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THE EFFECT OF ACUTE INFECTION BY TRYPANOSOMA CRUZI UPON THE RESPONSE OF MICE TO SHEEP ERYTHROCYTES

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SUMMARY

Mice infected with different amounts of **Trypanosoma cruzi** trypomastigotes, Talahuen strain, and groups inoculated with homogenate of the same **T. cruzi** strain epimastigotes, were inoculated simultaneously with SRBC. Rosette forming cells (RFC) against SRBC were studied by immunocytoadherence test (ICA) at spleen level and hemagglutinins against the same antigen were checked. No significant differences between test and control samples were detected by direct hemagglutination. However, by the ICA test, the number of splenocytes with rosette forming (RF) capacity found in infected mice differed from the number found in uninfected mice. In mice heavily infected, an increase was first observed, followed by a decrease, in the number of RFC against SRBC; in intermediate infections a decrease in the number of RFC was the only alteration seen, while no alteration in the immune response was established in mice with the lowest infection during the time of observation. These alterations of the immune response of mice acutely infected with **T. cruzi** and their possible mechanisms are discussed.

INTRODUCTION

Enhancement or depression of the immune response an antigenic stimulus by previous or concomitant experimental or natural viral or bacterial infection or by products from Gram negative bacteria have been reported by different Authors (FLOERSHEIN [15]; NETER [26]; SALAMAN [31,32,33]; TKACZCEVSKI et al.[39]). The patterns of incidence and distribution of African Lymphoma of childhood and their relationship with malaria have suggested that the parasite may produce a depression in the immune reactivity to the virus favoring the development of the Lymphoma (BURKITT [8] EDINGTON & MACLEAN [12]; BURKITT [9]; ZIEGLER et al.[45]). A significant number of Gambian children with chronic malaria had an impaired capacity to react to tetanus toxin (MC GREGOR & BARR [23]). SALAMAN et al. [32], BARKER [6] and GREENWOOD et al. [20] showed in mice infected with murine plasmodium, a depression in the immune response to sheep red blood cells (SRBC).

Immune depression by protozoan parasites, other than plasmodia, has been reported in hamster infected with **Leishmania donovani** (CLINTON et al. [10]) and in mice and rabbits infected with **Trypanosoma brucei** (GOODWIN [18]; GOODWIN et al. [19]). Furthermore, there is evidence that in mice concomitantly

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infected with **Toxoplasma gondii** and **Plasmodium yoeli,** each infection depressed the immune response to the other (STRICKLAND et al.[36]). The present investigation was designed to establish whether mice infected with **T. cruzi,** the causative agent of Chagas'disease, had any alteration in their immune response to SRBC, measuring the rosette forming cells (RFC) at the spleen level using the immunocytoadherence test (ICA), and the agglutinins against SRBC at humoral level by the direct hemagglutination test.

MATERIALS AND METHODS

Animals — Mice of the Pittsburgh strain of either sex weighing 30 grs (± 2) were used througout this study.

Parasites — Trypomastigotes of the Tulahuen strain of **Trypanosoma cruzi** maintained by serial passages in mice for over 20 years, were obtained by collecting blood after cutting the axillary vein of heavily infected mice and counted by the method of PIZZI et al.[28]. Blood either undiluted (5×10^6 parasites) or diluted with 0.15 M saline to obtain a concentration of 1×10^6, 1×10^5 or 1×10^4 trypomastigotes per 0.2 ml of suspension was used.

Sheep red blood cells (SRBC) — Sheep erythrocytes were collected in sterile Alsever's solution. They were washed twice in 0.15 M phosphate buffered saline (PBS), pH 7.2, resuspended in the same solution, counted in a Neubauer type chamber and diluted to obtain a concentration of 5 to 6×10^8 SRBC per 0.2 ml suspension.

T. cruzi antigens — T. cruzi homogenates were obtained from epimastigotes grown in a disphasic medium (VATTUONE & YANOVSKY[41]) and disrupted by the aid of pressure in a Ribi Cell Fractionator (GONZÁLEZ CAPPA et al.[16]).

Mice injected by intraperitoneal route (IPR) with the different doses of trypomastigotes in 0.2 ml suspension or with 0.5 ml of **T. cruzi** homogenate (prepared by resuspending in 0.5 ml of PBS, 300 mg wet weight of parasites obtained by centrifuging at 10 000 g for 10 minutes, and disrupted the suspension at 8.000 PSI in a Ribi Cell Fractionator) were inoculated by IPR 5 minutes later with 0.2 ml of the suspension of SRBC. Control groups of mice were injected with one of the following, respectively: SRBC, SRBC plus each 0.2 ml of undiluted or diluted normal mouse blood, **T. cruzi** homogenate, each dose of trypomastigotes and PBS.

Rosette forming cells (RFC) against SRBC were studied by the immunocytoadherence test (ICA) beginning on the third day after inoculation of sheep erythrocytes. The technique used for the ICA test was a modification of those described by NOTA et al.[27], and by ZAALBERG[43]. Mice were exsanguinated through the retroorbital sinus. The spleens were teased and dispersed with a potter homogenizer in PBS. The cell suspension was filtered to remove larger particles of debris before centrifuging for 10 minutes at 500 g. The cells were washed twice with PBS and diluted in the same solution. Suspensions of 5×10^5 spleen cells were incubated for 1 hour at 37°C after the addition of SRBC at a concentration of 2 erythrocytes/1 spleen cell. After incubation the mixture was gently shaken and a sample examined under phase-contrast microscopy (400 x) in a Neubauer type chamber. The cells exhibiting the adherence phenomenon were easily detected by their characteristic rosette pattern. Cells with at least 3 SRBC attached were considered as rosettes (without any characterization of the type of the reactive spleen cells (STORB & WEISER[35]). Each sample was examined at least four times and the results averaged. The results were reported as RFC per million spleen cells and represented the mean values from 3 to 5 mice (usually 4) either of the test or control groups. Except for the experiment with 5×10^6 parasites, which was performed twice, all assays were repeated at least three times.

Survival was recorded daily in additional groups inoculated with each trypomastigote dose used (5×10^6, 1×10^6, 1×10^5 and 1×10^4) (20 mice per group).

Parasitemia was established following PIZZI et al.'s[28] every time the ICA test was performed in infected mice. Due to inequality of variances among different assays a "t" test was carried out on the square root of the parasitic counts to compare parasitemia among test and control mice.

A "t" test was also performed at each ICA test on the square root with regard to RFC numbers, in order to compare test mice with controls.

Serum samples collected from each group of mice for ICA testing were pooled and stored at -20°C until used, to detect hemagglutinins against SRBC. Hemagglutination was performed using microtiter plates as reported by GOODWIN et al.[19]. Titers obtained were converted to $\log_2$, each log increment thus representing one dilution.

RESULTS

There were no significant differences in the hemagglutinin antibody titer detected in the pooled serum samples from normal mice given SRBC only, compared to sera from the different groups of test mice (Fig. 1). However, the ICA test has revealed that the number of splenocytes with RF capacity against SRBC in infected mice differed from the number in uninfected mice (Fig. 2). In the spleens of mice inoculated with 5×10^6 trypomastigotes, the number of RFC on the 3rd and 6th days after inoculation of SRBC and parasites was significantly higher than in those mice which received SRBC only. A similar phenomenon, but on the 6th day after infection, was observed in those mice injected with 1×10^6 trypomastigotes and given SRBC. However, by the 10th or 13th days respectively, the number of rosettes in mice infected with 5×10^6 or 1×10^6 trypomastigotes and inoculated with SRBC was significantly lower than in their unifected counterparts. The average survival time of groups of mice infected with similar number of parasites was 11.6 and 13.9 days respectively. No significant differences in the number of RFC between mice inoculated with 1×10^5 trypomastigotes plus SRBC and controls inoculated with SRBC only were observed except on the 16th day when the former group had fewer RFC (Fig. 2). Only 50% of the similarly inoculated mice survived after 20 days of infection, the average survival time of dead mice being 18.6 days.

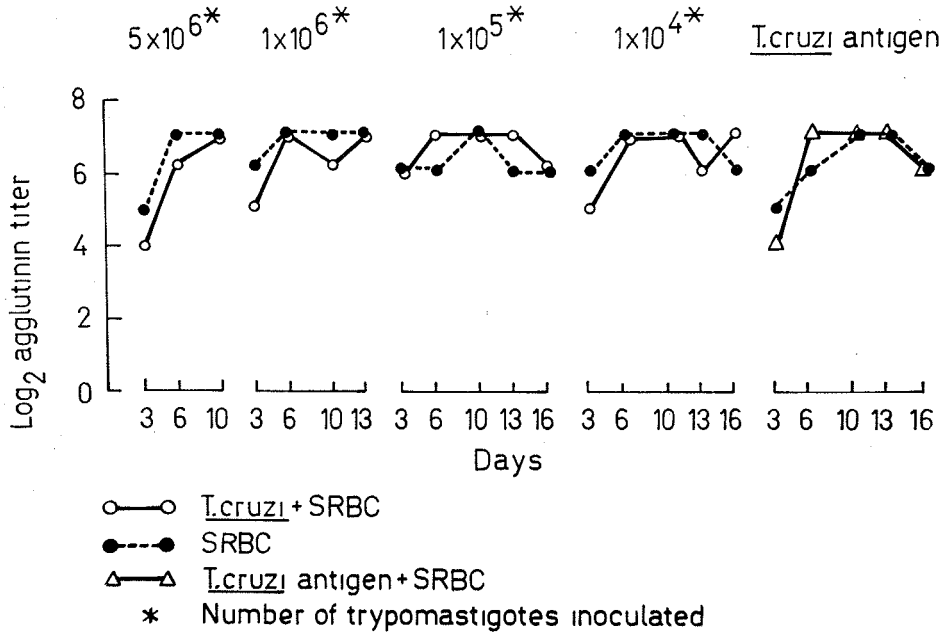

Fig. 1 — Direct agglutinin antibody titer against SRBC of mice infected or uninfected with **T. cruzi** or injected with **T. cruzi** antigen and inoculated with SRBC

There were no significant differences in the number of RFC among mice infected with 1×10^4 trypomastigotes and inoculated with SRBC and those given SRBC only (Fig. 2). Three out of the 20 similarly inoculated mice died during the first 30 days after infection.

The lower number of RFC which were

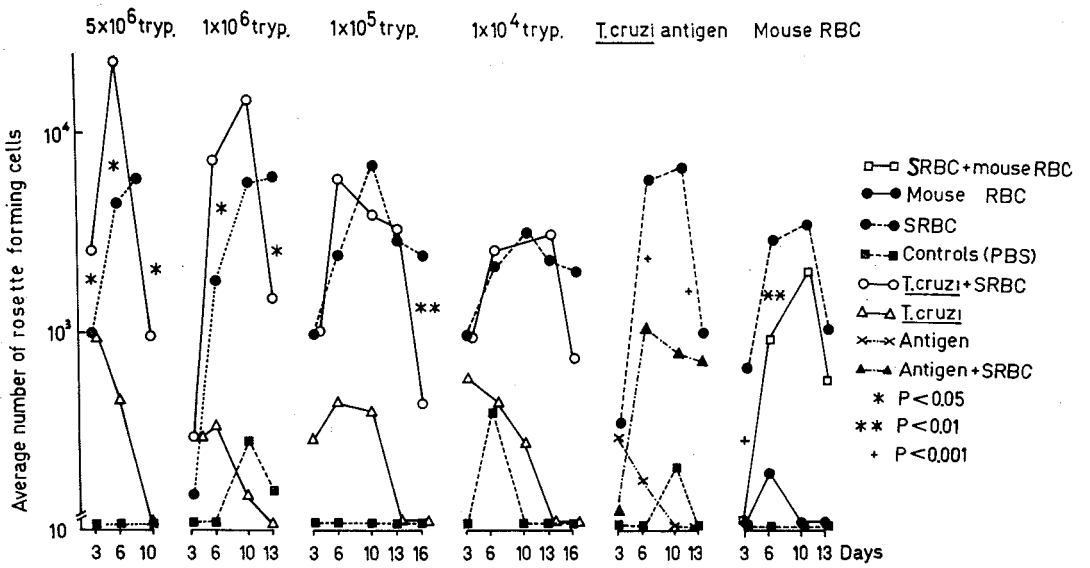

Fig. 2 — Average rosette forming cells per million spleen cells against SRBC in mice inoculated with **T. cruzi** trypomastigotes, **T. cruzi** antigen or mouse RBC plus SRBC, or given only SRBC, trypomastigotes, **T. cruzi** antigen, mouse RBC or PBS

able to react with SRBC observed in mice inoculated with trypomastigotes plus SRBC, was coincident with the peak of parasitemia reached in each particular group of mice (Fig. 3). Although parasitemia in infected mice which also received SRBC had a consistent tendency to be higher than in those mice only infected, differences were not statistically significant.

Those mice inoculated with **T. cruzi** homogenate plus SRBC had at the 3rd, 6th and 10th days a significant decrease in the number of RFC to SRBC if compared to mice given SRBC only.

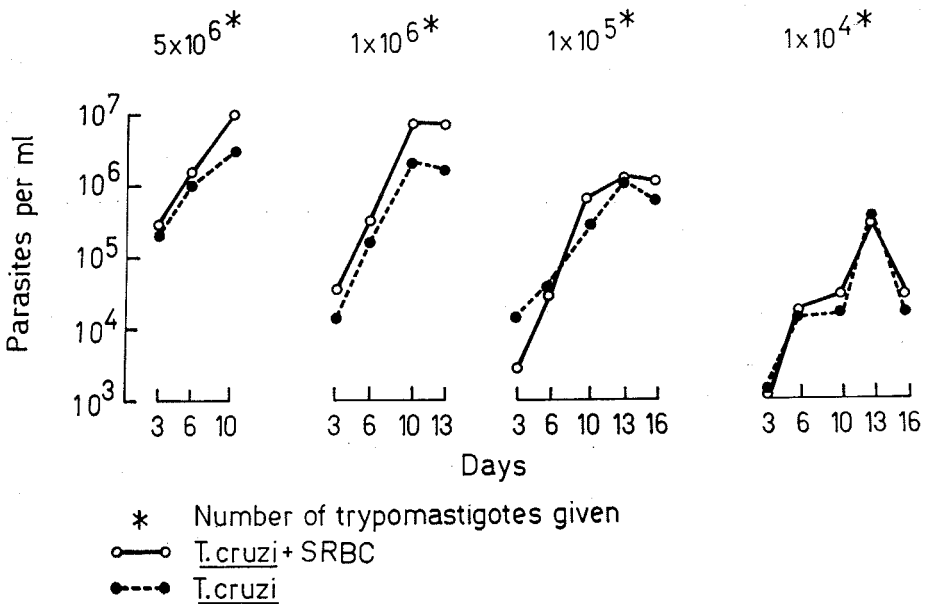

Fig. 3 — Average parasitemia of mice inoculated with SRBC and infected with different amounts of **T. cruzi** or only infected with **T. cruzi**

Those animals inoculated with undiluted normal mouse blood cells plus SRBC possess on the 3rd and 6th days a lower number of RFC against SRBC, than in controls given SRBC (Fig. 2). This decrease in the number of RFC was not noticed in the groups of mice inoculated simultaneously with diluted normal mouse blood and SRBC.

Although no hemagglutinins against SR BC were detected in the pooled sera of mice injected with different amounts of trypomastigotes only, some had spleen cells with RF capacity against SRBC with an average number less than 1000 per million of cells. This nonspecificity was higher than that shown by mice injected with PBS, which only gave an average background of RFC in the presence of SRBC of less than 500 per million spleen cells in a few pools (Fig. 2).

DISCUSSION

As the ICA test indicates differences in the number of RFC between test and control mice, we may conclude that in the test mice, under the experimental conditions used, and at spleen level, there has been an alteration of the immune response.

The altered immune response to SRBC detected in mice inoculated with 5×10^6, 1×10^6 or 1×10^5 trypomastigotes but not in those infected with 1×10^4 parasites indicates that this alteration during acute infection was related to the dose of parasites inoculated and consequently with the degree of infection.

The ability of the ICA test to establish differences in the number of RFC among the above groups (if RFC are considered essentially as antibody forming cells) STORB & WEISER [35], while no differences were detected through the hemagglutinin antibody titers, might only reflect the high sensitivity of the ICA test (BAKER et al. [5]; ZAALBERG et al. [44]). On the other hand, BEKIERKUNST et al. [7] have reported no increases in antibody response (hemagglutinins) when injecting cord factor and SRBC simultaneously but they noticed good stimulation when cord factor was injected 5 to 20 days before SRBC; in our experimental work, SRBC were injected immediately after inoculation of trypomastigotes, (it was done in this way because the groups with the heaviest infections died rather soon). But as the nature of the cells that are involved in the ICA test are yet under discussion, it is also possible that the altered immune response would not be dependent on modifications in the number of the antibody secreting cells, but on T cells (ARGYRIS et al. [2], BACH & DARDENNE [4]). Otherwise, we have used the ICA test to measure RFC only at the spleen level and even if these cells were mostly antibody secreting cells it may be possible that cells from other areas could compensate, at humoral level, the modification in number of RFC detected in the spleen. FERREIRA et al. [13] found that while mice treated with cortisone had 95% less antibody forming cells in the spleen, as measured by Jerne's technique, than untreated mice given only SRBC, both groups had similar titers of humoral antibodies. In their study, plaque forming cells in the bone marrow against SRBC in cortisone-treated mice were more numerous than those found in untreated animals. So, they concluded that the decrease in spleen plaque forming cells in cortisone treated animals may be compensated, at least partially, by the bone marrow. However, antibodies against SRBC have been reported to be formed mainly in the spleen (TLASKALOVA et al. [40]).

In the groups of mice given 5×10^6 or 1×10^6 parasites, two phases were established in the alteration of the immune response when measured by the ICA test. First, one immune stimulation which in the former group was so intense as to compensate for the immunosuppresive action of the concomitant inoculation of undiluted normal mouse red cells. One or several of the following possibilities may explain the increased immune reactivity; a) Common antigens between SRBC and trypomastigotes; thus, those mice inoculated with SRBC and parasites received a higher antigenic stimulus. This point of view is supported by the fact that an increased background of RFC against SRBC was observed in infected mice which did not received SRBC (Fig. 2). Furthermore, heterophyle antibodies against SRBC have been described in **T. cruzi** infected humans (MUNIZ & FREITAS [24]; MUNIZ & SANTOS [25]; AMATO NETO & PEREIRA DA SILVA [1]; SACKMAN MURIEL [30]); b) The existence of products from trypomastigotes with

capacity to stimulate the immune mechanisms. SENECA & PEER [34] have reported on a lipopolysaccharide obtained from epimastigotes with a markedly similar effect to that of the endotoxin from Gram negative bacteria. Such endotoxin is well known as a modifier of the immune response (NETER [26]). However, KIERZENBAUM & BUDZKO [22] were unable to obtain a functionally similar substance. Furthermore, there is no evidence that chemical extracts from trypomastigotes have such activity; c) the inflammatory reaction produced by the inoculation of the trypomastigotes was characterized in early stages of the infection by the proliferation of medium lymphocytes and macrophages (PIZZI [29]), cells which are involved in the chain of events which characterize the immune response.

Inoculation of mice with **T. cruzi** sharply increases the weight of the spleen (PIZZI [29]). The Authors have observed that mice inoculated with 5×10^6, 1×10^6 and 1×10^5, possess a significant splenomegaly when compared with the control groups, on day 6, of the infection (unpublished data). In groups inoculated with 1×10^4 parasites, splenomegaly began to be significantly different on day 10. Increase in spleen weight was observed in infected mice, whether they had been injected or not with SRBC. In mice infected with the 2 higher numbers of parasites the increase in spleen weight might be correlated with a higher number of cells which were able to play a role in the immune response. This possibility fits well with the point c) mentioned above and with the fact that the local granulomatous reaction in other experimental systems has been well correlated with antibody production (FISCHEL et al. [14]; ASKONAS & HUMPHREY [3]; BEKIERKUNST et al. [7])

The second phase of the alteration of the immune response to SRBC in mice receiving 5×10^6 or 1×10^6 parasites was represented by the immunodepression characterized by a decrease in the number of RFC. This was the only alteration observed in mice infected with 1×10^5 parasites plus SRBC. This last fact correlates well with the report of CLINTON et al. [11], who demonstrated immunosuppression against burro erythrocytes in **T. cruzi** infected mice using the hemolytic plaque assay. Furthermore it is consistent with those findings made in malaria (SALAMAN [31,32]; BARKER [6]; GREENWOOD et al. [20]), **L. donovani** (CLINTON et al.[10]) or **T. brucei** infections (GOODWIN et al. [19]) in which depression was also coincident with increased parasitemia and consequently with a massive antigenic load which may alter, by blockage or by antigenic competition, the function of cells involved in the immune response. Either of these possibilities may be also taken in account for the decreased number of RFC against SRBC observed in mice which received **T. cruzi** homogenates plus SRBC and in mice inoculated with 0.2 ml of normal mouse red cells plus SRBC. However, it should be considered that during the terminal stages of infection, the mice have initially a greatly reduced number of small lymphocytes in the spleen, and later an important lymphorrexis (PIZZI et al. [28]; TALIAFERRO & PIZZI [37]; TARATUTO et al. [38]; GONZALEZ CAPPA et al. [16]). Thus, it seems logical to correlate the decreased number of RFC found during terminal stages of infection with the alteration of the spleen population available for the immune response.

A lower degree of histological alterations in mice inoculated with 1×10^4 parasites has been reported (VILCHES et al. [42]). A high percentage of these mice survived the **T. cruzi** infection. A better preservation of the spleen cell population may explain why the groups similarly infected in the present study (1×10^4) do not show any decrease in the number of RFC to SRBC during acute infection when compared to controls.

Possibly more than one of the above mentioned hypotheses are involved in the altered response to SRBC as measured by the ICA test in the spleen of acute **T. cruzi** infected mice.

Although in murine malaria it has been demonstrated that a decrease in antibody production to SRBC may be coincident with normal immune response to other antigens (BARKER [6]; GREENWOOD et al.[20]) our finding of immune alteration to SRBC in **T. cruzi**-infected mice may well have a bearing on the correlation between human congenital Chagas' disease and concomitant susceptibility to other infectious agents (HOWARD & RUBIO [21]).

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Mechanisms of these immune alteration in mice acutely infected with **T. cruzi** must be investigated.

RESUMEN

El efecto de la infección aguda por Trypanosoma cruzi **sobre la respuesta de los ratones a los eritrocitos de carnero**

Utilizando la técnica de la inmunocitoadherencia (ICA) se estudiaron las células formadoras de rosetas (CFR) contra glóbulos rojos de carnero (GRC) a nivel del bazo y los títulos de hemaglutininas séricas en ratones inoculados simultáneamente con trypomastigotes o con homogenatos de epimastigotes de **Trypanosoma cruzi.**

Aunque no se pudieron detectar diferencias significativas entre los títulos de hemaglutininas de los ratones controles inoculados sólo con GRC y aquellos que recibieron GRC y trypomastigotes u homogenatos de epimastigotes, al realizar la ICA se estableció que el número de células formadoras de rosetas contra los GRC a nivel del bazo era diferente en los animales infectados en relación a aquellos que no lo estaban. En los animales infectados con 5×10^6 ó 1×10^6 parásitos se detectó primero un aumento en el número de células formadoras de rosetas, seguido por una disminución en el número de las mismas. En aquellos infectados con 1×10^5 sólo se observó este último hecho, mientras que en los infectados con 1×10^4 **T. cruzi** la ICA no pudo establecer ninguna alteración en la respuesta inmune contra los GRC.

Se discuten los posibles mecanismos que originan esta respuesta inmune alterada contra los GRC en ratones con infección aguda por **T. cruzi.**

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