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MODULATION OF PARASITEMIA AND ANTIBODY RESPONSE TO *TRYPANOSOMA CRUZI* BY CYCLOPHOSPHAMIDE IN *CALOMYS CALLOSUS* (RODENTIA, CRICETIDAE)

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SUMMARY

Calomys callosus a wild rodent, previously described as harboring **Trypanosoma cruzi**, has a low susceptibility to infection by this protozoan.

Experiments were designed to evaluate the contribution of the immune response to the resistance to **T. cruzi** infection exhibited by **C. calossus**. Animals were submitted to injections of high (200 mg/kg body weight) and low (20 mg/kg body weight) doses of cyclophosphamide on days -1 or -1 and +5, and inoculated with 4 x 10^3 **T. cruzi** on day O. Parasitemia, mortality and antibody response as measured by direct agglutination of trypomastigotes were observed. Two hundred mg doses of cyclophosphamide resulted in higher parasitemia and mortality as well as in suppression of the antibody response. A single dose of 20 mg enhanced antibody levels on the 20th day after infection, while an additional dose did not further increase antibody production. Parasitemia levels were not depressed, but rather increased in both these groups as compared to untreated controls. Passive transfer of hyperimmune **C. callosus** anti-**T. cruzi** serum to cyclophosphamide immunosuppressed animals resulted in lower parasitemia and mortality rates. These results indicate that the immune response plays an important role in the resistance of **C. callossus** to **T. cruzi**.

KEY WORDS: Calomys callosus: Cyclophosphamide; **Trypanosoma cruzi**; Parasitemia; Mortality; Antibody response.

INTRODUCTION

Calomys callosus is a wild silvatic rodent commonly found in South America and was described in Brazil harboring **Trypanosoma cruzi**[3,18,21]. Laboratory infections of **C. callosus** result in much lower parasitemias and mortality than those of mice[5]. In this respect, they resemble **T. cruzi** in infections in rats[22,27].

C. callosus are similar in aspect to mice, except for a shorter tail. Their fur is greyish brown. Size and weight are also comparable to that of mice for the first two months, with higher weight gain from then on (maximum weight about 40 g.). **C. callosus** have been adapted to breeding in animal facilities by several research groups, initially mainly to study viruses[17].

There are only a few papers about the immunological response of wild animal reservoirs of Chagas' disease[12,15,19] which represent an important link in the natural history of human infection. It seemed therefore of interest to gain more insight into the mechanisms involved in the relative resistance of **C. callosus** to **T. cruzi** challenge.

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Literature on the immune response of several animal species to **T. cruzi** is extensive. The mouse model has been studied more frequently. Besides Swiss mice, several inbred strains of mice have been used, and some of them, for instance the C57BL/10, are quite resistant to infection[31].

It is known that physiological and biological factors such as body temperature, vitamin deficiencies, hormonal or sex-linked characteristics and the major histocompatibility complex (MHC) play a role in the outcome of **T. cruzi** infection.

Both cellular and humoral immunity have been shown to operate in the resistance to **T. cruzi**. Antibodies develop during infection, and serology is always positive when the animals do not die. Passive antibody transfer is also associated with partial protection[13,16].

We therefore undertook this study to elucidate whether the immune response plays a significant role in the resistance of **C. callosus** to **T. cruzi** infection. The antibody response and its modulation by the immunosuppresive drug cyclophosphamide (Cy) were studied.

It was found that immune mechanisms are indeed an important component of the resistance to **T. cruzi** infection in this animal species, since immunosuppression by Cy enhances infection. A low dose of Cy slightly enhances specific antibody production. However this enhancement resulted in no additional protection, whereas the passive administration of specific hyperimmune serum reversed the susceptibility induced by a high dose of cyclophosphamide.

MATERIALS AND METHODS

Animals

C. callosus raised at the animal facilities of the Instituto de Medicina Tropical de São Paulo were used. The colony was initiated in 1981 by mating animals kindly provided by Dr. D.A. Mello (Núcleo de Medicina Tropical) from her colony at Universidade de Brasília. Male and female rodents aged 30 to 45 days and weighing 18 ± 2 g constituted all experimental groups.

Cyclophosphamide

Cyclophosphamide (Cy), Enduxan (Pravaz Division, Abbott Laboratórios do Brasil Ltda.) was suspended in twice-distilled water and injected i.p. at concentrations of 200 or 20 mg/kg body weight,as a 0.2 ml dose, one day prior to parasite inoculation, followed in some experiments by a second dose 5 days after infection.

Parasites

The Y strain (Silva and Nussenzweig[25]) of **T. cruzi** was maintained in **C. callosus** by weekly passages, inoculating 1 x 10^5 parasites in 30 day old animals. Parasites for agglutination were raised in mice, immunosupressed by injection of 200 mg Cy/kg one day before infection.

In experimental groups, animals were inoculated with 4 x 10^3 parasites i.p.. Parasitemia was measured by the method of Brener[7], counting parasites in 50 microscopic fields of a wet preparation with a 22 x 22 mm coverslip, contaning 5 µl tail blood. Counts were made three times a week, for 30 days, and mortality was observed daily for the same period of time. Controls consisted of an equal number of infected animals, without Cy, and an additional control was injected with Cy only. Parasitemia is expressed as the $\log_{10}$ of the median of parasite counts (per ml of blood) from the individual mice in each group.

At least 10 animals were used per experimental group, and experiments were repeated at least once. Observation of parasitemia and mortality was done on animals different from those bled for agglutinatin studies.

Agglutination test

Antibody titers were determined by the direct agglutination of trypomastigotes. Serum samples were collected through the retro-orbital plexus from each animal (under light ether anesthesia), on days 0, 5, 9, 14, 20, 27 and 37 after infection and stored individually at -70°C until used.

Parasite suspensions were obtained, bleeding mice on the 7th day of infection, defibrinizing blood with glass beads. After centrifugation at 1800 xg for 1 min., the blood was kept at 37°C for

15 min. The supernatants rich in parasites were collectted and the parasite number adjusted to 4 x 10^6/ml with PBS, after couting in Neubauer hemacytometer.

Serial serum dilutions in PBS, were started at 1:2; agglutination titers are expressed as the reciprocal of the last dilution containing aggregates of at least 3 parasites, as observed by light microscopy.

Agglutination was carried out on microscopic slides, mixing 20 µl of parasite suspension with 20 µl serum dilution. Incubation at 37°C for 40 min was carried out in a humid atmosphere.

Immune Serum

Immune serum was obtained by i.p. inoculation of **C. callosus** with 1 x 10^5 **T. cruzi**. They were challenged with the same number of parasites after 30 days, and bled 7 days later. Serum pools were kept at -70°C until used. In passive antibody transfer experiments, 0.3 ml of this serum was inoculated i.p. 3 hs before parasite challenge.

Statistical analysis

Since we have been working with animals from an outbred colony, the individual response to infection is quite variable within each experiment. Therefore the median rather than the mean values were used in all graphs and in statistical analysis. Percentiles 25% and 75% are indicated in Fig. 3 and this gives an indication of antibody titer median variations. The Kruskal-Wallis test[11] for independent samples was applied to parasitemia levels and antibody titers, comparing ranks for the different groups on individual days. Significant differences were obtained with the chi-square test, taking 0.05 as the minimum acceptable level.

RESULTS

C. callosus inoculated with 4 x 10^3 Y strain **T. cruzi** presented parasitemia peaking on the 7th day. Subsequently parasites were gradually cleared from circulation, becoming undetectable by day 11 (Fig. 1). The mortality rates ranged from 0-9.5% by day 30 (see text below and Table 1 group 5).

Table 1.
Effect of specific antibody transfer on mortality of immunosuppressed **C. callosus** infected with **T. cruzi.**

	Inoculated ip with[1]				Mortality[2] %	Nr. dead/total
Group	Cy	IS	NS	Parasites		
1	+	+	-	+	10.34	3/29
2	+	-	+	+	35.00	7/20
3	-	-	+	+	4.76	1/21
4	+	-	-	+	35.00	7/20
5	-	-	-	+	9.52	2/21
6	+	-	-	-	0	0/41

1. Cy = Cyclophosphamide 200 mg/kg on day -1, IS = Immune **C. callosus** serum 0.3 ml, 3 hs before parasite challenge. NS = Normal **C. callosus** serum 0.3 ml, 3 hs before parasite challenge. Parasites = 4 x 10^3 **T. cruzi** Y strain on day 0.
+ = injected - = not injected
2. Cummulative mortality by day 30, group (1 = 3 = 5) < (4 = 2) $p < 0.001$

Effect of 200 mg Cy/kg body weight on parasitemia and mortality

Fig. 1 shows that both one and two doses of 200 mg Cy/kg increased parasitemia and mortality significantly from the 8th day after infection onward ($p < 0.001$), the effect being more pronounced with two doses. In this experiment, cumulative mortality was 18.51% (5/27) with one dose of Cy, increasing to 90.9% (20/22) with two doses of Cy. Controls inoculated with parasites only, had no mortality (0/15), as well as those receiving one dose of Cy (0/41), while those receiving two doses of Cy had a 3.5% mortality rate (1/28), up to the 30th day.

Effect of 20 mg Cy/kg body weight on parasitemia and mortality

Fig. 2 shows that either one or two doses of 20 mg/kg Cy had similar effects on parasitemia, which was higher than that of controls at days 7 and 8 ($p < 0.02$ on day 8). There was no mortality in both experimental groups (group 1,0/22, group 2, 0/24), while in controls (group 3), mortality was 10% (2/19), up do day 30.

Effect of 200 mg Cy/kg and 20 mg Cy/kg body weight on antibody response

Fig. 3 shows that the injection of 200 mg Cy

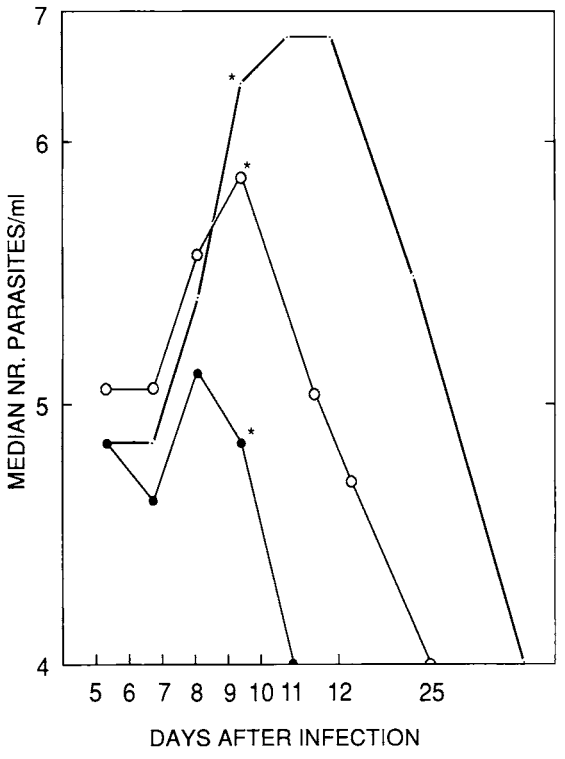

Fig. 1. Effect of 200 mgCy/kg body weight on parasitemia (log 10) of **C. callosus** inoculated with 4 x 10^3 **T. cruzi Y** strain, on day 0; O—O Group 1 200 mg Cy on day -1; •—• Group 2 200 mg Cy on days -1 and +5, ●—● Group 3 parasites only.
* - Group 3 < (1 = 2) $p < 0.001$.

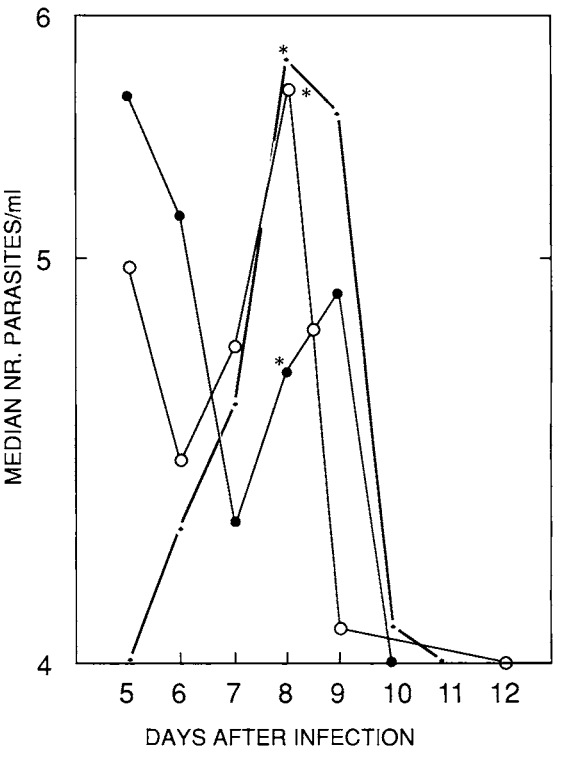

Fig. 2. Effect of 20 mg Cy/kg body weight on parasitemia (log 10) of **C. callosus** inoculated with 4 x 10^3 **T. cruzi** Y strain on day 0; O—O Group 1 20 mg Cy day -1; •—• Group 2 20 mg Cy day -1 and day +5; ●—● Group 3 parasites only.
* - Group 3 < (1 = 2) $p < 0.02$

results in significantly lower antibody titers than that of controls (group 4) on days 14 ($p < 0.02$) and 20 ($p < 0.05$). The administration of 20 mg Cy/kg on day -1 results in slight antibody enhancement, statistically significant only on day 20 ($p < 0.05$). No significant antibody enhancement was observed with the administration of two doses of 20 mg Cy (group 3), as compared with one dose.

Effect of hyperimmune anti-T. cruzi antibody on parasitemia and mortality of C. callosus inoculated with 200 mg Cy/kg body weight.

The inoculation of 0.3 ml hyperimmune **C. callosus** anti-**T. cruzi** serum three hours before parasite challenge in immunosuppressed animals reduced parasitemia significantly throughout the experiment when compared with those which received either normal **C. callosus** serum, or parasites and Cy only (Fig. 4) ($p < 0.001$ on day 9).

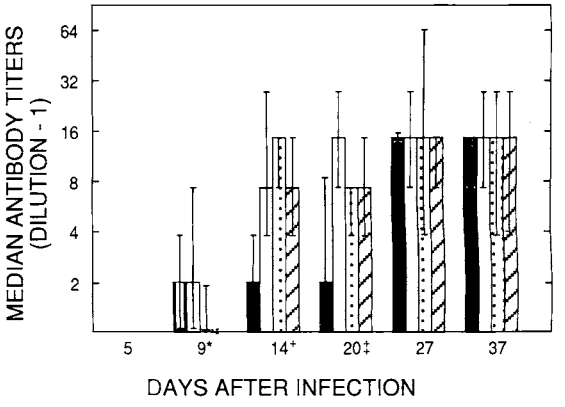

Fig. 3. Effect of Cy on direct agglutination titers of **C. calossus**, inoculated with 4 x 10^3 **T. cruzi** Y strain on day O; ■ Group 1 200mgCy/kg on day -1; □ Group 2 20 mg Cy/kg on day -1; □ Group 3 20mgCy/kg on days -1 and +5; ▨ Group 4 parasites only. Percentiles 25% and 75% are indicated.
* - Group 4 < (2 = 1) $p < 0.01$
+ - Group (4 = 2) > 1 $p < 0.02$
++ - Group 2 > (3 = 4) > 1 $p < 0.05$

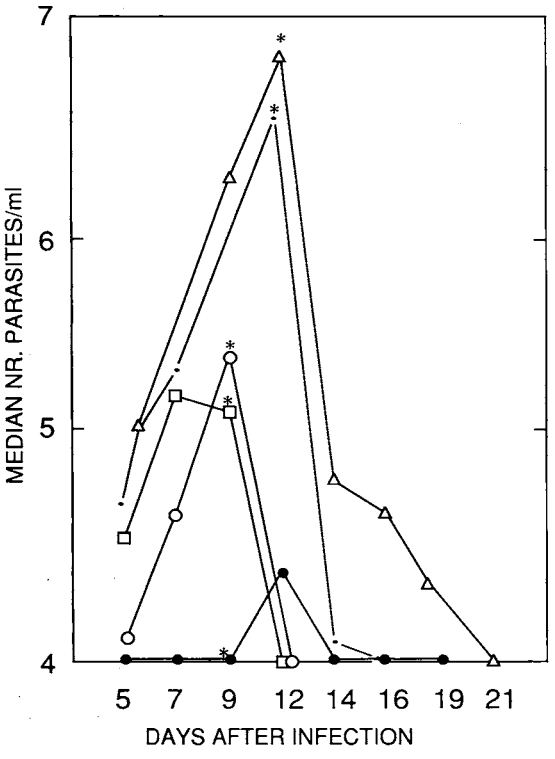

Fig. 4. Effect on parasitemia (log 10) of the injection of 0.3 ml hyperimmune anti **T. cruzi C. callosus** serum (IS) or normal serum (NS) 3 hs before parasite challenge with 4 x 10^3 **T. cruzi** strain on day 0 in immunosuppressed **C. callosus.** ●——● Group 1, 200 mg Cy day -1 + IS; Δ——Δ Group 2, 200 mg Cy day -1 + NS; □—□ Group 3, NS; •——• Group 4, 200 mg Cy day -1; O—O Group 5, parasites only. * - Group 1 < (5 = 3) < 2 = 4 p < 0.001

Hyperimmune serum reverted the effect of 200 mg Cy/kg on mortality of **T. cruzi** challenged animals. Mortality dropped from 35% (7/20 - group 4) to 10.34% (3/29 - group 1) (Table 1). Controls receiving parasites only (group 5) had a 9.52% (2/21) mortality rate. Normal **C. callosus** serum had no apparent effect on mortality of immunosuppressed animal (group 2), and the administration of Cy by itself did not kill any animals in group 6.

DISCUSSION

The Y strain of **T. cruzi**[25] is known to be highly pathogenic for most strains of mice, with high parasitemia and mortality rates. In constrast, even young **C. callosus** are able to clear parasitemia after a short period, and the majority of animals survive infection[5]. This is similar to infections in rats[27].

Cyclophosphamide has been used in many systems to modulate the immune response to T dependent antigens[30,32] including **T. cruzi** infections [1,2,8,26].

The immune response of **C. callosus** to sheep red blood cells (SRBC) and its modulation by Cy has been previously studied by us[6], and this served as a guideline for the drug a administration schedule.

Cy is known to be a potential suppressor of both B and T cell functions[24] and enhances **T. cruzi** infections in mice[2,14,26]. In our system, specific anti-**T. cruzi** antibodies were substantially lower in **C. callosus** immunosuppressed with one dose of 200 mg Cy/kg, up to the 20th day after infection. Parasitemia and mortality were also much higher than in controls. Two doses of 200 mg Cy/kg had a more pronounced effect both on parasitemia and mortality.

Anti-**T. cruzi** antibodies were determined for animals which received Cy on day-1 only. Antibody titers were extremely low in this animal group, up to the 20th day. Thus is seemed superfluous to follow up antibody production in twice injected **C. callosus**.

Low doses of Cy act mainly on those T cells responsible for suppresion. In humans, for instance, CD4+, 2H4+ suppressor-inducer T cells are selectively destroyed[4]. In mice, low doses eliminate Lyt 1,2+ T lymphocytes[23] which are regulators or precursors of the Lyt2+ suppressor cells.

Our experimental design did not elucidate the mechanism of the slight enhancement of anti-**T. cruzi** antibody with the administration of 20 mg Cy/kg.

T. cruzi infections are under the control of both the B and T cell compartment of the immune response. Suppression is known to exist in these infectios[20] and it is plausible that the drug interfered with the suppressor network. However the slight rise of anti-**T. cruzi** antibodies was not reflected on parasitemia levels. At the onset of infection, on the 5th day, when antibodies were not de-

tectable by direct agglutination, 20 mg Cy injected animals had lower parasitemias than controls. By the time of parasitemia peak - 7th and 8th day, both 1 dose and 2 doses 20 mg Cy injected animals had a significantly higher parasitemia than controls (Fig.2) ($p < 0.001$). Antibody levels may be insufficient or ineffective in protection, since for instance Takehara et al.,[29] found that passive protection against **T. cruzi** in mice was associated mainly with IgG_2, while IgG_1 seemed to have little activity. Brodskyn et al.[9] demonstrated that this higher efficiency of IgG_2 is due to a five-fold concentration of specific anti **T. cruzi** antibodies in this fraction as compared to IgG_1. The latter sub-class, in appropriate concentrations mediated clearance of **T. cruzi**, However, it must be borne in mind that T cell cytokines mediating cellular immune responses may also be depressed which could account for the higher parasitemia observed. Interleukin-2[10], interferon[20] and IL-4[33] have been shown to affect the course of infection both **in vitro** and **in vivo**.

In other animal models, similar effects of low doses of Cy have been observed. In BALB/c mice infected with the Y strain, the administration of 40 mg/kg Cy resulted in parasitemia similar to that of controls, besides intensification of myocarditis and higher mortality rate[26]. Much the same was found in dogs receiving low doses of Cy after having recovered from the acute phase of infection by the 125F or Colombiana strain[2].

Anti **T. cruzi** serum obtained from **C. callosus** was able to revert the effect of immunosuppression provoked by 200 mg Cy/kg, attesting to the importance of humoral immune response in the control of Y strain **T. cruzi** infection in **C. callosus**, much as in the mouse model.

T. cruzi laboratory infections tend to have high individual variations in parasitemia, with consequent high standard deviations when results are indicated as mean values of animal groups. Sogayar[28] compared mean, median and mode analysis in **T. cruzi** parasitemia of Wistar rats infected with the Y and FL strain. Since the mean is only significant when standard deviation is situated in low levels, resulting in a symetric distribution, he suggested that **T. cruzi** parasitemias should be indicated as the median. We adopted this criterion, and extended it to antibody levels.

C. callosus seems to be an adequate alternative animal model for the study of Chagas'disease in wild rodents. It breeds well in the laboratory, and due to the ease in handling and its smaller size it would be a more convenient and economical experimental animal than the rat. More sophisticated immunological experiments will soon be feasible, since we are raising inbred lines in our **C. callosus** colony.

RESUMO

Modulação da Parasitemia e da Resposta de Anticorpos ao Trypanosoma cruzi **pela Ciclofosfamida em** Calomys callosus (Rodentia, Cricetidae).

Calomys-callosus, roedor silvestre, que já foi encontrado naturalmente infectado pelo **Trypanosoma cruzi**, tem baixa suscetibilidade à infecção experimental por este protozoário.

Foram feitos experimentos para avaliar a contribuição da resposta imune a essa baixa suscetibilidade. Animais foram submetidos a injeção de doses altas (200 mg/kg peso corporal) ou doses baixas (20 mg/kg peso corporal) de ciclofosfamida nos dias -1 ou -1 e +5, e inoculados com 4 x 10^3 **T. cruzi** no dia O. Observou-se a curva de parasitemia, mortalidade e resposta de anticorpos medida por aglutinação direta de tripomastigotas. Doses de 200 mg resultaram em parasitemia e mortalidade mais elevada e supressão da resposta de anticorpos. Uma dose de 20 mg aumentou os níveis de anticorpos no 20º dia após a infecção, enquanto a administração de uma segunda dose não alterou significativamente a produção de anticorpos. Os níveis de parasitemia não diminuíram, mas pelo contrário, elevaram-se em relação aos animais testemunhos, em ambos os grupos. A transferência passiva de soro anti-**T. cruzi** de **C. callosus** resultou em parasitemia e mortalidade mais baixa nos animais imunossuprimidos. Estes resultados indicam que a resposta imune é um importante fator na resistência de **C. callosus** à infecção por **T. cruzi**.

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