferation and maturation by signals provided by antigen and cell interactions or by substances called mitogens or polyclonal lymphocyte activators (PLA) such as LPS [9].

ANDERSON et al. [1] have shown that one out of three splenic B lymphocytes reacts with LPS being activated to clonal growth and maturation to IgM secretion. Similar frequencies were found for B cells sensitive no **Nocardia** mitogen and mitogen compounds in fetal calf serum. Moreover, these Authors have shown that once activated a B cell displays receptors to other mitogens. This fact suggests that a large proportion of B lymphocytes reacts to mitogens found currently in nature such as LPS and **Nocardia.**

A mitogen pushing B cells towards proliferation and differentiation had been suggested in **T. gambiense** infections [13]. A correlation between high Ig levels and immunodepression was demonstrated in mice infected with **T. brucei** [14]. An exhaustion of $B^{2,8}$ and T cell potential[2] was also demonstrated in experimental African trypanosomiasis. Extracts prepared from **T. brucei** were claimed to have mitogenic activity over spleen cells "in vitro" [4,12,17]. It appears, consequently, that African trypanosomes provide such signal for B cell proliferation and differentiation.

In this paper we showed evidence that a similar phenomenon of polyclonal activation occurs in **T. cruzi** infected mice. Thus, the increase in spleen cell number and IgM secreting cells are quite similar to what occurs in **T. brucei** infections [14,16].

The existence of a mitogen in **T. cruzi** infections is suggested by the experiments performed with the extract prepared from circulating trypomastigotes. Thus, PFC against SRBC were developed in mice injected with 0.2 ml of TCE prepared from suspensions containing several amounts of parasites. Curiously the time course of the increase in PFC against SRBC was practically that verified in infected mice i. e. PFC peaked on day 4 in TCE injected mice and on day 3 in infected mice.

Since there was a possibility of cross reactivity between common determinants existing in trypanosomes and SRBC we looked for PFC against F1-SRBC. Inhibition experiments with free F1 confirmed that PFC were against that hapten and not SRBC determinants.

COUTINHO & MÖLLER [9] argue that a marked increase in background IgM PFC to a highly hapten-substitued target cell is in itself a good measure of polyclonal B cell activation. This enables us to conclude that TCE exhibits polyclonal B cell activation.

The results obtained with bone marrow CFU in spleen of TCE injected animals confirms the mitogenic activity in this extract. This result implies also that very immature cells in bone marrow are susceptible to this mitogen, a fact that may be related to clonal exhaustion of stem cells.

We still do not have any data about the biochemical characterization of the mitogen in TCE. Since the source of TCE is the infected blood a large contamination with serum protein does occur. This fact is important since suppression of humoral response to thymus dependent and independent antigens have been

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described in mice injected with serum harvested from infected animals [10] that might be due to mitogen contamination.

In conclusion: There is an evidence of polyclonal B cell activation in **T. cruzi** infections and that activity was found in an extract obtained from circulating trypomastigotes. Experiments are being trypomastigotes. Experiments are being carried out to verify if TCE acts independently of T cells. This polyclonal B cell activation may be related to the humoral immunosuppression verified in this infection.

RESUMO

Imunosupressão em camundongos infectados com Trypanosoma cruzi **(Chagas, 1909)**

I — Evidências de ativação B policlonal na infecção experimental e atividade mitogênica de um extrato de tripomastigotas

Camundongos Balb/C infectados com **T. cruzi** cepa Y mostraram evidências de ativação policlonal das células esplênicas do tipo B. Fenômenos semelhantes foram obtidos mediante a inoculação endovenosa de um extrato obtido de tripomastigotas circulantes. Células de medula óssea também foram suscetíveis à ação mitogênica do extrato que poderá estar relacionada com a imunosupressão verificada na infecção experimental.

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