Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Vol. *87* (4): 493-502, oct./dec. 1992

MACROPHAGE ACTIVATION AND HISTOPATHOLOGICAL FINDINGS IN *CALOMYS CALLOSUS* AND SWISS MICE INFECTED WITH SEVERAL STRAINS OF *TRYPANOSOMA CRUZI*

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Peritoneal macrophage activation as measured by H_2O_2 release and histopathology was compared between Swiss mice and Calomys callosus, *a wild rodent, reservoir of* Trypanosoma cruzi, *during the course of infection with four strains of this parasite.*

In mice F and Y strain infections result in high parasitemia and mortality while with silvatic strains Costalimai and M226 parasitemia is sub-patent, with very low mortality. H_2O_2 release peaked at 33,6 and 59 nM/2 x 10^6 cells for strains Y and F, respectively, 48 and 50 nM/2 x 10^6 for strains Costalimai and M226, at different days after infection. Histopathological findings of myositis, myocarditis, necrotizing artheritis and absence of macrophage parasitism were found for strains F and Costalimai. Y strain infection presented moderate myocarditis and myositis, with parasites multiplying within macrophages.

In C. callosus *all four strains resulted in patent parasitemia which was eventually overcome, with scarce mortality. H_2O_2 release for strains Y and F was comparable to that of mice-peaks of 27 and 53 nM/2 x 10^6 cells, with lower values for strains Costalimai and M226 – 16.5 and 4.6 nM/2 x 10^6 cells, respectively. Histopathological lesions with Y and F strain injected animals were comparable to those of mice at the onset of infections; they subsided completely at the later stages with Y strain and partially with F strain infected* C. callosus. *In Costalimai infected* C. callosus *practically no histopathological alterations were observed.*

Key words: macrophages – H_2O_2 release – *Trypanosoma cruzi* strains – *Calomys callosus* – Swiss mice – Chagas' disease – histopathology

Several aspects of the mechanism of pathological lesions and resistance to reinfection by *Trypanosoma cruzi* in the infected host have yet to be elucidated. Besides humoral immunity (Takehara et al., 1981; Krettli, 1984) cellular immunity also plays an important role in this resistance (Nogueira & Cohn, 1984; Reed et al., 1987; Hontebeyrie-Joskowicz, et al., 1987).

At the initial stages of infection macrophages support intracellular multiplication of several strains of *T. cruzi,* but with the progress of infection T cells are activated, producing several lymphokines, which in turn activate macrophages turning them into effectors of cellular immunity. When stimulated by IL 4 (Wirth et al., 1989) and γ INF (Reed et al., 1987; Plata et al., 1987), respiratory burst of macrophages is highly increased, resulting in H_2O_2, O_2 and/or OH– mediated intracellular killing of *T. cruzi* (Nogueira & Cohn, 1984).

Histopathological lesions in Chagas' disease have always been related to the presence of inflammatory macrophages (Andrade, 1983; Andrade et al., 1987). Recently Hontebeyrie-Joskowicz et al. (1987) have found evidence

This work was financed by Conselho Nacional de Desenvolvimento Cientifico e Tecnológico – CNPq, and Laboratório de Investigação Médica – LIM, Hospital das Clínicas, USP.

Received 6 January 1992.
Accepted 10 August 1992.

that many of the pathological lesions associated with chronic *T. cruzi* infection are mediated to a high degree by L3T4 T cells, through Delayed Type Hypersensitivity (DTH) function.

Wild rodents are natural reservoirs of Chagas' disease. Probably they have been harbouring these parasites for a longer period than man, since their interaction with *T. cruzi* is characterized by low morbidity (Deane, 1964; Pereira Barreto, 1965).

The experimental model of Chagas' disease has been mostly the mouse and experimental infection of wild rodents is little explored. The few experimental works with wild animal species have revealed unique aspects of host-parasite interactions, as for instance in opossums – *Didelphis marsupialis* (Deane et al., 1984; Jansen et al., 1985).

Calomys callosus (Rodentia-Cricetidae) is a reservoir of *T. cruzi.* These animals were originally adapted to laboratory breeding to study Machupo virus (Justines & Johnson, 1970) and during a research on pest in Brazil (Petter et al., 1967). Mello (1978) also studied the biology and breeding of *C. callosus,* and since then this animal species is being used in experiments with *T. cruzi* (Mello et al., 1979; Borges et al., 1982, 1989, a, b, 1992).

The present paper is a comparative study of *C. callosus* and Swiss mice infected with several strains of *T. cruzi.* Peritoneal macrophage activation of animals killed at different times after infection, measured by *in vitro* H_2O_2 release, as well as histopathological aspects of these animals are presented.

MATERIALS AND METHODS

Parasites and animals – T. cruzi strains: Y, originally isolated from a Chagas' disease patient (Silva & Nussenzweig, 1953), F originally obtained from P. A. D'Alesandro (Deane & Kloetzel, 1974), M226, isolated from a wild *C. callosus* (Mello et al., 1979) and Costalimai, isolated from a *Triatoma costalimai* (Mello & Borges, 1981).

Strains Y and F were maintained both in mice and *C. callosus* and strains M226 and Costalimai in *C. callosus* only.

Experimental animals were inoculated subcutaneously (s.c.) with 4 x 10^3 bloodstream parasites; in the histopathology studies inocula were intra peritoneal (i.p.) Trypomastigotes were obtained from the homologous species, except for mice which received strains M226 and Costalimai raised in *C. callosus,* since these strains, when inoculated in mice, result in subpatent parasitemias.

Calomys callosus were raised in animal facilities of Instituto de Medicina Tropical, in a colony originating from animals kindly provided by D. A. Mello, Núcleo de Medicina Tropical Universidade de Brasília (Mello, 1984). Swiss mice were raised in animal facilities of Faculdade de Medicina, São Paulo.

All experimental animals were approximately 30 days old males, weighing 20 ± 2g, on inoculation.

Groups of 50 to 70 animals were inoculated simultaneously for each strain, and animals were killed by ether anesthesia for macrophage or histopathological studies at points corresponding to pre-patency, initial parasitemia, its peak, drop of parasite counts and a final point 60 days after inoculation. These points varied for each parasite strain and animal species. Non-inoculated animals were kept simultaneously as controls.

Parasitemia and mortality – Parasites were counted placing 5 µl tail blood under a 22 x 22 coverslip and examining 50 microscopic fields as described by Brener (1962).

Counts represent the mean of 10 animals per *T. cruzi* strain. Parasitemia was also determined at the time of collecting organs for histopathology. Mortality was observed up to 90 days.

Macrophages – Cells were collected by injecting 3 ml ice-cold phosphate buffered saline (PBS) i.p. in ether anesthetized animals. After slight massage, liquid was withdrawn, collected in a siliconized tube, and maintained on ice. These cells were used for differential and total cell counts, as well as for H_2O_2 measurements.

Total cell counts were made in a Neubauer hemocytometer and cells were suspended at 2 x 10^6/ml for H_2O_2 assays.

Differential cell counts were performed adding 9v of the cell suspension to 1v of a crystal violet solution (containing 0.05% crystal violet in 30% acetic acid).

One drop of these cells was put under a coverslip and 100 cells were counted under oil immersion (x 1000) scoring polymorphs, lymphocytes, mastocytes and macrophages.

Hydrogen peroxide assay – The production of H_2O_2 by freshly explanted peritoneal cells was determined by the horseradish peroxidase-dependent (HRPO) phenol red oxidation method (Pick & Keisari 1980), adapted by Pick & Mizel (1981) for microassay.

The peritoneal cells from individual animals were centrifuged at 200 g 10 min in the cold, and resuspended in an ice-cold buffer-phenol red-peroxidase solution (140 mM NaCl, 10 mM potassium phosphate buffer, pH 7.0, 5.5 mM dextrose, 0.28 mM phenol red and 50 µl/ml horseradish peroxidase). 100 µl of cell suspension (2x 10^6/ml) were placed in each well of a 96 well flat bottomed tissue culture plate (Corning). Plates were incubated in humid chambers at 37 °C for 1 hr. The reaction was stopped with 10 µl/well of 1N NaOH. Absorbance was read at 620 nm on a Titertek Multiscan automatic reader (Flow Laboratories). Conversion of absorbance to nanomoles (nM) of H_2O_2 was deduced from a standard curve of H_2O_2 repeated for each test. All determination were performed in triplicate. Each point corresponds to the mean values of five controls and ten infected *C. callosus* or five infected mice.

Histopathology – Heart, spleen, liver, thigh muscle, colon and jejunum fragments were fixed in 10% formalin, collected on days corresponding roughly to H_2O_2 determination.

The paraffin-embedded 5 µm sections were stained by hematoxylin and eosin.

Statistical analysis – Student's t test was used to assess differences, with $p < 0.05$ considered significant.

RESULTS

Parasitemia and mortality – C. callosus and mice had different parasitemia patterns as illustrated in Fig. 1a, b. Y strain peaked on day 9 both for *C. callosus* and mice. *C. callosus* overcame the infection and parasitemia dropped, becoming negative by day 16, with no mortality up to 90 days. Mice presented a slight drop of parasitemia after the 9th day and no animal survived beyond the 22nd day.

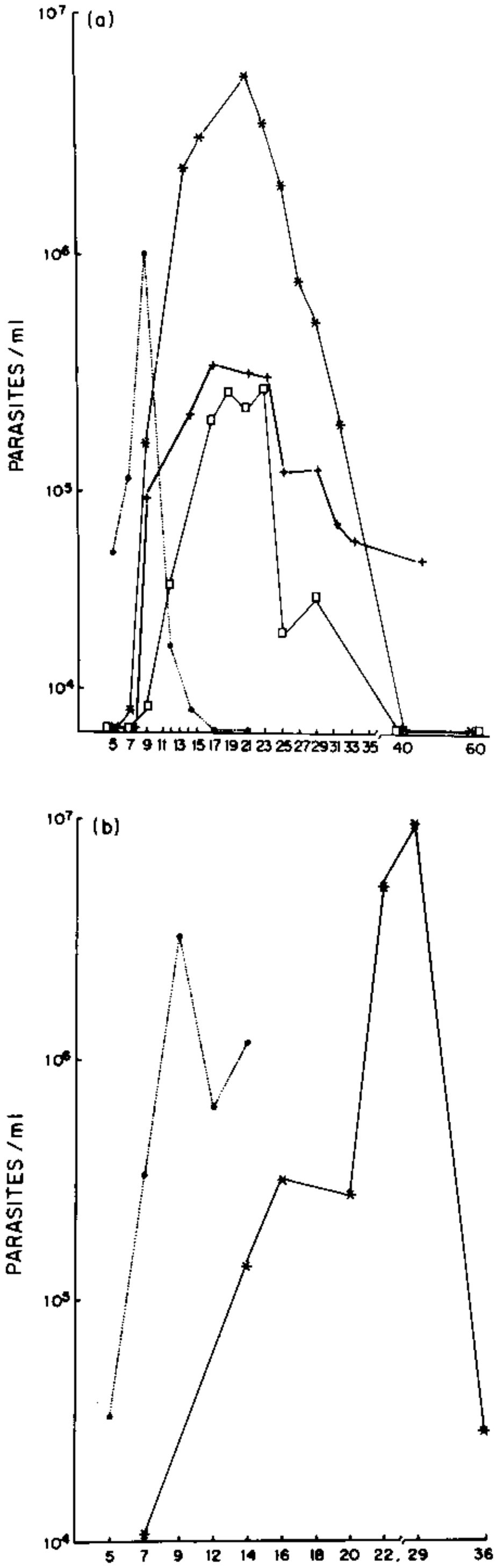

Fig. 1: parasitemia of (a) *Calomys callosus* and (b) Swiss mice infected with 4 x 10^3 blood trypomastigotes (s.c.) of several *Trypanosoma cruzi* strains. ● Y; ✳ F; + M226; ❑ Costalimai. Each point is the mean of 10 animals. No patent parasitemia observed for strains M226 and Costalimai in mice.

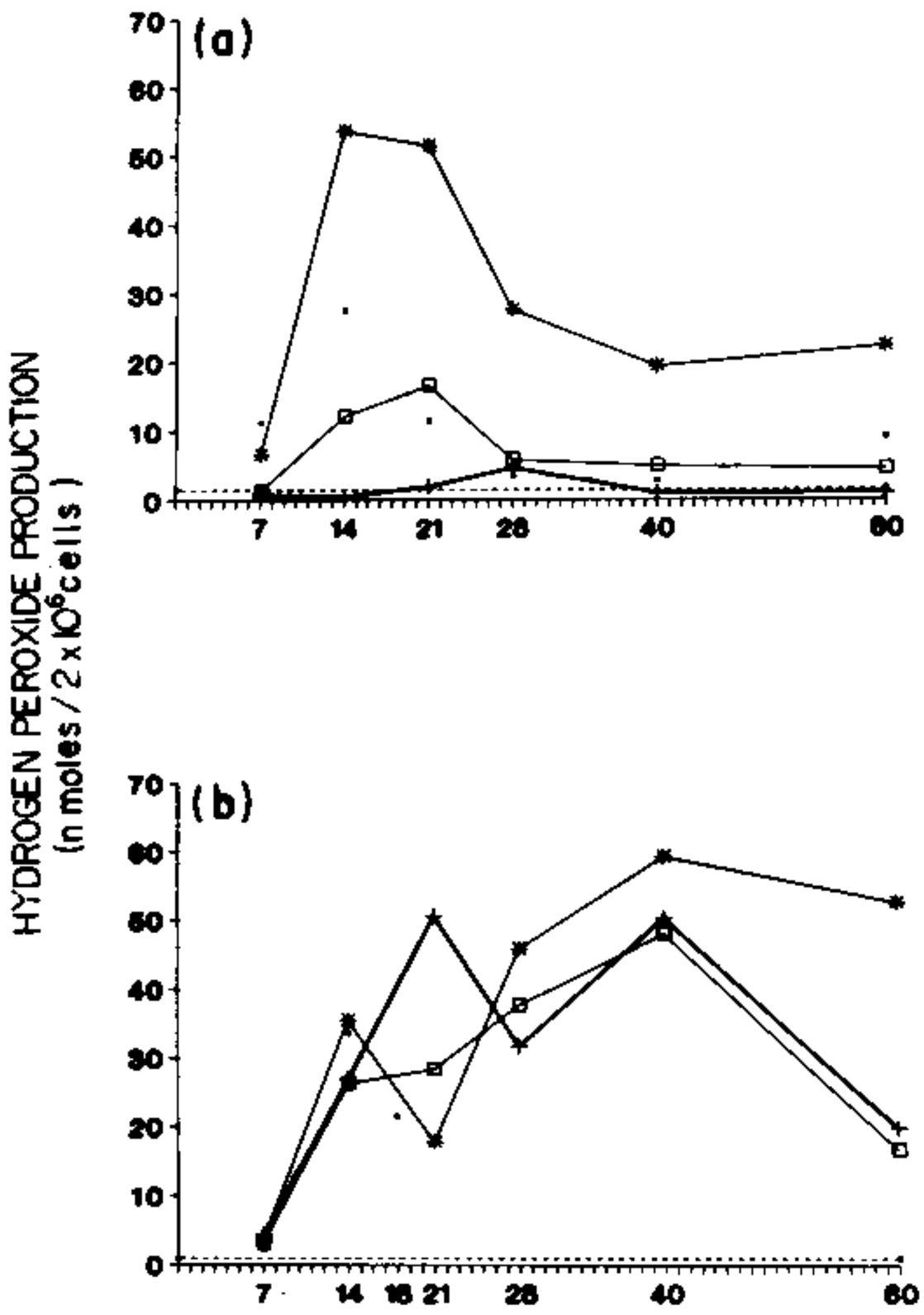

Fig. 2: H_2O_2 release by total peritoneal cells. Freshly harvested cells of (a) *Calomys callosus* and (b) Swiss mice inoculated with 4 x 10^3 blood trypomastigotes (s.c.) of several *Trypanosoma cruzi* strains. ● Y; ✳ F; + M226; ❑ Costalimai. Each point is the mean of 10 *C. callosus*, seven infected mice, or five controls, made in triplicate. (a) Strains F and Y $p < 0.005$ to < 0.0001 for all points. Strain Costalimai $p < 0.01$ to < 0.0001, except for day 7 (N.S.). Strain M226 differences N.S. except for day 28, $p < 0.03$; (b) $p < 0.0001$ throughout all experiments, except for day 7.

F strain peaked on day 21 for *C. callosus*, day 29 for mice, the curve being similar for both species, with a very low parasitemia by day 40. While no mortality was observed for *C. callosus*, up to 60 days, 5 mice out of 10 had died by the 36th day.

In *C. callosus* strains Costalimai and M226 provoked much lower parasitemia than F and Y. The peak was spread over days 18 to 23 no parasites being found by day 40, and all animals survived. No patent parasitemia and mortality below 10% was observed in mice, although parasites were found in tissues as will be discussed in histopathological findings.

Differential cell counts – Peritoneal cells of non-infected mice and *C. callosus* had a predominance of macrophages, ranging from 85-90%, while polymorphonuclears and monocytes never exceeded 2% each. Somewhat higher values were observed for lymphocytes, varying from 8-10% (data not shown).

Macrophage, polymorphonuclear and mastocyte counts were comparable to those of controls in mice and *C. callosus* infected with any of the four *T. cruzi* strains. Only a slight increase in lymphocytes was found with F strain infected mice and *C. callosus* on some days, ranging up to 18%, and in mice with the Y strain, with a maximum of 20%.

Hydrogen peroxide release – Hydrogen peroxide release varied with animal species and parasite strain, as can be seen in Fig. 2a, b and Tables I, II. The most noteworthy differences between the two models are observed with the

TABLE I

Parasitemia peaks and *in vitro* H_2O_2 release of peritoneal macrophages from *Calomys callosus* infected with 4 x 10^3 *Trypanosoma cruzi* strains F, Y, Costalimai and M226

T. cruzi strain	Maximum H_2O_2 release (n moles[a])	day	Parasitemia Paras./ml	day	Am.[b] in spleen and liver
F	53-51	14-21	5 x 10^6	21	–
Y	27. [4]	14	1 x 10^6	9	–[c]
Costa	16.5	21	2.5 x 10^5	19-21	–
M226	4.6	28	3.5 x 10^5	18-21	ND
Control	1.4	0-60	–	–	–

a: per 2 x 10^6 cells, determined by the Pick & Mizel (1981) method.
b: amastigotes.
c: except for one animal, with scarce amastigotes in macrophages on day 10.
ND: not determined.

TABLE II

Parasitemia peaks and *in vitro* H_2O_2 release of peritoneal macrophages from Swiss mice infected with 4 x 10^3 *Trypanosoma cruzi* strains F, Y, Costalimai and M226

T. cruzi strain	Maximum H_2O_2 release (n moles[a])	day	Maximum Parasitemia Paras./ml	day	Am.[b] in spleen and liver
F	59.3	40	3 x 10^7	29-36	–[c]
Y	33.6	14	3.3 x 10^6	9	+[d]
Costa	48	40	sub-patent		–
M226	50-35	21-40	sub-patent		ND
Control	0.9	0-60	–		–

a: per 2 x 10^6 cells, determined by the Pick & Mizel (1981) method.
b: amastigotes.
c: except for one animal, with scarce amastigotes in macrophages on day 10.
d: maximum macrophage parasitism on day 10.
ND: not determined.

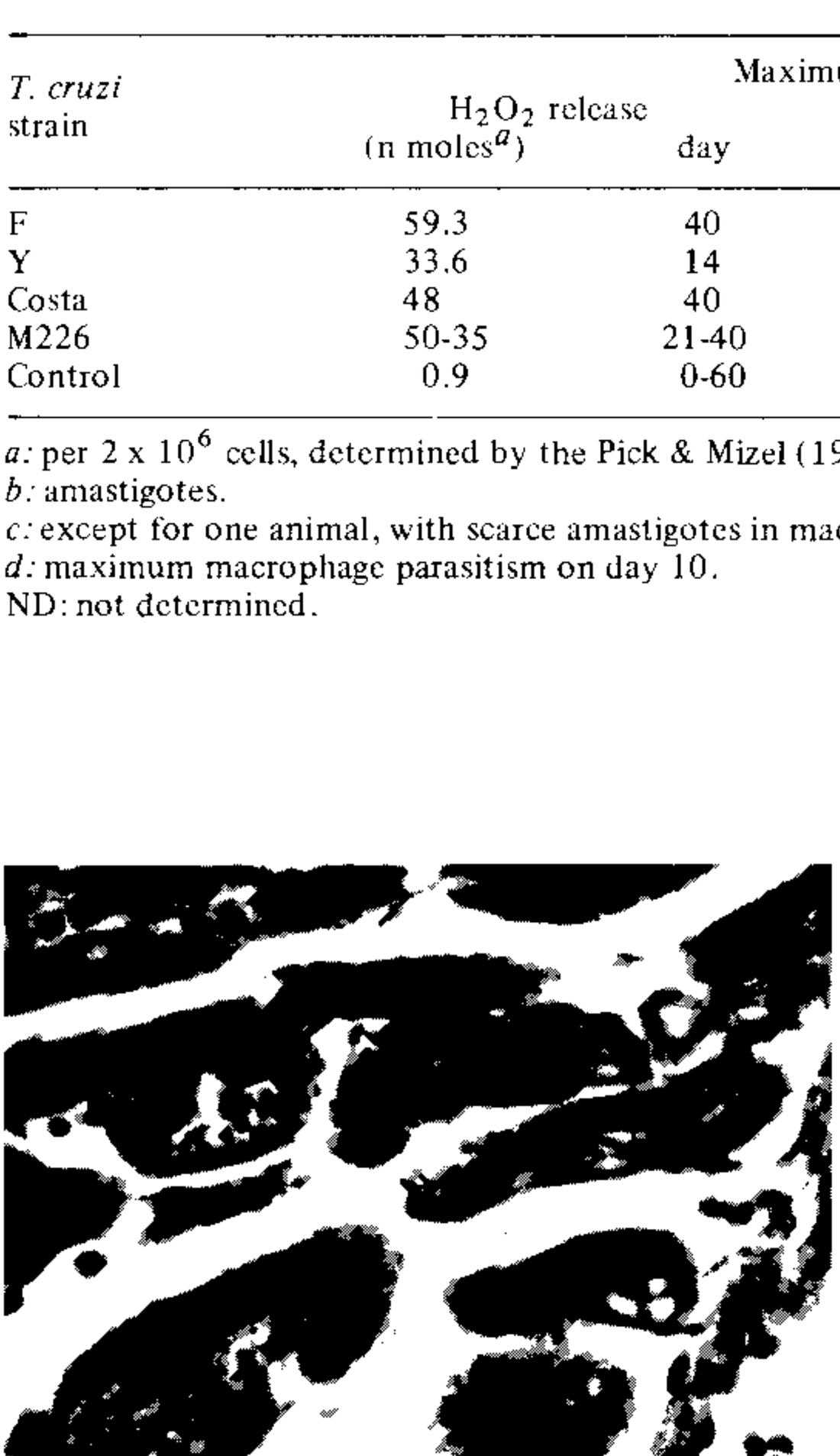

Fig. 3: *Calomys callosus* – 24th day of infection with the F strain of *Trypanosoma cruzi:* a: amastigote forms in cardiac myocell and mild myocarditis; b: interatrial septum showing intense inflammatory infiltration of the conduction tissue (Hiss bundle); (x 250, H E).

silvatic strains Costalimai and M226, where H_2O_2 release in mice was high, in spite of sub-patent parasitemia, with much lower values for *C. callosus,* which have patent parasitemia.

Histopathology – Histopathological alterations with the Y strain infection were similar in *C. callosus* and mice at the initial phase with low myocardial and macrophage parasitism, moderate myocarditis and myositis, up to the 10th day.

After the 20th day, when all mice were dead, lesions in *C. callosus* subsided, and had completely disappeared by the 40th day.

Calomys callosus inoculated with the F strain had a slow progressive tissue infection, increasing myocardidits and myositis from the 24th to the 38th day (Figs 3, 4). Myositis and parasitism of muscular fibers tended to be more intense than myocardial infection and persisted up to the 61rst day with lesser intensity. Macrophages were not infected. In mice, observations were similar to those of *C. callosus,* with necrotizing artheritis on the 61st day. In only one mouse on the 10th day of infection a scarce number of amastigotes were found in liver and spleen. These lesions are similar to those described in mice by Andrade (1974), for *T. cruzi* strains type III.

Calomys callosus inoculated with the Costalimai strain had no lesions from the 10th

Fig. 4: *Calomys callosus* – strain F, 24th day of infection: a: focal area of destruction of cardiac fibers with fibroblastic proliferation and mononuclear cells infiltration; b: skeletal muscle showing intense proliferation of intracellular forms of *Trypanosoma cruzi;* (arrows) (24 days, H E).

through the 61st day except for focal myocardial mononuclear infiltrate in one out of three animals on days 20, 26, 31, 38 and 61. No intracellular parasites were found in the organs examined. In contrast, mice infected with strain Costalimai presented intense myositis and large parasite nests with less intense myocarditis on days 26, 31 and 38. On day 17 and 24 some of the animals had pronounced myocarditis and myositis while in others these lesions were less intense (Fig. 5). Lesions in mice infected

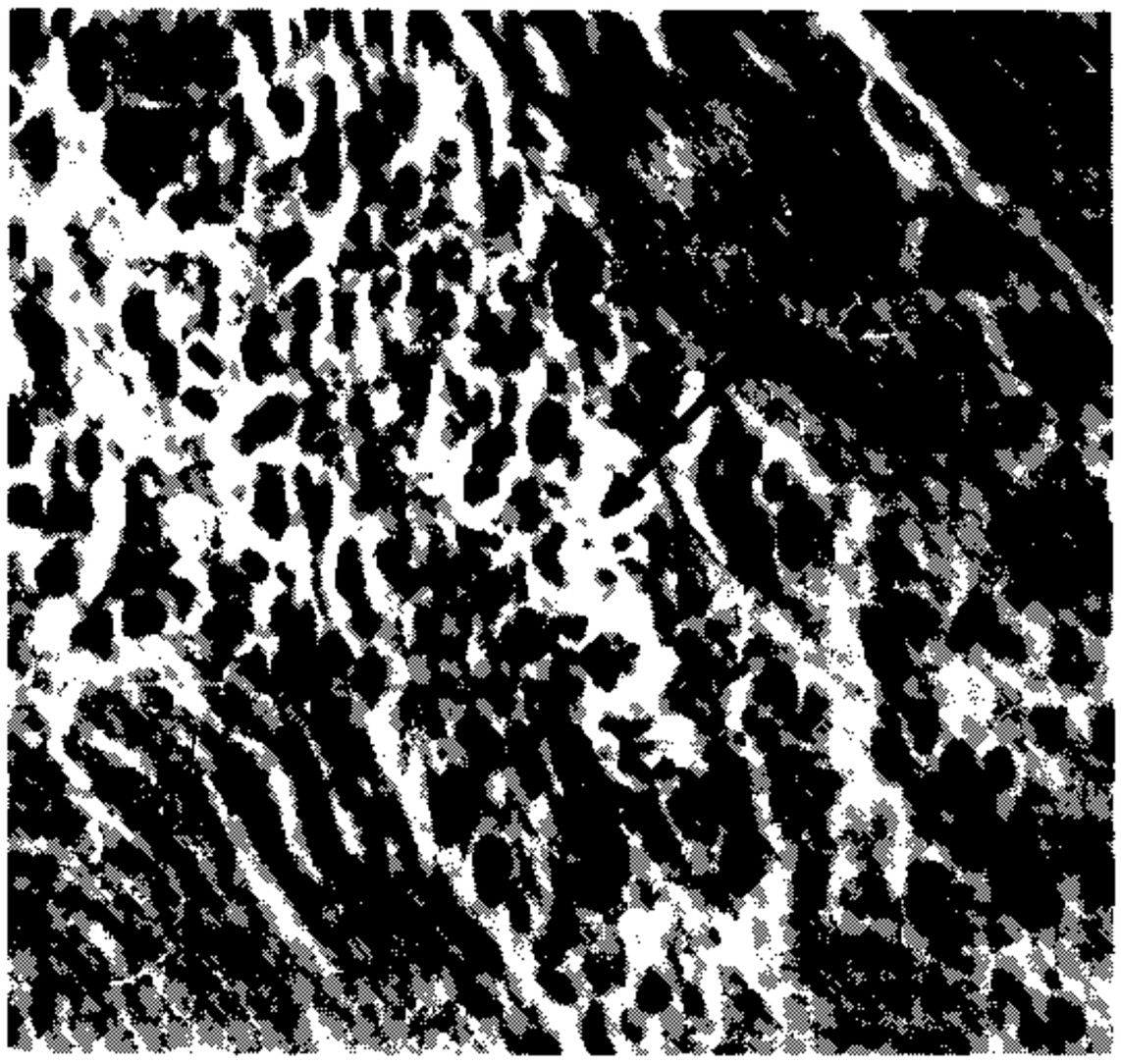

Fig. 5: mouse – 24th day of infection with the Costalimai strain of *Trypanosoma cruzi:* skeletal muscle showing extensive area of destruction, presence of intracellular forms of *T. cruzi,* (arrows) intense inflammatory reaction and fibroblastic proliferation (x 250, H E).

with strains Costalimai were also similar to those described for strains type III (Andrade, 1974).

DISCUSSION

Parasitemia and mortality differences between *C. callosus* and Swiss mice had already been reported for the Y (Borges et al., 1982), M 226 (Mello et al., 1979) and Costalimai (Mello & Borges, 1981) strains. Our parasitemia curves and mortality rates are somewhat different from those observed by other authors, probably due to different inocula size, or other experimental details.

A rather low inoculum, 4 x 10^3 parasites was elected, to allow a longer survival of susceptible mice. The s.c. route was chosen for part of the experiments to avoid direct stimulation of peritoneal macrophages. In fact, only rarely a peritoneal inflammatory reaction was found in these animals, as assessed by total cells count. Average cell numbers were always comparable to those of controls and differential counts were also practically identical to normals, the only indication of macrophage activation being an increased H_2O_2 release, as discussed below.

We confirmed a high resistance of *C. callosus* to *T. cruzi* infection, as compared with mice, for strains Y and F. Although parasitemia

curves are similar for both species, maximum counts are different. The more notable resistance refers to mortality. With the Y strain all mice died, while *C. callosus* are able to control the infection, parasitemia becoming negative by day 18 and no mortality was observed. For the F strain peaks were comparable between mice and *C. callosus*. Both animal species eventually cleared parasitemia, but 60% of mice died, while all *C. callosus* survived.

In spite of differences in parasite counts, peritoneal macrophage activation for Y strain as measured by H_2O_2 release, was similar for mice and *C. callosus*. A parallel between macrophage activation and histopathological findings was observed.

With the F strain H_2O_2 release corresponds roughly to parasitemia peaks: maximum H_2O_2 release was also similar for both species, differing only in timing. Histopathological findings corrobate macrophage activation, with a progressive inflammatory process in mice, quite active up to day 60, when H_2O_2 release is still high, while *C. callosus* present values only slightly above normal on days 40 and 60, a time when histopathologic lesions have subsided.

Comparing macrophage activation for strains Y and F, significantly higher values are found for the latter. F strain parasites did not multiply in reticulo-endothelial cells, since only rarely a very few number of them was found in liver and spleen of the animals in our experiments. This is in accordance with *in vitro* findings (Milder et al., 1977). Y strain has been considered as "reticulotropic" opposed to "non-reticulotropic" strains, which are not found within reticulo-endothelial cells in the infected host (Melo & Brener, 1978; Andrade, 1985).

Another striking difference was observed between mice and *C. callosus* with the two silvatic strains M226 and Costalimai. Paradoxically parasitemia in *C. callosus* was much higher than that of mice. In fact, only very rarely parasites were found on microscopic examination of mouse blood. However, these animals were infected, since examination of tissues revealed intracellular parasites. Also, a group of mice was inoculated s.c. with strain Costalimai and on day 17 peritoneal washings were examined. In most of these mice a low number of *T. cruzi* was found, while the examination of 10 µl tail blood resulted negative (data not shown).

In spite of the low parasitemia in mice, tissue aggression was intense with the Costalimai strain, corresponding to increase in H_2O_2 release, which was comparable to that found for the F strain. On the other hand, *C. callosus*, which do have patent parasitemia, had no histopathological alterations and macrophage activation as measured by H_2O_2 release was only slightly increased on day 21. For strain M226, increased H_2O_2 release was also observed for mice, while *C. callosus* had values close normal controls.

In vitro development in macrophages of blood trypomastigotes of M226 and Costalimai strains is being investigated, but histopathology seems to indicate that both in *C. callosus* and mice, macrophages are not infected. Therefore, in the case of silvatic strains, no direct correlation was found between macrophage activation as measured by H_2O_2 release and lack of multiplication of the parasite within these cells.

Histopathology of experimental Chagas disease has been extensively studied in mice (Andrade, 1974). Morphobiological behaviour of *T. cruzi* strains in Swiss mice led to their classification into three distinct types (Andrade, 1985). A comparative study of these strain types in several inbred strains of mice showed that some of them changed their expression according to the host (Andrade et al., 1985a). These autors have demonstrated that different inflammatory reactions occurred in mice of resistant or susceptible strains. Exudative lesions with polymorphonuclears occurred in the most resistant, while a scarce infiltrate with predominance of mononuclear cells was observed in the more susceptible mouse strain. The differences observed here between *C. callosus* and mice with silvatic strains of *T. cruzi* may be another expression of the host difference already observed among strains of mice.

The spleen index (spleen weight/body weight) has been correlated with increased cellularity of the spleen on histopathological examination (Andrade et al., 1985b), and this correlates with other histological lesions. We found that in general spleen index increased in both *C. callosus* and mice, in the acute phase of the infection and started decreasing with

the decrease in intensity of histological lesions (data not shown). This correlation was specially conspicuous in the case of strain Costalimai, where at the end of the experiment (60 days) *C. callosus* spleen index was close to controls with very slight histophatological lesions, while spleen index of mice was still above normal with histopathological lesions, as mentioned earlier.

The literature on intracellular development of *T. cruzi* in macrophages is quite extensive. Both development and destruction have been reported, depending on the parasite strain, the immune response of the host, and particularly macrophage activation via T cell products (Nogueira, 1983).

Intracellular parasite death has been related to H_2O_2 dependent and independent mechanisms. As instances of the former, Nathan et al. (1979) found a correlation between the capacity of macrophage H_2O_2 release after Phorbol Mystrate Acetate (PMA) stimulation and intracellular killing of *T. cruzi.* The down-regulation of oxidative metabolism by Transforming Growth Factor-β (TGF-β) in macrophages increases the intra-macrophage replication of *T. cruzi,* and blocks the inhibitory effect of INF-γ on the parasite (Silva et al., 1991).

Russo et al. (1989), working with different mouse and *T. cruzi* strains did not observe correlation between higher susceptibility to infection and lower H_2O_2 release. McCabe & Mullins (1990) described INF-γ activated macrophages inhibiting *T. cruzi* development by an H_2O_2 independent mechanism, in the presence of reactive oxygen scavengers, or after exhausting the respiratory burst with PMA.

More recently, the role of nitrogen oxide (NO) mediated killing of intracellular parasites in macrophages is being stressed. TNF, interferons and endotoxins stimulate NO production by several cells, including macrophages. These latter cells produce NO_2 and NO_3, derived from L-arginine; this is blocked by structural analogues, such as N^g-monomethyl-L-arginine (L-NMMA). *Toxoplasma gondii, Cryptococcus neoformans,* and *Leishmania major* have been shown to be killed in cytokine activated macrophages by this mechanism (Liew & Cox, 1991) The same has been described by Gazinelli et al. (1991) for *T. cruzi.* INF-γ activated macrophages killed this parasite in an arginine dependent manner, since the addition of NMMA and IL10, which also correlates wilth the inhibition of nitrite synthesis, inhibited this intracellular killing.

We therefore believe that macrophage activation as measured by H_2O_2 release in the case of *T. cruzi* infection is a more complicated phenomenon than was believed so far. Results depend both on animal models and parasite strains.

Probably a different mechanism results in macrophage activation when intense histopathological lesions are the outcome of *T. cruzi* infection and this may be related to parasite strains or the host's genetic constitution.

Is thus seems that no simple explanation for different levels of respiratory burst, parasite agression to the host, host and parasite survival, can be given.

Two points must be emphasized: (1) the parasite strain is important in the determination of histopathological lesions in *C. callosus;* (2) as compared with mice, *C. callosus* showed a higher resistance, controlling the infection with the possibility of self-cure. Factors connected with the parasite strain and with the genetic patterns of the host are important for the parasite's fate in the vertebrate cells.

Calomys callosus is proposed as an alternative model of experimental Chagas' disease, affording a unique opportunity of studying a wild rodent and its interaction with a *T. cruzi* strain isolated from the same species. We are also raising inbred lines of this animal species which will allow to explore other immunological aspects.

The role of several cytokines in this model is being investigate by us.

ACKNOWLEDGEMENT

To Dr Momtchillo Russo for helpful suggestions, Dr Heitor Franco de Andrade Jr for suggestions on statistical analysis, Marlene Iumy Ljichi for technical assistance, Elita R. Jesus for supervision of the computational task of word processing, and Almir Robson Ferreira for graphical illustrations.

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