Rev. Inst. Med. trop. São Paulo
26(2):67-77, *março-abril*, 1984

UDC 593.161.13
616.937.3

SUPPRESSION OF HUMORAL IMMUNITY AND LYMPHOCYTE RESPONSIVENESS DURING EXPERIMENTAL TRYPANOSOMA CRUZI INFECTIONS

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SUMMARY

C3H/He and C57B1/6 mice were inoculated with 500 **Trypanosoma cruzi** trypomastigotes (Strain Y). During the acute phase infected mice presented parasitemia and enlargement of lymph nodes and spleens and intracellular parasites were observed in the heart. Examinations of cells derived from spleen and lymph nodes showed increased numbers of IgM and IgG-bearing cells. During the peak of splenomegaly, about day 17 post-infections, splenic lymphocytes showed a marked decrease in responsiveness to T and B-cell mitogens, parasite antigens and plaque forming cells (PFC) to sheep red blood cells (SRBC). Unfractionated or plastic adherent splenic cells from mice, obtained during the acute phase were able to suppress the response to mitogens by lymphocytes from uninfected mice. During the chronic phase. Disappearence of parasitemia and intracellular parasites in the hearts as well as a decrease in spleen size, was observed. These changes preceded the complete recovery of responsiveness to mitogens and **T. cruzi** antigens by C57B1/6 splenic lymphocytes. However, this recovery was only partial in the C3H/He mice, known to be more sensitive to **T. cruzi** infection. Partial recovery of humoral immune response also occurred in both strains of mice during the chronic phase.

INTRODUCTION

Mice infected with **Trypanosoma cruzi** develop splenomegaly, lymph node enlargement and immunosuppression during the acute phase of the infection (CLINTON et al.[3]; ROWLAND & KUHN[21]; CUNNINGHAM et al.[4]; RAMOS et al.[18]; HAYES & KIERSZENBAUM[10]). Suppression of humoral and cellular immunity has been reported during the acute phase; however, controversy exists about the normalization of immune functions during the chronic phase in **T. cruzi** infected mice (HAYES & KIERSZENBAUM[10]; KIERSZENBAUM & HAYES[13]; CUNNINGHAM & KUHN[5,6]). Controversy exists also, about the mechanism of immunosuppression in **T. cruzi** infected mice. While some Authors described a T-lymphocyte suppressor mechanism (RAMOS et al.[18]), this has not been confirmed by others Authors (CUNNINGHAM & KUHN[5,6]).

In this study aimed at understanding the immunological changes occurring in mice during the acute and chronic phase of **T. cruzi** infections, we selected two strains of mice: C3H/He and C57B1/6, known to show differences in susceptibility to infection by **T. cruzi** (TRISCHMAN et al.[24]). A low dose of **T. cruzi** was used to obtain a relatively long survival of the infected mice to permit studies during the acute and chronic phase. In our work we correlate in the same animal and at the same time the effect of a given inoculum with spleen growth, amastigote pseudocysts in heart, plaque forming cells, response to parasite antigens and po-

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lyclonal activators, cell proliferation in lymph nodes and spleens of a given immunoglobulin bearing class. Our results provide evidence that support the immunocompetent recovery of patients (TEIXEIRA et al.[23]), as well as experimental animals with Chagas'disease.

MATERIALS AND METHODS

Animals — C3H/He and C57B1/6 mice weighing 19-22 gr (Jackson Laboratories, Bar Harbor, Maine) were used in all experiments. Purina Laboratory chow and water were supplied ad libitum. The mice were housed in a temperature controlled animal room in bonnet-covered plastic cages with six to eight mice in each cage.

Infections — Mice were infected intraperitoneally (i. p.) with 5 x 10^2 blood-form trypomastigotes of the Y strain of **T. cruzi.** This strain was obtained from a patient in Brazil in 1950 (SILVA & NUSSENZWEIG[22]) and maintained by serial passage in mice. In 1967 Dr. A. Velasquez introduced the strain in Venezuela and continued its passage in heterozygous mice. Since 1975 it was maintained in our Laboratory by serial passages in homozygous mice (C57B1/6, C3H/He and BALB/C). The LD_{30} at 30 days post infection was obtained by inoculating four groups of 20 mice each with 5 x 10^2, 5 x 10^3, 2 x 10^4 and 5 x 10^4 **T. cruzi** (REED & MUENCH[19]).

Kinetics of infections — Experimental mice were bled by clipping the end of tail every 3 days, until day 33 post-infection. Parasitemia was determined in 5 µl of blood taken with a disposable micropipete (microcaps, Drunmond Scientific Col.) placed under a 22 x 22 glass coverslip and the parasites counted in 100 fields of view at 400 X. To determine the intracellular parasites, hearts of mice were obtained at 5, 10, 17, 30, 60 and 90 days post-infection. The organs were fixed in 10% (v/v) formaldehyde in phosphate buffered saline solution (PBS, pH 7.4), embedded in tissue-Prep (Fisher Scientific, Co., N.° T-565) and stained with hematoxylin-eosin. Serial sections of each of five experiments were made and amastigotes as well as inflammatory infiltrates observed at 100 X. A focus of inflammatory infiltrate was defined as a conglomerate of more than 20 cells with lymphoid morphology. To assess splenomegaly of uninfected mice, the spleens were removed at different days post-infection (three/experiment) and weighed in sterile 35 x 10 mm disposable Petri dishes. Immediately the spleens were pooled, teased with forceps in RPMI 1640 culture medium (Gibco, Grand Island, New York) and the cells divided for biastogenic response and immunofluorescence studies.

Spleen and lymph node cells — Spleen cells were obtained as described above. Lymph node cells were obtained from normal and infected mice from the inguinal, axillary and brachial lymph nodes. The lymph nodes were processed as described for spleens.

Blastogenic assays — Two x 10^5 nucleated cells/well (Falcon N.° 3040) in a total volume of 200 µl of RPMI-1640 supplemented with 5% fetal bovine serum (FBS-Gibco), penicillin (100 µ/ml), streptomycin (100 µg/ml) and 25 mM hepes (Gibco), were used in triplicate for each assay. Cells were incubated at 37 C in 5% for 48 hr, after which 0.2 µCi of ^{3}H-thymidine (2.5 Ci/mmol, New England Nuclear, NEN) was added to each well and incubated for 18 hr until the time of harvest. The ability of spleen cells to proliferate in response to mitogens was assessed by adding suboptimal, optimal and supraoptimal doses of: Phytohemagglutinin (0.125, 0.25 and 0.5 µl/ml. PHA-Difco, Detroit, Michigan); Concanavalin A (0.5, 1.0 and 2.5 µg/ml Con A, Sigma); bacterial lipopolysaccharide (10, 25 and 50 µg/ml; **Escherichia coli** LPS, Sigma); and Dextran sulphate (5, 10 and 25 µg/ml, DS, Sigma). Cultures were processed with a multiple automated cell harvester (M 24, Brandel, Gaitherburg, MD). After drying, the glass fiber discs (Reeve Angel, grade 934) were placed in scintillation counting vials and counted in Packard scintillation fluid. Data are expressed as the difference in counts per minute (Δ CPM) between experimental (stimulated) and control (unstimulated) cultures or as % suppression, which was calculated as 1 {(experimental)/CPM (control) x 100}. Each point represents the average of three differents experiments ± standard deviation (SD).

Determination of immunoglobulin-bearing cells — Suspension of mononuclear cells derived from spleen and lymph nodes were sedimented in a cytocentrifuge (Shandon) at 900 g for 15 min. Duplicate slides were prepared for each experiment and stained with the appropriate anti heavy chain antibody.

Fluorescein conjugated goat anti-mouse IgG, IgM and IgA was purchased from Cappel Laboratories (Cochranville, PA). The specificity of each antibody was tested with the appropriate control before the assays. Each antibody was used at the appropriate dilution using PBS. The slides were processed for immunofluorescence staining as described before (CEBRA & GOLDSTEIN [2]). Cells were counted with the aid of a fluorescence microscope (Zeiss). The results are expressed as IgG and IgM-bearing cells of a total of 1 x 10^3 splenic or lymph node cells, and each value represents the average of three experiments ± SD (Fig. 5).

Heart tissues were also examined for immunoglobulin-bearing cells using cryostat sections of mice at different days post-infection. After counting the fluorescent cells, the tissue sections were stained with hematoxylin-eosin and the percentage of fluorescent/total lymphocytes in inflammatory infiltrates was calculated.

Preparation of T. cruzi antigens — T. cruzi, strain Y, was grown in a modified culture medium (O'DALY et al. [16]) containing 5% (v/v) GG free fetal bovine serum at 26, 30, 34 and 37C. 10^9 trypanosomes, at stationary phase of growth, were washed three times in 40 ml of PBS by centrifuging at 900 g for 20 min at 4 C, and sonicated for 5 min at 4 C in 10 mM PMSF (phenyl methyl sulfonyl fluoride, Sigma) in PBS containing 2% (v/v) 2 propanol (O'DALY, SERRANO & RODRÍGUEZ [17]).

After incubation, the crude extract was precipitated with an equal volume of 10% (w/v) trichloroacetic acid (TCA) and centrifuged at 100 g for 30 min at 4 C. The supernatant (low molecular weight antigens) was filtered through 0.22 μm Millipore membranes to avoid contamination with precipitated proteins, and extracted three times with 5 ml of diethylether to remove TCA. The final aqueous phase was liophylized, dissolved in saline solution and sterilized by 0.22 μm Millipore filters. The TCA precipitate (high molecular weight antigens) was washed twice with 5% (w/v) TCA finally dissolved in saline and sterilized through 0.22 μm Millipore filters. The pH of both preparations was adjusted to 7.2.

Fractionation of spleen cells — Spleen cells were passed through a column of Sephadex G-10 as described previously and reported to enrich the effluent fraction for T (non-adherent) lymphocytes (LY & MISHELL [14]). These cells will be referred to as non-adherent A (—) cells. Plastic adherent (A) cells were prepared according to ALBRIGHT et al. [1].

Hemolytic plaque assay — Two groups of 30 C3H/He and C57B1/6 male mice, 10-12 weeks of age, were infected with **T. cruzi** on day O. The primary splenic antibody response was studied at intervals after infection. Four days before the experiment, each mouse received an i.p. injection of 0.4 ml of a 10% sheep erythrocyte (SRBC) suspension in PBS. The splenic secondary responses were studied in mice infected, inoculated with SRBC, 4 and 14 days before the hemolytic plaque assay. A slide modification of the Jerne plaque assay (JERNE et al. [11]) was used. Spleens were teased in RPMI-1640 and cycled in a plastic syringe, first through a 23-gauge needle and then through a 26 gauge needle. Goat anti-mouse IgG (Cappel Laboratories) at 1:800 dilution in RPMI-1640 was used for the expression of 7S class antibodies. Guinea-pig serum diluted 1:30 in RPMI-1640 was used as a source of complement. Plaque forming cells (PFC) data were normalized with a log transformation (GOTTLIEB [9]). Uninfected mice immunized with SRBC as described above were used as controls.

Cocultured experiments — Unfractionated and plastic-adherent cells were obtained from infected mice and cocultured for 48 hr with splenic cells from uninfected mice for 48 hr in RPMI-1640 and the mitogen responsiveness determined as described above. The results are expressed as ΔCPM and compared to responsiveness shown by splenic cells of infected and uninfected controls.

RESULTS

Parasitemia and spleen size data during the acute and chronic phases of **T. cruzi** infection in C3H/He and C57B1/6 are shown in Fig. 1. Parasitemias reached a maximum at day 10 in both strains of mice and decreased progressively until parasites were not seen under the microscope at 25 (C57B1/6) and 33 days (C3H/He) after infection. Spleen size increased with a doubling time of approximately 5 days after infection. Maximum spleen size was found at day 17 post-infection with a subsequent decline

until the weight stabilized at approximately three times the normal.

The histological analysis of the heart of infected mice showed the presence of intracellular parasites and inflammatory infiltrates by day 17th post infection. While the numbers of pseudocysts/heart were greater in C3H/He (5.6 ± 1.5) as compared to C57B1/6 (one); the numbers of inflammatory foci observed at day 17th post infection were similar in C3H/He and C57Bl/6 mice (48 ± 4 and 50 ± 8). At this time, extensive pericarditis, proliferation of endocardial cells and mononuclear cell infiltrates in subendocardial spaces were observed. By day 30 post-infection the presence of pseudocysts and inflammatory foci had decreased in C3H/He (2 pseudocysts and 10 ± 1.5 inflammatory foci per heart) and C57B1/6 (1 pseudocyst and 8.5 ± 2.1 inflammatory foci per heart). At 60 days post infection, no parasites were observed in hearts of both C3H/He and C57B1/6; and the few inflammatory infiltrates (6/heart in both mouse strains) were predominantly formed by histiocytes and fibroblasts. It was not possible to find parasites nor inflammatory infiltrates by 90 days post-infection.

The LD_{50} measured 30 days post-infection was 17,500 trypanosomes for C3H/He and 27,000 trypanosomes for C57B1/6 mice.

The responsiveness of spleen cells to mitogens is shown in Fig. 2. The response to T and B-cell mitogens was lowest at 17 days post-infection. The response to T cell mitogens was recovered sooner (30 days post infection), than the response to B-cell mitogens, which was only partially recovered in C3H/He mice. Experiments designed to study the effect of splenic cells from infected mice on responsiveness to mitogens by uninfected syngeneic splenic cells

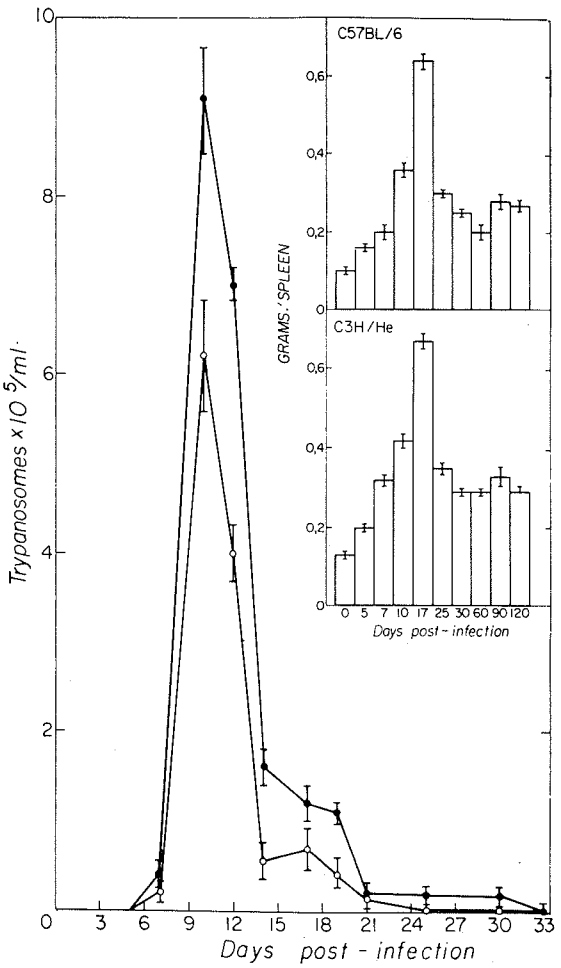

Fig. 1 — Parasitemia and splenomegaly in **T. cruzi** infected mice. Parasitemia was determined at different days post-infection of C57B1/6 (○—○) and C3H/He (●—●) mice as described in **Material and Methods.** The spleens size data at different days post-infection in both mouse strains are also presented. Each value represents the average of three experiments ± S.D.

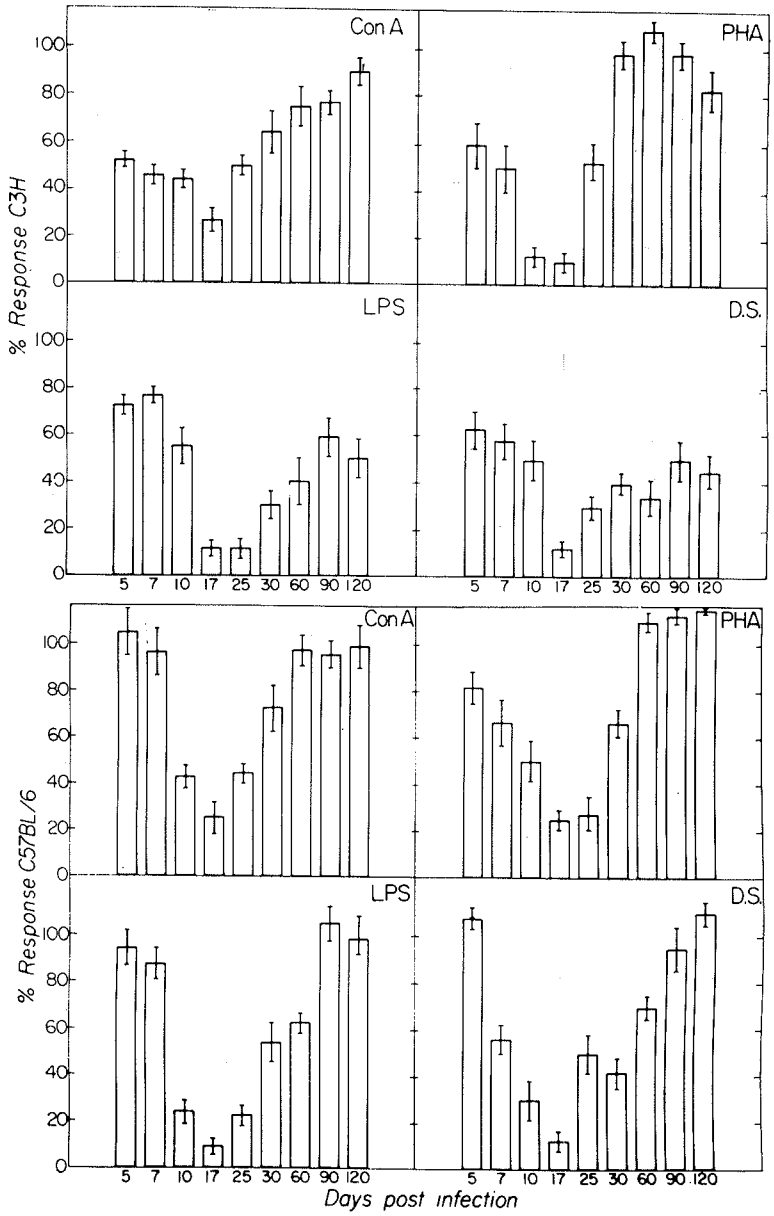

Fig. 2 — Blastogenic response of splenic cells to Con A, PHA, LPS and D.S. was determined at different days post-infection in both C57B1/6 and C3H/He mice. Results are expressed on % of C.P.M. values obtained with uninfected controls. Each value represents the average of 3 experiments ± S.D.

are shown in Fig. 3. The non-adherent A(—) fraction responded only to T-cell mitogens as expected, suggesting that this fraction was enriched in T-cells (LY & MISHELL [14]). These A(—) non adherent cells obtained at day 17 post-infection did not affect the responsiveness to T or B-cell mitogens displayed by spleen cells from uninfected (N) mice; when cocultured. However, unfractionated or plastic adherent (A) splenic cells obtained at 17 days post-infection were able to suppress the responsiveness of spleen cells from uninfected (N) mice. As expected plastic adherent cells alone (A) did not responded to T or B cell mitogens. Plastic adherent cells from uninfected mice cocultured with unfractionated spleen cells from uninfected mice (nA + C) responded normally to T and B-cell mitogens (Fig. 3). In no case trypanosomes were found in the culture wells used for the blastogenic assay. However, culture of these cells in liquid medium (O'DALY [15]) showed epimastigotes after 3 weeks cul-

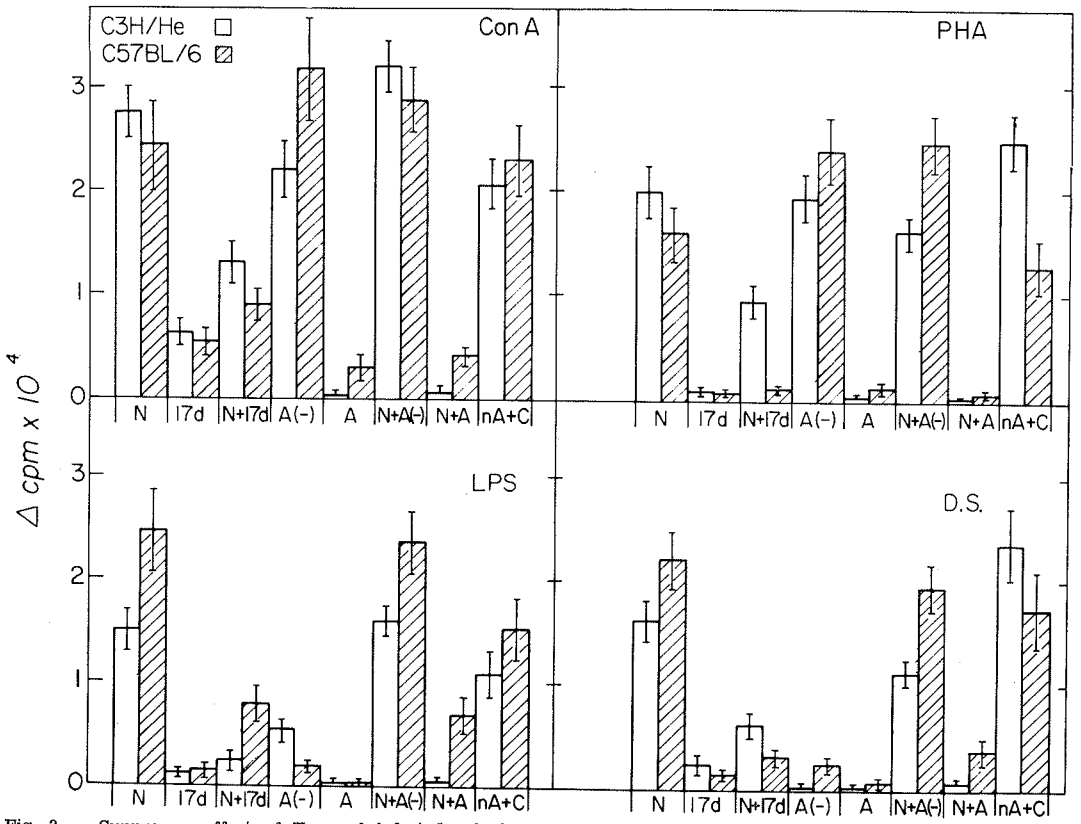

Fig. 3 — Suppressor effect of **T. cruzi** infected splenic cells on blastogenic response to mitogens exhibited by splenic cells from uninfected mice. Responsiveness to Con A, PHA, LPS and D.S. was measured in splenic cells from uninfected (N) and **T. cruzi** infected mice at 17 days (17 d) post-infection. The mitogens responsiveness of non-adherent [A(—)] and plastic adherent (A) splenic cells of 17 days **T. cruzi** infected mice are shown. The responsiveness of splenic cells of uninfected animals co-cultured with unfractionated (N + 17 d) non-adherent [N + A (—)] and plastic-adherent (N + A) syngeneic splenic cells from mice at 17 days post-infection are also shown. Controls where plastic-adherent spleen cells (nA) from uninfected mice were co-cultured with spleen cells also from uninfected mice (nA + C) are included. ΔCPM was determined using 10^5 cells as described in **Material and Methods.** Each value represents the average of 3 experiments ± S.D.

ture of the nonadherent (non-suppressive) cells. No parasites were observed after one month culture of the plastic adherent cells of infected mice.

Similar evidence for the recovery of the blastogenic response to low molecular and high molecular weight antigens in C57B1/6 mice is presented in Fig. 6. Between days 10 to 50 there was suppression of the blastogenic response and by day 90 the spleen cells responded again as normal cells. It should be noted that the high level of response against trypanosomal antigens at day 0 was sustained by days 5 and 7 after infection. Also at day 0, C57B1/6 responded twice as much as C3H/He mice, antigens from trypanosomes cultured at 34 C being the most efficient. The recovery of blastogenic response again was better with the high molecular weight antigens from trypanosomes grown at 34 C. In C3H/He mice the response to low molecular weight antigens was negligible and with high molecular weight antigens it remained suppressed after 10 days post-infection and up to 90 days.

The Jerne plaque assays showed suppression of primary and secondary response to SRBC at 17 days post-infection, as compared to uninfected mice. A progressive recovery in PFC activity was observed during the chronic infectious phase (Fig. 4).

The quantitation of IgG and IgM-bearing cells in spleens and lymph nodes of infected mice is shown in Fig. 5. The IgA-bearing cells

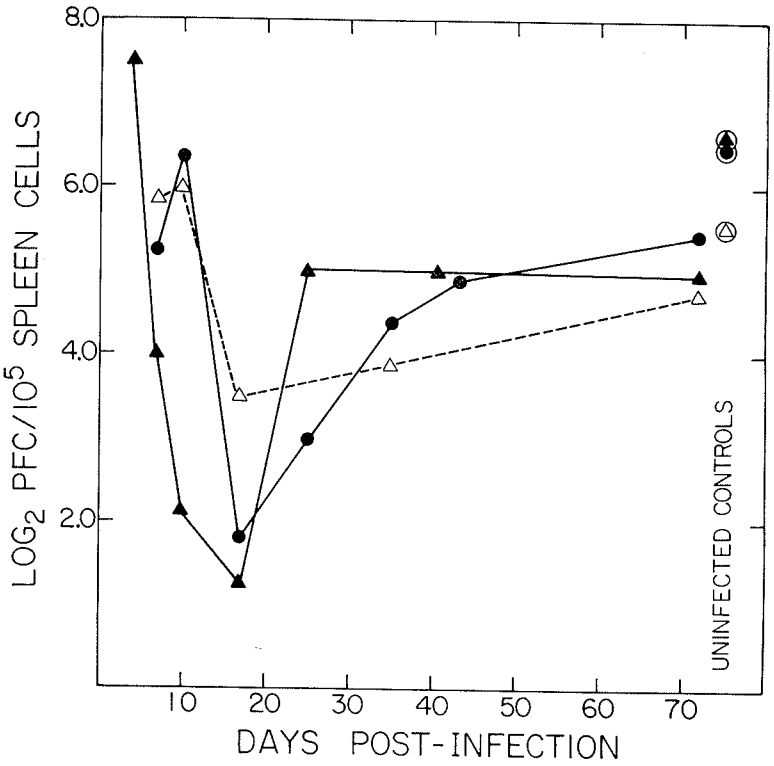

Fig. 4 — Plaque forming cells (PFC) activity to SRBC by splenic cells of uninfected and **T. cruzi** infected mice. Primary (▲—▲) and secondary (Δ- - -Δ) responses of C57B1/6 and primary response of C3H/He (●—●) mice at different days post-infection was determined as described in Materials and Methods. Primary response of uninfected C57B1/6 ▲ and C3H/He ● and secondary response of uninfected C57B1/6 (Δ) is also shown. Each value represents the average of 3 experiments. The data were normalized and expressed as Log_2 of PFC per 10^6 spleen cells.

are not shown, since they did not deviate from the counts performed on uninfected mice. As seen in Fig. 5, IgG and IgM-bearing cells increased in the spleens and lymph nodes of infected mice, reaching a maximum 17 days post-infection and coinciding with the time of maximum splenomegaly and suppression of response to mitogens. The increase in immunoglobulin bearing cells was higher in the spleen of C3H/He mice, than C57B1/6, during the acute infectious phase.

DISCUSSION

Experimental infection of mice with **T. cruzi** is followed by a series of events affecting immunocompetent cells. C3H/He mice show higher sensitivity to experimental **T. cruzi** infection as evidenced by higher parasitemias, higher mortality, a lower LD_{50} dose and greater number of intracellular parasites in the hearts (TRISCHMANN et al.[24]; CUNNINGHAM & KUHN[7]). Our observations are consistent with previous reports which indicate that experimental **T. cruzi** infection in mice are accompanied by splenomegaly, lymph node enlargement and immunosuppression (CLINTON et al.[3]; CUNNINGHAM et al.[4]; ROWLAND & KUHN[21]; RAMOS et al.[18]; HAYES & KIERSZENBAUM[10] and CUNNINGHAM & KUHN[7]).

The data indicate that decreased responsiveness is mainly limited to the acute phase of **T. cruzi** infection. Comparisons of mitogen responsiveness in C3H/He and C57B1/6 mice suggest similar sequence of changes in both strains. However, the more susceptible C3H/He mice showed greater suppression in response to T cell mitogens (Con A and PHA) than C57B1/6 mice. This difference between C3H/He and C57B1/6 mice is more dramatic when responsiveness to B cell mitogens is studied. While responsiveness to B-cell mitogens is completely recovered during the chronic phase in C57B1/6, C3H/He mice only show partial recovery. Our findings are in agreement with those of KIERSZENBAUM & HAYES[13] and HAYES & KIERSZENBAUM[10], who reported decreased immunoresponsiveness to mitogens only during the acute phase of **T. cruzi** infection in CBA/J mice.

In our experiments the antigens from **T. cruzi** induced in normal and infected animals at the begining of the infection (day 1 to 7) a

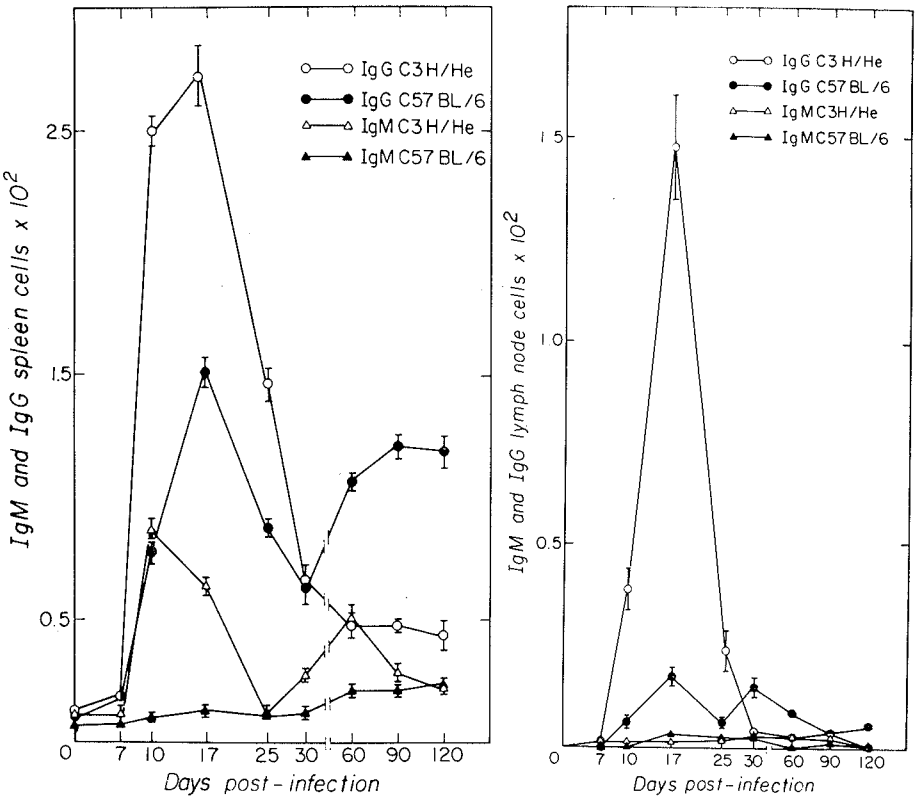

Fig. 5 — IgG and IgM-bearing cells in spleens and lymph nodes of mice infected with **T. cruzi.** Spleens and lymph nodes were taken from C57B1/6 and C3H/He mice at different days post-infection and stained for IgG and IgM markers as described in **Material and Methods.** The results are expressed as the number of IgG and IgM stained cells over a total of 1 x 10^3 lymph nodes or spleen cells. Each value is the average of three experiments ± S.D.

blastogenic response above the value of unstimulated controls. Mitogenic activities have been described in **T. brucei** on the spleen cells of normal and athymic mice (ESURUOSO[8]). In our experiments the trypanosomal antigens were as good mitogens as Con A, PHA, LPA and DS in both mouse strains (Compare Fig. 3, column N, and Fig. 6 at 0 time). The return to normal response of spleen cells in C57B1/6 when stimulated with trypanosomal antigens was similar to the findings of TEIXEIRA et al.[23] in patients with apparent Chagas'disease which recover responsiveness of delayed type skin response and MIF in peripheral blood leukocytes.

The same phenomena observed with polyclonal activators in C3H/He was obtained with trypanosomal antigens, which did not show a recovery back to normal of the blastogenic response. The antigens prepared from cultures at different temperatures were obtained from parasites grown in the presence of 5% GG-free serum (O'DALY et al.[16]). All the cultures showed epimastigotes in the exponential and stationary phase of growth at all temperatures. The lack of recovery of response with **T. cruzi** antigens suggests that B-cells or essential assistant cells present in the adherent cell population from spleen are more susceptible to trypanosomal antigens in these mouse strains which might add to their increased parasitemia, amastigotes pseudocysts, and higher mortality.

Our results indicate that decreased responsiveness to mitogens observed during the acute phase is mediated by splenic plastic-adherent cells with "macrophage-like" characteristics. These results do not support the findings of a T-cell mediated suppression mechanism in experimental **T. cruzi** infection (RAMOS et al.[18]). Increased response against **T. cruzi** in-

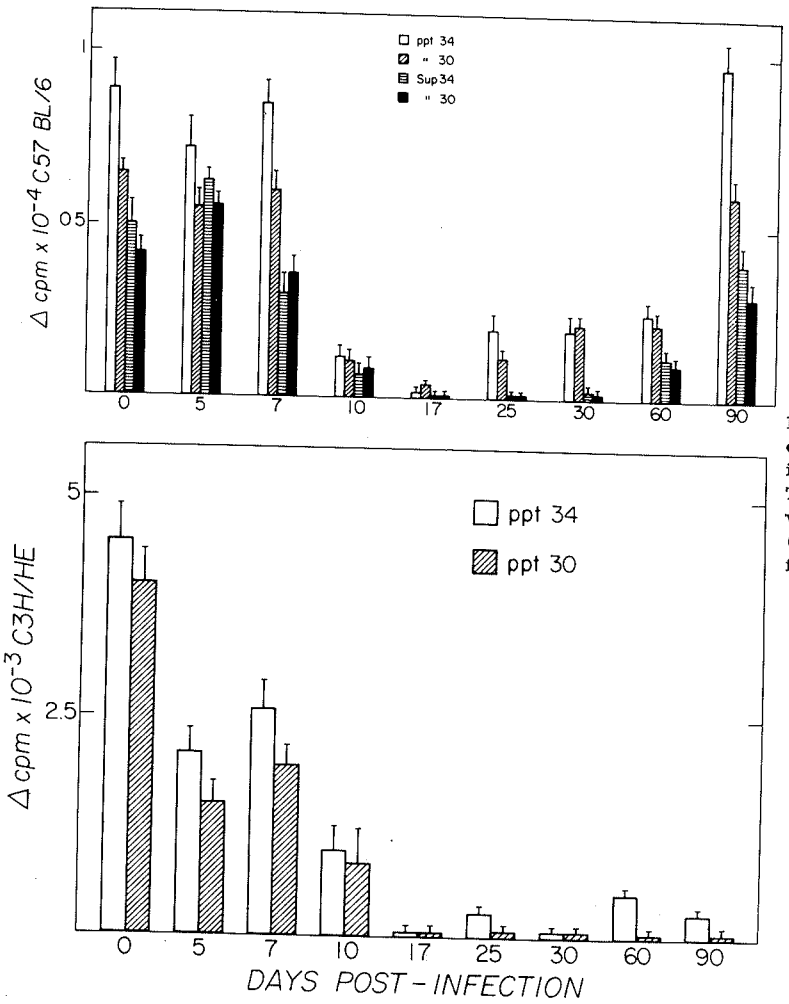

Fig. 6 — Blastogenic response to **T. cruzi** antigens at differents times after infection. Antigens prepared from TCA precipitate (High molecular weight antigens, ppt) or supernatant (low molecular weight antigens, sup), from parasites cultured **in vitro** at 30C and 34C, respectively.

fection has been observed in athymic (nu/nu) mice after neonatal thymus transplant (KIERSZENBAUM & PIENKOWSKY [12]). In addition partial protection against **T. cruzi** infection mediated by T-cells transfer from immunized donors have been reported (REED [20]).

Our studies of the humoral immune response, as determined by PFC assays, indicate that during acute **T. cruzi** infection a significant suppression occurs. This is followed by recovery during the chronic phase. CUNNINGHAM & KUHN [6], CUNNINGHAM et al. [4], described a decreased humoral response during acute and chronic **T. cruzi** infection and possibly a lower parasite inoculum was the reason for the recovery of humoral immunity observed by us during the chronic phase.

The increase in IgG and IgM-bearing cells in spleens and lymph nodes correlated with the onset of parasitemia. Analysis of spleen cells for other markers is necessary in order to determine the contribution of each cell subpopulation to splenomegaly. An absolute increase in B-cells has been described in spleens during **T. cruzi** infection (HAYES & KIERSZENBAUM [19]). The significant increase in immunoglobulins bearing cells observed in spleens and lymph nodes, contrasts with the inflammatory foci observed in the heart, where less than 1% of the infiltrate cells have B cells markers.

The appearance of amastigotes in the heart, together with cell proliferation against the intracellular parasite (see text) at day 17 post-infection coincides with the decrease in blood-form trypomastigotes, and immunoglobulin

producing cells. After day 60 of infection the animal recovered its responsiveness to **T. cruzi** dependent and independent antigens and polyclonal activators (with exceptions in the susceptible C3H/He strain) probable as response to antigens found in the amastigote form. Also the histological lesions typical of the inflammatory infiltrates disappeared and it was not possible to find intracellular parasites nor round cell inflammatory infiltrates in the heart.

RESUMEN

Supresión de la inmunidade humoral y de la respuesta linfocitaria durante la infección experimental con Trypanosoma cruzi

Ratones C3H/He y C57B1/6 inoculados con 500 tripomastigotes de la cepa Y de **T. cruzi** muestran durante la fase aguda de la enfermedad, parasitemia, aumento del bazo y ganglios linfáticos así como parásitos intracelulares en el corazón. Análisis de las células presentes en ganglios linfáticos y bazo presenta aumento de células IgM e IgG. Cuando la esplenomegalia es mayor, alrededor del día 17 postínfección, los linfocitos esplénicos mostraron un descenso marcado en la respuesta a mitógenos de células B y T, antígenos de **T. cruzi** y células formadoras de placas contra glóbulos rojos de carnero. Células de bazo o células esplénicas adherentes a plástico, obtenidas de ratones durante la fase aguda de la infección suprimen la respuesta a mitógenos de linfocitos normales. Durante la fase crónica, desaparece la parasitemia, los parásitos intracelulares en el corazón y disminuye la esplenomegalia. Estos cambios preceden a la recuperación de la respuesta a mitógenos y antígenos de **T. cruzi** en linfocitos esplénicos de C57B1/6. Esta recuperación es solo parcial en C3H/He, los cuales son más sensibles a la infección. También se encuentra recuperación de la respuesta humoral durante la fase crónica.

ACKNOWLEDGMENTS

We thank Dr. Francisco Castillo and Dr. José Azocar for discussion and help in writing the manuscript and Miriam de León for excellent secretarial assistance.

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Recebido para publicação em 10/1/1983.