Humoral Immune Response Kinetics in *Philander opossum* and *Didelphis marsupialis* Infected and Immunized by *Trypanosoma cruzi* Employing an Immunofluorescence Antibody Test

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Philander opossum *and* Didelphis marsupialis *considered the most ancient mammals and an evolutionary success, maintain parasitism by* Trypanosoma cruzi *without developing any apparent disease or important tissue lesion. In order to elucidate this well-balanced interaction, we decided to compare the humoral immune response kinetics of the two didelphids naturally and experimentally infected with* T. cruzi *and immunized by different schedules of parasite antigens, employing an indirect fluorescence antibody test (IFAT). Both didelphids responded with high serological titers to different immunization routes, while the earliest response occurred with the intradermic route. Serological titers of naturally infected* P. opossum *showed a significant individual variation, while those of* D. marsupialis *remained stable during the entire follow-up period. The serological titers of the experimentally infected animals varied according to the inoculated strain. Our data suggest that (1) IFAT was sensitive for follow-up of* P. opossum *in natural and experimental* T. cruzi *infections; (2) both* P. opossum *and* D. marsupialis *are able to mount an efficient humoral immune response as compared to placental mammals; (3) experimentally infected* P. opossum *and* D. marsupialis *present distinct patterns of infection, depending on the subpopulation of* T. cruzi, *(4) the differences observed in the humoral immune responses between P. opossum and* D. marsupialis, *probably, reflect distinct strategies selected by these animals during their coevolution with* T. cruzi.

Key words: *Trypanosoma cruzi - Philander opossum - Didelphis marsupialis* -immunoglobulins

Didelphid marsupials, which originated in the late Cretaceous and are considered the most ancient terrestrial mammals, are extremely adaptable to different environments and are considered an evolutionary success. Paradoxically, the marsupials' immune system was thought to be less complex than that of other mammals (Wirtz & Westfall 1967, Rowlands & Dudley 1968, Rowlands 1970, Marx Jr et al. 1971). However, the marsupial *Didelphis marsupialis*, an important wild reservoir of *Trypanosoma cruzi*, maintains an harmonic interaction with this parasite (Deane et al. 1984) with no important tissue lesions (Carreira et al. 1996); depending on the inoculated strain, it may present a high level of immunoglobulins (Jansen et al. 1985, 1991).

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Received 7 August 1998
Accepted 3 February 1999

Previous reports had already shown that the indirect fluorescent antibody test (IFAT) is a sensitive test for the diagnosis and follow-up of experimental and natural *T. cruzi* infections in *D. marsupialis* (Jansen et al. 1985), contrary to Miles (1979), Minter-Goedbloed et al. (1980), and Luckins and Miles (1982). Specific antibodies were directly correlated with the control of circulating blood parasites in the *D. marsupialis* experimentally infected with F or sylvatic strains. However, the early control of infections in opossums inoculated with Y strain probably occurs by nonspecific mechanisms (Jansen et al. 1991).

Another important reservoir of *T. cruzi*, *Philander opossum*, lives in the same sylvatic habitat as *D. marsupialis* and also efficiently controls parasitism by *T. cruzi*, although histopathological studies in this marsupial have shown more lymphomacrophagic infiltrates than *D. marsupialis* (Pinho et al. 1996). *D. marsupialis* differs from *P. opossum* in experimental infections (Jansen et al. 1991, Pinho et al. 1995), but data on the humoral immune response of this species have not been

shown in detail. The search for insight into the humoral mechanisms involved in natural and experimental infections in these two didelphids infected by *T. cruzi*, led us (1) to adapt an IFAT for the diagnosis of *T. cruzi* infections in *P. opossum* and (2) to follow the kinetics of humoral immune responses in both didelphids.

MATERIALS AND METHODS

Didelphids - Naturally infected *P. opossum* (*n*=5) and *D. marsupialis* (*n*=5) were captured in the Caleme area near Teresópolis (State of Rio de Janeiro, Brazil). For experimental infection and immunization, newly weaned animals (*n*= 18) obtained from females born and reared in our laboratory were used. All animals were caged individually and fed on dog food, fruit, and eggs.

Parasites - The *T. cruzi* strains used in the experiments were as follows: Y (isolated from a human patient by Silva & Nussenzweig 1953); C13 (isolated from naturally infected *P. opossum*), F (originally isolated as *T. lewisi* and afterwards identified as *T. cruzi* by Deane & Kloetzel 1974). All strains were maintained by (a) cyclical passages through triatomines, opossums, and LIT medium and (b) successive LIT medium passages.

Inoculation schedules - Experimental infections (Table): all animals were infected subcutaneously in the inner part of the right thigh. Two litters (three specimens/litter) of *P. opossum* were inoculated with either Y or C13 metacyclic trypomastigotes (500 parasites/g of body weight), and one litter (three specimens) of *D. marsupialis* were inoculated with strain C13 (1000 parasites/g of body weight). Immunization (Table): one litter (three specimens/litter) of *P. opossum* and *D. marsupialis* were immunized with strain F total antigen as follows: 1st dose - 1.5 mg protein (ptn) and Complete Freunds' Adjuvant; 2nd dose - 1.5 mg ptn and Incomplete Freunds' Adjuvant; 3rd dose - 5 mg ptn and Incomplete Freunds' Adjuvant.

Parasitological follow-up - Briefly, fresh blood smears of infected *P. opossum* and *D. marsupialis* were examined after inoculation every two days, and patent parasitemia was followed by counting parasites in a Neubauer chamber. Animals with negative blood smears were submitted to hemocultures in NNN medium with a LIT overlay. Hemocultures were examined every two weeks over a period of two months. Weekly examination for parasites of the scent glands was performed by gentle manual squeezing of the glands.

IFAT - An indirect fluorescent antibody test, as described elsewhere (Jansen et al. 1985), has been adapted to follow-up *P. opossum* infected by *T. cruzi*. The antigen, consisting of epimastigote forms of *T. cruzi* F strain, was adjusted to 40 parasites by microscopic field examination (40x) and stored at -20ºC. *P. opossum* and *D. marsupialis* sera from the natural and experimental infections were obtained from blood samples taken from the tail vein. Positive control serum was obtained from *P. opossum*/*D. marsupialis* immunized with parasite antigens, and negative control serum was obtained from uninfected animals, born and kept in captivity. Rabbit antisera to *P. opossum*/*D. marsupialis* immunoglobulins (Ig) and a fluorescein conjugated anti-rabbit Ig (Sigma) were used.

RESULTS

Fig. 1 demonstrates the follow-up of the humoral immune response kinetics of *P. opossum* and *D. marsupialis* naturally infected with *T. cruzi*. Fig. 1a shows an important serological variation in the naturally infected *P. opossum*, where the total anti-*T. cruzi* Ig levels varied from 1:80 to 1:5120. On the other

TABLE

Inoculation shedules

Experiment	Specie	Source of inoculation	No. of inoculation	Inoculation route
Experimental infection	*Philander opossum*	C13	01 (500MT/b.w.)	SC
		Y	01 (500 MT/b.w.)	SC
	Didelphis marsupialis	C13	01 (1000MT/b.w.)	SC
Immunization	*Philander opossum*	F	03 (soluble fraction)	ID
		F	03 (soluble fraction)	SC
		F	03 (soluble fraction)	IP
	Didelphis marsupialis	F	03 (soluble fraction)	ID
		F	03 (soluble fraction)	SC
		F	03 (soluble fraction)	IP

MT/b.w.: metacyclic trypomastigotes per gram of body weight; SC: subcutaneous; ID: intradermic; IP: intraperitoneal.

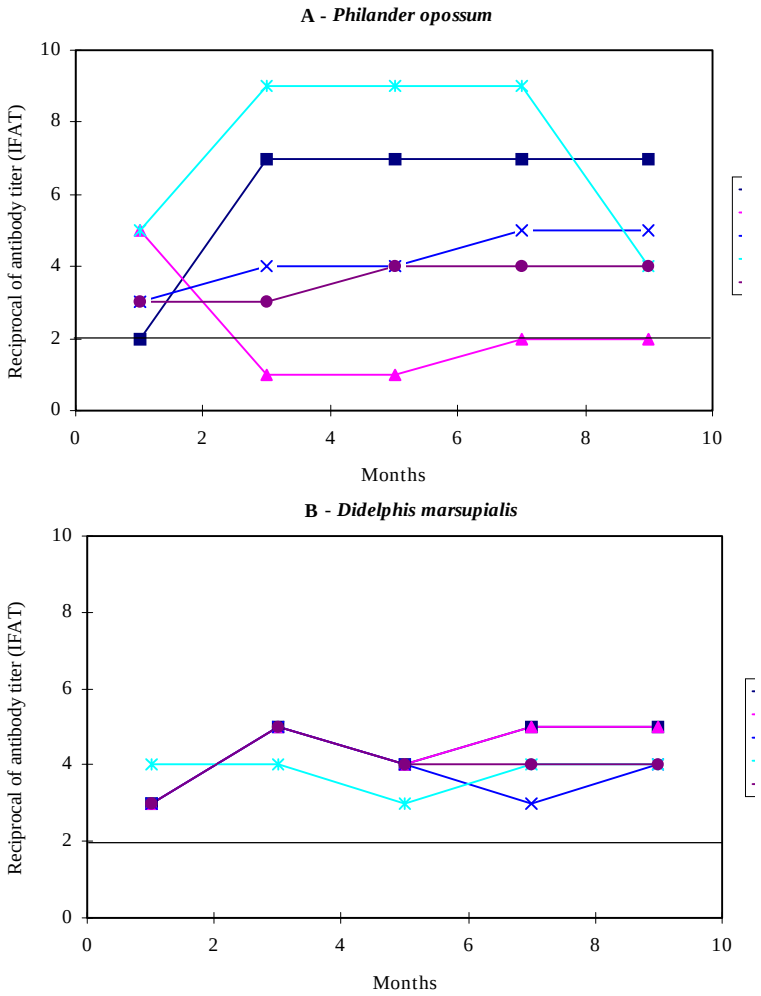

Fig. 1: levels of total anti *Trypanosoma cruzi* antibodies in five *Philander opossum* (A) and *Didelphis marsupialis* (B) naturally infected with *T. cruzi*. Immunoglobulin levels were detected by an indirect immunofluorescence antibody test, and curves were plotted as log 2 of the dilution titers. The line parallel to the X axis (2) indicates the lowest diagnostic titer.

hand, naturally infected *D. marsupialis* displayed serological titers from 1:80 to 1:160 (Fig. 1b).

Serological follow-up of experimentally infected opossums showed that *P. opossum* and *D. marsupialis* presented similar antibody titers (1:80-1:160) when inoculated with the C13 strain. However, *P. opossum* inoculated with the Y strain presented the highest serological titers (1:1280) (Fig. 2).

Sera from immunized didelphids showed high levels of total Ig, regardless of the immunization route. On the other hand *D. marsupialis* reached the serological peak in the 2nd week after the first antigen dose, earlier than *P. opossum* (Fig. 3).

The natural infections were subpatent and stable: 41% (*P. opossum*) and 30% (*D. marsupialis*) of hemocultures performed, during the follow-up, were positive.

The experimental infections in both didelphids resulted in a low patent parasitemia with scarce positive fresh blood smears, and no parasites were observed in the lumen of the scent glands. During follow-up, 33% and 94% (C13 and Y strain, respectively) of the hemocultures of *P. opossum* and 12% of those from *D. marsupialis* (C13 strain) were positive.

DISCUSSION

As described for *D. marsupialis* (Jansen et al. 1985), IFAT is also sensitive in the diagnosis of *T. cruzi* infection in this other reservoir of *T. cruzi*: *P. opossum*. Infection detected by the serological test (IFAT) could be confirmed, in all cases, by positive hemocultures. The characterization of the isolates were revealed by eletrophoretic profile of isoenzymes (Pinho et al. 1997) and the non-tran-

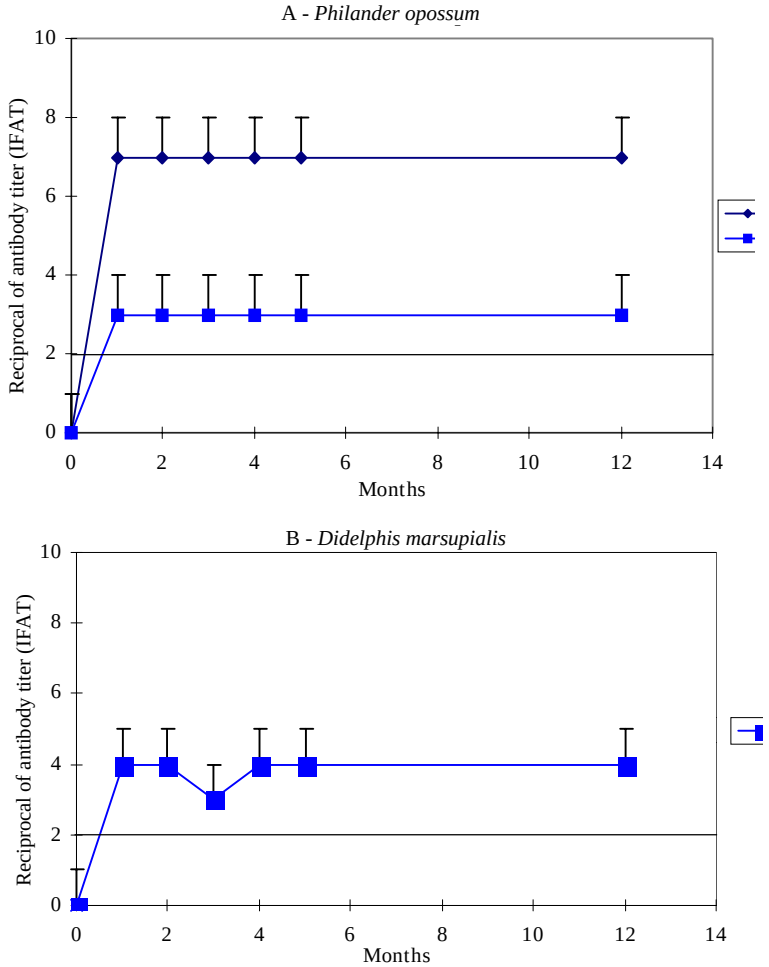

Fig. 2: levels of total anti-*Trypanosoma cruzi* antibodies in five *Philander opossum* from a litter infected with the Y and C13 strains (A) and *Didelphis marsupialis* infected with the C13 strain (B). Each point represents mean values for the litter. All other data as in the caption to Fig. 1.

scribed spacer of the mini-exon gene (Fernandes et al. 1998). Moreover, uninfected laboratory born and reared *P. opossum* presented negative IFAT.

The serological variation in naturally infected *P. opossum* (Fig. 1) suggests the following: (1) reactivation of a chronic infection or a recent infection in the two animals with rising serological titers; (2) control of the infection in an opossum resulting in a decrease in the serological titer; and (3) stable infection reflected by constant titers during the follow-up of two *P. opossum*. Alternatively, the serological variation could be the consequence of the interaction of *P. opossum* with different *T. cruzi* subpopulations, since *P. opossum* is not a strict "biological filter", in contrast to *D. marsupialis* (Pinho et al. 1995). This hypothesis can be confirmed by our data from naturally infected *D. marsupialis* that presented constant levels of immunoglobulins during the whole infection. Similar results were obtained by Jansen et al. (1985). Differences in the pattern of infection in naturally infected opossums could also be observed in ally infected opossums in recent report.

Differences in immune response in experimental infections (Fig. 2) suggest peculiarities in the interaction between *T. cruzi* and these two didelphid species rather than any distinguishing trait of the didelphids' immune response, since immunized *P. opossum* and *D. marsupialis* (Fig. 3) showed the same humoral immune response. We did not note any differences in the antibody production in the patent and subpatent phases. This homogeneous humoral response displayed by the experimentally infected *P. opossum* and *D. marsupialis* reinforce this hypothesis.

The experimental infection with strain C13 resulted in a similar serological pattern in *P. opossum* and *D. marsupialis*, despite the two-fold higher in-

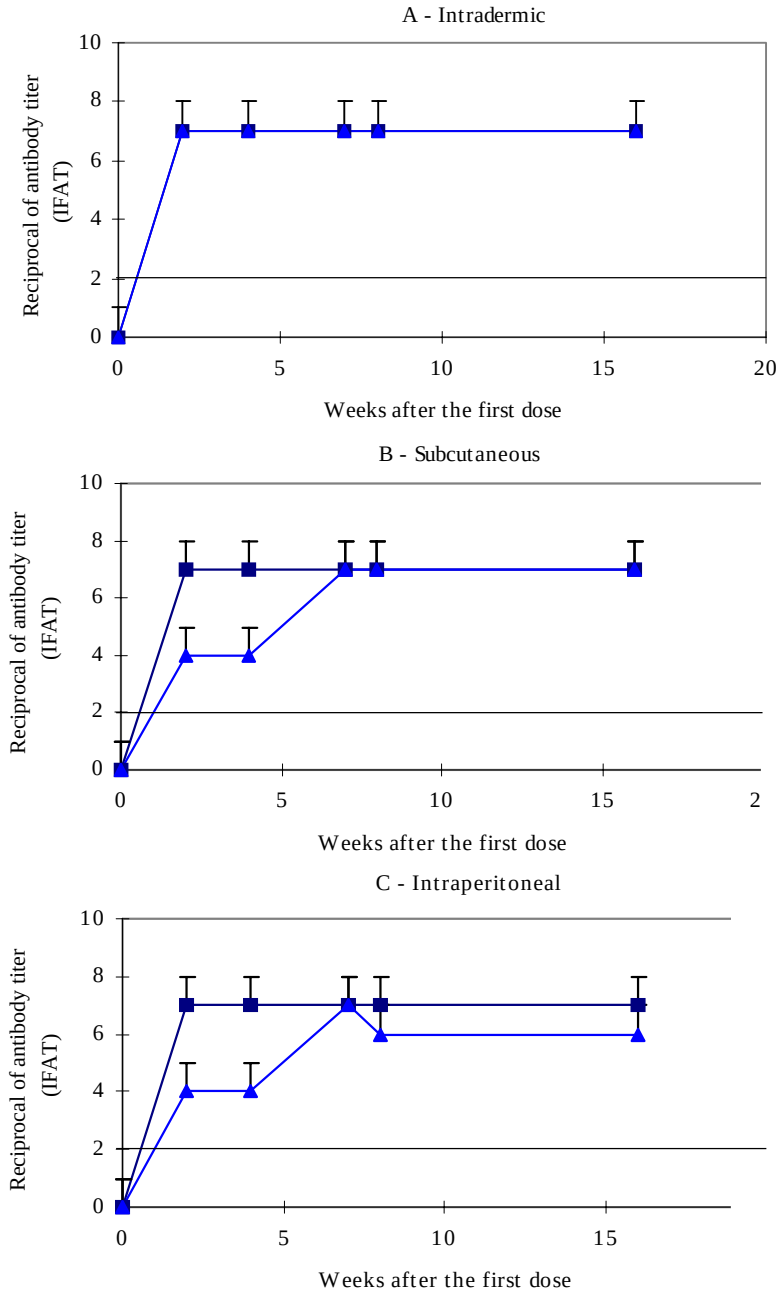

Fig. 3: levels of total anti-*Trypanosoma cruzi* antibodies in *Philander opossum* and *Didelphis marsupialis* immunized by the intradermic (A), subcutaneous (B), and intraperitoneal (C) routes with total antigen of *T. cruzi*. All other data as in the caption to Fig. 1.

oculum in the latter. However *P. opossum* infected with Y strain, displayed significantly higher serological titers than previously described for *D. marsupialis* (Jansen et al. 1985, 1991). These findings point to the peculiarities in the *T. cruzi-P. opossum* and *T. cruzi-D. marsupialis* interactions and also suggest that *D. marsupialis* seems to control the *T. cruzi* infection more efficiently than *P. opossum*.

The correlation in serological and parasitological results could be confirmed by the follow-up of the experimental infection. The animals displayed a significantly lower antibody titers and positive hemocultures suggesting a lower parasitic burden in these host.

All these findings confirm that the marsupial immune response is comparable to that of

placentals and that the IFAT is considered sensitive for following natural and experimental infections in didelphids, contrary to the prevailing opinion several years ago (Miles 1979, Minter-Goedbloed et al. 1980, Luckins & Miles 1982).

Our data indicate that maternal antibodies transferred during lactation could probably confer protection to young *P. opossum* in the pouch, since newly weaned animals control this parasitism in experimental infections as do naturally infected young animals. It has been reported that marsupials acquire antibodies only a few hours after suckling for the first time (Hindes & Mizell 1976), and that *D. marsupialis* maternal antibodies confer partial protection to the young (Jansen et al. 1994).

Although highly speculative, it is tempting to hypothesize that *P. opossum* and *D. marsupialis*, two closely related species, selected distinct strategies to efficiently control the parasitism by *T. cruzi*, during their coevolution. Our result also suggest that this well-balanced interaction of *T. cruzi* with didelphids is more complex than previously belived.

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