Cutaneous leishmaniasis in Bolivia. A study of 185 human cases from Alto Beni (La Paz Department), isolation and isoenzyme characterization of 26 strains of *Leishmania brasiliensis brasiliensis*

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Abstract

A clinical, serological, parasitological and therapeutic study of cutaneous leishmaniasis was carried out in a low sub-andean area (250-800 metres) of the La Paz Department, Bolivia. A team of seismic prospectors (350 workers) was surveyed for 12 months. Of 200 suspected cases of cutaneous leishmaniasis, 185 were serologically or parasitologically confirmed (incidence 52-8%). Those exposed to the greatest risk of infection were working in a virgin forest environment. Leishmanial organisms were isolated from 26 of the workers, either by *in vitro* cultivation or inoculation into hamsters. Isoenzyme characterization of the organisms by cellulose acetate electrophoresis showed them to be *Leishmania brasiliensis brasiliensis*. The results of treatment of 168 patients with a pentavalent antimonial drug are also reported.

Introduction

Detailed studies on cutaneous leishmaniasis (clinical, serological and epidemiological) have been performed in Bolivia only since 1973: in the Santa Cruz Department, Yapacani area (De Muynck et al., 1978; Recacochea, 1980) and in the La Paz Department (Yungas valleys joining the Amazonian slopes of the Andes Cordillera) Walton et al., 1973; Desjeux, 1974, 1976; Desjeux et al., 1974; Walton & Chinel, 1979). The lower sub-Andean region of Alto Beni, an eastern extension of Yungas, also proved to be an important focus for cutaneous leishmaniasis. The disease represents a serious public health problem because of the creation of settlement zones (with settlers coming from the altiplano highlands), the opening of new roads, the extension of gold and petroleum prospecting, etc.

In the present study, we performed a complete clinical, serological and parasitological survey of the prospecting teams of a petroleum company (350 workers) during one year, from August 1983 to August 1984, in the above-mentioned area. We found that *Leishmania brasiliensis brasiliensis* is the main etiological agent for cutaneous leishmaniasis in Alto Beni, and that the global rate of infection over one year is 52.8%.

Material and Methods

Geography of the studied area

The study area is located in the furthest sub-andean gully, orientated NW-SE, situated west of the village of Kurrenabaque (14°1’S, 63°35′W) (Fig. 1). The ombrothermal diagram of this station (Fig. 2) shows it to have a subtropical climate, with an annual rainfall of 2000 mm and a mean annual temperature of about 25°C. The whole area is covered with primary forest but includes a great variety of biotopes due to the uneven terrain.

From Quiquibay River to Alto Madidi in the north-east,

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straight prospecting paths 20 to 60 km long, lined with camps and heliports every 2-5 km, cross the area. Some prospecting also occurred on the eastern slopes of the Marimonos mountain in high Alto Madidi. The depression of the gully is located 200-300 meters above sea level, with bordering serranias reaching 800 to 1500 metres. Prospecting was carried out on straight paths, irrespective of the unevenness of the ground, which forced prospectors to cross all the existing biotopes: river banks, cliffs, mountain crests, bamboo bushes, marshes, water-logged forest, terra firma, etc.

Detection of patients in the field and clinical study

Two permanent Bolivian physicians were present in the main camp of the Company in the Alto Beni. They were able to contact by radio, every day, the teams working in the forest. Each team had a nurse (1 nurse to 20 workers). When a new suspected case of cutaneous leishmaniasis was noticed by the nurse, he was sent to the main camp with the physician’s agreement. Previous history, general data (age, sex) and clinical characteristics of the lesions (location, number, aspect) were recorded on a standard observation form.

SEROLOGICAL STUDY

This was carried out in the IBBA laboratory in La Paz for each clinically suspect patient, using an indirect immunofluorescence (IIF) test with *L. b. brasiliensis* antigen, according to the method of Guimaraes et al. (1974). The IIF test was standardized using 30 sera from Bolivian patients living in non-endemic areas (Alitiplano) and sera from 10 European people. Repeat tests were made just after finishing specific antimonial treatment.

Parasitological study

Parasite isolation and culture. Small tissue samples were removed by punch-biopsy from the indurated edges of the cutaneous lesions. After triturating in phosphate-buffered saline, the fragment was either cultured directly in modified NNN medium (Decker-Jackson & Honberg, 1978) or inoculated into the paws of a hamster (*Mesocricetus auratus*). Hamsters were examined weekly; if they became infected reisolation was attempted by incising the lesion with a lancet, aspirating with a Pasteur pipette and culturing on biphasic
3 times. The sediments were frozen at -80°C. Immediately before electrophoresis, an equal volume of hypotonic enzyme stabilizer was added (GODFREY & KILGOUR, 1976). The organisms were then lysed by freezing and thawing 3 times.

**Electrophoresis.** Electrophoretic methods on cellulose acetate were those of TIBAYRENC & LE RAY (1984), adapted from LAMHAM et al. (1981). Glutamate oxaloacetate transaminase (GOT) activity was detected by the method of KREUTZER et al. (1980), and HR Helena buffer (ionic strength 0.75) was used as the running buffer.

The following enzymes were used for biochemical characterization: malate dehydrogenase (E.C.1.1.1.37, MDH), malate dehydrogenase (oxaloacetate decarboxylating NADP+) (E.C.1.1.1.40, ME), isocitrate dehydrogenase (E.C.1.1.1.42, ICD), 6-phosphogluconate dehydrogenase (E.C.1.1.1.44, 6PGDH), glucose-6-phosphate dehydrogenase (E.C.1.1.1.49, G6PD), glutamate dehydrogenase (NAD+), glutamate oxaloacetate transaminase (E.C.2.5.1.1, GOT), phosphoglucomutase (E.C.2.7.5.1, PGM), aminopeptidase (substrate: L-leucyl-leucyl-alanine) (E.C.3.4.11.1, PEP 1), aconitate hydrolase (E.C.4.2.1.3, ACON), mannosephosphate isomerase (E.C.5.3.1.5, MPI) and glucosephosphate isomerase (E.C.5.3.1.9, GPI)

Six reference strains were used, named according to LAINGSON'S & SHAW'S (1972) classification for New World Leishmania (Table 1).

**Treatment**

Patients confirmed serologically (IIF test) or parasitologically (isolation of Leishmania) were treated by intramuscular injection of meglumine antimoniate (Glucantime), under a physician's control. The usual dose was 14 mg/Kg/day, followed by a pause of 10 days. The response to Glucantime treatment was followed clinically and serologically, and further ten-day courses being given if necessary to produce complete scarring. After finishing the treatment, patients were sent back to the forest, and then checked every 15 days by the permanent physicians, and once a month by IBBA investigators. Three patients who failed to respond to antimony were treated with Ketoconazole at 400 mg/day for 3 months.

**Table 1—Reference strains of Leishmania**

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Host</th>
<th>Origin</th>
<th>WHO Code</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. braziliensis brasiliensis</em></td>
<td><em>Homo sapiens</em></td>
<td>Brazil</td>
<td>MHOM/BR/75/M-2904</td>
</tr>
<tr>
<td><em>L. braziliensis guyanensis</em></td>
<td><em>Homo sapiens</em></td>
<td>Brazil</td>
<td>MHOM/BR/75/M-4147</td>
</tr>
<tr>
<td><em>L. braziliensis panamensis</em></td>
<td><em>Homo sapiens</em></td>
<td>Panama</td>
<td>MHOM/PZ/75/M-4037</td>
</tr>
<tr>
<td><em>L. mexicana amazonensis</em></td>
<td>Lutzomyia flaviscutellata</td>
<td>Brazil</td>
<td>IFIL/BR/67/PH-8</td>
</tr>
<tr>
<td><em>L. mexicana mexicana</em></td>
<td>Nyctomys sumichrasti</td>
<td>Belize</td>
<td>MNYC/BZ/62/M-379</td>
</tr>
<tr>
<td><em>L. mexicana pifanoi</em></td>
<td><em>Homo sapiens</em></td>
<td>Venezuela</td>
<td>MHOM/VE/77/L-20</td>
</tr>
</tbody>
</table>

**Table 2—Results of indirect immunofluorescence tests carried out before treatment**

<table>
<thead>
<tr>
<th>Title</th>
<th>Number of sera</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14</td>
<td>7.6</td>
</tr>
<tr>
<td>1/20</td>
<td>48</td>
<td>25.9</td>
</tr>
<tr>
<td>1/40</td>
<td>62</td>
<td>33.5</td>
</tr>
<tr>
<td>1/80</td>
<td>57</td>
<td>30.8</td>
</tr>
<tr>
<td>&gt;1/80</td>
<td>4</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>185</td>
<td></td>
</tr>
</tbody>
</table>

**Cultivation and preparation of material for electrophoresis.** 200 ml culture flasks were inoculated with growing cultures from 10 ml tubes. Late log-phase cultures were harvested by centrifugation at 2500 rpm, 4°C for 10 min, and then washed medium. Tubes incubated at 23°C were examined every week and discarded if they were negative after one month. If organisms developed, cultures were routinely subcultured each week and growth characteristics noted after each passage.

**Fig. 1.** Map of north-western region of Bolivia, showing the study area.

1 = western cordillera, 2 = altiplano, 3 = eastern cordillera, and 4 = sub-andean region. The area surveyed is cross-hatched.

**Fig. 2.** Ombrothermal diagram for Rurrenabaque. Precipitation curve, ------- Temperature curve.
Clinical study

350 employees of the oil prospecting company were surveyed prospectively for one year. 200 had lesions likely to be cutaneous leishmaniasis, of which 185 were confirmed parasitologically or serologically, an infection rate of 52.8% over one year. In most cases (168 = 90.8%), the cutaneous lesions were single (71.9%), more rarely double (18.9%).

The lesions were located as follows: lower limbs, 141 (52.4%), upper limbs, 64 (23.8%), trunk, 57 (21.2%) and head, 7 (2.6%). Most commonly, the lesions were extensive ulcers with a raised indurated erythematous border (181 cases 87.8%); exceptionally, there was a nodular infiltrative lesion (2 cases) or a verucose lesion with hyperkeratosis (2 cases).

Serological study

The humoral response was determined by IIF (Table 2).

Of 14 negative results in the first test, 10 had become positive at the second test, just after finishing antimonial treatment. The remaining 4, although still serologically negative, were confirmed parasitologically.

Parasitological study

Isolation of the parasite. We performed 72 biopsies, followed in 53 cases by direct inoculation into culture medium, and in 19 cases by inoculation into a hamster. We obtained 22 positive cultures (41.5%), and 13 hamsters developed lesions allowing isolation of the parasite (68.4%). Of these 35 isolations, 9 were later lost by contamination, allowing us finally to characterize 26 isolates.

Inoculation to the hamster. The 13 hamsters developed characteristic lesions at the inoculation sites, with local swelling and moderate inflammation with induration. However, the incubation period was usually very short: 2 to 3 weeks, with only a very few cases requiring 2 to 3 months. The evolution of the lesions was usually progressive regression of the swelling, and spontaneously cure; more rarely, the lesion simply stopped growing. We never observed metastases. Visceralization was not systematically looked for, but, in the few animals examined, parasites were found in the liver and spleen. CUBA CUBA et al. (1985) reported visceralization as a constant feature of L. b. brasilienis in hamsters.

Electrophoresis, isoenzymic characterization of strains

Electrophoresis revealed common patterns for 10 of the 13 enzymes studied, in all 26 strains, which were identical to those of the reference strain of L. b. brasilienis (MHOM/BR/75/M-2904). Some variation was observed in only 3 enzyme systems: MDH, ME and ICD.

Treatment

168 patients were treated with Glucantime. Usually 2 to 4 ten-day courses of treatment were necessary for clinical and parasitological cure. 3 patients interrupted their treatment. In 145 cases (87.9%), we achieved complete healing of the lesions. In 2 cases the parasite was re-isolated in culture after 4 ten-day courses, and after 5-5 ten-day courses, respectively, of Glucantime.

3 patients in whom antimonial treatment failed, and for whom there was no clinical nor serological improvement, were treated with Ketoconazole. This resulted in complete healing (clinically and serologically) for all 3. No hepatic toxicity was observed; nephrotoxicity was not looked for.

Discussion

The high rate of infection (52.8% over a year) indicates a situation with a high risk of transmission. The reasons for this include (1) the workers were operating in the forest (intrusion phenomenon); (2) during the day, they were lightly clad (wearing only shorts, short-sleeved shirts, and sandals or boots), due to the hot and humid climate; and (3) during the evening and night, the teams gathered in primitive camps, which were lighted, and were protected only with mosquito nets.

In these almost uninhabited areas, a sylvatic cycle of leishmaniasis transmission is usually occurring. During their continuous movements, the teams crossed a variety of biotypes, passing through more intense transmission zones (resulting in the appearance of numerous new cases), but it is difficult to determine exactly which parts of the ecosystem had the highest risk. We could not establish a correlation between intensity of transmission and climate, new cases being reported all year long, in both wet and dry seasons.

The predominance of lower limb localization (52.4%) is due to the mode of infection (AMUNARRIZ, 1982) and a consequence of professional activity (day bites of phlebotomine sandflies disturbed by the clearing of forest and the sawing of trees). Among the trunk lesions, 4 were encountered on the anus, pubes or perineum, these infections probably occurring during defaecation in the forest. In the same area, a new anthropophilic species of sandfly was recently observed: Phebotomus yucumensis LE PONT et al. (1986a), and two strains of L. b. brasiliensis indistinguishable from human strains from the same area were isolated from P. llanos-martini and from P. yucumensis, allowing us to conclude that these two sandfly species, considering their aggressive anthropophilic behaviour, are involved in the transmission of cutaneous leishmaniasis caused by L. b. brasiliensis in this lowland sub-andean region of Bolivia (LE PONT et al., 1986b).

The fact that 72% of the cases presented a single lesion is interesting, since LLanos-CUENTAS et al. (1984) and AGUILAR et al. (1984) reported prevalences of 68% and 66% respectively. It has been reported that Leishmania-infected sandflies probe more frequently than uninfected flies, thus increasing the chances of producing multiple lesions (BEACH et al., 1984; 1985), but this does not seem to occur in our study zone.

The predominance of ulcerative lesions (97.8%) is comparable to the results obtained by LLanos-CUENTAS et al. (1984) in the Tres Braços focus in Bahia (Brazil), where all strains have been identified as L. b. brasiliensis, and where 87% of the lesions were of an ulcerative crateriform type (3.5% being verucose and 4.5% indurative plaques). In Venezuela, AGUILAR et al. (1984) reported 98% of ulcers in a L. b. brasiliensis focus, 1% frambeuesoid and 1% verucose lesions.
The humoral response was rather low in most cases, probably because patients were seen early in their infection, when their lesions were still small, and were rapidly treated.

Reisolation of the parasite after inoculation into hamsters was much more successful than direct inoculation of culture medium. This was especially so under field conditions where the chances of bacterial and fungal contamination were extremely high. Inoculation of hamsters was previously proposed as an easy method for isolation of strains from human cases (Marsden & Nonata, 1975; Mayrnick et al., 1979), and Grimaldi et al. (1984) obtained a 90% yield when reisolating the parasite from the hamster between 5 and 7 days after inoculation.

The extrinsic characteristics of our strains differed from some of the usual criteria of _Leishmania_ classification (Lainson & Shaw, 1972). Whereas the slow multiplication in vitro and difficult adaptation to monophasic medium suggested the _L. braziliensis_ complex, the rapid growth in culture was in the harlequin hamster suggested otherwise: Cúba Cúba et al. (1985) reported, for _L. braziliensis_ strains in Tres Brácos, a delay of 175 days. A possible explanation could be the extreme virulence of the Bolivian strains, which was verified by clinical observations. When one of the authors was infected, within 2 weeks 3 distinct papules 1 cm apart had formed, which coalesced in one month to form a single ulcerative lesion.

Isoenzyme characterization showed that _L. b. braziliensis_ predominates in the low sub-andean region, widening its distribution to the entire andean foothills from 250 to 1800 meters. We have identified 20 other strains from the Yungas valleys and Alto Beni as _L. b. braziliensis_, with the same isoenzymic pattern. Up to now, no strain of the _L. mexicana_ complex has been isolated in this area of Bolivia.

The therapeutic study confirmed the efficiency of pentavalent antimonials (87-9% success rate) on cutaneous leishmaniasis with _L. b. braziliensis_. If a cure is not obtained after 4 or 5 series of 10-day treatment it seems useless to extend the treatment. Substitute therapy must be applied using Ketonconazole, although this requires strict observation of liver and kidney functions, hepatic toxicity being greater in patients previously treated by antimonials.

Our cure rate was comparable with the 89% reported by Llanos-Cuentas et al. (1984) in the _L. b. braziliensis_ focus of Tres Brácos.

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**Short Report**

Leishmaniasis in Burkina Faso

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During a field programme aimed at controlling the bacteriological quality of water in Burkina Faso (Monjou et al., 1986a, b), a rural endemic focus of cutaneous leishmaniasis was discovered at Koutougou, 40 km north of Aribinda, in the province of Soum. In this preliminary survey, 6 patients, 4 related to the Fulsé and 2 to the Peuhl ethnic groups, were found with lesions suggestive of leishmaniasis. 4 males and 2 females, aged 10-35 years, had cutaneous sores (growing papules in 3 cases, ulcerations with rolled indurated edges in 3) on exposed skin of the forearms and legs. The ulcerations had appeared less than 3 months before examination. In all patients, secondary infection was present and enlargement of the draining lymph nodes were found in 2. Due to the presence of endemic syphilis (bejel) in the region, one patient with 3 cutaneous sores was treated with long-acting penicillin. The other patients remained untreated.

In this village, where no sandflies could be found in January 1987 (cool nights, dry and windy season) and where oriental sore was previously unknown, a diagnosis of cutaneous leishmaniasis was proved in 2 patients (1 male, 1 female). Material aspirated with a fine hypodermic needle introduced through normal skin to the margin of the lesion (Vouldoukis et al., 1987) contained amastigotes. To our knowledge, these are the first cases of authenticated cutaneous leishmaniasis reported in Burkina Faso.

This study has been carried out in co-operation with the Ministry of Health, Republic of Burkina Faso.

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