

## Epidemiological and Immunological Aspects of Human Visceral Leishmaniasis on Margarita Island, Venezuela

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Sixty-five patients were diagnosed with visceral leishmaniasis (VL) on Margarita Island in the decade from 1990 to 1999; 86.2% were  $\leq 3$  years old. All were leishmanin-negative at diagnosis. Evaluation of 23 cured patients in 1999 revealed that 22/23 had converted to leishmanin-positive; five had persisting antibodies to rK39 antigen, with no clinical evidence of disease. Leishmanin tests were positive in 20.2% of 1,643 healthy individuals from 417 households in endemic areas. Of the positive reactors, 39.8% were identified in 35 (8.4%) of the households, 15 of which had an antecedent case of VL, a serologically positive dog or both. Weak serological activity to rK39 antigen was detected in 3 of 488 human sera from the endemic areas. The presence of micro-foci of intense peri-urban transmission and the apparent absence of other Trypanosomatidae causing human disease offer a unique opportunity for the study of reservoirs, alternative vectors and evaluation of control measures on the Island.

Key words: American visceral leishmaniasis - rK39 antigen - leishmanin reactions - control - Margarita Island - Venezuela

American visceral leishmaniasis (AVL), a potentially fatal systemic infection, is caused by several species of intracellular protozoa belonging to the genus *Leishmania*. Most cases of AVL are caused by *Leishmania chagasi* (= *L. infantum*). *L. amazonensis* has been isolated sporadically from leishmaniasis with visceral involvement (Barral et al. 1986, 1991). Isolation of *L. colombiense* and *L. mexicana* from human AVL in individuals without known immunodeficiency has been reported by Delgado et al. (1993) and Monroy-Ostria et al. (2000), respectively.

AVL is widely distributed in the Americas, from the northern provinces of Argentina to the United States (Grimaldi et al. 1989). More than 90% of the reported human cases of AVL in the Americas are from Brazil, corresponding to nearly 2,330 cases annually in the period from 1984 to 1999 (WHO 1999), but accurate information for most if not all of the Western Hemisphere is very limited. During the period from 1995 to 2000, 251 cases of human AVL have been reported in widely distributed foci in Venezuela (O Zerpa and others, unpublished data). The principal reservoir of human infection is assumed to be the domestic dog. *L. chagasi* has been detected by PCR in single specimens of a black rat, *Rattus rattus* and an opossum, *Didelphis marsupialis*, in the eastern mainland focus of AVL (Zulueta et al. 1999). While the principal vector of AVL in Venezuela is presumed to be *Lutzomyia longipalpis*, *Lu. evansi* has been implicated in transmission of AVL in both Northern Colombia (Travi et al. 1996) and Venezuela (Feliciani et al. 1999).

One of the active areas of AVL in Eastern Venezuela is Margarita Island, geographic coordinates 10°51'50"-11°11'06" N, 63°46'06"-64°24'32" W, which occupies an area of 1085 km<sup>2</sup> at a distance of 21.5 km off the north-eastern coast of Venezuela. The current estimated population is 384,800 inhabitants. Velásquez et al. (1965) reported the first known case of human AVL on the island. A recent study, motivated by the small but increasing number of human AVL infections in recent years, revealed an average infection rate of 21.6% in domestic dogs in eight endemic communities on the island (Zerpa et al. 2000). Rats, opossums and other possible wild reservoirs on the island apparently have not been studied. Two isolates of *Leishmania*, one from a young patient and the other from a dog, have been identified as *L. infantum*, zymodeme MON-1 (Zerpa et al. 2001), which has also been reported in Western Venezuela as well as many areas throughout the world.

The objective of this study has been to evaluate epidemiological aspects of human AVL on the island as well as to study immunological reactivity to *Leishmania* in the healthy population of endemic areas. Margarita Island represents a particularly interesting focus for study, since no autochthonous cases of American cutaneous leishmaniasis or Chagas disease have been reported on the island, in contrast to the presence of these human trypanosomatid infections in areas where AVL has been reported on the Venezuelan mainland. Additionally, well-defined geographic and climatic features are prominently associated with disease distribution on the island.

### MATERIALS AND METHODS

**Patients** - Sixty-five well-documented cases of human AVL have been diagnosed on Margarita Island, Nueva Esparta State in the decade from 1990 to 1999. Patients with a presumptive diagnosis of AVL were hospitalized in the Luis Ortega Hospital in Porlamar, Nueva Esparta for confirmation of the diagnosis and treatment. Presumptive

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diagnosis was based on the clinical signs of prolonged fever, anemia and hepatosplenomegaly. Parasitological confirmation was based on the observation of amastigotes in Giemsa-stained bone marrow aspirates, particularly before 1998. More recently, strongly positive serological reactivity [optical density (OD) > 0.7 in ELISA reactions] with the highly specific rK39 antigen derived from *L. chagasi* by Reed (1996) has been used extensively to confirm infection, particularly to reduce traumatic intervention in infants. Patients with confirmed diagnosis are reported to the National Registry at the Institute of Biomedicine, Caracas. This data base has provided the information on incidence, age, and sex of these patients.

In March 1999, 23 patients who had completed treatment in previous years were evaluated by the following criteria: ELISA with rK39 antigen; delayed-type hypersensitivity to leishmanin, abdominal sonogram, routine hematology, and blood chemistry. The protocols for these studies in patients as well as studies in healthy individuals have been approved by the Ethical Committee of the Institute of Biomedicine and were carried out after informed consent of the individuals or the parents or legal guardians of children.

**Healthy individuals from endemic and inactive areas** - Skin tests with leishmanin were applied during the first four months of 1998 to 1,643 healthy individuals, 713 males and 930 females, from 417 households in four communities where cases of AVL have been diagnosed in the last decade. Further analysis of the data was carried out on two arbitrarily defined subgroups in which households were divided into those with fewer than three leishmanin-positive individuals (382 households with 1,443 individuals) and those with three or more individuals with positive leishmanin reactions (35 households with 200 individuals). Blood samples were randomly taken from 488 people from three of these communities for serological testing. A "control" group of 69 persons (21 households) from San Francisco, a town on the large peninsula of Macanao which forms the western part of Margarita Island, were also skin-tested. Since there is no clinical evidence of active transmission of AVL in Macanao at present, this group was included to evaluate leishmanin reactivity which might be attributed to persistence of *L. chagasi* in the area or the presence of other trypanosomatids. Blood samples were also taken from 21 dogs in this community for serological testing.

**Skin tests** - Leishmanin skin tests were performed in the group of 1,643 individuals as described previously (Convit et al. 1989) by the intradermal injection of 0.1 ml of an autoclaved preparation containing  $6.25 \times 10^6$  promastigotes of *L. pifanoi*/ml in the ventral surface of the forearm. A group of 141 individuals from one of the endemic communities studied here as well as the control group received a second simultaneous injection on the other forearm with leishmanin prepared as described with promastigotes of *L. donovani*, MHOM/IN/80/DD8. Induration at the skin-test sites was read at 48 h using the ballpoint pen technique described by Sokal (1975); reactions  $\geq 10$  mm were considered positive.

**Serological tests** - ELISA tests were carried out with as described previously (Zerpa et al. 2000). Briefly,

microtiter plates were sensitized overnight with  $1 \times 10^6$  formalin-inactivated *L. donovani* promastigotes or 40 ng rK39 antigen/well, blocked and treated with 1:300 or 1:100 test serum dilutions, respectively, followed by a 1:5000 dilution of anti-human IgGAM conjugated with horseradish peroxidase. Reactions were revealed with  $H_2O_2$  and o-phenylenediamine dihydrochloride and OD was read at 490 nm. Canine serum samples were tested with rK39 as described above, using a 1:2000 dilution of anti-dog IgG conjugated with peroxidase as the second antibody. Correlation between reactivity to rK39 and DD8 antigens was determined in 44 sera from patients with active or treated AVL. Based on previous studies in the laboratory, results with rK39 were grouped as follows: OD < 0.0690 negative; 0.0700-0.1000 doubtful; 0.1005-0.4000 weakly positive; 0.4005-0.7000 moderately positive and > 0.7000 strongly positive. The corresponding values with promastigotes were negative < 0.2000; doubtful, 0.2005-0.3000; weak, 0.3005-0.5000; moderate, 0.5005-0.8000 and strong > 0.8000.

**Statistical analysis** - Many of the data are descriptive, and average values  $\pm$  standard error of the mean (SEM) are indicated where relevant. Statistical significance was determined using the Student *t* test for unpaired samples, the Mann-Whitney test for nonparametric data, determination of correlation coefficient and one-way analysis of variance (ANOVA) where appropriate. GraphPad software, version 3.02 (InStat, San Diego, CA), was used for data analysis.

## RESULTS

**Patients** - As shown in Fig. 1, the incidence of AVL on Margarita Island has shown an irregular increase during the 1990-1999 decade. Prior to 1998, parasitological confirmation in bone marrow smears was carried out in 42 patients; in 1998-1999, strongly positive ELISA reactivity to rK39 (OD > 0.7) was used to confirm diagnosis (23 patients). Of these 65 well-documented cases, two fatalities were reported in 1998. The age and sex of these patients are presented in Fig. 2. As shown, 86.2% of the patients were  $\leq 3$  years old; only three were > 5 years of age.

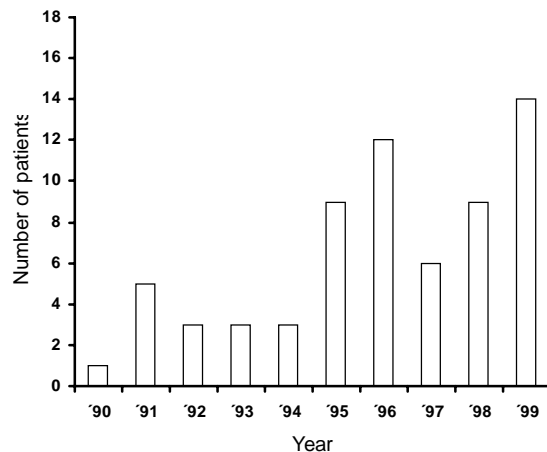


Fig. 1: incidence of human visceral leishmaniasis on Margarita Island, 1990-1999.

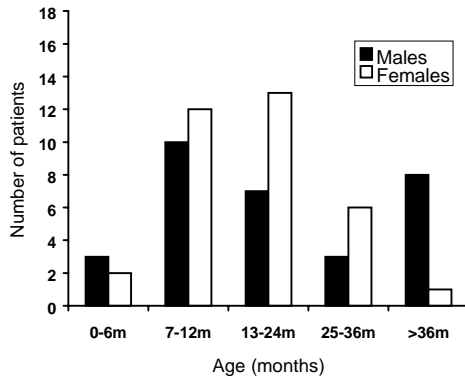


Fig. 2: age and sex of 65 patients diagnosed with American visceral leishmaniasis on Margarita Island, 1990-1999.

Of the 23 patients, 16 females and 7 males, who had been diagnosed and treated from 1990 to 1998 and re-evaluated in March 1999, 21 were  $\leq 2$  years old at the time of diagnosis, average age 12.7 months, and two were 4 years old. All were leishmanin-negative at diagnosis. At re-evaluation, 22/23 had become leishmanin-positive, average reaction  $18.2 \pm 1.06$  mm, range 7-29 mm. The single patient with a reaction less than 10 mm in diameter, diagnosed in 1996 when he was 4 years old, is clinically inactive but with persistent reactivity to rK39 antigen, OD 0.6430. Eighteen of the re-evaluated patients gave weak, doubtful or negative ELISA reactions using rK39 antigen, OD  $< 0.40$ . Three patients diagnosed in 1991, 1993 and 1996 were still moderately positive, average OD  $0.5482 \pm 0.0494$  and two, diagnosed in 1996 and 1998, were strongly positive, OD 0.7370 and 0.7120. Abnormalities in abdominal sonograms and in laboratory tests were not reported, and no clinical signs of persisting infection such as fever or weight loss were present.

*Healthy individuals from endemic and control areas* - As we reported previously, the distribution of AVL on Margarita Island is very strongly related to geographic features. Essentially all cases are limited to semi-urban communities on the western and southern slopes and dry plains which are separated from the east coast by a low north-to-southwest mountain chain. The frequency of positive leishmanin skin tests in 1,643 individuals, 713 males and 930 females, in four communities from the endemic area, as well as the control group from San Francisco, Macanao peninsula, is shown in Table I. Of the 332 positive reactors, 156 (47%) were males and 176 (53%) females. The overall percentage of positive reactors in the inactive control area, Macanao, was 20.3%, nearly identical to the endemic-area population, but a single individual of 38 tested who were  $\leq 15$  years old was positive in Macanao, compared to 15.4% of this age group from endemic communities. Of the 21 canine sera from the control area, one was weakly reactive with rK39 antigen, OD 0.1600, and the remainder were negative.

The frequency of positive skin reactions in 1643 individuals from endemic communities, tested early in 1998, increased from 12.7% in the youngest age group, 0-10

TABLE I  
Leishmanin reactivity in healthy individuals from endemic areas of visceral leishmaniasis in Margarita Island

Community	No. positive/ No. tested (%)	No. positive $\leq 15$ years/ No. $\leq 15$ years
Achípano I	108/420 (25.7%)	51/249 (20.5%)
Achípano II	37/141 (26.2%)	16/90 (17.8%)
La Vecindad	65/524 (12.4%)	22/270 (8.1%)
La Pista	122/558 (21.9%)	53/312 (17%)
Subtotal	332/1643 (20.2%)	142/921 (15.4%)
San Francisco (inactive area)	14/69 (20.3%)	1/38 (2.6%)

years, to 32.9% in individuals  $> 40$  years old. These data and the extrapolated prevalence/1000 are shown in Table II. Differences in the average sizes of positive leishmanin reactions among different age groups were not statistically significant,  $p = 0.1696$  (ANOVA).

In the subgroup of 35 households with three or more leishmanin-positive reactors, 66% of the 200 skin-tested individuals were leishmanin positive; 39.8% of all the positive reactors were detected in just 8.4% of the households. Each of the communities studied was formed by 50 to 200 or more small houses on individual plots of land rarely exceeding an area of 50 m<sup>2</sup>, and every household was studied. Simple maps showed the presence of clusters of three or four and occasionally as many as 10 neighboring houses with three or more positive reactors. The average age,  $14.1 \pm 1.1$  years and male/female ratio, 1:1.76, in households with three or more reactors were not significantly different from the rest of the population ( $16.8 \pm 0.7$  years; M/F ratio 1:1.47). Positive skin tests in these individuals from dwellings with three or more positive reactors averaged  $17.00 \pm 0.57$  mm, compared to positive reactions of  $14.57 \pm 0.39$  mm in households with fewer than three reactors ( $p < 0.0001$ , Mann-Whitney test).

Canine serology and skin tests in humans were not synchronized in the studies on the island, and the results in dogs have been published previously (Zerpa et al. 2000). These overlapping studies permitted the identification of six households with more than three leishmanin-positive individuals and the presence of a serologically positive dog (OD  $> 0.70$  with rK39); nine households with three or more reactors reported a previous case of human VL. In

TABLE II  
Leishmanin skin test reactivity in healthy individuals in different age groups in the endemic areas of American visceral leishmaniasis

Age group (years)	No. tested	No. of positive reactions	Prevalence/ 1000
0-10	703	89	127
11-20	362	86	238
21-30	214	54	252
31-40	161	53	329

an additional house, an inactive human case and a serologically positive dog were identified. Of 50 skin-tested healthy individuals in the nine households with a previous case of human AVL, 82% were leishmanin-positive. In contrast to these micro-foci of intense reactivity, the presence of serologically positive dogs was not associated with multiple human reactors or with reported human cases of AVL in most households.

When we simultaneously compared skin test reactivity using leishmanin prepared from *L. pifanoi* and *L. donovani* promastigotes in one of the Achípáno barrios, no significant differences were observed. The average reaction size in 142 individuals was  $7.3 \pm 0.6$  mm and  $6.9 \pm 0.6$  mm with leishmanin prepared with *L. pifanoi* and *L. donovani*, respectively; the average size of 38 positive reactions was  $15.4 \pm 1.2$  mm with *L. pifanoi* and  $14.65 \pm 1.3$  mm with *L. donovani*,  $p = 0.1968$  (*t* test). In the control area, San Francisco, positive reactions averaged  $11.0 \pm 0.4$  mm and  $11.0 \pm 0.3$  mm with *L. pifanoi* and *L. donovani* leishmanins, respectively.

To compare the reactivity of rK39 and DD8 promastigotes in human sera, 44 sera from patients with inactive ( $n = 9$ ) or active ( $n = 35$ ) disease were studied. The average OD with rK39,  $0.5211 \pm 0.0717$ , was not significantly different from the OD with DD8,  $0.4756 \pm 0.0487$  ( $p = 0.3197$ ). The correlation coefficient between the two was  $r = 0.7854$  ( $p < 0.0001$ ). These two antigens were used in ELISA tests to screen 488 serum samples from healthy individuals from endemic communities. Reactions with *L. donovani* promastigote antigen were weakly ( $n = 15$ ) and moderately ( $n = 3$ ) positive, average OD  $0.3951 \pm 0.0258$ , in 18 (3.7%) of the 488 blood samples taken from the healthy test group. When these were retested with rK39, only three (0.6%) gave positive reactions, average OD  $0.4315 \pm 0.1772$ . The leishmanin reactions in these three persons were 15, 17 and 0 mm. These three individuals showed no clinical evidence of active infection when tested nor in subsequent follow-up.

## DISCUSSION

The incidence of human AVL on Margarita Island has shown a modest increase in the last decade. The disease is most frequent in very young children, the case-fatality ratio of 3% is low, and previous studies of canine infection as well as identification of *L. infantum* zymodeme MON-1 in both an infected dog and a young girl clearly suggest important similarities between AVL on Margarita Island and the disease which occurs in the Mediterranean Basin of the Old World.

As reported many years ago (Pampiglione et al. 1975) and confirmed in other studies including this one, positive leishmanin reactions are much more frequent than clinical disease in endemic areas of visceral leishmaniasis. This suggests a high frequency of subclinical infection with little or no potential for developing active disease in healthy humans, particularly as age increases. This observation is usually attributed to low pathogenic potential of *L. chagasi* in persons with mature immune systems and competent immune reactivity, but other explanations should be considered. The leishmanin skin test reagent is a complex antigenic mixture and cross-reactivity

induced by infections with other Trypanosomatidae is widely recognized. We are unaware of any reports of cutaneous leishmaniasis, Chagas disease or other human infections caused by this group of micro-organisms on the Margarita Island. With a single exception, leishmanin skin tests were negative in children and adolescents on the Macanao peninsula, where the last known case of AVL was reported 15 years before. At present there are no data to suggest the induction of leishmanin-positive reactions by other Trypanosomatidae on Margarita Island; a literature search did not reveal investigations to detect trypanosomatids in other animals including mammals, reptiles, and insects, nor in plants.

Recent studies in a murine model with *L. major* have shown that components of saliva injected by the bites of uninfected sand flies induce protective immunity to subsequent infection by the parasite (Kamhawi et al. 2000, Valenzuela et al. 2001). The interesting possibility that this type of immunity might be involved in high rates of subclinical human infection remains to be explored. A preliminary study of the phlebotomine sand fly population by Felicciangeli et al. (1998) in three endemic communities on Margarita revealed very small numbers of *Lu. longipalpis*, but the frequency of other sand flies which might be involved in bite-induced immunity was not reported.

Positive serological reactions with rK39 antigen were rare in healthy individuals from endemic areas, but persisted in 5/23 (22%) of the clinically cured patients, all but one of whom had become leishmanin-positive. While this might suggest persistence of parasites in some patients, none have any clinical evidence of infection.

The low frequency of positive serology in healthy individuals from endemic areas appears to differ markedly from the situation in Brazil, where positive serology was frequent in family members and neighbors of patients with AVL (Badaró et al. 1986). The use of the specific rK39 antigen in many aspects of this study as well as the apparent absence of autochthonous infections in humans by other Trypanosomatidae on Margarita Island may be important variables in the two studies. Comparative studies of vector density and activity as well as different strains and hosts of *L. chagasi* in the Brazilian and Venezuelan foci would be of interest.

We observed multiple households with three or more individuals with positive leishmanin reactions within a very limited geographic region. Of these 35 households, 43% had a serologically positive dog or history of an earlier human AVL infection. Neighboring houses separated by less than 10 m often presented multiple leishmanin-positive individuals, suggesting a high level of transmission in micro-foci of infection. The presence of a previous case of human AVL was associated with leishmanin-positive reactivity in 82% of co-inhabitants of these houses, which supports the observations of Costa et al. (2000), that infected humans, particularly very young children, may be competent reservoirs of infection. These niches offer a valuable opportunity for further study of factors which influence disease transmission, as well as evaluation of targeted control measures.

The recent increase in co-infections with HIV and visceral leishmaniasis, particularly in Southern Europe (Alvar et al. 1997) is a cause for concern in all geographic areas where both infections occur. The Department of Epidemiologic Surveillance, Ministry of Health and Social Development, reported an average annual morbidity of 39 HIV infections and 15 cases of AIDS on Margarita in the period from 1996 to 2000. Mortality averaged 18 deaths/year in the period from 1997-1999. No HIV-visceral leishmaniasis co-infections have been reported on the island. Nevertheless, epidemiologic surveillance is clearly warranted. One of the measures which has been suggested to implement surveillance for co-infection is to establish baseline reactivity to leishmanin in HIV-infected individuals (Alvar et al. 1997).

While the incidence of human AVL on Margarita Island is relatively low, the apparent absence of other human infections caused by Trypanosomatidae, high frequency of canine infection and well-defined, restricted geographical distribution of the disease are factors which offer a unique opportunity for the study of other reservoirs, of phlebotomine vector activity or other forms of transmission and of targeted control measures.

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