Parasitol Res (2000) 86: 504–508

ORIGINAL PAPER

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Molecular and biologic characterization of *Leishmania* parasites implicated in an epidemic outbreak in northwestern Argentina

Received: 6 October 1999 / Accepted: 22 October 1999

Abstract *Leishmania (Viannia) braziliensis* and its variants were implicated in the epidemic outbreak of mucocutaneous leishmaniasis that occurred in Salta, northwestern Argentina, in 1985. A total of 24 suspected, untreated cases were evaluated clinically and parasitologically. Four of five stable isolates were consistent with the reference strain of *L. (V.) braziliensis* as determined by monoclonal antibodies and indirect immunofluorescence or radioimmunobinding assays. Zymodeme analysis in agarose gels showed a close relationship with *L. (V.) guyanensis* and *L. (V.) panamensis*. All zymograms obtained with polyacrylamide gels belonged to the subgenus *Viannia*; the patterns were different from, but very closely related to, the reference strains of *L. (V.) braziliensis* as determined by dendrogram analysis. Hamsters infected with two isolates showed a pattern consistent with *L. (V.) braziliensis*. The pattern of development in the gut of *Lutzomyia longipalpis* was consistent with members of *Viannia*.

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This article is dedicated to the memory of Néstor Juan (deceased in August 1991)

Introduction

Approximately 12 million people become infected with *Leishmania* parasites each year throughout the world, and 350 million others have been estimated to be at risk of infection (WHO 1990). In Argentina, leishmaniasis, which was first reported in 1916, occurs in nine northern provinces (Mazza 1926; Bernasconi 1930; Rivero et al. 1983). The dominant clinical form is mucocutaneous leishmaniasis, but some isolated visceral cases have also been described (Mazza 1926; Grimaldi et al. 1989).

Reporting of leishmaniasis is mandatory in Argentina. National records indicate that the mean number of cases occurring per year was less than 90 during the 1970s and until 1984. Concurrent with a clear worldwide increase in the incidence of leishmaniasis, in 1985 the number of reported cases increased dramatically as the result of an epidemic outbreak occurring in the provinces of Salta and Jujuy, the first ever documented in Argentina. Moreover, most of the leishmaniasis cases occurred in the rural-urban interphase of the town of Pichanal. The biologic behavior of some isolates in hamsters, examined at Instituto Nacional de Parasitología Dr Mario Fatala Chaben (formerly INDIECH), suggested that the implicated parasites were *L. braziliensis* and other variants of the subgenus *Viannia* (M. Fayat de del Prado et al., unpublished results). However, the identification of *Leishmania* is often difficult when based only on biologic criteria. The correct iden-

tification of species is of paramount importance for the treatment and subsequent management of the patient.

Until the early 1980s the taxonomy of New World *Leishmania* was based largely on the clinical and epidemiologic features of the diseases that they produced in humans, on the geographic distribution of such diseases, and on the biologic characteristics of the parasites in laboratory animals and sandflies (Grimaldi et al. 1989). However, many of the New World *Leishmania* are capable of producing a wide spectrum of disease in humans, and development of the parasite in animal and insect hosts shows a wide variability that is likely related to their genetic constitution. Moreover, the known geographic distribution of many parasites has been extended as a consequence of changes in land use and deforestation, and new species of reservoir hosts, sandfly vectors, and *Leishmania* species have been described. In this study, in an effort involving multiple research centers, we defined the parasitologic and taxonomic characteristics of the *Leishmania* species implicated in the epidemic outbreak occurring in Salta in 1985 using serodeme analysis with monoclonal antibodies, zymodeme analysis, inoculation of hamsters, and experimental infection of sandflies.

Materials and methods

Study area

The outbreak occurred in Pichanal, Embarcación and General Mosconi (22 30′–24 10′S, 63°10′–64°25′W), northwestern Salta, Argentina. A detailed map of the area can be found elsewhere (Sosa Estani et al. 1998). The region presents two phytogeographic environments: a subtropical humid forest in the west and xerophytic open and low woods in the east. The climate is temperate and includes a dry period in winter and a rainy season between November and April along with an annual average rainfall of 800 mm. The average monthly temperature ranges from 14.80 °C in July to 26.30 °C in January. Work activities in the area include oil and timber exploitation, fishing, hunting, and agriculture close to the forest border. Intense deforestation started in the 1970s. According to the 1991 census, approximately 47,000 people resided in the area.

Survey design

Personnel from INDIECH and from the Servicio Nacional de Chagas carried out a cross-sectional survey during August 1985. The local primary health care (PHC) staff was trained to detect cases and send suspected *Leishmania*-infected individuals directly to the local hospital. Trained physicians examined the patients and obtained biopsies from the lesions. Of 32 patients that were examined clinically, 24 had not received previous treatment against *Leishmania* and were therefore included in the subsequent studies.

Parasitologic diagnosis

Material was collected from the edge of the lesion and divided into several parts, which were then inoculated in diphasic culture, into golden hamsters; and used for histopathologic analysis and smears for Giemsa staining.

Primary parasite isolates were obtained by addition of the homogenized tissue fragments and aspirated material into culture media (Walton et al. 1977; Hashiguchi et al. 1991). The culture media used were NNN, LIT (liver infusion tryptose) and Difco blood-agar base supplemented with 20% defibrinated rabbit blood, penicillin, and streptomycin (at 100 U and 100 μg/ml, respectively).

Most of the *Leishmania* isolates were characterized by indirect radioimmunobinding assay (RIA) using specific monoclonal antibodies and enzyme electrophoresis. Reference strains recommended by the World Health Organization (WHO) were used to compare and characterize the parasites.

Isolates INCH 1, INCH 2, INCH 3, and INCH 9 were classified by radioimmunobinding assay using whole-parasite lysate as the antigen of *Leishmania* at Yale University School of Medicine according to procedures described elsewhere (Grimaldi et al. 1987). The monoclonal antibodies used were B-2, B-5, B-8, B-11, B-16, B-18, B-19, T-2, T-4, T-6, T-8, M-2, M-4, M-7, M-8, M-11, D-13, D-16, and XLIV 2E9E7.

Identification of isolates INCH 1 and INCH 4 to species using monoclonal antibodies and indirect immunofluorescence assay (IIFA) was carried out at the University of Brasilia according to previously described procedures (Cuba Cuba et al. 1985). We used monoclonal antibodies B-2, B-5, B-16, B-17, and B-18 for *L. (Viannia) braziliensis*, B-19 for *L. (V.) guyanensis*, M-4 and M-2 for *L. (L.) amazonensis*, and M-7 and LXVIII 4D12D for *L. (L.) mexicana*. Parasites had previously been cultured in Schneider's medium.

Zymodeme analysis was carried out on a total of five different isolates harbored by five patients. Three isolates (INCH 2, INCH 3, and INCH 4) were analyzed by electrophoresis in agarose gels by H. Momen at Instituto Oswaldo Cruz according to previously described procedures (Momen et al. 1985). A phenetic numerical analysis was performed on those isolates for which data corresponding to at least 11 enzyme loci were available, including malate dehydrogenase (MDH; E.C.1.1.1.37), malic enzyme (ME; E.C.1.1.1.39), isocitrate dehydrogenase (IDH; E.C.1.1.1.40), 6-phosphogluconate dehydrogenase (6PGDH; E.C.1.1.1.44), glucose 6-phosphate dehydrogenase (G6PD; E.C.1.1.1.49), phosphoglucomutase (PGM; E.C.2.7.5.1), nucleoside hydrolase (NH; E.C.3.2.2.2, two loci), peptidase (PEP1; E.C.3.4.11), proline dipeptidase (PEP D; E.C.3.4.13.9), and glucose phosphate isomerase (GPI; E.C.5.3.1.9).

Zymodeme analysis of isolates INCH 1, INCH 2, INCH 3, INCH 4, and INCH 9 was performed by E. Montamat and G. De Luca d'Oro at Universidad Nacional de Córdoba. Extracts were resolved on polyacrylamide gel electrophoresis using the micromethod described by Ogita and Market (1979), and the electrophoretic mobilities of promastigote isoenzymes were obtained using procedures described elsewhere (Miles et al. 1980, 1981; Montamat and Arauzo 1987). After electrophoresis the gels were stained for aspartate amino transferase (ASAT; E.C.2.6.1.1), alcohol dehydrogenase (ADH; E.C.1.1.1.1), G6PD, ME, PGM, GPI, and MDH.

The biologic behavior of INCH 1 and INCH 4 in golden hamsters (*Mesocricetus auratus*) was studied at the Laboratory of Protozoology of the University of Brasilia. Two 200-g outbred golden hamsters were inoculated intradermally in the hindfeet dorsum with 5×10^6 promastigotes in 0.1 ml of phosphate-buffered saline (PBS). The hamsters were examined weekly. Material obtained by needle aspiration of the borders of the lesions was cultured in Difco blood-agar and Schneider's media. The tubes were incubated at 23 °C and examined every week for 2 months. Necropsy specimens obtained from each organ (lymphatic node, spleen, liver, bone marrow) were examined for metastasis and visceralization. Samples were considered positive if promastigotes were observed at 10 days after inoculation.

The growth of *Leishmania* isolates INCH 1 and INCH 4 in the digestive tract of experimentally infected *Lutzomyia longipalpis* was carried out at the Laboratory of Protozoology, University of Brasilia, according to standard procedures (Ward et al. 1978). A total of 40 sandflies were exposed to and fed on the nodular lesion of a hamster that had been infected with isolate INCH 4 or had been infected with cultured promastigotes of INCH 1 by artificial xenodiagnosis (Ward et al. 1978).

Results

Most (85%) of the 34 patients examined during the 1985 outbreak were from Pichanal, and a large proportion of them (85%) lived close to or within the forest (Table 1). Nearly half (56%) of the patients were women, and the average age of the patients was 30.4 years. A total of 19 (56%) patients had multiple cutaneous ulcers, which ranged from 2 to 32 weeks of evolution (mean 9.6 weeks). Only one patient presented with mucocutaneous leishmaniasis, and another one had a lesion in the nasal area. Most lesions occurred in the lower or upper limbs.

The most sensitive method for parasite detection was direct examination, which yielded 10 positive results among the 24 patients thus examined. Isolates were less frequently obtained by culture of aspirated materials (in 3 of 20 patients examined) or histopathology (in 2 of 11 patients). Of the 12 patients found to be positive by any method, 83% were determined to be positive by direct examination; 25%, by culture; and 17%, by histopathology.

Eight parasite stocks were finally obtained, six by culture and two by inoculation into hamsters. Five of the *Leishmania* isolates were stable and were identified as MHOM/AR/85/INCH 1, INCH 2, INCH 3, INCH 4, and INCH 9 according to the international WHO codes. Inflammatory lesions of the skin were predominantly characterized by lymphocytes, macrophages, and plasma cells; leukocytes were only rarely observed. All patients showed granuloma formation of different degrees of progress and reparative processes as evidenced by fibrosis. Vasculitis and fibrinoid necrosis, typical of acute lesions, were not commonly observed. *Leishmania* amastigote nests were scarce, being detected only in two patients.

Isolates INCH 1, INCH 2, and INCH 3 showed a positive reaction with monoclonal antibodies B-2 and B-5 [which in RIA cross-react with *L. (V.) panamensis*], B-16 and B-18 [which are specific for *L. (V.) braziliensis* from Brazil but do not usually react with *L. (V.) braziliensis* from Colombia or Belize; Table 2). Isolate INCH 9 gave weak reactions with monoclonal antibodies T-2 and T-4, which are sometimes suggestive of *L. (L.) venezuelensis*, but these results were not considered definitive. Isolates INCH 1 and INCH 4 corresponded to *L. (V.) braziliensis* as determined by a positive reaction with monoclonal antibodies B-16, B-17, and B-18 and by IIFA (Table 2).

Zymodeme analysis of isolates INCH 2, INCH 3, and INCH 4 in agarose gels showed a close relationship with two members of the subgenus *Viannia* (*braziliensis* complex) – *L. (V.) guyanensis* and *L. (V.) panamensis* (Table 2). All zymograms obtained with polyacrylamide gels were identical for all enzymes and corresponded to a member of the subgenus *Viannia* different from, but very closely related to, *L. (V.) braziliensis* as determined by dendrogram analysis.

Both hamsters inoculated with INCH 1 showed nonulcerated erythematous nodules, the first evidence of infection, at 5 weeks after inoculation. At the 6th week, both hamsters had subcutaneous erythematous parasitic nodules with incipient ulcers. Enlargement of the lymph

Table 1 Characteristics of 34 patients examined during the leishmaniasis outbreak in Salta, Argentina, in 1985

Variable	Frequency	%
Source locality of patients		
Pichanal	29	85
Others	5	15
Distance from the forest (m)		
< 200	25	74
> 200	4	12
Within	4	12
No information	1	3
Age (years)		
Mean (range)	30.4 (1–70)	
SD	23.6	
Gender		
F	19	56
M	15	44
Clinical form		
Multiple cutaneous ulcer	19	56
Single cutaneous ulcer	13	38
Mucosal lesion	1	3
Mucocutaneous lesion	1	3
Localization of lesion		
Legs	16	47
Arms	4	12
Face or trunk	3	9
Combined[a]	11	33
Period of evolution (weeks)		
Mean (range)	9.6 (2–32)	
SD	6.7	

[a] Combination of limbs and face or trunk. Two patients had nasal lesions

Table 2 Summary of the results of the use of biochemical, immunologic, and biologic methods for the characterization and identification of *Leishmania* isolates from Salta, Argentina, in 1985 [*L(V) L. (Viannia)*, *L(V)b L. (V.) braziliensis*, *L(V)g/p L. (V.) guyanensis* or *panamensis*, *L(L)v L.(L.) venezuelensis*, *ND* not done]

Method	Isolate				
	INCH 1	INCH 2	INCH 3	INCH 4	INCH 9
Monoclonal antibodies					
RIA	L(V)b	L(V)b	L(V)b	ND	L(L)v ??
IIFA	L(V)b	ND	ND	L(V)b	ND
Zymodemes					
Agarose gel	ND	L(V)g/p[a]	L(V)g/p[a]	L(V)g/p[a]	ND
Polyacrylamide gel	L(V)b[a]	L(V)b[a]	L(V)b[a]	L(V)b[a]	L(V)b[a]
Hamster	L(V)	ND	ND	L(V)	ND
Sandfly	L(V)	ND	ND	L(V)	ND

[a] Pattern related to, but different from, the corresponding WHO reference strain

nodes, liver, and spleen was found at necropsy in one animal. Both hamsters infected with INCH 4 had evident parasitic nodules at 2 weeks after inoculation, and by the 6th week, both had completely necrotized and ulcerated parasitic nodules. The histopathologic observations of the infected hamsters showed viscerotropism with viable amastigote invasion of the lymphatic nodes, spleen, liver, and bone marrow (tibial, hind leg) as determined by culture. No metastasis was detected in any other cutaneous site. Skin lesions were small, and the histopathologic examination showed amastigotes. The infections showed a discrete pattern of progress involving the formation of parasitic nodules of slow development, ulceration and necrosis of the lesions, and hairless erythematous areas.

The pattern of development in the gut of *L. longipalpis* was consistent with peripylarian parasites, members of the subgenus *Viannia* (Table 2). Both short promastigote forms attached by the flagella to the wall of the pyloric triangle and rounded paramastigotes with short flagella were observed. Invasion of the midgut area by free flagellar forms arranged in clusters or rosettes of promastigotes was also observed. The sandfly infection rate was 10.3% (3 of 29 dissected flies) for INCH 1 and 13.3% (4 of 30 dissected sandflies) for INCH 4.

Discussion

The leishmaniasis outbreak that occurred in Salta in 1985 was caused by *Leishmania (Viannia) braziliensis* as determined by multiple molecular and biologic methods. The most frequently observed clinical forms during the outbreak involved cutaneous leishmaniasis with multiple or single ulcers. Only 3% of the patients presented with mucocutaneous leishmaniasis, a likely consequence of early detection and prompt treatment of the disease. No case similar to the anergic diffuse cutaneous form was found. Despite some reports of *L. major* in neighboring Paraguay and Brazil (Momen et al. 1985; Yamasaki et al. 1994), we found no evidence of the *L. mexicana* complex in the area. Moreover, serodeme and zymodeme analyses of eight isolates of human origin recovered in 1992 from the Province of Santiago del Estero, some 500 km to the south of Pichanal, showed that they were *L. (V.) braziliensis* (Cuba Cuba et al. 1996). However, at least one isolate from Argentina has been characterized by enzyme electrophoresis as *L. (V.) guyanensis* (Cupolillo et al. 1994).

Of 24 suspected, untreated patients, 12 were parasitologically diagnosed as having leishmaniasis. As in Colombia (Weigle et al. 1993), direct examination was the most sensitive method, followed by in vitro culture and histopathology. All lesions showed nonspecific inflammation, in accordance with features described in persons infected with *L. (V.) braziliensis* (Ridley et al. 1980), and sparse nests of *Leishmania* amastigotes (Magalhaes et al. 1986). The limited effectiveness of parasitologic methods in the detection and isolation of parasites was likely related to the characteristic poor growth of *L. (V.) braziliensis* in artificial media (Walton et al. 1977; Lainson and Shaw 1979; Rey et al. 1990).

Serodeme analysis with monoclonal antibodies revealed antigens specific for *L. (V.) braziliensis*. No positive reaction with other members of the *Viannia* subgenus [i.e., *L. (V.) guyanensis* or *L. (V.) panamensis*] was detected. The pattern observed with isolate INCH 9 was defined as that of *Leishmania* by histopathology and excluded *L. (L.) mexicana*, *L. (L.) amazonensis*, *L. (V.) braziliensis*, *L. (V.) panamensis*, and *L. (L.) donovani*. However, the precise species of INCH 9 was not determined by monoclonal antibodies.

Zymodeme analysis in polyacrylamide gels demonstrated that all five isolates belonged to the subgenus *Viannia*, showing a pattern distinct from, but very closely related to, the WHO reference strains of *L. (V.) braziliensis*. Zymodeme analyses of three isolates using agarose gel electrophoresis identified them as being closer, although not identical, to *L. (V.) panamensis* or *L. (V.) guyanensis*. In both cases the enzymes ALAT and MPI, which help distinguish between *L. braziliensis* and *L. guyanensis* (Miles et al. 1981), were not used. Therefore, zymodeme analyses clearly indicated that the isolates were members of the subgenus *Viannia* but did not provide a consistent identification to the species level.

L. (V.) braziliensis has been described as a metastatic pathogen in the hamster (Wilson and Lollini 1980). The biologic behavior of the two study isolates in both hamsters and sandflies was typical of the subgenus *Viannia*. In the hamster the progress of infection involved the formation and slow development of parasitic nodules followed by ulceration and necrosis of the lesion, as in other reference strains of *L. (V.) braziliensis* (Cuba Cuba et al. 1985). A visceralizing infection was also observed in hamsters infected with both isolates. This behavior is characteristic of *L. (V.) guyanensis* and *L. (V.) panamensis* (Martinez et al. 1991) but has also been observed for *L. (V.) braziliensis* from Bahia State, Brazil (Cuba Cuba et al. 1985) and for another isolate from Salta in 1990 (Sinagra et al. 1997).

L. braziliensis, the most widely distributed agent of New World leishmanias (Grimaldi et al. 1989), is extremely heterogeneous and shows considerable enzyme polymorphism (Saravia et al. 1998). In the study area, *Lutzomyia intermedia*, a markedly anthropophilic species with domestic or peridomestic distribution, was the sandfly most commonly captured with Shannon traps, with CDC light traps, and on horse or human bait slightly after the outbreak and in subsequent years (Salomón et al. 1995). No wild or domestic animal reservoir of *Leishmania* sp. has yet been identified in the area. The epidemic outbreak in 1985 slowly retreated in 1986–1987 to return to a stable endemic level in subsequent years, though slightly greater numbers of cases were observed both in Salta and in the whole country (Sosa Estani et al. 1998). This probably resulted from an increase in the awareness and training of the local health

services and the initiation of surveillance measures. As generalized by Mott et al. (1990), the most likely determinants of the outbreak were the uncontrolled deforestation and expanding urbanization of low-income population groups concentrated in periurban compounds close to the leishmaniasis zoonotic cycle, concurrent with favorable environmental conditions.

Acknowledgements All experiments conducted in this study comply with the current laws of Argentina. This study was supported by the National Minister of Health and Social Action of Argentina. We thank the staff at the Instituto Nacional de Parasitología "Dr. Mario Fatala Chabén" for providing active support throughout this study. Ricardo E. Gürtler provided helpful comments on an earlier version of this manuscript.

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