LOCALIZED CUTANEOUS LEISHMANIASIS DUE TO *LEISHMANIA DONOVANI* AND *LEISHMANIA TROPICA*: PRELIMINARY FINDINGS OF THE STUDY OF 161 NEW CASES FROM A NEW ENDEMIC FOCUS IN HIMACHAL PRADESH, INDIA

NAND LAL SHARMA, VIJAY K. MAHAJAN, ANIL KANGA, ANURADHA SOOD, VISHWA M. KATOCH, ISABEL MAURICIO, CHAUHAN D. SINGH, UTTAM C. PARWAN, VIJAY K. SHARMA, AND RAMESH C. SHARMA

Departments of Dermatology, Microbiology, and Community Medicine, Indira Gandhi Medical College, Shimla, India; Central JALMA Institute of Leprosy and Other Mycobacterial Diseases, Agra, India; Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, United Kingdom

Abstract. Localized cutaneous leishmaniasis (LCL) in India is due mostly to *Leishmania tropica*. It is mainly endemic in the deserts of Rajasthan. Recently, Himachal Pradesh has been identified as a new endemic focus for the disease. In the last few years, the number of new cases has been increasing almost to epidemic proportions. This report presents the preliminary findings of clinico-epidemiologic and investigative results of 161 new localized cases of LCL seen between May 2001 and December 2003. The study population was composed of 80 males and 81 females between 10 months and 75 years of age. All were indigenous to the sub-alpine valley along the Satluj River in the mountainous region of the Kinnaur District (altitude = 700–2,900 meters). Most patients were seen from April to September and had 1–8 lesions (duration = 1–6 months) that involved mainly the face. Tissue smears were positive for amastigotes in 37% and histopathology showed non-caseating epithelioid cell granuloma in 77% of the cases. Analysis by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) of the ribosomal gene region of 10 biopsy specimens showed amplicons indistinguishable from *L. donovani* in eight cases and *L. tropica* in two cases. *Leishmania* was cultured on modified Nicole-Novy-McNeal (NNN) medium containing RPMI 1640 medium and heat-inactivated fetal bovine serum from 13 of 38 biopsy samples. Three of these isolated strains were identified as *L. donovani* while a fourth was *L. tropica* by PCR-RFLP of the ribosomal internal transcribed spacer region. One strain had a gp63 sequence identical to that of east African strains. Another strain had a unique gp63 sequence that has not been found in *L. donovani* complex strains. Sand flies trapped in the cattle sheds of a few patients were identified as *Phlebotomus longiductus* (Parrot 1928). Treatment with intraleisonal sodium stibogluconate was effective in all patients without any major side effects. One patient developed lupoid leishmaniasis that responded to a higher dose of sodium stibogluconate. Though rarely reported as a cause of LCL, *L. donovani* seems to be the predominant pathogen in this new focus of cutaneous leishmaniasis. *Phlebotomus longiductus* is a possible vector, albeit based on circumstantial evidence.

INTRODUCTION

Localized cutaneous leishmaniasis (LCL) is a vector-borne disease commonly caused by the flagellate parasites *Leishmania tropica* or *L. major* in the Old World. The human host of *L. major* is in most cases an accidental victim, with gerbils being the sylvatic reservoir. Infection with *L. tropica* is largely considered to be an anthropophilic disease, but may be transmitted from rodents or dogs.

Localized cutaneous leishmaniasis may occur in arid, rural areas, tropical forests, sub-alpine valleys, and urban environments, but is mainly endemic in tropical and subtropical regions. The geographic range of this disease is limited by a disease-endemic area. In northern India, cases have been reported from Calcutta, Calcutta, and the extreme northern part of the state of Uttar Pradesh (in the region of the Satluj River). Localized cutaneous leishmaniasis was not known to occur in this state prior to the late 1980s, but a few sporadic cases were detected between 1988 and 1995. We recently reported 36 new cases without any systemic involvement among the indigenous and migrant populations. However, the incidence of the disease is still increasing, and 161 additional cases of LCL without any systemic involvement have since been recorded. In this report, we present preliminary results of clinico-epidemiologic and investigative studies of these cases identified in this new focus.

MATERIALS AND METHODS

Disease cases. The medical records of the 161 new cases diagnosed with LCL between May 2001 and December 2003 were maintained in the Dermatology Department of Indira Gandhi Medical College (Shimla, Himachal Pradesh, India). Demographic features and disease profiles were recorded. The study was reviewed and approved by Institutional Ethical Committee.

The diagnosis of LCL was made mainly by clinical findings and confirmed by demonstration of amastigotes in Giemsa-stained tissue smears. Skin biopsy specimens were obtained (after obtaining informed consent) using aseptic precautions, and the specimens were divided into two portions. Tissue smears were prepared from one portion for histopathologic
**Biopsy samples from 10 patients were fixed in buffered formalin and processed at the Central JALMA Institute of Leprosy and Other Mycobacterial Diseases in Agra, India. Primers (P1 upstream: 5'-CTGGATCATTTTCCCAGTTGAT-TAC-3' and P2 downstream: 5'-ACACTACAGGTCTG-TAAAC-3') targeting a 1.1-kb fragment of ribosomal internal transcribed spacer (ITS) region of *Leishmania* were used for amplification of DNA fragments of 938–1109 basepairs. The PCR mixture was composed of 1× PCR buffer, (10 mM Tris HCl, [pH 9.0], 2.0 mM MgCl₂, 50 mM KCl, 0.01% gelatin), 10 mM dNTPs, 1 μM of each primer, and 2.5 units of *Taq* polymerase. Amplification was performed using the following steps: denaturation at 94°C for 5 minutes; 35 cycles at 94°C for 1 minute, 48°C for 1 minute, and 72°C for 2 minutes; and a final extension at 72°C for 5 minutes. The PCR amplicons were subjected to electrophoresis on 1.5% agarose gels for four hours. Amplicons were digested with *Hinfl* I (5 units/10 μL of ampiclon in a 30-μL reaction) and digested fragments were subjected to electrophoresis (3.8 V/cm for 5 hours) on 2% agarose gels.

**Culture and characterization of causative organisms.**Culture of *Leishmania* was attempted on biopsy specimens of cases reported between May and December 2003. The culture media used were 1) modified Nicole-Novy-McNeal (NNN) medium, 2) modified NNN medium plus RPMI 1640 medium and 10% heat inactivated fetal bovine serum (HIFBS), 3) modified NNN medium plus medium 199 and 10% HIFBS, 4) RPMI 1640 medium plus 30% HIFBS, and 5) Schneider’s *Drosophila* medium plus various concentration of HIFBS. Cultured organisms preserved in ethanol or DNA extracted from five isolates were sent to the London School of Hygiene and Tropical Medicine for identification and characterization. The ribosomal ITS was initially amplified with primers described by Cupolillo and others and PCR conditions used were those of Stothard and others. A second PCR with internal primers (ITS1F 5'-GCAGCTGGATCATTTTCCC-3' and ITS2R 5'-ACACTACAGGTCTG-TAAAC-3') and the initial PCR products was performed using the same conditions. The PCR products (15 μL) were digested with 2 units of *Bst* UI or *Cfo* I in a total volume of 20 μL and subjected to electrophoresis on a 2% agarose gel for 2 hours at 120V for species identification by comparison with reference strains. Strains were further characterized by direct sequencing of PCR products of the *gp63* gene and the glucose phosphate isomerase (GPI) gene (Mauricio I and others, unpublished data). Products from the first PCR or a nested PCR were sequenced in an ABI 3730 DNA analyzer with BigDye V3 (Applied Biosystems, Foster City, CA).

**Vector studies.**Sand fly collection was attempted in domestic and peridomestic areas with adhesive sheets and manual aspirators. The flies collected were sent to the Medical Entomology Division of the National Institute of Virology in Pune, India for identification.

**Treatment.**All patients were treated with intralesional sodium stibogluconate (SSG) injected on alternate days for (three injections) and repeated once a month until healing was complete. The parameters for a favorable therapeutic response were disappearance of induration and resolution of lesions. The patients were followed up once a month for clinical cure or repeat SSG therapy.

### RESULTS

**Geographic distribution and topography of the study area.**All patients lived in a sub-alpine valley along the Satluj River that leads to the mountain deserts of the tribal district of Kinnaur. The altitude ranges between 700 and 2,900 meters above sea level. The terrain of the Kinnaur District is dry and sandy with loose rocks. It shares its northeastern border with China. The average temperature at the district headquarters (Kalpa) is 1.5°C and 16.2°C, respectively, in the months of December and June. There is no rainfall from November to February, and rainfall ranges from 4.9 mm to 83.5 mm from February to October with an annual average of 28.1 mm. Snowfall ranges from 64 mm to 1,399 mm from October to April. For the last few years, major construction activities in the area have included telecommunication work, road construction, and hydroelectric projects.

**Demographic features.**Of the 161 LCL cases, 80 (49.7%) were males and 81 (50.3%) were females. The mean age was 26.4 years, with the oldest patient being a 75-year-old woman and the youngest a 10-month-old girl (Table 1). None of our patients reported having been out of the state or district during the three years preceding onset of cutaneous lesions. Most patients lived in a rural area and were engaged in cattle rearing and agricultural and horticultural activities. Where present, in most instances the cattle sheds were situated on the ground floor, with wooden planks separating them from the residential upper floor. The gaps between wooden planks were large enough for free movement of the vector. The number of new cases has been increasing: there were 36, 40, and 97 new cases, respectively, in 2001, 2002, and 2003. The majority (66%) were seen during the summer months.

**Time between onset and diagnosis.**In most (61.5%) cases, there was a 1–6-month interval (mean = 7.8 months) between onset of the lesion and the time when LCL was diagnosed (Table 2). Only four cases were diagnosed in less than one month since the time of onset.

**Number of lesions and body sites involved.**There were 254 (range = 1–8) lesions observed in 161 patients. Most (105) had a single lesion and 36 had 2 lesions. Only one patient each had 6 and 8 lesions and 18 patients had ≥3 lesions. The lesions were mostly nodulo-ulcerative plaques with or without crusting. Their distribution and the body sites involved are shown in Table 3. Exposed body parts, face, and neck were affected in all patients. Extremities, particularly the upper arm and sex distribution of patients with localized cutaneous leishmaniasis

<table>
<thead>
<tr>
<th>Age groups (years)</th>
<th>Males</th>
<th>Females</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>1–10</td>
<td>13</td>
<td>17</td>
<td>30</td>
</tr>
<tr>
<td>11–20</td>
<td>18</td>
<td>15</td>
<td>33</td>
</tr>
<tr>
<td>21–30</td>
<td>18</td>
<td>16</td>
<td>34</td>
</tr>
<tr>
<td>31–40</td>
<td>17</td>
<td>14</td>
<td>31</td>
</tr>
<tr>
<td>41–50</td>
<td>12</td>
<td>11</td>
<td>23</td>
</tr>
<tr>
<td>&gt;51</td>
<td>2</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>80</td>
<td>81</td>
<td>161</td>
</tr>
</tbody>
</table>
limbs, and the trunk had a total of 52 lesions. Of the facial lesions, 31 were mucocutaneous, mainly involving the lips or angle of mouth. None of our patients had systemic involvement.

**Tissue smears and histopathology.** Reports of tissue smears were available for 129 cases and amastigotes were visualized only in 48 (37%). Smear positivity was higher in recent lesions (duration of 1–6 months, Table 2) and in mucocutaneous lesions. Available histopathology reports of 48 patients (Table 4) showed non-caseating epithelioid cell granuloma in 37 (77%) patients. Only 12 (25%) patients demonstrated amastigotes in both direct tissue smears and histologic sections.

**Analysis of biopsy specimens by PCR.** Eight of 10 biopsy specimens subjected to PCR-RFLP analysis of the ribosomal gene region showed amplicons resembling *L. donovani* (Figure 1), while in other two, the amplicons resembled *L. tropica*. Amastigotes were identified in tissue smears of seven of these patients.

**Culture studies and characterization of Leishmania strains.** A *Leishmania* sp. could only be isolated in culture from 13 of the 38 biopsy specimens. Maximum success (12 of 36) was obtained in modified NNN medium containing RPMI 1640 medium and HIFBS for primary culture. We could not maintain these strains in any of these media. Five cultured/DNA specimens were sent to the London School of Hygiene and Tropical Medicine, but one sample spoiled in transit. Strain MHOM/IN/2003/NAV-131 did not produce a product after ITS amplification, but the DNA sequence of the nested PCR product of the GPI gene (AJ862829) was very similar to those of *L. tropica* isolates. The other three strains (MHOM/IN/2003/NAV-122, -132, and -135) were indistinguishable from the *L. donovani* reference strain when analyzed by the ITS PCR-RFLP. Strains MHOM/IN/2003/NAV-122 and MHOM/IN/2003/NAV-132 had a partial *gpl63* sequence (AJ864519 and AJ864520, respectively) that was more similar to those of *L. donovani* strains than *L. infantum* strains, but distinct from that of the common Indian zymodeme, MON-2, based on a National Center for Biotechnology Information (Bethesda, MD) BLAST search. The most similar sequences to that of NAV-122 were from *L. donovani* strains MHOM/SU/1984/Marz-Krim (AJ495007), MCAN/IQ/1977/Bumm3 (AJ495006), and MCAN/IQ/1981/Sukkar2 (AJ495005), with 99% identities of 1,187 nucleotides. Conversely, the most similar sequence to that of NAV 132 was that of MHOM/CN/0000/WangJie1 (AJ495004, 99% identity of 1,052 nucleotides). The *gpl63* gene could not be amplified from the MHOM/IN/2003/NAV-135 strain, but a partial GPI sequence (AJ862828, 1,237 nucleotides at the 3′ end) was indistinguishable from that of strain NAV-122 (AJ862827), very similar to those of *L. donovani* reference strain MHOM/ET/1967/HU3 (LV9) (AJ620639) and *L. infantum* MHOM/FR/1978/LEM75 (AJ620617), both with 99.92% identity, and less similar to that of MHOM/IN/0000/DEVI (AJ620621), MON-2, (99.51% identity).

**Vector studies.** Fifty-four adhesive traps were laid overnight in domestic and peri-domestic areas of 17 houses in 5 villages where patients lived. However, when checked, none of the adhesive traps contained any sand flies. Simultaneously, collection was also attempted by manual aspiration from houses at dusk. Seventeen female sand flies were caught with the aspirators in 5 cattle sheds. Eleven sand flies contained a visible blood meal and 10 were inoculated into culture media (NNN medium plus RPMI 1640 medium and HIFBS). However, all of these cultures became contaminated. Six sand flies were identified as *P. longiductus* (Parrot 1928) using the criteria of mouth parts and genitalia.

**Treatment.** Intral esional infiltration of 1–5 mL (100 mg/mL) of SSG was given to all patients on alternate days for three days once a month, and resulted in complete healing by the end of second month in most cases. A larger dose and ≥3 schedules were needed for multiple or larger lesions. One female patient treated with this schedule developed multiple, discrete, granulomatous lupoid lesions around the scar of healed primary lesion two months later. She was re-treated with intramuscular SSG, in addition to intral esional SSG. The total dose of 800 mg/day was divided into two parts and given for five consecutive days every month for two months. She showed complete resolution of lesions after three months and is being followed up. She lives in another valley along the Beas River, which is slightly about 50 kms. from the delineated endemic focus.

**DISCUSSION**

The fact that all our patients had contracted the disease indigenously is suggestive of a local vector and probably a...
Histopathological features

<table>
<thead>
<tr>
<th>Chronic granulomatous inflammation</th>
<th>Number of cases</th>
<th>LD positive by histopathology</th>
<th>LD positive in tissue smears</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelioid cell granuloma with Langhans’ giant cells, lymphplasmacytic infiltrate, histiocytic infiltrate present in a few cases, and no caseation necrosis</td>
<td>37 (77%)</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>Chronic non-specific inflammation Dense lymphplasmacytic infiltrate, occasional eosinophils present, and a non-granulomatous reaction</td>
<td>7 (14.5%)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Lymphohistiocytic infiltrate without granuloma formation</td>
<td>4 (8.3%)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>48</td>
<td>12</td>
<td>18</td>
</tr>
</tbody>
</table>

*a* LD = Leishmania donovani bodies.
gotes in histologic sections of six tissue smear-positive cases is similar to the observation of Bahamdam and others. They also postulated that Leishmania organisms appear larger in direct tissue smears and are scarce in samples for histopathologic analysis due to repeated processing of biopsy specimens with dehydrating solutions.

The strains of Leishmania isolated in this study grew best on a biphasic modified NNN medium with RPMI 1640 medium and 10% HIFBS as liquid phase. Different strains of the protozoa probably have specific nutritional requirements that require selective culture media. This is also evident because we failed to subculture and maintain these strains in any of the media used.

We have identified three of the four cultured strains causing LCL in this region as L. donovani and eight more as L. donovani from biopsy samples by PCR-RFLP in two independent laboratories. Previously, it was thought that only L. tropica was responsible for LCL, but reports have now appeared of isolated cases due to L. donovani from Kenya, Iraq, and Sri Lanka. The newly identified strains of L. donovani that cause LCL have sequences in their GPI and gp63 genes that are distinct from those of MON-2 strains that commonly cause visceral leishmaniasis in the plains of India. However, these areas are in eastern part of India and not related to the geographic areas studied. Instead, some of the studied strains had sequences similar to those of other L. donovani genetic groups and others had unique sequences. The study of more strains is required to establish the exact phylogenetic origin of this focus.

The large number of cases caused by L. donovani suggests that this is the predominant pathogen of LCL in the study area. However, the presence of L. tropica makes it a mixed focus. Recognition of the mixed nature of this focus is important when considering treatment and control measures because two different parasite species have distinct biologic properties and epidemiologic features.

Although we have yet to establish the identity of the vector, all circumstantial evidence suggests it to be P. longiductus. It would be premature to speculate on the life cycle of this Leishmania sp. unless additional vector and animal studies are carried out. We do believe that some animal reservoir exists in the region because the focus is new, the strains are different from those already known to exist in India, and there is a definite intrusion into the forests by humans for road construction and hydroelectric projects. However, this is purely conjectural and needs to be investigated. Furthermore, the epidemic seems to have spread rapidly downstream along the Satluj River in recent years.

Systemic use of pentavalent antimony compounds for treating LCL often results in toxicity due to a need for higher doses. However, intralesional SSG therapy has been recommended by the World Health Organization, and in our patients has been consistently effective without any major side effects except for painful injections.

Financial support: The study was supported by a grant provided by the Tribal Development Department of the Government of Himachal Pradesh. The American Committee on Clinical Tropical Medicine and Travelers’ Health (ACCTMTH) assisted with publication expenses.

Authors’ addresses: Nand Lal Sharma, Vikram K. Mahajan, and Ramesh C. Sharma, Departments of Dermatology, Venereology, and Leprosy, Indira Gandhi Medical College, Shimla, India, Telephone: 91-177-288-3404, Fax: 91-177-265-8339, E-mail: nandlals@hotmail.com. Anil Kanga, Anuradha Sood, and Vijay K. Sharma, Department of Microbiology, Indira Gandhi Medical College, Shimla, India. Uttam C. Parwan, Department of Community Medicine, Indira Gandhi Medical College, Shimla, India, Vishwa M. Katoch and Chauhan D. Singh, Central JALMA Institute of Leprosy and Other Mycobacterial Diseases, Agra, India. Isabel Mauricio, Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London WC1E 7HT, United Kingdom.

Reprint requests: Nand Lal Sharma, Department of Dermatology, Indira Gandhi Medical College, Shimla 171001, Himachal Pradesh, India, Telephone: 91-177-288-3404, Fax: 91-177-265-8339, E-mail: nandlals@hotmail.com.

REFERENCES


Received May 17, 2004. Accepted for publication November 21, 2004.

Acknowledgments: We thank Dr. S. M. P. Khurana and Dr. I. D. Garg (Central Potato Research Institute, Shimla, India) for their support and help in the DNA extraction procedures. We also thank Dr. Dharmendra Singh (JALMA Institute of Leprosy and Other Mycobacterial Diseases) and Dr. P. V. M. Mahadev (National Institute of Virology, Pune, India) for their technical help in this study.

CUTANEOUS LEISHMANIASIS IN HIMACHAL PRADESH, INDIA 823