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Identification and biochemical characterization of *Leishmania* strains isolated in Peru, Mexico, and Spain

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Abstract

Eight *Leishmania* promastigotes were isolated from different geographical areas: three (LP1, LP2, and LP3) from the provincial department La Libertad and the fourth (LP4) from the department of Cajamarca (northern Peru); another three (LM1, LM2, and LM3) in the province of Campeche (Mexico); and the last (LS1) from a clinical case of a dog in Madrid (Spain). The isolates were characterized by carbohydrate cell-surface residues using agglutinations with four purified lectins, by isoenzyme analysis using different isoenzymes, by analysis of kinetoplast DNA (kDNA) restriction fragment length polymorphism using four different restriction endonucleases and by the final metabolite patterns after in vitro culture. These isolates were compared with four reference strains and typified as: *Leishmania* (*Leishmania*) *donovani*, two strains of *L.* (*L.*) *infantum*, and one species of *L.* (*Viania*) *peruviana*. According to our results and the statistical study, the Peruvian isolates represent three different strains: one would be *L.* (*V.*) *peruviana*, another the strain isolated in Cajamarca (LP4) and the third would include the three strains from the department of La Libertad (LP1, LP2, and LP3), these latter three isolates being phylogenetically closer to the reference strain *L.* (*L.*) *donovani*. Meanwhile, the three isolates from Mexico form a group with close phylogenetic relationships to each other. The isolate from Spain belongs to the species *L.* (*L.*) *infantum*. Thus, a close correlation was drawn between the identity of each strain and its geographical origin.

Index Descriptors and Abbreviations: *Leishmania* species; In vitro culture; Lectin agglutination: Con A, lectin from *Canavalia ensiformis*; VV, lectin from *Vicia villosa*; WGA, lectin from *Triticum vulgaris*; PNA, lectin from *Arachis hypogae*; Isoenzyme electrophoresis: ME, malic enzyme [EC 1.1.1.40]; MDH, malate dehydrogenase [EC 1.1.1.37]; IDH, isocitrate dehydrogenase [EC 1.1.1.42]; GPI, glucose phosphate isomerase [EC 5.3.1.9]; PGM, phosphoglucomutase [EC 2.7.5.1]; SOD, superoxide dismutase [EC 1.15.1.1]; kDNA restriction pattern: kinetoplast deoxyribonucleic acid; ^{1}H NMR, proton nuclear magnetic resonance spectroscopy; MEM, minimal essential medium; EDTA, ethylenediaminetetraacetic acid; NaCl, sodium chloride; DNA, deoxyribonucleic acid

1. Introduction

Human leishmaniasis, caused by protozoan parasites of the genus *Leishmania*, constitutes a serious public health problem in several countries, according to the World Health Organization (W.H.O., 1997). In human hosts, the clinical profile of different *Leishmania* species can vary from a single cutaneous lesion, which may undergo spontaneous cure, to mucocutaneous lesions that can become grossly disfiguring. Severe diffuse cutaneous lesions, that is, extremely difficult to treat, can also occur. Moreover, the disease can evolve to visceral forms that are lethal in most cases (Ferreira et al., 2003).

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Clinicians are confronted with steadily higher numbers of leishmaniasis patients not only in countries where the disease is endemic but also in countries where these parasites are not endemic. This increased incidence is partly due to geographical expansion of the disease, changing patterns of international travel and population migration, non-immune people moving into endemic regions, or infected people moving into non-endemic regions (Desjeux, 2001).

Leishmania species are morphologically very similar and species identification is possible using standard biochemical methods (lectin agglutination, isoenzyme analysis, analysis of kDNA restriction fragment using different restriction endonuclease, etc.). As has been demonstrated in many works where these techniques have been satisfactorily used to characterize *Leishmania* isolates (Andrade and Saraiva, 1999; Belhadj et al., 2003; Sampali et al., 2003; Shamsuzzaman et al., 2000), the ability to distinguish between *Leishmania* species is crucial when prescribing treatment as well as when determining possible control measures in epidemiological studies. Frequently, *Leishmania* species are identified based on their geographical distribution and on clinical manifestations of the resulting disease. However, geographical origin is an inadequate criterion in non-endemic areas, as well as endemic regions where multiple species of *Leishmania* may co-exist. Identification of the infecting species based on clinical symptoms can be problematic, since several species cause both cutaneous and mucocutaneous disease, while others cause visceral and cutaneous disease (Schönian et al., 2003).

In the present work, we characterized eight *Leishmania* isolates from different areas of Latin America (Peru and Mexico) as well as from the Mediterranean region (Spain), using interaction of the parasites with lectins together with electrophoretic analysis of their isoenzyme profiles and analysis of kDNA restriction fragment. Morphologically, all these have been considered to be members of the genus *Leishmania*. For comparison, we used four isolates from human cases characterized as: *L.* (*L.*) *donovani*, two strains belonging to *Leishmania* (*L.*) *infantum* and another characterized as *Leishmania* (*Viania*) *peruviana*. In addition, we made a comparative study of the major end-products excreted into the culture medium by the parasites.

2. Materials and methods

2.1. Parasite isolation and in vitro culture

The eight *Leishmania* were isolated from different areas of Peru, Mexico, and Spain. Three of these isolates were from cutaneous cases in the central zone (LP1 and LP2) and north-eastern part (LP3) of the department of La Libertad (Peru); a fourth isolate (LP4) from a mucosal lesion was isolated in the zone of Cajamarca, in north-eastern Peru (near the border of Ecuador and Colombia). Three isolates (LM1, LM2, and LM3) were from cutaneous lesions in Campeche (Mexico) during the period 2000–2002. The final isolate (LS1), from the area of Madrid (Spain), was taken from a dog. For comparison, we also included: *L.* (*L.*) *donovani* (LCR-L 133, *Leishmania* Reference Center Jerusalem, Israel) isolated in a human case of kala-azar in Begemder (Ethiopia); *L.* (*V.*) *peruviana* (MHOM/PE/84/LC26) and two strains of *L.* (*L.*) *infantum*: I and II, characterized as MCAN/ES/2001/UCM-10 and MCAN/2000/UCM-1 isolated in Spain, respectively. (The reference strains have been maintained for several years in our laboratory by successive passes in cultures of NNN medium modified with a liquid phase of minimal essential medium (MEM) plus 10% inactivated foetal bovine serum kept in an air atmosphere at 28 °C. To maintain infectivity, a subculture was approximately every two weeks and at least every six months, was inoculated in the Syrian golden hamster, *Mesocricetus auratus*, and parasites are isolated from the spleen 30 to 45 days post-inoculation.

The isolates from hamsters were cloned and cultured in vitro as previously described (Sánchez-Moreno et al., 1995). Different monophasic cultures were tested: RPMI-1640, MTL, and TC medium (Gibco, Karlsruhe, Germany) and MEM (Sigma, St. Louis, MO).

The epimastigote forms of *Trypanosoma cruzi* strain Maracay were cultured in vitro using Grace's medium (Sigma) (Sánchez-Moreno et al., 1995).

2.2. Lectin-agglutination test

Lectins from *Canavalia ensiformis* (Con A), *Vicia villosa* (VV), *Triticum vulgaris*, a wheat-germ agglutination (WGA), and *Arachis hypogaea* (PNA) were used (Sigma, St. Louis, MO). Parasites were washed three times with phosphate-buffered saline, pH 7.4, and collected by centrifugation ($600g \times 10$ min, 4 °C), and resuspended in phosphate-buffered saline, pH 7.4, containing 0.5% inactivated foetal bovine serum. Tests were performed in duplicate in 96-well plates (Flow Laboratories). The parasite suspension (50 μl) was incubated with equal volumes of lectins at different concentrations for 1 h at room temperature (final lectin concentrations of 1, 10, 20, 50, 100, 150, 500, 750, and 1000 μg/ml). Agglutination was determined by microscopic observation according to (Zubiaur and Alonso, 1985). Controls for specific agglutination were performed using 0.1 M α-D-glucosyl for Con A, 0.1 M *N*-acetyl-D-galactosamine for VV, 0.1 M β-galactose for PNA, and 0.1 M *N*-acetyl-D-glucosamine for WGA.

2.3. Isoenzyme characterization

Crude homogenates were obtained from 250 ml of culture medium containing 2×10^7 cells/ml. Cells were harvested by centrifugation at $1500g \times 10$ min, washed twice in a phosphate-buffered saline (pH 7.4), and resuspended in a hypotonic enzyme stabilizer solution containing 2 mM dithiothreitol, 2 mM *E*-aminocaproic acid, and 2 mM EDTA (Fernandez-Ramos et al., 1999). The samples were frozen at −80 °C for 15 min and thawed at 25 °C. After several freezing–thawing cycles, cell lysates were centrifuged at