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Eco-Epidemiological and Immunological Features of Localized Cutaneous Leishmaniasis in Southeastern Mexico: Thirty Years of Study

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Abstract

The Yucatan Peninsula is considered an important endemic area of localized cutaneous leishmaniasis (LCL) caused by *Leishmania (Leishmania) mexicana* and mainly the states of Campeche and Quintana Roo where 41.5% of all new cases in Mexico were reported in 2015. People were affected due to the lack of the resources for early diagnosis and treatment and although many aspects of the disease are known, control of LCL is absent in this region. Thus, better case detection and epidemiological surveillance are required. The presence of emerging focus and changes in the clinical form suggest the importance of continuing the eco-epidemiological studies, which could lead to the implementation of a sustainable control on the disease. In this review, we focus on the results of our multi-disciplinary studies carried out in the southeastern Mexico, including LCL burden, clinical aspects, causal agents, vectors, reservoirs and the host immune response to *Leishmania (L.) mexicana* infection.

Keywords: ecology, epidemiology, immune response, localized cutaneous leishmaniasis, southeastern Mexico

1. Introduction

American cutaneous leishmaniasis (ACL) is a vector-borne protozoan zoonotic disease widely spread in Latin America. At least 12 different *Leishmania* species cause ACL. The disease occurs

in countries from the United States to Argentina, except in Uruguay and Chile. ACL is an important health problem affecting the poorer population and is thus one of the most neglected diseases. Depending mainly on the *Leishmania* species and the host immune response, the spectrum of clinical forms includes localized cutaneous leishmaniasis (LCL), disseminated and diffuse cutaneous leishmaniasis (DCL) and mucocutaneous leishmaniasis (MCL). The former is the most commonly occurring disease with approximately 0.7–1.2 million new cases every year [1, 2].

In south-eastern Mexico, the Yucatan Peninsula is an important endemic area of LCL, locally known as the "chiclero´s (gum collectors) ulcer". The LCL was first described by Seidelin in 1912, who classified the agent as morphologically indistinguishable from *Leishmania tropica* [3]. Since then, the humid forest of the Yucatan Peninsula has been documented as an endemic focus of LCL [4–6].

The purpose of this chapter is to review the most relevant studies performed in the last 30 years in the Laboratory of Immunology of the Autonomous University of Yucatan. This research has covered the characterization of the "chiclero´s ulcer", its diagnosis and treatment, and the identification of risk factors as well as the *Leishmania* vectors and reservoir species that are important to be known in order to develop control strategies.

2. Epidemiology of LCL in southern Mexico

2.1. Incidence and prevalence

Leishmaniasis control is usually hampered by ignorance of the true incidence/prevalence of infection, thus underestimating human suffering and disability caused by the disease. After parasites are inoculated by a sand fly, the infection outcomes might be either an asymptomatic infection or a clinically manifested infection. Most studies done in Latin America with reference to ACL have been focused on incidence/prevalence of the disease (clinical infection).

In the first approach and after diagnostic tools were implemented, a total of 63 cases of LCL were recorded in the state of Campeche between 1982 and 1987. The most common clinical presentation was a chronic ulcerated lesion (with an evolution time longer than 10 years), located predominantly on the ear (39%). Single lesions were found in 49/63 (78%) cases affecting men working in the field [7].

Based on these data, a program for the study and surveillance of LCL in collaboration with health services from the state of Campeche was established. First of all, a study to determine the incidence of LCL was carried out in seven rural health communities of the state of Campeche from January to December, 1987. Montenegro skin test (MST) was carried out on a sample batch of 449 persons randomly selected from men aged 15–45 years. Risk factors including age (15–45 years old), sex (male) and exposure (working in the field) had been identified previously [8]. MST-positive response ranged from 24 to 90% among the communities studied. These wide-range results could reflect differences in endemicity of LCL in the state of Campeche. A total of 56 new LCL cases with both a positive parasitological diagnosis

(smear, isolation-culture and/or biopsy) and MST-positive response were recorded during 1987. In summary, an annual incidence rate of 0.5 per 1000 inhabitants was reported [9].

Asymptomatic infection is the term used to refer to those individuals living in endemic areas of LCL, exposed to sand fly bites, presenting a MST-positive response but without signs and symptoms of the disease. Based on the criteria given above, a study to determine the prevalence of asymptomatic infection was performed in four rural communities from Campeche. From January to December 1999, a total of 22/116 (18.9%) men of 15–45 years of age and working in the field showed a MST-positive response in the absence of signs and symptoms [10]. Asymptomatic infection by *Leishmania* is the most common outcome after parasite inoculation. It must be highlighted that studies of the biological, immunological and epidemiological significances of the asymptomatic infection have been neglected in Latin America. Therefore, this important challenge should be addressed.

In the state of Yucatan, cases of LCL were restricted to villages located in the South, near to the characterized endemic areas from Campeche and Quintana Roo. Recently, a new outbreak of LCL was reported in the municipality of Tinum, Yucatán. This village is located in the West of the state and no cases had been reported before. In 2015, 17 new cases were recorded by the health services of the Yucatan State in comparison with the only case reported in 2014. From those cases, 11 were from Tinum. This increased incidence is alarming and suggests possible changes in the epidemiological patterns of leishmaniasis in the Yucatan Peninsula that need to be studied [11].

2.2. Clinical picture

The clinical picture of LCL in the Yucatan Peninsula was characterized through a study performed between January 1990 and December 1995 [12]. A total of 683 patients with cutaneous lesions suggestive of LCL were examined. Parasite demonstration by smear, biopsy and/or isolation-culture was positive in 445 cases (65.1%). From these samples, *Leishmania (Leishmania) mexicana* was successfully isolated, cultured and identified by either isoenzyme characterization and/or monoclonal antibodies in 136/445 cases (30.5%). The LCL clinical picture was limited to these 136 cases in which *L. (L.) mexicana* was identified. Males (94.1%) between 10 and 40 years of age (85.3%) were mainly affected. The most common lesions were single (84.5%), rounded (52.6%), ulcerated (72.5%) and located on the ear (39.9%). A total of 72.8% cases detected were classified as acute with less than 3 months of evolution. Since in those years an active surveillance program for LCL was implemented in the state of Campeche most cases were acute. In summary, the clinical picture of LCL caused by *L. (L.) mexicana* is characterized by a commonly single, rounded, painless ulcerated lesion, without lymphangitis and/or adenopathy; with the absence of mucosal involvement, and when located on the ear (the most common location) tends to become chronic if left untreated, causing the destruction of the pinna and disfiguration.

The importance of the active surveillance program was highlighted by the observation of changes in the clinical form with time. The manifestation of LCL has evolved, during the last years, from the typical single, rounded, small and ulcerated lesion worldwide recognized as "benign" (**Figure 1A–C**), to nodular lesions with an increased diameter as well as the

appearance of multiple lesions (**Figure 1D–F**). Those findings are suggestive of changes in pathogenicity of the parasite that need to be studied.

Figure 1. Clinical spectrum of the LCL in southeastern Mexico. (**A**) Acute ulcerated lesion on ear; (**B**) typical small, rounded, ulcerated lesion in forearm; (**C**) chronic lesion on ear; (**D and E**) nodular infiltrated lesions; (**F**) multiple ulcerated lesions in forearm.

2.3. Histopathological picture

From the previous clinical study, 73 biopsies were taken to characterize the histopathology of LCL caused by *L. (L.) mexicana*. The histopathological picture observed varied widely impairing classification into a meaningful pattern. Magalhães classification [13] identified a total of five histopathological patterns: type I) exudative-cellular reaction due to infiltration of histiocytes, lymphocytes and plasma cells, without granuloma; type II) exudative-necrotic reaction, characterized by cellular infiltration, necrosis and no granulomatous response; type III) exudative and necrotic-granulomatous reaction (unorganized granuloma) corresponding to pattern described as chronic granulomatous inflammation with necrosis; type IV) exudative granulomatous reaction (unorganized granuloma) without necrosis characterized by the presence of an unorganized granulomatous reaction; type V) exudative tuberculoid reaction in which a typical tuberculoid granuloma (organized) is formed. According to those histopathological patterns, 28.7% of type III and 43% of type IV were most commonly found. Parasite identification was positive in 68.5% of the biopsies. The size of the lesion was directly correlated with the time of evolution; however, an inverse correlation between the lesion size and abundancy of amastigotes was detected [14]. Therefore, the histopathology of LCL caused by

L. (L.) mexicana as in other leishmaniasis is characterized by a chronic granulomatous inflammatory response to this obligated intracellular protozoan infecting the macrophages.

2.4. Treatment

An investigation on the response of LCL to treatment with pentavalent antimonials (Sb5$^+$) was carried out between January 1990 and December 1994 [15]. This study was not designed to be a controlled clinical trial, but rather to evaluate the response of the chiclero´s ulcer to treatment with meglumine antimoniate. Patient eligibility for the study included a confirmed diagnosis of acute LCL (time of evolution lesser than 12 months) based on both clinical diagnosis and parasite visualization by smear, biopsy and/or isolation-culture, as well as no previous treatment with any antileishmanial drug, absence of any serious concomitant disease, and to be available for a 12-month follow-up. In all the 105 cases presented, at the end of the treatment, a complete re-epithelialization of all lesions occurred without both residual erythema and relapse during a 1-year monthly follow-up. The mean number of injections required for complete re-epithelialization of chiclero's ulcer was 25.1 (range = 5– 60), with a daily dose of one ampule. Since then LCL caused by *L. (L.) mexicana* in the Yucatan Peninsula has been successfully treated with a daily dose of meglumine antimoniate for 20 days, average 10 mg/kg/day. The dose seems lower dose compared to international recommendation of 20 mg/kg/day but it is effective. Moreover, the lack of report on resistance to the treatment is important to point out.

3. Ecology of the endemic area

The Yucatan Peninsula is a discrete biotic province of approximately 143,500 km^2. The region is a broad, flat shelf of marine limestone of geologically recent formation (Paleocene to Recent). The peninsula includes the states of Yucatan, Campeche, Quintana Roo and a portion of Tabasco east of the Rio Usumacinta and north of the Sierra del Norte de Chiapas. The peninsula is surrounded on three sides by water and bounded on the south by highlands that isolate this region from the rest of Central America. An interesting observation is that because of its geographic isolation, the Yucatan Peninsula is an area of mammalian endemism with fauna that differs markedly from the rest of Mexico. The climate is subtropical with a relative humidity of 80%, an unpredictable rainy season (annual rainfall over 1401 mm) mostly during the summer, and an average temperature of $27 \pm 5°C$ [16].

3.1. Parasites

Epidemiological studies and molecular characterization of the New World leishmaniases have revealed that the genus *Leishmania* Ross, 1903 (Protozoa: Trypanosomatidae) is by far much more complex than originally thought. The genus is comprised of 30 species infecting a wide variety of mammalian hosts (wild or domestic) and vectors. Each of the New World species of *Leishmania* has unique ecological and geographical distributions [17]. From an epidemiological point of view and disease-control stand-point, to know whether an organism, causing the

disease in a given area, is of the same species as that found in suspected mammalian reservoirs and insect vectors, is very important [18].

Based on both the clinical and epidemiological features of the disease, as well as on the biological characteristics of the parasite in laboratory animals [19–21], *L. (L.) mexicana* Biagi, 1953 emend. Garnham, 1962, was considered the main agent of LCL in the Yucatan Peninsula. Nevertheless, its characterization at the genus and sub-genus level was not done in those studies.

Therefore, from January 1990 to July 1992, 153 patients with LCL determined by both clinical diagnosis and parasite visualization (smear, biopsy and/or isolation-culture) were studied. All of them were infected in the state of Campeche. Parasite isolation by needle aspirates taken from the edge of the lesions was positive in 49%. Isolates were characterized by isoenzyme markers (glucose phosphate isomerase, mannose phospate isomerase, nucleoside hydrolase, phosphoglucomutase, 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase). Seventy (93.3%) were identified as *L. (L.) mexicana* Biagi, 1953 emend. Garham, 1962 and 5 (6.7%) as *Leishmania (Viannia) braziliensis* Viannia 1911 emend. Matta, 1916 [22].

Later on, a study to identify *Leishmania* parasites isolated from humans and wild rodents from the state of Campeche, using IFA with Mabs was carried out. The main purpose was to determine if the parasites of both types of hosts were of the same biotype. All the isolates obtained from wild rodents reacted with monoclonal antibodies M7 and M8 and were thus identified as *L. (L.) mexicana*. No differences in reactivity patterns were found among the different strains of *L. (L.) mexicana* from humans and wild rodents [22].

Finally, to assure that *L. (L.) mexicana* is the main agent causing LCL in the state of Campeche, a study leading to the identification by PCR of *L. (L.) mexicana* in the potential vectors *Lutzomyia olmeca* and *Lutzomyia cruciata* was performed [23].

3.2. Vectors

Phlebotomine sand flies (Diptera: Psychodidae: Phlebotominae) are insects of medical and veterinary importance since they are involved in the transmission of diverse pathogens [24]. The blood-feeding females are usually considered as the only natural vectors of protozoan *Leishmania* species. Among the phlebotomine sand flies recorded in the New World, 56 species, all belonging to the genus *Lutzomyia*, are involved in the transmission of the *Leishmania* spp. [25].

To determine the transmission of leishmaniasis in south-eastern Mexico, vectors were captured near La Libertad, municipality of Escárcega, Campeche (<200 m) and in a medium-sized sub-perennial forest located 8 km southeast of this village. Near La Libertad, nine sand flies species were collected using CDC light traps, a Shannon trap and a mouth aspirator; the most abundant species were *Lutzomyia deleoni* (61.3%) and *Lu. cruciata* (13.7%). The highest number of sand flies was obtained with CDC traps (46.5%) followed by direct searching in natural shelters (37.0%) and in the Shannon trap (16.5%). The highest peak of abundance of *Lu. cruciata* was obtained in March. The population peak of *Lu. cruciata* was related to the low temperature (21–22°C), high levels of humidity (≥80%) and low rainfall. The anthropophilic species like

Lu.cruciata, Lu. ovallesi, Lu. panamensis, Lu. shannoni had the lowest densities around the village. In contrast, at 8 km southeast of La Libertad in the forest, 16 species were caught using CDC light traps and a Shannon trap. The most abundant species were *Lu. olmeca* (21.7%), *Lu. cruciata* (19.2%), and *Lu. ovallesi* (14.1%). *Lu. olmeca* had the highest leishmania infection rate of all sand flies collected in the forest. In both study areas, more females than males were captured. The low densities of anthropophilic sand flies species captured near the village and the abundance of *Lu. olmeca* females (zoophilic species) suggested the sylvatic transmission of *Leishmania* in the Yucatan peninsula [26, 27].

Further entomological studies were carried out at 150 km, east of La Libertad in the village of La Guadalupe and the nearby village of Dos Naciones, municipality of Calakmul, Campeche. Using Shannon traps, Disney traps and CDC light traps, 15 sand fly species (*Brumptomyia* and *Lutzomyia*) were caught. In both study locations, the number of sand fly species caught was very similar but the predominant species differed. In the Dos Naciones village, *Lu. panamensis* (1682), *Lu. ovallesi* (504), *Lu. cruciata* (332), and *Lu. olmeca* (329) had the highest abundance; while in La Guadalupe *Lu. cruciata* (754) and *Lu. olmeca* (244) were most abundant. In both locations, the numbers of sand flies attracted to Shannon traps peaked between 18.00 and 22.00 hours. Given the abundance *of Lu. olmeca* in the collections made with Shannon and Disney traps (it was the only species caught in the latter), this species was confirmed as the primary vector of *L. (L.) mexicana* in Calakmul county. Dos Naciones and La Guadalupe differ in terms of vegetation structure, with much more severe and extensive deforestation around La Guadalupe than around Dos Naciones. Destruction of habitats by humans has led to the decrease in sand fly diversity and abundance. However, some species of medical importance have adapted to deforested habitats becoming closely associated with human settlements. Moreover, there is evidence that the deforestation could increase domestic transmission of *Leishmania* parasites [28].

Although the abundance of sand fly vectors was determined in three foci, the species of *Leishmania* in vectors had yet to be determined. Using the PCR method, kDNA of the parasite was amplified from sand flies collected in the forest. Because only two species of *Leishmania* have been reported in the Yucatan Peninsula, specific primer for *Leishmania* genus, namely 13 A (GTGGGGGAGGGGCGTTCT) and 13 B (ATTTTACACCAACCCCCAGTT), B-4 (TCGTACTCCCCGACATGCCTC) for the subgenus *Viannia*, and M1.1 (CCAGTTTC-GACCGCCGGAGC) for the subgenus *Leishmania* were used. Both *Lu. olmeca* and *Lu. cruciata* were found infected by *L. (L.) mexicana* [23].

3.3. Reservoirs

Leishmaniases is a complex of zoonotic diseases, which are infections transmitted from animals to humans. Identifying a reservoir of such zoonosis requires extensive ecological, entomological, mammalian, parasitological and epidemiological studies. The World Health Organization enumerated five criteria to incriminate a primary reservoir [17]. Thus, a step-by-step method is needed to investigate a primary reservoir of a zoonosis.

The first step is to identify animals that harbour the parasite. The first attempt to find leishmanial hosts close to the Yucatan Peninsula was carried out in Belize in the early 1960s

[29–31]. However, the south of Belize has to be considered as a different endemic area of the disease since this area is not part of the Biotic Province of the Peninsula of Yucatán [32]. In the Yucatan Peninsula, *L. (L.) mexicana* was identified by PCR in the base of tails of two heteromid rodents *Heteromys desmarestianus* Desmarest, 1817 and *Heteromys gaumeri* J.A. Allen and Chapman, 1897; and five cricetid rodents *Ototylomys phyllotis* Merriam, 1901, *Peromyscus yucatanicus* J.A. Allen and Chapman, 1897, *Oryzomys melanotis* Thomas 1893, *Sigmodon hispidus* Say and Ord, 1825, *Reithrodontomys gracilis* J.A. Allen and Chapman, 1897; and one marsupial *Marmosa mexicana* Merriam, 1897 [18, 33, 34]. Thus, many small terrestrial mammals of the Peninsula seemed to be able to harbour the parasite. However, the sand-fly vector is absent from the driest and unstable habitats such as corn- and bean-fields [27]. Due to *Sigmodon*'s preference for those disturbed areas, this species is not a potential reservoir of *L. (L.) mexicana* in the Yucatan Peninsula. The endemic area of *L. (L.) mexicana* is limited to the humid medium-size forest (height < 12 m), which characterizes southern Campeche and Quintana Roo states and the low-stature forest (height < 8 m) from eastern and southern Yucatan state and northern Campeche. This area occupies 19,839 km^2 [16].

The second step to identify a primary reservoir is that the species, which is relatively abundant in the focus to provide a food source for sand flies. In southern Campeche, *H. gaumeri* was the most abundant rodent in four of the five studied foci (total 46%), followed by *O. phyllotis* (19%) and *P. yucatanicus* (13%). Hence, the least abundant species such as *H. desmarestianus, Oryzomys* and *Reithrodontomys* as well as the marsupial *Marmosa* cannot be primary reservoirs of *L. (L.) mexicana* in the Yucatan Peninsula [34]. Because of the geographic isolation of the Yucatan Peninsula, two species of rodents exist only in the Yucatán Peninsula, the Gaumer's spiny pocket-mouse, *H. gaumeri* and the Yucatán deer-mouse, *P. yucatanicus*; two of the three potential reservoirs of *L. (L.) mexicana* in the area [32].

In the Yucatan Peninsula, a well-defined transmission season has been demonstrated, which is limited to the coolest months of the year, from November to March [35]. Thus, an important step to demonstrate a primary reservoir in an area of seasonal transmission is that the individuals of the reservoir species survive the infection and keep the parasite until the next transmission season, which is more than 7 months. In the field, *S. hispidus* and *O. melanotis* do not live for more than 6 months while *Heteromys* spp., *O. phyllotis* and *P. yucatanicus* can live for more than 2 years [34]. However, in order to survive such a long time, the course of infection has to be relatively non-pathogenic. In other words, the immune system of the reservoir must react to the presence of the parasite in such a way that while preventing it from doing any irrevocable damage, the parasite is not eliminated. The infection by *L. (L.) mexicana* in small rodents is mild and painless lesions situated at a non-vital part of the animal, the tail, and thus do not weaken their survival in the wild [33]. Subclinical infections, detected by PCR in the absence of visible lesion, are common [34]. Moreover, the existence of cryptic parasites in *P. yucatanicus* during the warmest months and the presence of a low-temperature trigger of clinical infection placed the Yucatán deer-mice in the best position as a primary reservoir of *L. (L.) mexicana* [36]. This type of infection either asymptomatic or with a mild pathology results from an ancient parasitic association in a well-balanced host-parasite relationship [37].

The next step to implicate a primary reservoir is that the proportion of animals infected is high enough (20%) to infect the vector during the transmission season (OMS 1984). In the state of Campeche, the seasonal prevalence of infection of *H. gaumeri* range from 88% ($n = 32$) to 29% ($n = 7$), *O. phyllotis* from 100% ($n = 33$) to 27% ($n = 17$), and *P. yucatanicus* from 18% ($n = 17$) to 50% ($n = 14$). Those alarming prevalence in the municipality of Calakmul, Campeche, indicate the need to study each focus individually in order to assess their consequences on human health [34].

The last step to identify the primary reservoir is that the species of parasite is identical in all hosts (reservoir, vector and human) thus, the geographic and temporal distributions of humans and the transient micro-habitat of reservoir and vector need to overlap. Leishmaniasis existed first among wild animals and sand flies and the forest was not very populated by humans. However, with the human displacements due to overpopulation of some areas, new settlements appear constantly deep in the forest. A deforested ring around village limits the contact with the reservoir species and vectors. However, due to the Mayan slash-and-burn agriculture human enters deep into the forest exposing themselves to the bites of infected sand flies. Moreover, subsistence hunting takes place during the night mainly when the agricultural season is over [38]. Moreover, with the ecological protection of large forests, such as the Calakmul Biosphere Reserve in the State of Campeche, the incidence of wild zoonotic diseases might increase.

In conclusion, *O. phyllotis* and the two endemic rodents *H. gaumeri* and *P. yucatanicus* have been incriminated as *L. (L.) mexicana* reservoirs based on their geographic and temporal distributions and the overlap of reservoir, vector and human habitats. All three rodent species are long-lived and the course of the infection is relatively non-pathogenic. Human encroachment into wild areas increases their contact with the infected vector.

3.4. Seasonal transmission

To know the timing of the transmission cycle in each focus of leishmaniasis is very important because high-risk seasons might be restricted. Thus, intervention measures such as prevention through medical education could be conducted before the high-risk period. Moreover, epidemiological and ecological studies could be limited to that season and consequently the cost of research could be diminished.

Based on this rationale, all the results of previous research were analysed focusing on the timing of transmission of *L. (L.) mexicana* in the state of Campeche, Peninsula of Yucatan, Mexico [35]. The study included the timing of incidence of LCL in humans during 1993–1994, as well as the rate and time of infection in rodents and sand flies between February 1993 and March 1995. Rodents and sand flies were found infected between November and March, when men carried out their field activities and were exposed.

In summary, the median-size humid forests of the Yucatan Peninsula have ideal ecological conditions for *L. (L.) mexicana* transmission, particularly in the winter season when high humidity and low temperature, support the growth of sand fly population and trigger the appearance of lesion in the reservoir. This transmission cycle occurs when men enter the forest

for agriculture, hunting and gum collecting. Although the number of human cases of LCL reported each year has peaked from March to July, if the incubation period is considered, there is a strong correlation with the abundance, rates and timing of infection of both reservoirs and vectors (**Figure 2**). Based on these results, for the first time in the world, a seasonal transmission (from November to March) of LCL caused by *L. (L.) mexicana* in the sylvatic region of the state of Campeche was determined.
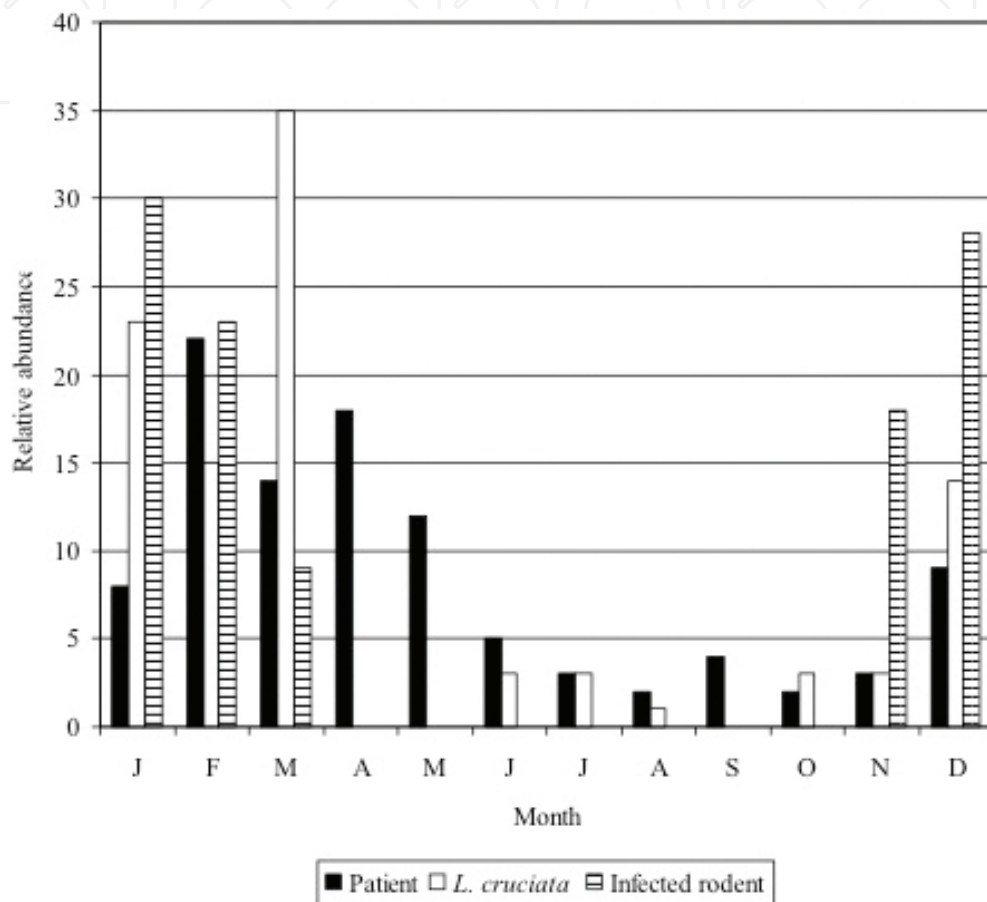

Figure 2. Monthly percentage of patients and rodents infected by *Leishmania (Leishmania) mexicana*, and relative abundance of *Lutzomyia cruciata* in a forest at 8 km from the village of La Libertad, Campeche, Mexico.

4. Immune response to *L. (L.) mexicana*

The skin is the first immune barrier against *Leishmania* promastigotes inoculated by the sand fly vector. The immune response against *Leishmania* spp. is highly complex. Macrophages are the main host cells of *Leishmania* and also responsible for parasite elimination. The activation of the macrophage microbicidal mechanisms depends on the cell-mediated immune response [39]. Thus, the nature and intensity of cellular immune response and their mediators (i.e. cytokines and chemokines) in the lesions are of primary importance for the disease outcome.

Based on this rationale, the characterization of the cytokine expression profile was studied in 13 LCL lesions caused by *L. (L.) mexicana* from the Yucatan Peninsula [40]. Age of the patient ranged from 10 to 29 years and the lesion evolution time from 8 days to 18 months. Lesions were classified as of early (≤2-month duration) and late (≥4-month duration) evolution. Skin biopsies were taken from the border of the lesion to analyse cytokine gene expression by RT-PCR. *Leishmania* amastigotes were present in 8 of the 11 histological sections. Intra-lesional cellular infiltrate was made up of equal proportion of macrophages and plasma cells. Lymphocytes represented 50% of the cellular infiltrate in three of four of the early lesion evolution but only in one of seven of the late-lesion evolution. The analysis of the *in situ* cytokine gene expressions revealed a concomitant presence of Th1 (IL-1α, IFN-γ, TNF-α) and Th2 (IL-6, IL-10, TGF-β) cytokines in all biopsies. The high expression of IFN-γ, cytokine related with macrophage activation, in both early and late evolution suggested that the presence of this cytokine was not sufficient for parasite elimination and control of the disease. A significant increase in IL-1α, TNF-α, Il-10, and TGF-β expressions was observed in late-lesion evolution compared with that in early lesions suggesting the role of these cytokines in the chronicity of LCL caused by *L. (L.) mexicana*. Both IL-10 and TGF-β down-regulate macrophage functions [41, 42]. Thus, the intra-lesional expressions of these cytokines could promote the persistence of the intracellular parasites in the skin. On the other hand, the presence of TNF-α in cutaneous lesions caused by New World *Leishmania* species has been related with lesion formation and loss of integrity of the infected tissue [43]. Further studies to confirm the role of TNF-α in the immunopathogenesis of LCL caused by *L. (L.) mexicana* are needed.

Another study of 20 LCL patients was carried out to analyse the role of IL-12 in the protective immune response to *L. (L.) mexicana* infection. The correlation of IL-12 with its counter-regulatory cytokine, IL-10, was also evaluated [44]. The patients were 10–48 years old and their lesions ranged from 10 days to 20 months of evolution. Cytokine expressions were evaluated by RT-PCR. Intra-lesional expression of both IL-10 and IL-12 was present in most of the 20 patients. The more chronic, non-healing lesions had higher levels of IL-12 mRNA indicating that the expression of this cytokine alone was not sufficient to induce healing. The IL-10 expression correlated with both IL-12 and IFN-γ, suggesting that IL-10 promotes disease persistence by a direct inhibition of macrophage activation rather than by suppression of the Th1 response.

Epidemiological studies detected many individuals from the endemic area of LCL without suggestive signs of the disease but with a delayed hypersensitivity skin test (DHT) positive to *Leishmania* antigens. Asymptomatic infection is the most common outcome to the infection in the Yucatan Peninsula [10, 45]. From the immunological point of view, asymptomatic infection is explained as the elicitation of an appropriate immune response capable of controlling parasite replication and maintaining tissue integrity [46]. Therefore, the characterization of this protective immune response in asymptomatic individuals becomes imperative for vaccine designs. Thus, the *in situ* cytokine (IL-4, IL-10, IL-12, IFN-γ) and chemokine (MCP-1, MIP-1α) mRNA expressions were analysed in biopsies of the DHT area of asymptomatic individuals ($n = 6$) and subjects with healed lesions ($n = 9$) and compared with biopsies from active lesions ($n = 11$) [47]. The expression was highly variable. Neither IL-4 nor MIP-1 α was

detected in any biopsy. IL-12 was detected in all three groups without significant differences in the median. MCP-1, chemokine that stimulates oxidative burst activity in macrophages thus killing intracellular amastigotes, was expressed in all three groups being significantly higher in active lesions. The most surprising finding was the absence of IFN-γ in both healed lesions and asymptomatic infection. Taken together, these results suggested that IL-12 and MCP-1 in the absence of IFN-γ might be playing a crucial role in the infection outcome at the skin level. Further studies are needed to identify the cytokine and chemokine network and their cell sources in asymptomatic infection. This knowledge is primordial to understand mechanisms involved in immune protection against *L. (L.) mexicana* and to develop better preventive and therapeutic strategies.

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