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Interferon-gamma levels during the course of *Trypanosoma cruzi* infection of *Calomys callosus* (Rodentia-Cricetidae) and Swiss mice

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Abstract Serum levels of interferon-gamma (IFN-γ) were evaluated in *Calomys callosus* and Swiss mice during the course of infection by four strains of *Trypanosoma cruzi*. All strains stimulated the production of this interleukine; however, the timing of its onset and permanence varied among strains and between the two animal models. When chronically infected animals with no detectable serum IFN-γ were challenged with the homologous strain, they produced quantities comparable with those obtained during the acute phase of infection. In *C. callosus* there was a correlation between H_2O_2 liberation by peritoneal macrophages and serum IFN-γ levels, whereas no such correlation was found in mice. *C. callosus* had a higher capacity to heal histopathological lesions, whereas lesions in mice were progressive. The results obtained suggest that *C. callosus* develops well-adapted immune mechanisms that may be important for its role as a reservoir of *T. cruzi*.

Introduction

Resistance and susceptibility to several infections, including those with *Trypanosoma cruzi*, are controlled at least partially by T-cells (Schmunis et al. 1971; Trischman 1983; Gonzalves da Costa et al. 1984; Reed 1988), both CD4+ and CD8+ cells being involved in the acute phase (Minoprio et al. 1987; Russo et al. 1988; Tarleton 1990). In the chronic stage, T-cell activity may play a role in promoting disease (Laguens et al. 1981; Hontebeyrie-Joskowisz et al. 1987).

Interferon-gamma (IFN-γ), a cytokine preferentially secreted by Th1 cells (Scott et al. 1989; Sher and Coffman 1992), is extremely powerful in mediating in vitro killing of *T. cruzi* by mouse peritoneal macrophages (Wirth et al. 1985; Plata et al. 1987). This anti-*T. cruzi* activity has been correlated with increased oxidative burst and production of nitric oxide (Nathan et al. 1979; Reed et al. 1987; Gazzinelli et al. 1992) and tumour necrosis factor (TNF; Titto et al. 1986; Black et al. 1989). IFN-γ acting on macrophages and fibroblasts is critical in reducing the number of intracellular *T. cruzi* organisms in the acute phase of infection (Plata et al. 1984). On the other hand, in the chronic stage the continuous T-cell activity with IFN production may result in autoimmune reactivity (Engleman et al. 1981; Campbell et al. 1988) driven by increased major histocompatibility complex (MHC) gene expression (Behbehani et al. 1981). Thus, the levels and nature of cytokine production contribute to regulate disease severity. It becomes clear that IFN may play a central role in the regulation of the immune response, but at the moment it appears to be quite difficult to define its exact position in the complex network of the immune system.

Studies suggest that the relationship between the immune system and *T. cruzi* is complex and that different kinds of models will probably be necessary to understand it. The genetic background and age of the host as well as peculiarities of the parasite strain contribute to the disease outcome. Only those individuals with the ability to regulate both the parasite load and the immune response, reaching an appropriate balance, will overcome infection and survive with minimal damage.

Calomys callosus (Rodentia-Cricetidae) is a silvatic rodent that has been described as a resistant model for Chagas' disease study. These animals survive high inoc-

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ula of virulent *T. cruzi* strains. For instance, 90% of *C. callosus* survive inocula of the Y strain, which kill 100% of mice, and there is an almost complete regression of their tissue lesions (Borges et al. 1992a, b).

Little is known about the immunoregulatory mechanisms involved in the outcome of Chagas' disease in silvatic animals. This holds true for Th1- or Th2-cell products in *C. callosus* and their involvement in the control of *T. cruzi* infection. We studied levels of IFN-γ in the serum of *C. callosus* as compared with Swiss mice after their infection with several strains of *T. cruzi* of different origins. We attempted to correlate this Th1-associated cytokine with the control of infection in vivo. We suggest that IFN-γ may be an important mediator of the immune control of infection in both models, more efficiently so in *C. callosus*.

Materials and methods

Parasites

Bloodstream trypomastigotes of the Y strain, originally isolated from a patient with Chagas' disease (Silva and Nussenzweig 1953); the F strain, originally obtained from P.A. D'Alesandro (Deane and Kloetzel 1974); the M226 strain, isolated from a wild *Calomys callosus* (Mello et al. 1979); and the Costalimai strain, isolated from a *Triatoma costalimai* (Mello and Borges 1981) were used. The Y and F strains were maintained in mice and strains M226 and Costlimai, in *C. callosus*.

Animals

C. callosus were raised in facilities of the Instituto de Medicina Tropical de São Paulo. Swiss mice were raised at the Faculdade de Medicina, São Paulo. Male rodents aged 30–45 days and weighing 18±2 g constituted all experimental groups.

Experimental groups

Animals were inoculated s.c. with 4×10^3 parasites, and immune serum was obtained by bleeding from the retro-orbital plexus of individual animals (under light ether anesthesia) at periods corresponding to the onset of infection, the intermediate time and the final drop in parasitemia. Experiments with chronic animals were done by challenging them with 10^5 parasites of the homologues strain at 3–4 months after the initial inoculation. Individual serum samples were collected at 7 days after challenge.

Parasitemia

Parasites were counted as described by Brener (1962) by placing 5 µl tail blood under a 22- ×22-mm coverslip and examining 50 microscopic fields.

IFN-γ assay

Antiviral activity was titrated as previously described (Pereira et al. 1984) on L929 cells by using a standard encephalomyocarditis (EMC) virus cytopathic-effect-inhibition assay. Titres were expressed as the reciprocal of the serum dilution neutralizing 50% of the virus, cytopathic activity. The sensitivity of this test was 10 pg rMuIFN-γ/ml (Boehringer). To characterize the antiviral activity, serial dilutions of serum samples were incubated with anti-murine IFN-γ monoclonal antibody (Holland Biotechnology). In all experiments the antiviral activity was tested in parallel in *C. callosus* and mouse sera.

H_2O_2 burst of peritoneal macrophages

Macrophages were obtained from peritoneal washings of infected animals and plated in 96-well flat-bottom tissue-culture plates (Corning). H_2O_2 production was determined by the peroxidase-dependent (HRPO) phenol red oxidation method (Pick and Keisari 1980) as adapted by Pick and Mizel (1981) for microassay.

Histopathology

Mice and *C. callosus* were killed at different days post-infection. Hearts and thigh-muscle fragments were fixed in 10% formalin. The paraffin-embedded 5-µm sections were stained by the Harris hematoxylin and eosin method (0.5% hematoxylin, 0.05% eosin) according to Thomson and Hunt (1966).

Results

Parasitemia

Different parasitemia patterns were observed in *Calomys callosus* and mice as illustrated in Fig. 1.

IFN activity of different parasite strains

All strains tested stimulated IFN production in *C. callosus* and Swiss mice (Fig. 2). Differences in the timing of the first appearance and persistence of significant IFN levels were observed between the various parasite strains. The highest serum IFN levels were obtained in *C. callosus* inoculated with strain F (Fig. 2a). The high peak of IFN was reached as early as day 7, with titres maintaining a plateau until day 28 and dropping after that to baseline levels. Strains Y and M226 also stimulated secretion of high levels of IFN; however, their kinetics were different, with maximal amounts being found on days 14 and 28, respectively (Fig. 2a).

In mice, strains F and Y stimulated high levels of the cytokine, the kinetics differing from those of *C. callosus*. With strain F, IFN peaks were somewhat delayed, occurring between days 21 and 28 after infection, with a subsequent drop. Strain Y stimulated increasing levels of IFN starting on day 7. Most animals died after day 14, and the IFN values of the few survivors dropped almost to the control level on day 18 (Fig. 2b).

Both animal species had the same kind of IFN kinetics with strain M226; however, *C. callosus* levels were 6 times those of mice (Figs. 2a, 1b). The lowest values for IFN were found with strain Costalimai in both *C. callosus* and mice, never rising above 80 U/ml, close to those of non-infected controls, which did not exceed 20 U/ml.

Characterization of serum IFN

Sera obtained on day 24 post-infection from *C. callosus* or mice inoculated with strain F were pooled and tested

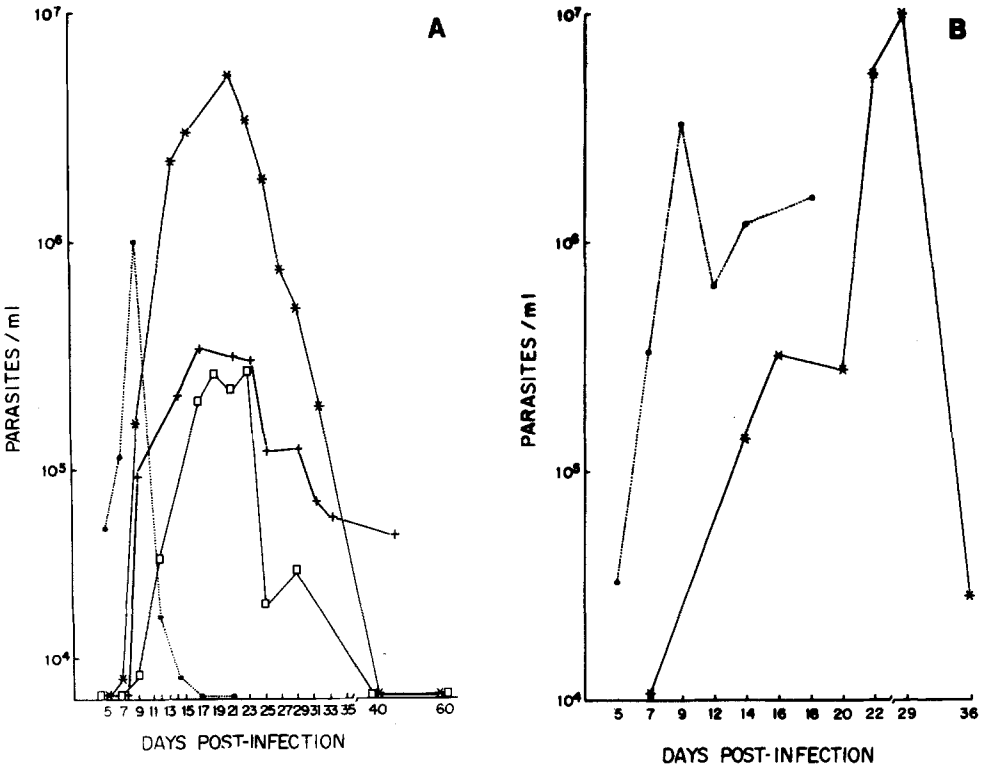

Fig. 1A, B Parasitemia of **A** *Calomys callosus* and **B** Swiss mice infected with 4×10^3 blood trypomastigotes (s.c.). (*—* F strain, ●—● Y strain, □—□ Costalimai strain, +—+ M226 strain). Each point is the mean value for 10 animals. No patent parasitemia was observed for strains M226 and Costalimai in mice

in the presence of anti-murine IFN-γ monoclonal antibody. The protective effect on cytopathic activity conferred by infected animal sera was abolished 100% by this monoclonal antibody, confirming that the protection had been provided by IFN-γ. The neutralization effect also implies cross-reactivity between IFN-γ of mice and that of *C. callosus* (data not shown).

Secondary IFN-γ response

At 3 months after the primary infection, IFN-γ was no longer detected in the sera of *C. callosus* inoculated with strains M226 or F. At 7 days after a new parasite challenge, IFN-γ levels were comparable with those of the primary response. The same applied to mice inoculated with the F strain (data not shown).

Correlation between serum IFN-γ levels and peritoneal-macrophage H_2O_2 burst in *C. callosus*

A parallel between IFN-γ titres and the H_2O_2 burst of peritoneal macrophages (Fig. 3) could be established in *C. callosus* infected with strain Y, all of which survived the infection. In mice, IFN levels were high early in the infection, with the H_2O_2 burst being low. After day 10, animals started dying; the data represent the few survivors. The same applies to animals infected with strains F and M226 (data not shown). A coincidence of IFN-γ titre and H_2O_2 burst was observed with strain Costalimai only in the acute phase of infection. This was the strain with the lowest-level stimulation of the cytokine.

Intensity and evolution of histopathological lesions in heart and skeletal muscle

The intensity and evolution of histopathological heart and skeletal muscle lesions varied with the animal model and parasite strain (Table 1). In *C. callosus*, strains Y, F and M226 generally induced more intensive lesions than those of Costalimai, albeit with an almost complete regression. In mice, lesions were more pronounced and progressive.

With Y-strain infection, up to the 11th day, lesions evoluted in a similar fashion in both animal models. In *C. callosus* they subsided almost completely by the 40th day, whereas all mice died in the acute phase of infection with intensive histopathological alterations.

F-strain-injected *C. callosus* had a slow and progressive infection marked by lesions and fibrosis in both heart and skeletal muscle. This was followed by an almost complete regression, and at the end of the observation period, only a slight myocardial inflammatory infiltrate was found. Mice had a similar initial evolution but showed no sign of regression.

Alterations were early and intensive in strain-M226-infected *C. callosus* as compared with mice, involving intense fibrogenesis and a dense mononuclear infiltrate, with fibroblasts substituting for destroyed heart fibres. After 3 weeks the lesions subsided, and only slight inflammatory infiltrations were observed in the heart muscle after 60 days, with no alteration being found in the skeletal muscle. Mice inoculated with the same strain had a progressive evolution of diffuse and focal myocarditis up to the end of the observation period, whereas no lesions was found in their skeletal muscle.

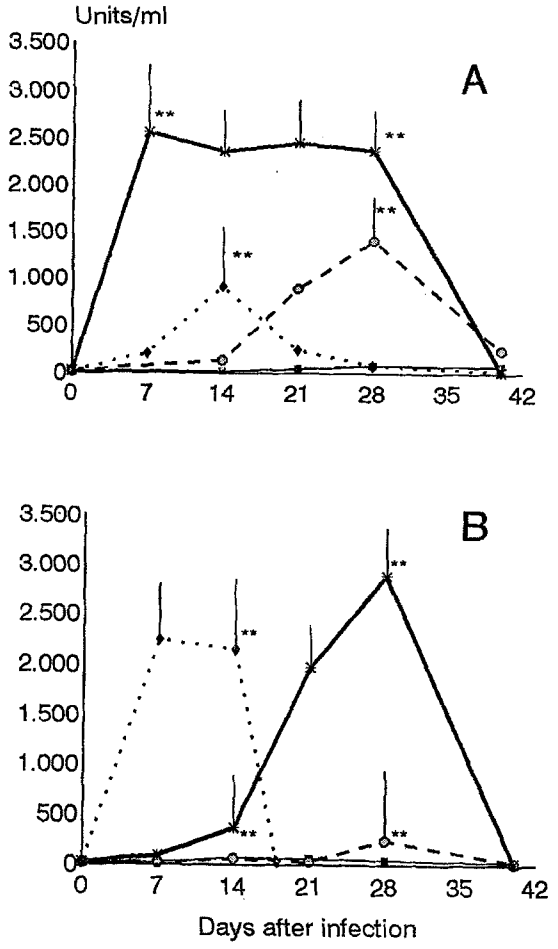

Fig. 2A, B Serum IFN levels measured after s.c. infection with 4×10^3 bloodstream trypomastigotes. The antiviral effect of serial serum dilutions was tested on L929 cells. **A** *C. callosus*. **B** Swiss mice. (*—* strain, F ◆—◆ Y strain, □—□ Costalimai strain, ○—○ M226 strain). Each point represents the mean value±SD for 10 animals/group; all determinations were made in duplicate. ** $P<0.001$; significant differences found between groups by multiple analysis of variance

In Costalimai infections a different course was observed in each animal model. *C. callosus* was resistant, developing hardly any tissue lesions, whereas in mice a progressive evolution of myocardial and skeletal mucle lesions was observed. A more detailed description of histopathological findings with strains Y, F and Costalimai has been published elsewhere (Borges et al. 1992a).

Discussion

Models of susceptibility and resistance in *Trypanosoma cruzi* infections have not been well characterized. However, there are indications that Th1-lymphocyte products such as IFN-γ participate in the control of this disease.

One of the factors complicating the study of Chagasic infection is the variability of protocols used, including the nature of the antigens and animal models chosen. However, most in vivo and in vitro experiments point toward IFN-γ as one of the main mediators in the control of this infection (Plata et al. 1987; Reed 1988; McCabe et al. 1991).

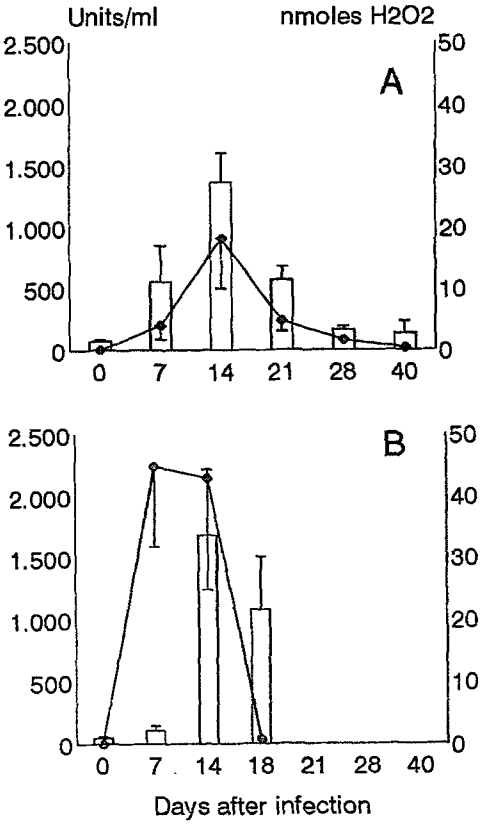

Fig. 3A, B Association between serum IFN-γ levels (*lines*) as tested by the antiviral effect on L929 cells and H_2O_2 production by peritoneal cells (2×10^6/ml; *bars*) as tested by the horseradish peroxidase method. Animals were infected s.c. with 4×10^3 bloodstream trypomastigotes of several *Trypanosoma cruzi* strains. **A** *C. callosus*. **B** Swiss mice. Error bars represent 1 SEM for 10 animals

Table 1 Intensity[a] and evolution[b] of histopathological lesions in *Calomys callosus* and Swiss mice inoculated with four strains of *Trypanosoma cruzi*

Strain	*C. callosus*		Swiss mice	
	Myocardium	Skeletal muscle	Myocardium	Skeletal muscle
Y	$(+++)R_1$	(–)	(++)[d]	(++)[d]
F	$(++)R_1$*	$(+++)R_2$	(+++)[Δ]	(+++)[Δ]
Costalimai	$(+)R_1$[c]	(–)	$(++)R_2$[c]	$(+++)R_2$
M226	$(+++)R_1$*	(–)	(+)[Δ]	(–)

[a] Key: –, No alteration; +, slight change; ++, moderate alteration; +++, intensive change

[b] Key: R, regression by day 60 (1, total; *, total with slight inflammatory infiltrate; 2, partial); Δ, no regression

[c] Occasional lesions

[d] Observed until day 11 only, when high mortality set in

In our experiments, in both *Calomys callosus* and Swiss mice, *T. cruzi* infection was associated with the presence of IFN kinetics varying with the parasite strain and animal model. Serum IFN titres were associated

with the acute phase of infection, dropping later to undetectable levels (Fig. 2).

C. callosus produced high levels of IFN with all strains tested except for Costalimai, which resulted in low levels of this interleukine. Although the parasitemia of Costalimai was comparable with that of M226 (Fig. 1a), tissue aggression was more intensive in animals infected with the latter, which may account for the higher IFN titres observed with the latter strain.

In mice, high levels were associated only with strains F and Y in correlation with parasitemia. With silvatic strains M226 and Costalimai, serum levels of this cytokine were always very low. No patent parasitemia was found in mice inoculated with the silvatic strains (Fig. 1b); however, intracellular forms were found in the heart muscle (data not shown). The presence of IFN was brief in the serum of all infected animals except for *C. callosus* inoculated with strain F, which maintained a high plateau for 3 weeks.

The abolition of antiviral activity observed after the addition of anti-murine IFN-γ monoclonal antibody to the system with sera obtained during the acute phase of infection from both *C. callosus* and mice attested to the presence of IFN of the γ-type and also showed an at least partial homology between murine and *C. callosus* IFN.

Antiviral activity, sometimes characterized as IFN-α, has been described in some in vitro and in vivo experiments with *T. cruzi* early in the infection (Sonnenfeld and Kierszenbaum 1981; Kierszenbaum and Sonnenfeld 1984; Plata et al. 1987). This kind of activity may have occurred in our animals, since in some experiments with *C. callosus* and mice inoculated with strain F or M226, an uncharacterized IFN-like activity was detected in both sera and peritoneal washings at 24–72 h after infection (data not shown).

The varying IFN-γ kinetics promoted by each of the parasite strains used in our experiments may be associated with antigenic differences among the strains as well as with the amount of circulating antigen, since parasitemia varies with strain and animal model (Borges et al. 1992a). In the case of mice inoculated with strains M226 and Costalimai, where isolated parasites are only occasionally found in circulation, tissue parasitism has been observed (Borges et al. 1992a). The IFN-γ present in these mice may have been stimulated by products of these parasites.

In vitro antigen shedding of *T. cruzi* membrane components has shown a quantitative and qualitative difference in polypeptides between strains (Gonçalves et al. 1991). The same phenomenon may be occurring in vivo, being responsible for different activation of T-lymphocytes and, consequently, of IFN-γ production. Besides this, some authors suggest that *T. cruzi* infection may promote differences in lymphocyte proliferation and IFN-γ production in cells obtained from either lymph nodes or the spleen (Curotto de Lafaille et al. 1990; Hoft et al. 1993; Vandekerckhove et al. 1994). Therefore, one could imagine that strains may select different lymphoid compartments in each animal model. Strains Y and F promoted the highest levels of circulating IFN-γ in both *C. callosus* and mice, and they were also the most pathogenic for both animal models, producing intensive tissular parasitism (Borges et al. 1992a). The lowest levels of IFN-γ were recorded over the whole course of the disease with silvatic strain Costalimai, whereas strain M226 behaved differently in the two animal models, stimulating high IFN-γ levels in *C. callosus* and very low ones in mice. In unpublished experiments conducted in our laboratory the course of infection was not modified in splenectomized *C. callosus* infected with strain M226, and this may also be attributed to different lymphoid compartments being responsible for the control of infection.

IFN-γ is a potent activator of macrophages, which, on the one hand, may control infections. However, on the other hand, chronic prolonged production of IFN-γ may lead to autoimmune reactions, thus contributing to immunopathology (Heremans et al. 1978).

There was a close correlation between the in vivo presence of IFN-γ and H_2O_2 production by *C. callosus* macrophages (Fig. 3), which did not take place in mice. The mechanism by which IFN-γ activates macrophages, increasing their microbicidal capacity, in *T. cruzi* infections has not been clarified, but there is evidence for a direct action, increasing the synthesis of enzymes that mediate the respiratory burst (Nathan 1983; Reed et al. 1987), and for an increase in class II MHC antigen expression (Behbehani et al. 1981).

Macrophage activation by IFN-γ may be one of the mechanism for the control of *T. cruzi* infections in both animal models. However, it should be noted that in *C. callosus* there was a reduction in or total regression of lesions, with only slight inflammatory infiltrates being seen by the end of the observations period, whereas despite their high production of IFN-γ and H_2O_2 when infected with the same parasite strains, mice did not recuperate (Table 1).

In a previous study (Borges et al. 1992a) it was shown that macrophage activation (as measured by H_2O_2 production) occurred in *C. callosus* in the initial phase of acute infection and generally tended to be reduced to levels close to those of controls after a parasitemia drop. This did not happen in mice, where activation set in later and increased progressively during the course of infection. A more detailed description of histopathological findings with strains Y, F and Costalimai has been published elsewhere (Borges et al. 1992a).

Parasite clearance in *C. callosus* seems to be correlated with the presence of activated macrophages. In animals infected with strain F, these were found in lesions in direct contact with basal membranes of myoblasts and contained numerous lysosomes and degenerated parasites, attesting to the direct action of these effector cells (Andrade et al. 1994). Small amounts of IFN-γ may be produced after the acute phase, stimulating macrophages. It has been shown that in vitro, amounts as small as 0.14 U IFN-γ/ml increase the capacity of these cells to destroy intracellular parasites (Murray et al. 1985).

The influence exerted by IFN-γ in vivo has been proven (Plata et al. 1987; McCabe et al. 1991). Of course, this is only one of the aspects of parasite control. The role played by antibodies, which for many years were the only immune-response factor studied in Chagas' disease, should not be minimized (Brener 1980). However, IFN-γ also acts on B-cells, which produce antibodies (Cox and Liew 1992). Other mechanisms must be involved in control of the immune response, since both susceptible and resistant animals produce IFN-γ during the course of infection (Silva et al. 1992; Hoft et al. 1993), which is in accordance with our findings. Interleukines such as interleukine 10 (IL-10) and transforming growth factor-beta (TGF-β), described as regulators of macrophage activation by IFN-γ, have been found in animal models susceptible to *T. cruzi* infection (Silva et al. 1991, 1992).

We hypothetize that negative-feedback mechanisms that regulate the presence of IFN-γ, avoiding an uncontrolled amplification of the immune response, may have occurred in *C. callosus*. This idea is supported by the following evidence: (1) strain Costalimai, one of the most pathogenic in mice, was the least so in *C. callosus*; (2) with strain-Y infection, tissue lesions subsided completely in these animals in the chronic phase of disease; (3) strain-M226 infection, although resulting in early myocardial lesions more intensive than those of mice, subsided to a point where only slight mononuclear infiltration was seen; and (4) strain-F infection provoked severe myocardial and skeletal muscle lesions that subsided to focal residual fibrosis later in the disease (Andrade et al. 1994), whereas mice maintained intense inflammatory myocardial and skeletal muscle reactions (Borges et al. 1992a) throughout the observation period.

Taking into consideration these data together with our previous publication (Borges et al. 1992a), one can conclude that during the acute phase of this infection, *C. callosus* develops a T-cell-dependent immune response (IFN-γ). This is regulated to low levels after a parasitemia drop, being sufficient to control tissue parasitism without resulting in host-tissue aggression. Thus, a parasite-host equilibrium is reached, which is fundamental for this animal species' role as a natural reservoir of *T. cruzi*. As a consequence, parasites can be harboured for long periods, as has been shown by xenodiagnosis in chronically infected *C. callosus*, which were positive at 6 months after infection (Prado et al. 1993; Andrade et al. 1994).

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