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RESEARCH BRIEF

Leishmania (*Leishmania*) *chagasi:* Clinical and Parasitological Observations in Experimentally Infected *Didelphis marsupialis*, Reservoir of New World Visceral Leishmaniasis

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Index Descriptors and Abbreviations: kinetoplastida; *Leishmania chagasi; Marsupialia; Didelphis marsupialis;* experimental infection; visceral leishmaniasis; Colombia; opossum (op); amastigotes (am); promastigotes (pr); intradermal (id); intracardial (ic); salivary glands (SG); alanine transferase (ALT); aspartate aminotransferase (AST); postinfection (pi).

Didelphis marsupialis has been considered an important reservoir of several infectious agents of which trypanosomatids are the most frequently reported (Travi *et al.* 1994). The majority of reports of naturally infected opossums, either with tegumentary or visceral leishmaniae, were originated in Brazil (Arias and Naiff 1981; Grimaldi *et al.* 1991; Sherlock *et al.* 1984). The natural infection rate of *D. marsupialis* with *Leishmania chagasi* in two different Colombian foci of visceral leishmaniasis was found to be 32.4 and 22.7% (Corredor *et al.* 1989; Travi *et al.* 1994), indicating that this mammal could be a potential reservoir. On the other hand, attempts to infect *D. marsupialis* with *L. chagasi* have been scarce and difficult to accomplish (Sherlock *et al.* 1988). Therefore, by means of clinical, parasitological, histopathological, and entomological methods, we evaluated the host–parasite–vector relationships in experimentally infected opossums.

Four young, recently weaned *D. marsupialis* and their mother, a wild female trapped in a nonendemic area for either cutaneous or visceral leishmaniasis, were used in this experiment. A negative blood culture ruled out the presence of trypanosomes. The strain of *L. chagasi* (MCAN/COL/95/NILO) used in this experiment was isolated from a dog with visceral leishmaniasis and was subsequently reinoculated in an opossum. Parasites recovered from this animal were cultured in NNN medium and in the U937 promonocytic cell line. A total of 13.5 $\times$ 10^7 amastigotes (am) derived from infected U937 cells or 5.6 $\times$ 10^7 promastigotes (pro) from the stationary phase of growth were inoculated into different individuals.

To test the exacerbation effect of sandfly saliva on *Leishmania* virulence, salivary glands (two per opossum) of colonized *Lutzomyia longipalpis* were incorporated in some of the inocula. The infection protocol, which contemplated the intradermal (id) or intracardial (ic) routes, with or without salivary glands (SG), was as follows: op 755 (adult female) pro ic; op 754 (young male) am ic; op 751 (young male) am ic + SG; op 752 (young male) am id; op 753 (young female) am id at the base of the tail + SG.

Parasite burden was roughly estimated by the density of promastigotes observed in cultures at different time points. The search for *Leishmania* DNA in skin samples was also carried out by PCR using specific primers for *L. chagasi* (DB8/AJS3) designed by Smyth *et al.* (1992), at 1 month postinfection and at the time of sacrifice. A specific biotin-labeled probe (BioPrime, Gibco, BRL) was used to hybridize the products transferred to a nylon membrane and detected according to the instructions of the manufacturer (Photogene, Gibco, BRL).

Only female 755 showed symptoms suggestive of visceral leishmaniasis during the second month postinfection; i.e. it became progressively adynamic and the food intake diminished concomitantly; also, the fur was dull and the skin showed signs of dehydration and incipient desquamation, and for this reason it was sacrificed with an overdose of anesthesia followed by intracardial injection of magnesium sulfate.

Culture of spleen aspirates in NNN medium indicated that both promastigote and amastigote inocula were infectious to opossums. Only opossum 755 showed amastigotes in Giemsa-stained impression smears of spleen (24 parasites/100 cells), liver (4.6 parasites/100 cells), and lymph node (1.3 parasites/100 cells). Semiquantitative estimates of parasite densities in culture revealed that the spleen had the highest

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parasite burden of all tissues studied (Table I). Lymph nodes were the second most frequently infected tissues, and the livers of subclinically infected individuals were either negative or showed a small number of parasites. On the other hand, the liver of opossum 755 was intensely colonized by amastigotes (Table I). Blood was consistently culture negative, with the exception of opossum 755 in which a small number of parasites was detected. Contamination of cultures from skin samples of the ears prevented the evaluation of parasite colonization of these tissues. In our hands PCR was less sensitive than xenodiagnosis for detecting amastigotes in the skin (Table I).

The histopathological analysis demonstrated that subclinically infected individuals had discrete inflammatory changes in the liver, lymph nodes, and spleen. On the other hand, the animal with full blown disease showed abundant parasitized histiocytes, extensive necrosis of the spleen, and hepatic steatosis.

Aspartate aminotransferase (AST) and alanine transferase (ALT) were evaluated only at the time of sacrifice. All infected juveniles had AST (104.8 $\pm$ 16) and ALT (13 $\pm$ 3.5) values similar to that of control (AST, 105; ALT, 13), whereas in female 755 both enzymes (AST, 1425; ALT, 51) were elevated. Also, in the latter specimen albumin production was below normal values, compared with the other opossums (1.8 vs mean 2.5 g/dl, respectively). The histopathological and biochemical results, considered together, indicated that *L. chagasi* infection did not affect liver functions in subclinically infected individuals and that the general clinical and biochemical condition of these specimens remained within normal limits, as opposed to the opossum with overt disease.

Colony bred *Lu. longipalpis* were used for xenodiagnosis on anesthesized opossums at 1, 2, and 4 months postinfection. Between the fourth and ninth day postengorgement sandflies were individually dissected, searching for either attached or motile promastigotes within the intestinal tract. In general, a low proportion of specimens were infected through feeding (Table I). One month postinfection two of four infected opossums, one inoculated id and the other ic, had amastigotes in the skin as detected by xenodiagnosis. At this point in time, juveniles that were inoculated with the addition of *L. longipalpis* salivary glands were xenodiagnosis negative. By the second and fourth month postinfection, no amastigotes could be detected in the skin of subclinically infected opossums, as determined by sandfly dissections. Opossum 755, which was inoculated ic with promastigotes, could only be subjected to xenodiagnosis on the second month pi, a few days before sacrifice. The proportion of sandflies that resulted infected after feeding on opossum 755 (5/52) suggested that this specimen harbored the highest density of amastigotes in the skin (Table I).

We speculate that the high infection rate achieved in this experiment could have been due to parasite adaptation, since the strain used in this study, which was originally isolated from a symptomatic dog, had a low infectivity for a first group of opossums. It was interesting to find that injecting parasites through the dermis, a more "natural" route, was as effective as the artificial approach of delivering large numbers of parasites directly into the blood. Though limited in number, this experiment suggests that overt disease would be an uncommon event in *D. marsupialis.* Based on xenodiagnosis and culture data we could not demonstrate an exacerbation effect of the sandfly salivary glands, as described by other authors (Titus and Ribeiro 1988; Hall and Titus 1995). Failure to increase *L. chagasi* virulence was also reported in dogs infected with *L. chagasi* (Paranhos *et al.* 1993) and Rhesus monkeys infected with *Leishmania amazonensis* (Amaral *et al.* 1996).

The absence of positive xenodiagnosis and PCR from skin samples at later stages of infection suggests that parasite distribution and consequently reservoir capacity varies with time of evolution (Table I). Cultures in NNN medium showed that *L. chagasi* amastigotes persist mainly in lymphoid organs (spleen, lymph nodes) as it has been observed in other mammal hosts. It is not surprising that the specimen with overt disease (opossum 755) had the highest amastigote load in all the tissues that were studied and that its abnormal skin was the most infectious to the vectors, an observation similar to that reported in dogs by Vexenat *et al.* (1994). The results of xenodiagnosis in our group of opossums are similar to those observed in dogs infected with *L. infantum,* in that subclinically infected individuals also were capable of transmitting leishmaniae to sandflies (Molina *et al.* 1994). This trait could be considered a biological indicator of reservoir capacity for both domestic (dog) and wild hosts (opossum). In terms of biomass, *D. marsupialis* represents the most abundant small mammal in the visceral leishmaniasis focus of northern Colombia (Adler *et al.,* in press) and has demonstrated to have the highest attraction rate for the

TABLE I

Parasitological Results of Opossums Experimentally Infected with *Leishmania chagasi*

Opossum inoculation protocol	Xenodiagnosis			PCR		Culture				
	Positive flies/dissected			Skin		Spleen		LN	Liver	Blood
Months p.i.:	1	2	4	1	4	1	4	4	4	4
752 am id	1/33	0/41	0/51	+	Neg	+++	+	++	Neg	Neg
753 am id + sg	0/30	0/27	0/16	Neg	Neg	Neg	+[a]	+[a]	Neg	Neg
754 am ic	2/50	0/46	0/39	Neg	Neg	+	++	+[a]	+[a]	Neg
751 am ic + sg	0/31	0/30	0/31	Neg	Neg	++	++	Neg	Neg	Neg
755 prom ic	ND	5/52	NA	+	+[b]	+++	+++[b]	+++[b]	+++[b]	+[b]

Note. ND, not done; NA, not applicable; am, amastigotes; prom, promastigotes; id, intradermal; ic, intracardial; sg, salivary gland; +++, large numbers of promastigotes by the 15th day of culture; ++, discrete numbers of promastigotes by the 15th day of culture; +, scarce numbers of promastigotes by the 15th day of culture.

[a] Promastigotes observed by subculture.

[b] Results were obtained by the second month postinfection at the time of sacrifice.

vector *Lutzomyia evansi* of all the species trapped in the area (Travi *et al.*, unpublished), characteristics that, in addition to its natural infection with *L. chagasi* (Corredor *et al.* 1989; Travi *et al.* 1994), further strengthen its reservoir role.

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