Parasitol Res (1998) 84: 100–105 

ORIGINAL PAPER

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# Influence of female gonadal hormones on the parasitemia of female *Calomys callosus* infected with the "Y" strain of *Trypanosoma cruzi*

Received: 27 April 1997 / Accepted: 5 July 1997

**Abstract** *Calomys callosus* is a wild rodent found infected with *Trypanosoma cruzi* in nature. Groups of female *C. callosus* were subjected to ovariectomy or sham operation or served as intact controls. At 1 month after surgery, animals were inoculated intraperitoneally with 4000 blood trypomastigotes of the "Y" strain of *T. cruzi*. Parasitemia during the course of infection was significantly higher in ovariectomized animals as compared with sham-operated rodents and controls. On steroid hormone replacement the parasitemia of ovariectomized animals dropped to levels close to those of controls. High or low doses of progesterone, estrogen, or a combination of both exerted similar effects. Splenocyte proliferation of ovariectomized animals was unresponsive to stimuli with concanavalin A and lipopolysaccharide as compared with that of control and sham-operated groups. The results show that gonadal hormones play a fundamental role in the defense against *T. cruzi* infection. The influence of these procedures on the immune defense in experimental Chagas' disease is being further investigated.

## Introduction

Chagas' disease continues to constitute an important health problem, affecting more than 18 million people in Latin America (WHO 1991). In spite of great efforts expended to understand and control the pathology of the disease caused by the intracellular protozoan *Trypanosoma cruzi*, many aspects remain to be clarified.

Acute infection with *T. cruzi* or its African relatives is frequently accompanied by manifestations of immunological dysfunction. In recent years a significant increase in studies relating to the function of the immune system and its involvement with other systems has been emphasized by many researchers.

The connection between the fields of endocrinology and immunology appears to be hormonally regulated, and the hormones involved originate from the thymus, the hypothalamus-pituitary unity, and the gonads (Grossman 1985). This immunoregulatory process may explain the different susceptibility of males and females to several parasitic infections as demonstrated by numerous authors (Brabin and Brabin 1992).

There is little information in the literature on hormonal influence on *T. cruzi* infection, and the reports that are available display conflicting results. Hauschka (1947) was the first investigator to show that higher levels of parasitemia, more extensive tissue invasion, greater weight loss, and shorter survival were more frequent in males than in females. At that time, one of the possible reasons for these phenomena was supposed to be based on differences in metabolic processes.

In an attempt to confirm these differences, Webster male and female rats were injected with culture forms of *T. cruzi* (Brazil strain). Each group of animals was treated with hormones characteristic of the opposite sex during the 2-week period following infection. Within groups of the same age and sex, no significant difference in mortality was observed between treated and control animals, leading to the inference that substances other than steroid hormones were involved (Goble 1952).

Studies on the susceptibility of C.F.W. mice to trypomastigotes of the Tulahuen strain of *T. cruzi* seemed to indicate that the profiles, displayed by the infected male and female mice were quite similar. Eventually observed differences were attributed to discrepancies between the weight of males and that of females of the same age (Kagan and Norman 1960).

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The influence of steroid hormones on the susceptibility of female mice to *T. cruzi* infection was clearly demonstrated in ovariectomized animals, which had a lower level of resistance as compared with intact mice (Chapman et al. 1975).

For some time it has been well known that during the acute phase of Chagas' disease, clones of B- and T-cells are activated (Minoprio et al. 1986). Besides this, a transitory suppression of the immune responsiveness against non-specific antigens not related to the parasite as well as against specific mitogens for these cells was detected (Kierszenbaum et al. 1994). For these reasons, immunosuppression has been postulated as a specific feature of acute Chagas' disease, irrespective of the tendency of the illness toward lethality or chronicity.

In our laboratories the lower susceptibility of females to chagasic infection has long been noted in mice and, recently, in the wild rodent *Calomys callosus*. This animal has been used as an alternative experimental model due to its involvement in the epidemiological cycle of several pathogenic microorganisms, such as Machupo virus, the etiologic agent of hemorrhagic fever (Johnson et al. 1965); *Yersinia pestis* (Almeida 1973); and *T. cruzi* (Ribeiro 1973; Mello and Teixeira 1977).

*C. callosus* has also been experimentally infected with other parasites such as *Schistosoma mansoni*, *Plasmodium berghei*, *Leishmania mexicana amazonensis*, *L. donovani chagasi*, and *Paracoccidio des brasiliensis* (Borda 1972; Mello 1979; Mello and Teixeira 1984; Junqueira-Kipnis et al. 1991).

The objective of this study was to check the influence of gonadectomy of female *C. callosus* on the course of infection with the "Y" strain of *T. cruzi* as compared with non-covariectomized animals and the role played by female steroid hormones in the level of parasitemia of these animals as well as their influence on the immune system.

## Materials and methods

### Animals

*Calomys callosus* aged 30–40 days and weighing 20–25 g, raised in the Animal Facilities of the Instituto de Medicina Tropical de São Paulo, were used. The animals were subjected to constant checkup, including occasional hematological controls (Prado and Kloetzel 1995) to guarantee a high health standard. They were kept in plastic cages, with water and food being provided ad libitum. They were treated according to *Principles of Laboratory Animal Care* (NIH publication 86-23, revised 1985) and to *Principles of Ethics in Animal Experimentation* (COBEA – Colegio Brasileiro de Experimentação Animal, 1991). Animals were divided into three groups: ovariectomized sham-operated, and control.

### Ovariectomy

The animals were anesthetized with an intraperitonel injection of tribromoethanol (Aldrich, 0.1 ml of a 2.5% solution/10 g body weight, and subjected to ovariectomy or sham operation. In the sham-operated group an incision was made in the peritonial cavity without removal of the ovaries. Animals of the same age and weight served as controls.

### Parasites

The "Y" strain of *Trypanosoma cruzi* was used in all experiements. This strain was isolated from a human patient by Silva and Nussenzweig (1953) and is maintained in our laboratory by weekly mouse passages.

### Infection and parasitemia

At 30 days after ovariectomy the animals were infected intraperitoneally with $4 \times 10^3$ blood trypomastigotes. The parasite counts were performed by Brener's method (1962), and the final adjustment of the number of parasites to be inoculated was done after counting in a Neubauer chamber. Blood samples were drawn via tail puncture on days 5, 7, 9, 12, 14, and 16 after infection, and levels of parasitemia were determined by Brener's method.

### Hormone reposition

At 1 week before infection the ovariectomized group was subjected to daily subcutaneous injection of either estradiol benzoate (Progynon, Schering), progesterone (Sigma), or a combination of both hormones (Ginecoside, Darrow). Due to the nonexistence of species-specific hormones, the reposition was carried out with human steroid hormones. To test the efficacy of human hormones in *C. callosus* and to select a suitable dose as close as possible to that meeting their physiological needs, we created a dose-response curve, taking as a reference the doses used in rats. The doses tested were 0.01, 0.1, and 1.0 μg/10 g body weight (b.w.) for estrogen and 0.83, 0.166, and 1.66 μg/10 g b.w. for progesterone. The response was almost the same following low, medium, and high doses of hormones. Final experiments were conducted using doses of 0.1 μg/10 g and 1.66 μg/10 g b.w., respectively. In the sham-operated and control groups, the same volume of diluent (corn oil) was given. The hormone therapy was maintained until 3 days after the negativation of parasitemia.

### Splenocyte proliferation

The splenocyte culture was carried out in polystyrene 96-well flat bottom plates using $5 \times 10^5$ cells/well in RPMI medium supplemented with 10% bovine fetal serum and 2-β-mercaptoethanol at $5 \times 10^{-2}$ *M*, with or without the addition of either concanavalin A (ConA) at 20 μg or lipopolysaccharide at 200 μg. Cultures were incubated for 48 h in wet chambers at 37 °C in an atmosphere containing 5% $CO_2$. Subsequently, tritiated thymidine was added (1 μCi/well per 20 μl of medium). After an additional 24 h of incubation, cells were lysed by the addition of hipotonic medium, filtered in special glass filters, and collected in a cell harvester (Titertek). The amount of radioactivity incorporated in the cellular DNA was assessed using scintillation liquid, and the counts were represented by counts per minute. Each test was carried out in triplicate. The results were analyzed using a stimulation (relation between the counts per minute recorded for infected cells and those recorded for noninfected control cells).

### Statistical analysis

The significance of differences between groups was determined by analysis of variance followed by the Neuman-Keuls test for multiple comparisons.

## Results

Parasitemia levels peaked on the 9th day after infection in all groups. The ovariectomized animals had

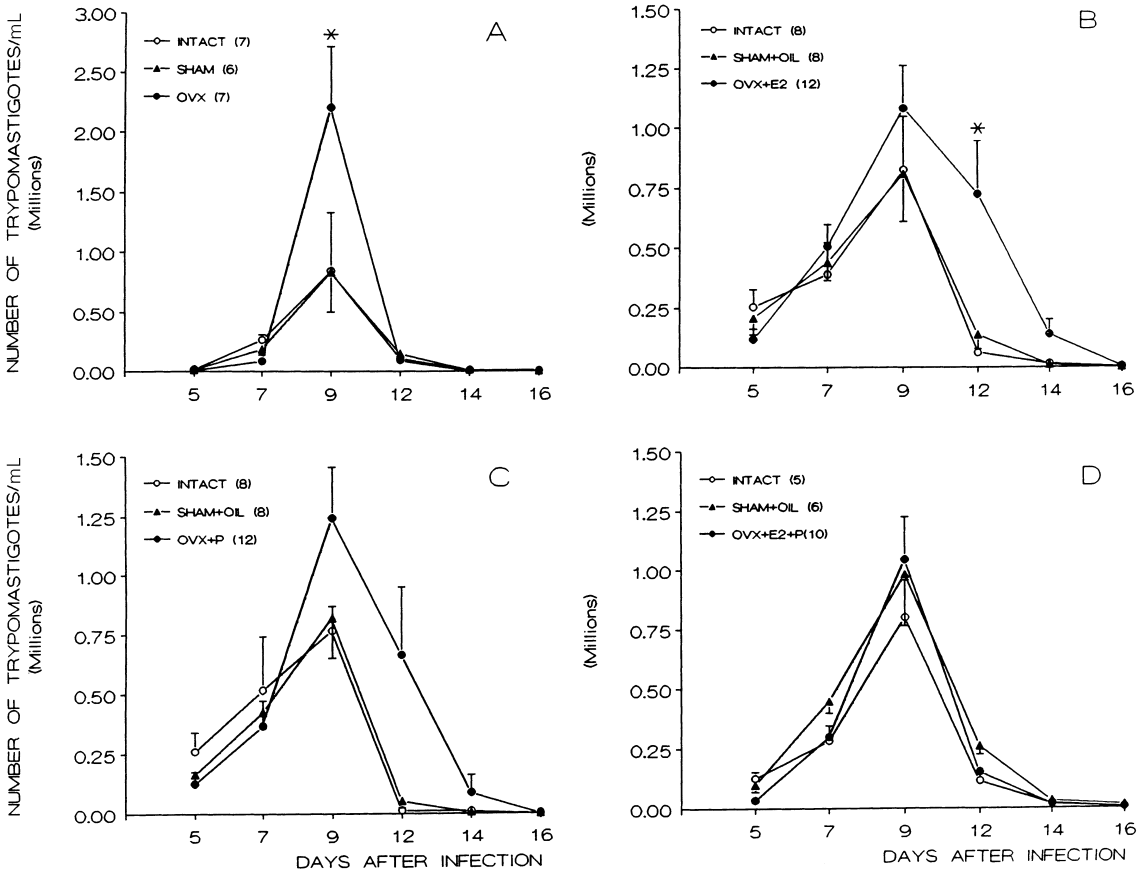


**Fig. 1A–D** Effect of ovariectomy (*OVX*) on parasitemia (mean value +SD) of *Calomys callosus* infected with 4000 blood trypomastigotes of the "Y" strain of *Trypanosoma cruzi* followed or not followed by hormonal reposition (corn-oil suspension). Comparison with a sham-operated group (injected with oil) and intact controls. **A** No hormone **B** Estradiol (*E2*, 0.1 μg/10 g body weight). **C** Progesterone (*P*, 1.66 μg). **D** E+P. Animal numbers are given in *parentheses*. $*P < 0.05$ as compared with sham-operated and control values

significantly higher parasitemia levels as compared with intact and sham-operated animals (Fig. 1A). After the 9th day peak the drop in parasitemia was somewhat slower in ovariectomized animals treated with estradiol reposition as compared with the intact and sham-operated groups (Fig. 1B). This difference was statistically significant on the 12th day after infection. The level of parasitemia was slightly higher in the progesterone reposition group (Fig. 1C) than in intact controls, whereas in animals receiving the combination of both hormones (Fig. 1D) it was slightly higher than in the intact and sham-operated groups. However, these differences were not statistically significant.

A comparison of the results recorded for groups subjected to the hormonal reposition schemes (Fig. 2) clearly shows that no additive effect was exerted by the combination of estradiol and progesterone.

The splenocyte-proliferation study showed an early stimulation of the immune response, detected as a higher degree of blastogenesis on the 5th and 12th days after infection in control and sham-operated animals (Fig. 3A, B). On the 9th day, when parasitemia was at its peak, immunosuppression was observed in the control and sham-operated groups (Fig. 3A, B), although ovariectomized animals displayed a lack of responsiveness, with a slight difference being seen in the group stimulated with ConA at 20 μg (Fig. 3C); however, this difference was not statistically significant.

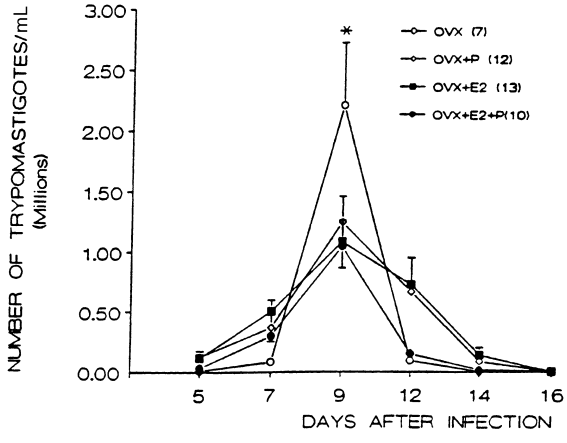


**Fig. 2** Comparison of the hormonal reposition effects of estradiol (*E2*), progesterone (*P*), or a combination of both on the parasitemia of ovariectomized (*OVX*) *C. callosus* infected with 4000 blood trypomastigotes of the "Y" strain of *T. cruzi* . Animal numbers are indicated in parentheses. $*P < 0.05$ as compared with the three other groups

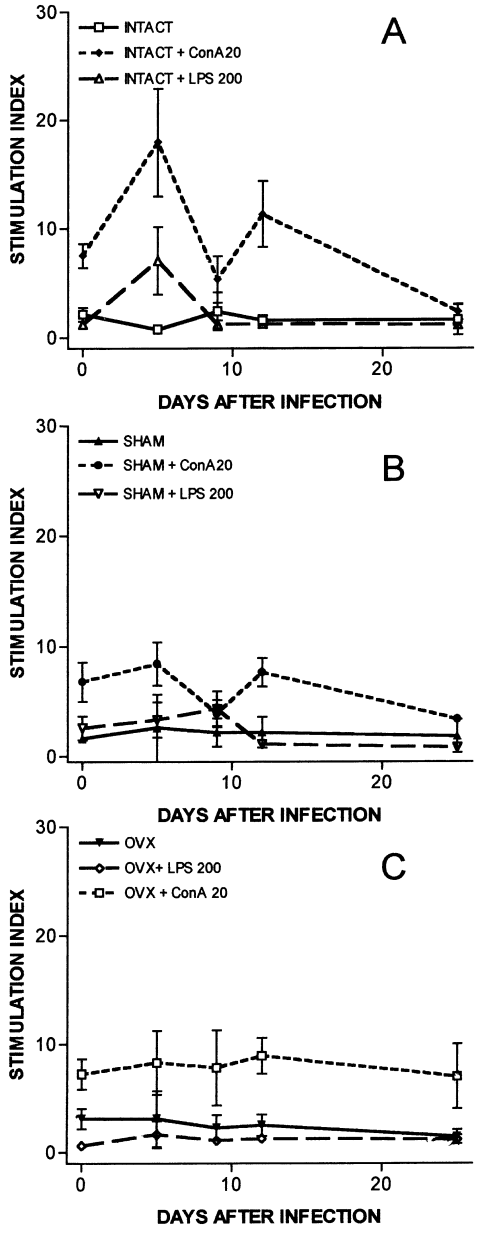


**Fig 3**. **A–C** Stimulation index of splenocyte cultures of *C. callosus* infected with 4000 blood trypomastigotes of the "Y" strain of *T. cruzi*, comparing **A** intact controls, **B** a sham-operated group, and **C** an ovariectomized group treated without stimulus and with ConA at 20 μg and LPS at 200 μg during the evolution of experimental trypanosomiasis

## Discussion

In this work it is evident that gonadectomy affects the course of *Trypanosoma cruzi* infection of females, with significantly higher parasitemia levels being seen in ovariectomized animals as compared with the control and sham-operated groups. The reposition of hormones led to a decrease in the numbers of blood trypomastigotes to levels similar to those observed in control animals.

In our experience, female *Calomys callosus* infected with different strains of *T. cruzi* have lower parasitemia levels than males. We followed the hematological alterations during the course of this infection, and, again, females displayed a faster recovery, reaching normal values earlier than males (unpublished results).

Reports concerning the role of sex hormones in host susceptibility to *T. cruzi* infection are conflicting (Hauschka 1947; Goble 1952; Kagan and Norman 1960). Clinical data on Chagas' disease in humans indicate that its incidence and severity are higher in men than in women (Goble 1970). Similarly, male CF1 mice have been found to be more susceptible to acute infection with *T. cruzi* than females as evidenced by the finding of significantly lower numbers of parasites in the latter as compared with males (Chapman et al. 1975).

Historically, the fields of reproduction and immunology have been classified as separate biological disciplines, but researchers from all over the world have begun to place greater emphasis on the interactions between the endocrine and immune systems. In several mammalian species, including humans, both IgM and IgG serum levels are usually higher in females than in males. The thymus seems to be involved in the process responsible for enhancing humoral antibody formation (Eidinger and Garret 1972). The addition of physiological concentrations of estradiol to pokeweed mitogen-stimulated cultures of human blood lymphocytes significantly increases IgM production as well as B-cell maturation, whereas nontoxic concentrations of testosterone do not influence in vitro B-cell maturation. These observations provide a cellular basis for the differences in immunoreactivity of males and females (Paavonen et al. 1981).

The effect of sex hormones acting on peripheral blood mononuclear cell cultures from healthy seronegative donors infected in vitro with LAV-1 or HIV-1 has been tested. Supernatants from these cultures were assayed by enzyme-linked immunosorbent assay (ELISA) for the presence of antigen P24, a product of HIV-1. When estradiol was added to the cultures at physiological and supraphysiological concentrations (up to 8 μ*M*), HIV replication was not affected as evidenced by in vitro P24 production as compared with that seen in cultures without hormone treatment. However, the addition of progesterone at concentrations of 5–30 μ*M* to these cultures inhibited P24 production. These data seem to indicate an inhibitory effect of progesterone on HIV-1 (Cavert et al. 1991).

Splenocytes are involved in immunosuppression during the experimental infection of mice with *T. cruzi*. Specific responses against parasite antigens have been studied in different lymphoid compartments, and it has been observed that T-cell-specific proliferative responses can be detected in the lymph nodes throughout the acute phase of infection, although the splenocytes of these animals do not react to antigen or lectin stimulation. This study shows the importance of the different lymphoid cell compartments other than the

spleen and also demonstrates that immunosuppression may not be a phenomenon common to all organs involved in the response to the parasite infection (Lafaille et al. 1990).

It seems that the mechanisms involved in immunosuppression are complex and multifactorial. Although the ovariectomized group displays an apparent lack of reactivity, *T. cruzi* infection is controlled, probably in a slower way, as can be seen in the parasitemia profile. However, if ovariectomized *C. callosus* are incapable of controlling the intense intracellular multiplication of the parasites during the acute phase, this may be due to modifications of their immune cells as a consequence of the absence of gonadal female steroids. Besides this, it should be kept in mind that the evolution of the infection also depends on factors related to the parasite. It is well known that the infection of mice with different parasite strains results in a variety of pathological lesions (Brener 1965; Andrade et al. 1985). The same applies to *C. callosus* infection with *T. cruzi* (Borges et al. 1992; Andrade et al. 1994). We chose to study the Y-strain infection in this system in more detail, however, in our first trials we used other strains of *T. cruzi* (Costalimai and M-226) and the modification of the parasitemia profile related to interference with the hormonal system varied from one strain to another, suggesting that the different strains of *T. cruzi* must exert different pathological actions, probably due to characteristics of the parasite as well as its tropism for determined tissue systems. This may be the object of further studies.

It seems that in the sophisticated web of events that participate in the immune response, other factors are involved besides the absence of female gonadal steroids. This interaction is being intensively studied in several parasitic diseases. Immunity against *Toxoplasma gondii* infection, for example, requires the participation of $CD8^+$ T-cells. These cells have estrogen receptors, and the administration of estrogen is effective in reducing the number of $CD8^+$ T-cells and their functional activity, thus contributing to the greater susceptibility of female mice to *T. gondii* infection. $CD4^+$ cells are also involved in the development of effective immunity against this parasite, probably through interleukin 1 (IL-1) secretion. Another example is *Leishmania mexicana* infection. Despite the general consensus that males have a better-developed cell-mediated immunity than females, estrogen may increase interferon-gamma (IFN-$\gamma$) production, mediating resistance against the parasite, whereas male hormones can decrease the production of this cytokine, which is often absent in susceptible male mice (Roberts et al. 1996). We are now investigating in more detail the interaction between the immune system and steroid hormones in *T. cruzi* infection.

**Acknowledgements** The authors wish to thank Dr. Waldemar M. Rocha Barros for the graphics elaboration. This work was partly financed by Conselho National de Desenvolvimento Cientifico e Tecnológico – CNPq. and LIM-49 – H.C.

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