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# *Trypanosoma cruzi* in marsupial didelphids (*Philander frenata* and *Didelhis marsupialis*): differences in the humoral immune response in natural and experimental infections

## *Trypanosoma cruzi* em marsupiais didelfídeos (*Philander frenata* e *Didelphis marsupialis*): diferenças na resposta imune humoral nas infecções naturais e experimentais

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***Abstract*** Philander frenata *and* Didelphis marsupialis *harbor parasitism by* Trypanosoma cruzi *without developing any apparent disease and on the contrary to* D. marsupialis*,* P. frenata *maintains parasitism by* T. cruzi *II subpopulations. Here we compared the humoral immune response of the two didelphids naturally and experimentally infected with* T. cruzi *II group, employing SDS-PAGE/Western blot techniques and by an Indirect immunofluorescence assay. We also studied the histopathological pattern of naturally and experimentally infected* P. frenata *with* T. cruzi*.* P. frenata *sera recognized more antigens than* D. marsupialis*, and the recognition pattern did not show any change over the course of the follow up of both didelphid species. Polypeptides of 66 and 90kDa were the most prominent antigens recognized by both species in the soluble and enriched membrane fractions.* P. frenata *recognized intensely also a 45kDa antigen. Our findings indicate that: 1) there were no quantitative or qualitative differences in the patent or subpatent phases in the recognition pattern of* P. frenata*; 2) the significant differences in the recognition pattern of parasitic antigens by* P. frenata *and* D. marsupialis *sera suggest that they probably "learned" to live in harmony with* T. cruzi *by different strategies; 3) although* P. frenata *do not display apparent disease, tissular lesions tended to be more severe than has been described in* D. marsupialis; *and 4) Both didelphids probably acquired infection by* T. cruzi *after their evolutionary divergence.*

***Key-words****:* Trypanosoma cruzi. *Humoral response. Marsupial didelphid.*

***Resumo*** Philander frenata *e* Didelphis marsupialis *mantém* Trypanosoma cruzi *sem desenvolver aparentemente nenhuma doença. Ao contrário de* Didelphis marsupialis*,* Philander frenata *mantém subpopulações do grupo* T. cruzi *II. Aqui, nós comparamos a resposta imune humoral de dois didelfídeos natural e experimentalmente infectados com o grupo* T. cruzi *II, empregando as técnicas de SDS-PAGE/Western blot e por um método de imunofluorescência indireta. Também estudamos os padrões histopatológicos de* P. frenata *infectado natural e experimentalmente por* T. cruzi*. O soro de* P. frenata *reconheceu mais antígenos que o de* D. marsupialis*. Polipeptídeos de 66 e 90kDa foram os antígenos mais reconhecidos por ambas espécies nas frações enriquecidas de* membranas solúveis. P. frenata *reconheceu intensamente o antígeno de 45kDa. Nossos resultados indicam que: não existem diferenças quantitativas e qualitativas nas fases subpatente e patente no padrão de reconhecimento de* P. frenata; *as diferenças no padrão de reconhecimento de antígenos de parasitas pelos soros de* P. frenata *e* D. marsupialis *sugerem que provavelmente essas espécies "aprenderam" a viver em harmonia com* T. cruzi *por estratégias distintas; embora* P. frenata *não apresente doença aparente, lesões tissulares tendem a ser mais severas do que as descritas para* D. marsupialis*; ambos didelfídeos provavelmente adquiriram a infecção por* T. cruzi *depois de sua divergência evolucionária.*

***Palavras-chaves:*** Trypanosoma cruzi. *Resposta humoral. Marsupial didelfídeo.*

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*Trypanosoma cruzi* circulates in sylvatic environments among dozens of species of mammalian reservoirs in complex transmission cycles[9 15]. The significant heterogeneity of the species observed based on molecular biological and biochemical markers recognized in the subpopulations of *T. cruzi* two main groups: *T. cruzi* I (associated mainly to the sylvatic transmission cycle) and *T. cruzi* II (associated mainly to the domestic transmission cycle)[2].

The marsupials *Philander frenata* and *Didelphis marsupialis* (Marsupialia, Didelphidae), are probably the most ancient sylvatic reservoirs of *Trypanosoma cruzi*[23]. In nature these two didelphid marsupials share the same ecotope and display similar alimentary habits. *P. frenata* and *D. marsupialis* are both endemic species from the Atlantic Coastal Rain Forest. Both species maintain the parasitism by *T. cruzi* from an early age onward[7 14], without clinical signs of disease. Moreover, *D. marsupialis* support without tissular lesions *T. cruzi* II infection and only scarce monocytic infiltrates were described in *T. cruzi* I experimentally infected *D. marsupialis*[5]. Nevertheless significant differences have been described in the course of the natural and experimental infections by *T. cruzi* of *D. marsupialis* and *P. frenata*[19]. *D. marsupialis* controls experimental infections with Y strain (*T. cruzi* II) from an early age while still marsupium dependent. Indeed, the *T. cruzi* II strains experimentally infected animals display a patent parasitemia only detectable during a short period through rare positive fresh blood smears examinations and hemocultures become rapidly and consistently negative a few weeks after inoculation. No parasitism in the scent glands is observed and the serological titers evaluated by Indirect Fluorescent Antibody Test (IFAT) are low[12 13]. Moreover some animals are able to eliminate the infection. Another picture is observed in *D. marsupialis* experimentally infected with F and other *T. cruzi* I strains. In this case the animals present a stable and long-lasting infection with high parasitemias and percentages of positive hemocultures during follow up. Furthermore, parasitism of the scent glands is detected in high percentages. A correlation between the rise of serological antibodies and the drop of parasitemia (that reaches a peak of $10^6$ parasites/ml) was observed and suggested to the authors that the early control of Y strain infection was probably achieved independently from the humoral immune response, but this was important for the control of infections with *T. cruzi* I strains[13]. The same authors observed also through immunoblottings of sera of experimentally and naturally infected animals that *D. marsupialis* recognized significantly fewer *T. cruzi* antigens than conventional laboratory animals and humans.

*P. frenata* was demonstrated to be able to maintain both *T. cruzi* I and *T. cruzi* II. It seems therefore that this species is not so strict a selective filter as *D. marsupialis*. Experimental infections can result in high parasitemia but parasitism in scent glands had never been recorded[19].

In order to better understand the different features of the interaction of *T. cruzi* with *P. frenata* and *D. marsupialis* its probable most ancient reservoir, we endeavored to compare the humoral immune response of both species in natural and experimental infections. We also studied the histopathological pattern of naturally and experimentally infected *P. frenata*.

## MATERIAL AND METHODS

**Animals**. Young recently weaned *P. frenata* and *D. marsupialis* 100 to 120-day-old animals reared in our laboratory were used in experimental infections. Naturally infected *P. frenata* and *D. marsupialis* were captured in the Caleme area near Teresópolis (Rio de Janeiro, Brazil).

**Parasites**. The *Trypanosoma cruzi* strains used in the experiments were as follows: MHOM/BR/53/Y (*T. cruzi* II), isolated from a chagasic patient[22], MDID/BR/92/C13 (*T. cruzi* II), isolated from a naturally infected *P. frenata*, Teresópolis, RJ, MDID/BR/92/G645 (*T. cruzi* I) isolated from a naturally infected *D. marsupialis*, M000/BR/00/F (*T. cruzi* I). The epimastigote forms were grown in LIT medium (Liver Infusion Tryptose Medium) supplemented with 10% fetal calf serum. For antigen preparation epimastigotes were harvested in the log phase. The metacyclic trypomastigote forms were obtained after spontaneous metacyclogenesis in Agar-LIT, counted in Neubauer chamber and adjusted so as to yield 500 or 1,000 parasites per gram of body weight to inoculate in didelphids. The molecular characterization of the isolates (*T. cruzi* I and II groups) were done by Dr. Octavio Fernandes, Departamento de Medicina Tropical/IOC/FIOCRUZ.

**Antigen to IFAT**. The antigen, consisting of epimastigote forms of *T. cruzi* F strain, incubated at 27°C, were centrifuged (3,000g, 20 minutes), the sediment washed twice in PBS (Phosphate Buffered Saline- 0.15M, pH 7.2), re-suspended in about the initial culture volume of one percent formalin in PBS. The stock antigen was kept in refrigerator and adjusted to 40 parasites per microscopic field examination (40x) in final preparations.

**Preparations of SDS-PAGE/Western blot antigens**. *Soluble fraction*: cultured epimastigotes from Y, C13 and G 645 strains were washed in PBS (3,000g, 20 minutes), sonicated (Branson Ultrasonics Corporations, USA) and lysed in Methanol and dry ice, alternately, in six cycles of 20 minutes in lysis buffer (20mM Tris, 40mM NaCl, 10mM EDTA, 2mM Iodoacetamyde, 1.6mM 1.1 Phenantroline, 1mM PMSF), centrifuged (4,000 rpm, 10 minutes in Eppedorf microcentrifuge) and the supernatant was maintained frozen at -20°C;

*Enriched membrane fraction*: the same cultured epimastigotes were in PBS (3,000g, 20 min), disrupted at 1,500 psi pressure in $N_2$ atmosphere, centrifuged (50,000g, 30 minutes 4°C), the pellet was re-suspended in lysis buffer and maintained frozen at -20°C.

**Inoculations**. All animals were subcutaneously inoculated in the inner part of the right thigh. One litter (five specimens) of *P. frenata* were inoculated with C13 (*T. cruzi* II) metacyclic trypomastigotes (500 parasites per gram of body weight), one litter (five specimens) of *P. frenata* inoculated with Y (*T. cruzi* II) metacyclic trypomastigotes and one litter (five specimens) of *D. marsupialis* were inoculated with C13 strain (*T. cruzi* II) (1,000 parasites per gram of body weight). (*Observation*- In another paper, Jansen et al[10,14] studied opossums inoculated with Y strain, so we did not repeat that experiment. In this paper we inoculated another strain (C13) in *D. marsupialis* belonging to the same group (*T. cruzi* II).

**Parasitological follow-up**. Parasitemia was followed up by fresh blood smears examination every other day. Monthly hemocultures were performed in NNN with a LIT overlay. Weekly examination for parasites of the scent glands was performed by gentle manual squeezing of the glands.

**Sera**. *P. frenata* and *D. marsupialis* sera from natural and experimental infections were obtained from blood samples taken from the tail vein. The sera from experimental animals were collected before *inocula* and at 30-day intervals *pos inocula* during the follow up. The sera were stored frozen at -20°C and used in Indirect Fluorescent Antibody Test and Western Blot assays.

**Indirect Fluorescent Antibody Test (IFAT)**. An IFAT was performed according to Camargo et al[4]. Positive control serum was obtained from *P. frenata*/ *D. marsupialis* immunized with *T. cruzi* antigens, and negative control serum was obtained from uninfected animals, born and kept in captivity. Diluted (1:10 up to 1:5120) naturally and experimentally infected and control didelphids sera, rabbit antisera (1:40) to *P. frenata*/ *D. marsupialis* immunoglobulins (Ig) and a fluorescein conjugated anti-rabbit Ig (Sigma) diluted with Evans blue (1mg%) were used. The sera, antisera and conjugate were incubated for 40 minutes at 37°C, twice washed in PBS for five minutes and mounted with a coverslip and buffered glycerin (pH 8.0). The fluorescent reactions were read under a 40x objective binocular microscopic Zeiss (HBO50W) and barrier filters.

**Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE)**. Soluble and enriched membrane antigens from *T. cruzi* I and II groups were solubilized by boiling them in an equal volume of a final sample buffer (0.0625M Tris-HCl-pH 6.8, 2% SDS, 10% glycerol, 5% 2 mercaptoethanol and 0.001 % bromophenol blue), electrophoreses in the 4.5% (stacking) and 10% (separation) polyacrylamide (30% acrylamide, 0.8% N, N'-bis-methylene acrylamide) gel containing 0.1% SDS in a electrophoresis buffer (25mM Tris, 250mM glycine, 0.1%SDS[13]. A pertained molecular weight markers ranging from 205 to 29 kDa (Sigma) were used.

**Western blot**. Electrophoresis antigens were transferred[26] to nitrocellulose membranes (0.45mm pore size-BIORAD) overnight at 0.17 A, 4°C, in a transfer buffer (39mM glycine, 48mM Tris base, 0.037% SDS, 20% methanol). The transferred antigens were immediately stained for 10 minutes with 0.2% Ponceau S in 3% acetic acid and 97% tri-distilled water. After blocking (5% nonfat dried milk, PBS 0.15M, pH 7.2, 0.5% Tween 20), the membrane were incubated with respectively naturally, experimentally and non infected didelphids sera, laboratory reared (diluted 1:500 in PBS, 0.5% Tween 20). After a second incubation with rabbit anti-*P. frenata* or anti-*D. marsupialis* serum (1:500), immune complexes were incubated with an anti-rabbit IgG-peroxidase conjugate (Sigma) and revealed with a freshly prepared substrate solution (15mg 3-3' diaminobenzidine tetrahydrochloride, 60ml citrate-phosphate buffer (0.01M $C_6H_8O_7$).H2O, 0.02M $Na_2HPO_4$) pH 5.0 and 40ml of 30% $H_2O_2$). In all steps the membranes were washed in PBS and 0.05% Tween 20. The molecular weight of polypeptides was estimated by a calibration curve. The curve was done plotting the log of molecular weight standard proteins (BIORAD) x relative mobility-*Rf* (*Rf* = distance of protein migration/distance of dye migration).

**Histopathology**. The naturally and experimentally infected *P. frenata* by *T. cruzi* were killed by injection of Ketalar (ketamine cholridrate. The following viscera were collected: heart, stomach, striated muscle, liver. Tissue samples were fixed in phosphate-buffer formaldehyde, processed for paraffin embedding and stained with hematoxylin/eosin (HE).

## RESULTS

**Parasitological Follow Up**. *Experimental infections in P. frenata with C13 strain resulted in a long period of patent parasitemia (70 days)*: the parasitemia peak reached $2x10^4$ parasites/ml and 33% of the hemocultures performed during the follow up of the subpatent phase were positive (*Table 2*). No patent parasitemia was observed in *P. frenata* inoculated with Y strain but 95% of the hemocultures performed during the follow up were positive (Table 1). Experimental infection in *D. marsupialis* with C13 resulted in patent parasitemia detectable only through rare positive fresh blood smears examination. Only 16.6% of the hemocultures performed during the follow up were positive (Table 2).

*Naturally infected P. frenata and D. marsupialis* (Table 2) *showed 45% and 16.6% positivity, respectively, in the hemocultures performed during the follow up*. No parasites were observed in the lumen of the scent glands.

**Serological follow up**. *Experimentally infected P. frenata and D. marsupialis with the C13 strain showed comparable Ig titers (1:80)*. *P. frenata* inoculated with the Y strain displayed significant higher serological titers (1:1280) (Table 2);

Naturally infected *P. frenata* by *T. cruzi* showed significantly higher Ig titers (1:5120) than naturally infected *D. marsupialis* (1:160) (Table 2).

*Table 1 - Serological and parasitological data on* P. frenata *and* D. marsupialis *subcutaneously inoculated with culture metacycle forms of* Trypanosoma cruzi *(200 metacyclics per gram of body weigth).*

| Inocula origin | Prepatent period X days | Duration of patency X days | Parasitemic peak X days | Peak time X days | Hemocultures during subpatent phase/total | Time follow up X months | Scent glands | IFAT maximum titer serological |
|---|---|---|---|---|---|---|---|---|
| *P. frenata* | | | | | | | | 1/1280 |
| Y | - | - | - | - | 18/19 | 7 | - | |
| C13 | 8 | 70 | $2x10^4$ | 13 | 10/30 | 12 | - | |
| *D. marsupialis* | | | | | | | | |
| C13 | - | - | - | - | 4/24 | 13 | - | 1/60 |

X – mean values

*Table 2- Serological data and parasitological follow up of naturally infected* P. frenata *and* D. marsupialis *by* T. cruzi.

| Isolate | Hemocultures during follow up/total | Group | Parasites in opossum scents glands | Time follow up (months) | IFAT maximun titer serological |
|---|---|---|---|---|---|
| *D. marsupialis* | | | | | |
| 648 | 4/13 | *T. cruzi I* | - | 24 | 1/160 |
| 655 | 1/8 | *T. cruzi I* | - | 23 | 1/80 |
| *P. frenata* | | | | | |
| C2 | 2/8 | *T. cruzi II* | - | 24 | 1/1280 |
| C22 | 1/7 | *T. cruzi II* | - | 14 | 1/5120 |

**Reactivity of infected marsupial didelphids sera with *T. cruzi* antigens**. Although both didelphids species recognized antigens in a range of 29-116kDa of *T. cruzi* I and *T. cruzi* II isolates, the pattern of recognition was significantly different. *P. frenata* recognized more intensively a larger number of *T. cruzi* antigens than *D. marsupialis* (Figures 1 and 2). Concerning the number of bands and intensity of recognition, both didelphids marsupial species recognized more antigens in experimental infections (Figure 1) than natural ones (Figure 2). Experimentally and naturally infected *P. frenata* and *D. marsupialis* by *T. cruzi* recognized more antigens from soluble fraction than membrane fraction ones (data not shown).

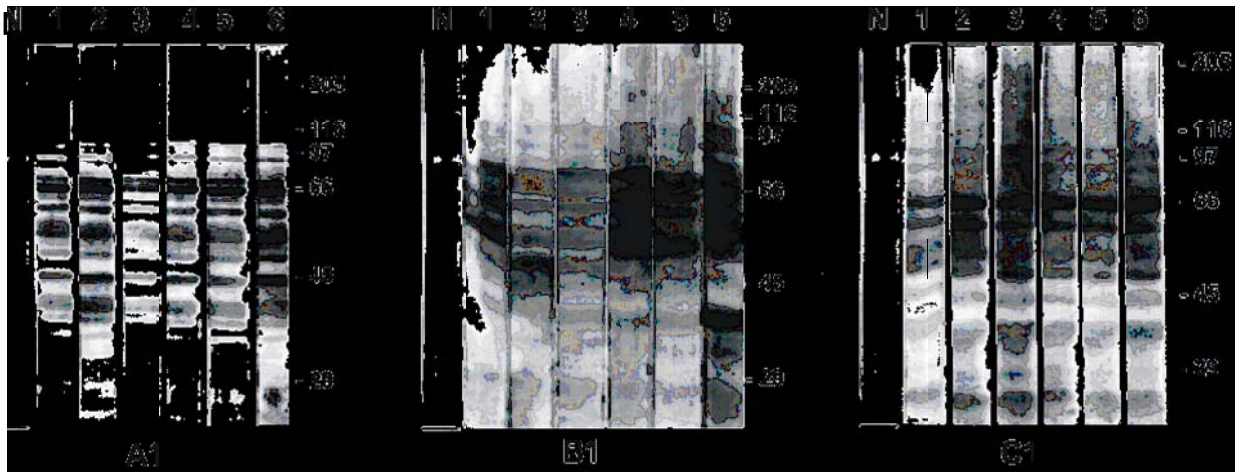


*Figure 1 - An immunoblotting showing* Trypanosoma cruzi *polypeptides recognition (Soluble fraction) by experimentally infected* Philander frenata *(A, B) and* Didelphis marsupialis *© sera. Inoculated strain: A1, C1-C13 (*T. cruzi *II group), B1- Y strain (*T. cruzi *II group). Antigens: A1,C1 – C13 strain, B1-Y strain. Follow up: 1- one month pos inoculum, 2- two months pos inoculum, 3- three months pos inoculum, 4- four months pos inoculum, 5 – five months pos inoculum, 6- one year pos inoculum, N- negative didelphid sera.*

Sera from *P. frenata* and *D. marsupialis* recognized more intensively the following antigens: 66 and 90kDa in both soluble and enriched membrane fractions and 45kDa on soluble fraction.

**Histopathology**. *Natural infections*: a moderate interstitial myocarditis with involvement of the subendothelial layer was observed in one of the naturally infected *P. frenata*. The inflammatory reaction predominated in the striated muscular fibers and was focal mononuclear cells without fibrosis and not related to the tissue parasitism. *Experimental infections*: the experimentally infected *P. frenata* with Y strain displayed

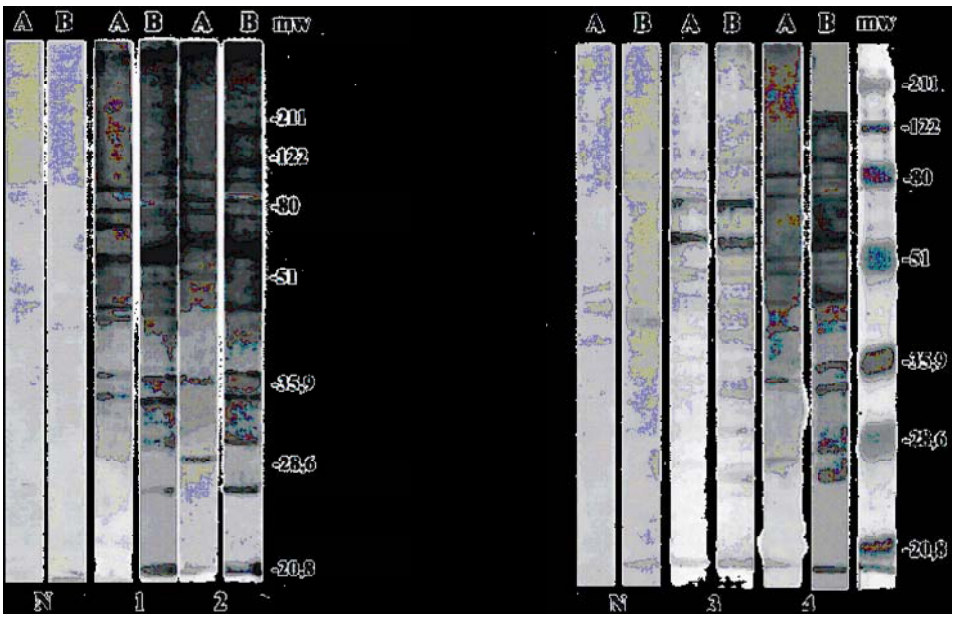


*Figure 2 – An immunoblotting showing* Trypanosoma cruzi *polypeptides recognition ( soluble fraction) by naturally infected* Didelphis marsupialis *(1,2) and* Philander frenata *(3,4) sera, n=negative didelphid sera antigens, a: (G645)* T. cruzi *I group, b: (Y)* T. cruzi *II group.*

a similar histopathological pattern of the naturally infected animals. The infection resulted in intense inflammatory reactions and a mild muscular fiber destruction in heart sections. An intense inflammatory reaction was observed in muscular fibers and in gastric nervous plexus of the stomach.

## DISCUSSION

Marsupials are known to be one of the most ancient mammals, since they appeared in the late cretaceous period (80 million years ago) and are probably the most ancient reservoirs of *T. cruzi*[21]. In the sylvatic environment *P. frenata* and *D. marsupialis* share the same habitat but only *D. marsupialis frequent* human dwellings. Although previous studies described marsupials as dull and primitive mammals with a weak immune response, recent reports together with the present study do not confirm these aspects. Indeed opossums are very adaptable animals since they resist several pathogenic microorganisms, new world snake venoms and take advantage of human colonization. Their immune response is also comparable to that of placental as described in more recent reports[13 18].

Although the two species present subpatent parasitemia, when inoculated with Y strain the follow up of *P. frenata* resulted in significantly higher hemoculture rates than was described for *D. marsupialis*[13 14].

Although some common features were seen, significant differences in the recognition pattern of *T. cruzi* antigens between *D. marsupialis* and *P. frenata* could also be observed.

A noticeable difference in *T. cruzi* infection antigen recognition pattern between *P. frenata* and *D. marsupialis* was that *D. marsupialis* species recognized less intensively a fewer number of antigens after a two fold higher inoculum of a *T. cruzi* II strain. A previous work[13] demonstrated that *D. marsupialis* recognizes fewer antigens on Y strain, also a *T. cruzi* II strain, than on F strain (*T. cruzi* I). This intense antigen recognition by *P. frenata* sera could explain the more severe lesions observed in histopathological studies in this species (Figure 3).

The more intense recognition of *T. cruzi* antigens in the experimentally infected didelphids than in the naturally infected animals is probably due to a larger inoculum in experimental conditions than one would expect to occur in nature. Moreover it should also be considered that the route of infection is a determinant of differences in the recognition pattern – it is worthy of mention that in nature animals are probably infected by predatory habits.

A common feature was the early recognition of the total spectrum of *Trypanosoma cruzi* antigens although experimentally infected *P. frenata* recognized twofold

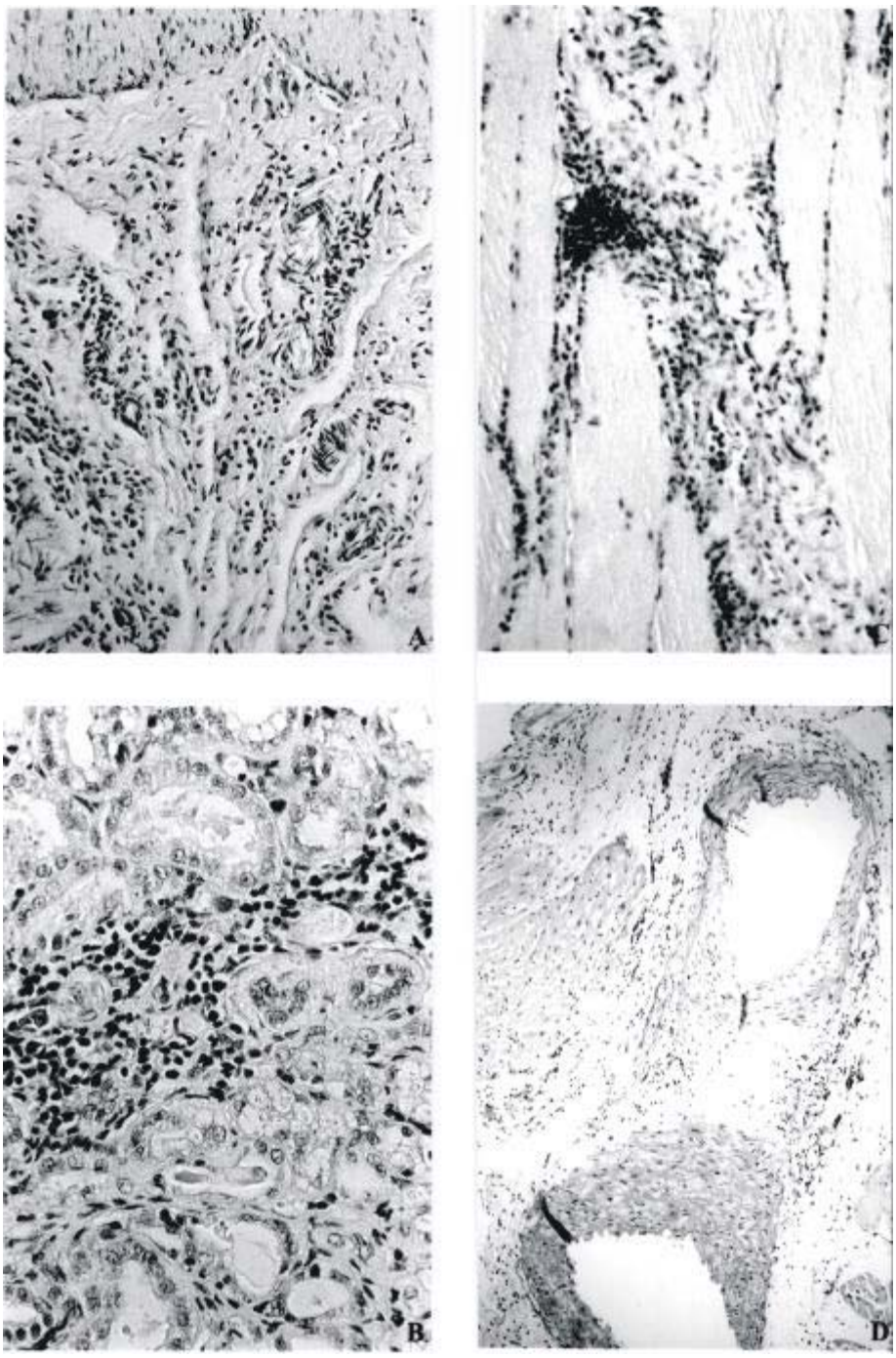


*Figure 3 – Hystopatology of experimentally infected* P. frenata *with Y strain (*T. cruzi *II group). Sections: A: intestine (x200); B: skeletal muscle (x200), C: kidney (x400); D: heart (x100).*

more antigens than experimentally infected *D. marsupialis*. Similar experiments in mice concluded that distinct mouse strains differed significantly concerning the spectrum of recognized antigens during the course of infection. Moreover, the authors correlated these differences with susceptibility or resistance[3 10]. Significant differences in the antigen recognition pattern between early infection stage sera samples and late

infection stage sera samples were also observed in infected human[6 11 24 27 28 30 31].

The delayed and lesser *T. cruzi* antigen recognition by *D. marsupialis* sera in Y (*T. cruzi II*) infection[13] is not a peculiarity of inoculated opossums with *T. cruzi* II group, since in C13 (*T. cruzi* II) infections started four weeks *pos inocula*. These data have changed the concept regarding the role of the humoral immune response in *T. cruzi* II infections. Apparently antibodies control the circulating parasites in opossums in C13 infections.

Since both Didelphidae marsupials control parasitemia efficiently, the differences in recognition pattern results more probably from the peculiarities inherent to each marsupial species in managing the infections by *T. cruzi*. The higher selectivity concerning *T. cruzi* subpopulations observed in *D. marsupialis*[19] reinforces this hypothesis. Moreover humoral response (*Table II*), concerning Ig levels, are distinct in *P. frenata* and *D. marsupialis* as described recently by Legey et al[18].

The most prominent trypanosome antigens recognized by *P. frenata* and *D. marsupialis* were those with molecular weights of 90kDa and 66kDa. Additionally an antigen of 45kDa was more intensively recognized by *P. frenata* than *D. marsupialis*. The recognition of an antigen of 45kDa was correlated with the effectiveness in controlling the parasitemia by resistent mice[16]. This antigen, described in the epimastigote form of *T. cruzi,* did not display homology with cruzipain, another *T. cruzi* antigen of similar molecular weight[1 8 20].

The recognition of a 66kDa seem to be a peculiarity of Didelphidae hosts since we did not find references for this recognition by other *T. cruzi* natural or experimentally infected animal species. Probably, this polypeptide is important in the resistance mechanisms since it was recognized earlier by all didelphids sera.

The second most commonly recognized antigen by didelphids, 90kDa, was recognized by the experimentally infected *P. frenata* and *D. marsupialis*. Yoshida et al[30] described this antigen in trypomastigote forms and related it to resistance and protection. Apparently this polypeptide is not related to the parasites in the scent glands[13], since none of the animals in the experiments presented parasites in this organ.

*P. frenata* did not display lesions as severe as those described in mice and other animal hosts studied. However lesions in *P. frenata* were significantly more intense than described in naturally or experimentally infected *D. marsupialis*[21] independently of the inoculated *T. cruzi* strain (Figure 3).

*Didelphis marsupialis* (the earliest fossil attributed with certainty to *Didelphis* is from Ensenadan age layers in the Middle Pleistocene between 1-2 million years ago in Argentina) and is considered the most ancient reservoir of *T. cruzi*. *Philander* (it was first found in beds of Montehermosan in Early Pliocene in Argentina, dating back five million years. Fossils of the living *Philander* are recorded from late Quaternary cave deposits in Brazil[29]. *Philander* should be considered similar, moreover its divergence precedes that for *D. marsupialis* by five million years. Consequently, both didelphids probably acquired infection by *T. cruzi* after their evolutionary divergence.

Our preliminary results strongly suggest that the interaction of *P. frenata* with *T. cruzi* is modulated by distinct factors to those of *D. marsupialis*. The more intense severity of the lesions in the naturally infected animals were probably a result of variables other than the strain, since milder lesions were observed in the experimental infections with an *Philander* isolate obtained from an animal captured in the same area.

Regarding these data as a whole we are tempted to speculate that *D. marsupialis* and *P. frenata* marsupials, considered to be the most ancient mammalian hosts for *T. cruzi*, selected different mechanisms to control the parasitism by this flagellate.

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