Studies on New World Leishmaniasis and its Transmission, with Particular Reference to Ecuador

1992
Research Report Series No. 3

Our ultimate triumph over leishmaniases will have to depend on a further concerted effort through understanding and cooperation of all workers in every discipline. It is no longer possible for any one specialist to grasp the complexity and diversity of the diseases, the causative agents and all the avenues of investigation. (K.-P. Chang and R.S. Bray, 1984, *Leishmaniasis*, Elsevier)

Studies on New World Leishmaniasis and its Transmission, with Particular Reference to Ecuador

edited by

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Representative of an Overseas
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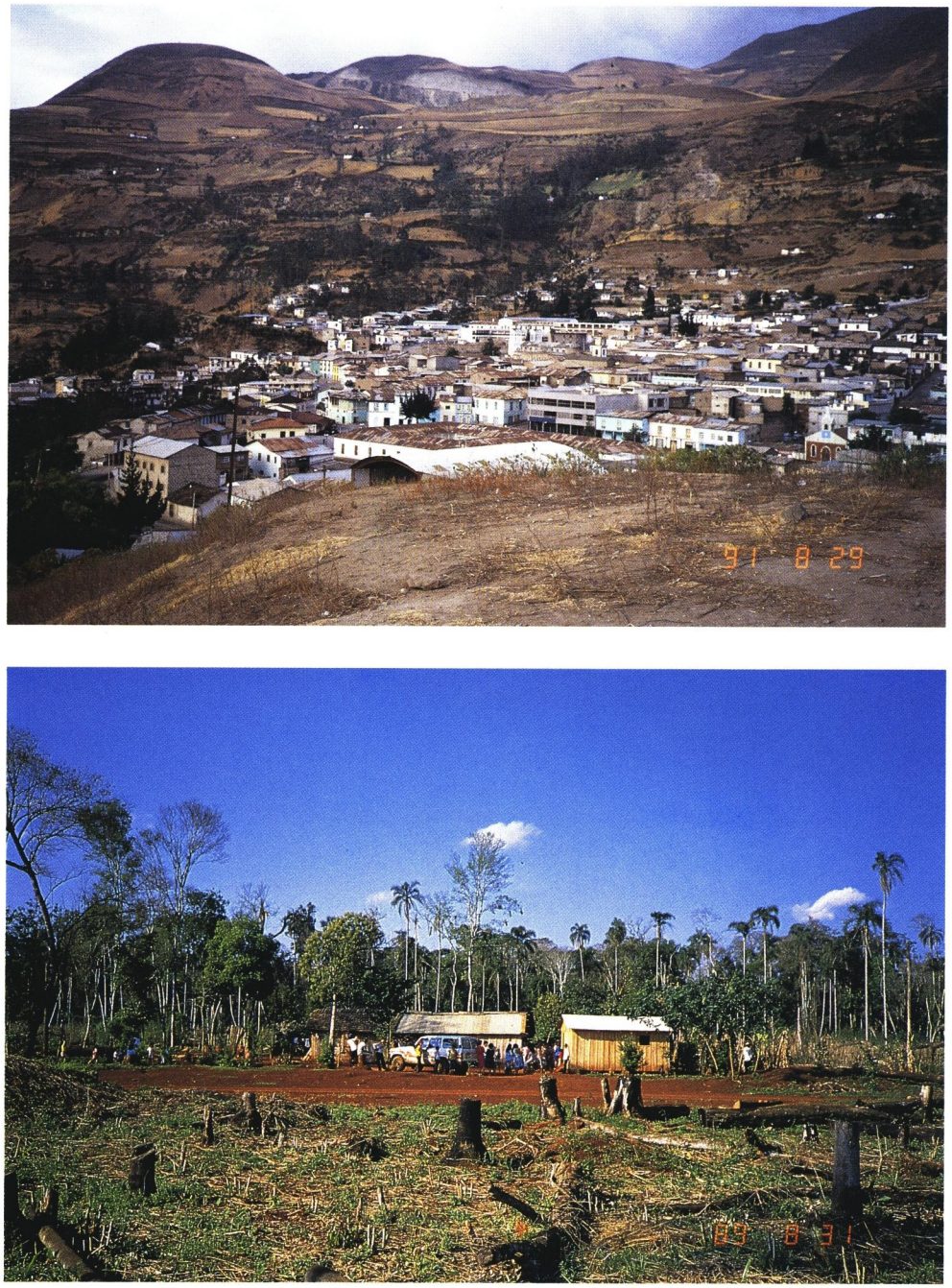

Plate 1. Showing leishmaniasis-endemic areas of Andean highlands of Ecuador (above) and hyper-endemic areas of Paraguay (below). **Above**, the town of Alausi (2,300-2,500 m above sea level), Department of Chimborazo; **below**, houses of a small community newly established after felling a dense tropical primary forest at Limóy (300 m a.s.l.), Department of Alto Parana, very close to the Brazilian border.

Plate 2. An assembly held at leishmaniasis-endemic areas of Ecuador (above) and Paraguay (below). **Above**, Dr. Y. Hashiguchi and Mr. R. Sud present foot-balls and school things to a teacher of primary school, "19 de Julio", Azuay, Ecuador ; **below**, Drs. D. Maciel and O. Arias explain our research activities to get support from the inhabitants at Limóy, Alto Parana, Paraguay.

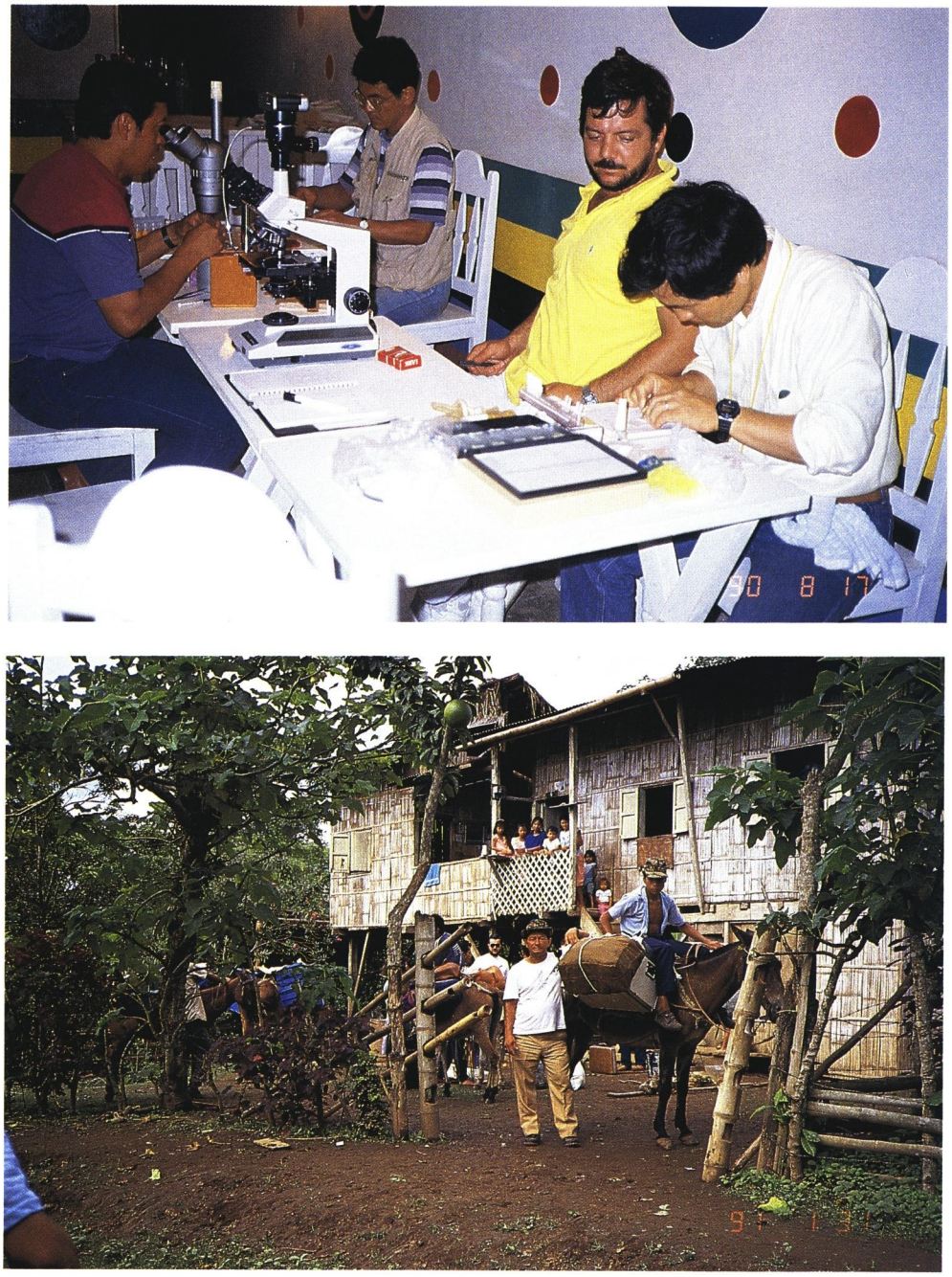

Plate 3. Showing field research activities. **Above**, sandfly dissections to search for natural infections with *Leishmania* parasites, and also to collect materials for the application of molecular techniques at Muisne, Department of Esmeraldas, Ecuador, from left: Dr. E. A. Gomez L., Dr. M. Furuya, Dr. L. Martini and Dr. Y. Eshita; **below**, our special transportation system of laboratory equipments (microscopes, medicines etc.) at remote areas endemic for leishmaniasis, and many sandflies were captured either inside or outside the house from dusk to dawn.

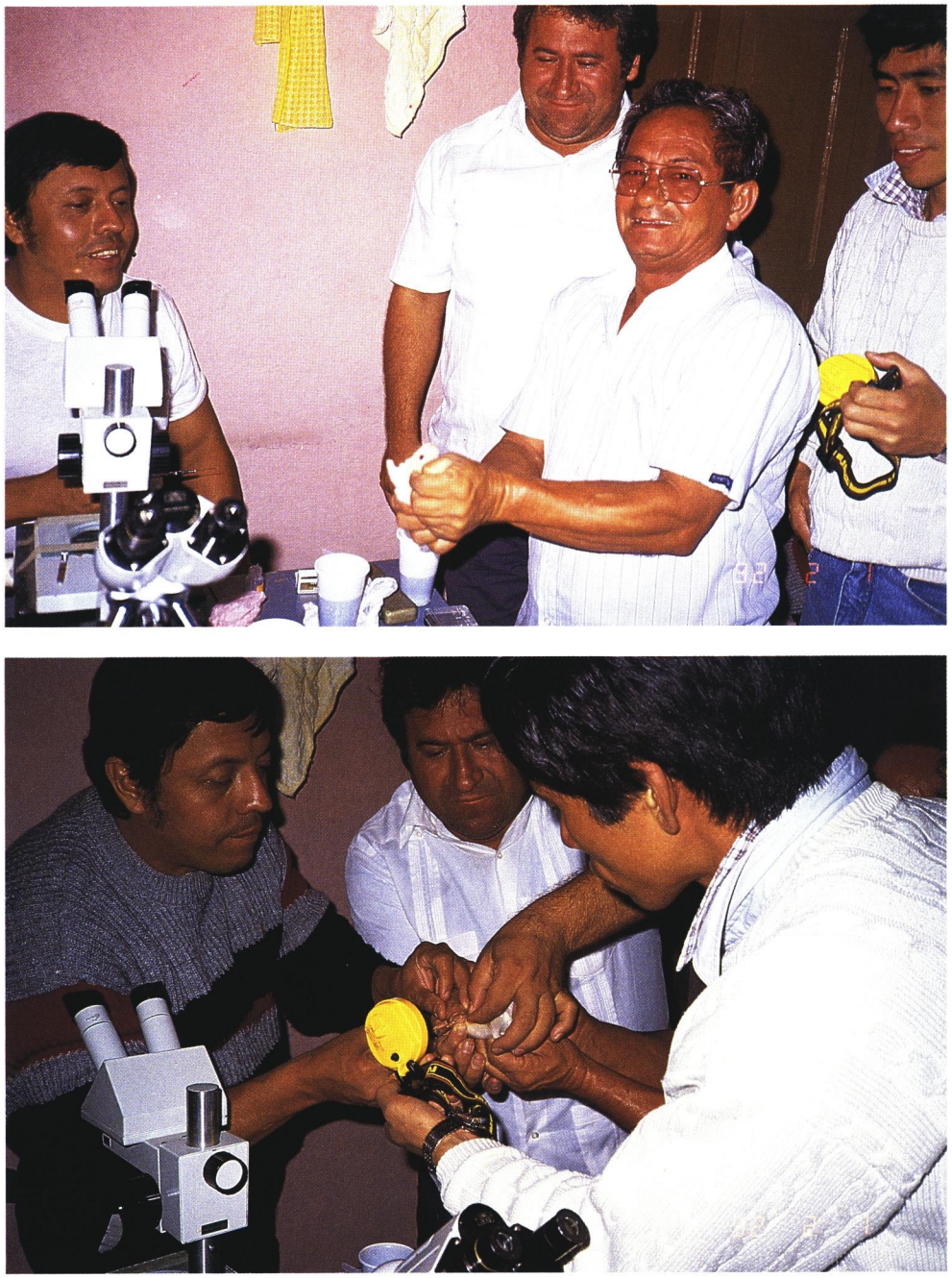

Plate 4. Showing laboratory works at the Andean leishmaniasis-endemic area (Alausi, Chimborazo, Ecuador). **Above**, all the members are very happy at midnight when they discovered for the first time the infected sandfly, *Lutzomyia ayacuchensis* with *Leishmania* promastigotes in the gut, from left: Dr. E. A. Gomez L., Dr. H. M. Jurado S., Mr. Roberto Sud A. and Dr. Y. Matsumoto; **below**, inoculation of the parasites into the nose and footpads of hamsters by the same members.

Plate 5. Examinations of inhabitants at a primary school "Escuela de San Sebastián". All the persons came from a remote area where no transportation system is available.

Plate 6. Registration, taking life history of the examinees at a hospital "Hospital de Junin" (above), and dermatological examinations of patients at a primary school "San Sebastián" (below).

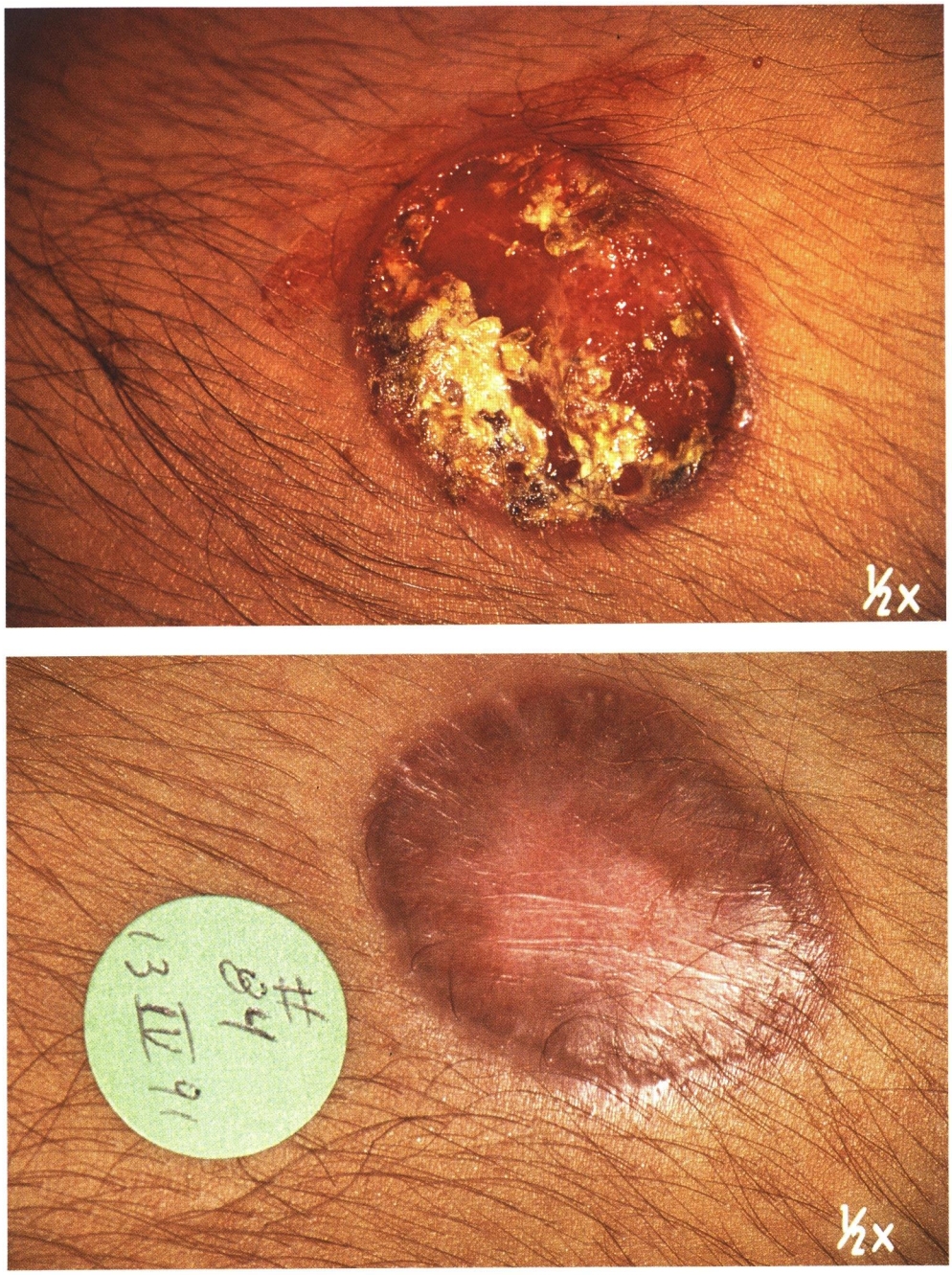

Plate 7. A cutaneous leishmaniasis case (14 year-old female, No. CT-84, M.C.S.) before and after the topical treatment with 2% paromomycin ointment (see Chapter 7.3). **Above**, a large ulcer (20×25 mm) before treatment; **below**, the same lesion cured completely.

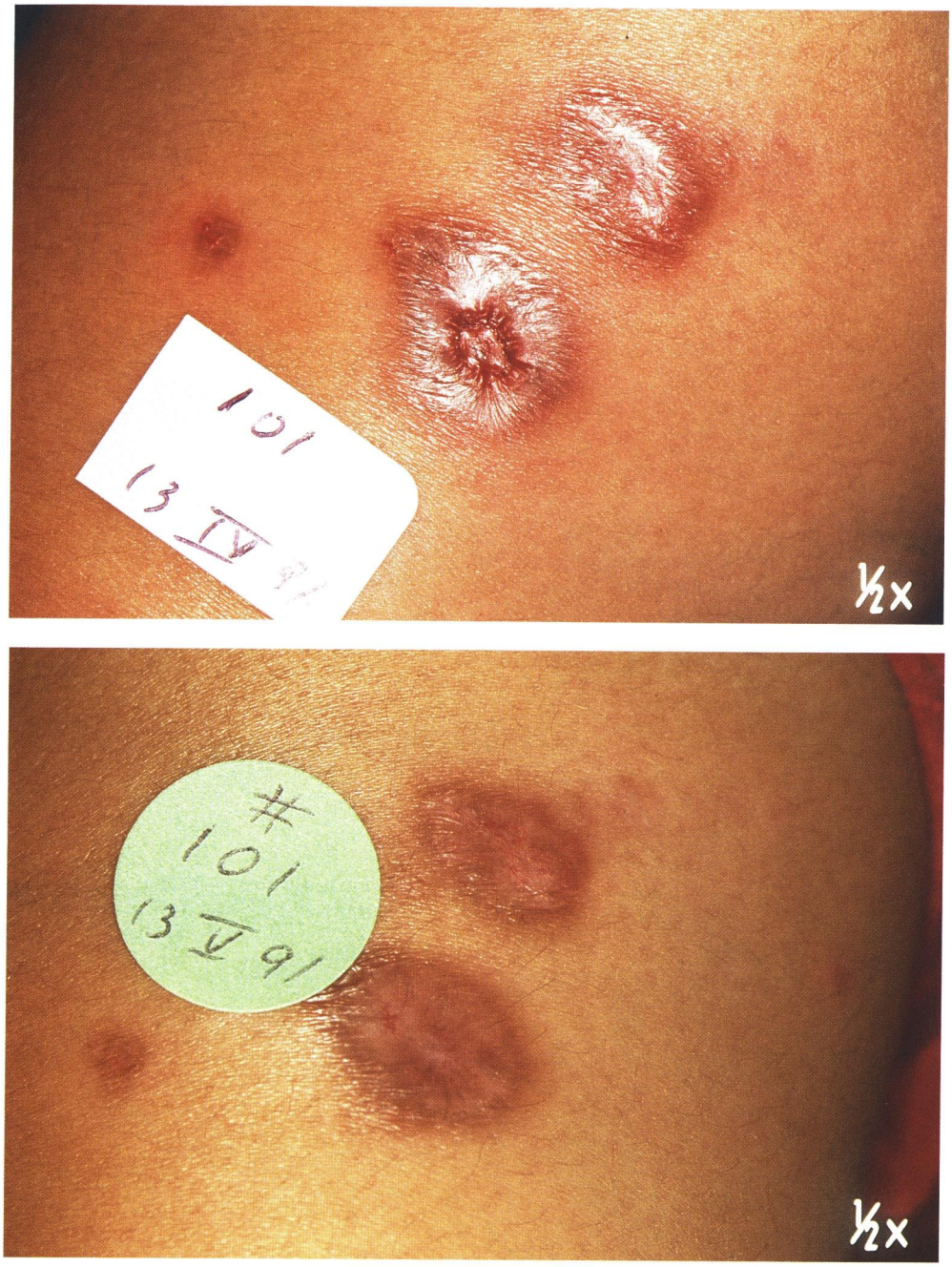

Plate 8. A slightly resistant case (26 year-old female, No. CT- 101, G.Z.) against 10% paromomycin ointment (above). The patient, therefore, received meglumine antimonate (Glucantime®) lotion topically and cured completely (below).

Foreword

La leishmaniasis como muchas de las otras enfermedades transmitidas por vectores ha ido en aumento en Latinoamérica y muy en especial en el Ecuador, fenómeno que puede ser explicado por la falta de programas tendientes al control de vectores, educación a las poblaciones expuestas, el diagnóstico oportuno y su tratamiento.

En el año de 1982 se inició un programa de investigación colaborativa entre el gobierno de Japón y el del Ecuador, siendo el investigador responsable el Dr. Yoshihisa Hashiguchi, de la Kochi Medical School, y como contraparte ecuatoriana, personal del Instituto Nacional de Higiene y Medicina Tropical "Leopoldo Izquieta Pérez", en especial el Dr. Eduardo A. Gómez L. Han transcurrido diez años y es mucho lo que se ha investigado sobre esta enfermedad, a costa de grandes sacrificios personales, riesgo de contraer la enfermedad, de exponerse como cebos y otras muchas vicisitudes, que son conocidas por quienes realizamos trabajos de campo y a no dudarlo por el amigo lector, pero el resultado de estos largos años de trabajo constante han dado sus frutos, los mismos que son altamente satisfactorios, y que se han reportado en forma minuciosa en tres publicaciones.

Este trabajo producto de un grupo multidisciplinario de investigadores en las distintas ramas de especialización, nos han permitido conocer los aspectos entomológicos, parasitológicos, dermatológicos, epidemiológicos, etc., teniendo en los actuales momentos una clara visión de los vectores más importantes y el mecanismo que juegan en la transmisión, así como los reservorios naturales en Costa, Sierra y Oriente Ecuatoriano, teniendo una cabal idea de la incidencia y prevalencia de la enfermedad, en un mapeo geográfico epidemiológico de la mayoría de las provincias ecuatorianas donde se presentan con más intensidad, así como la tipificación de cepas de *Leishmania* que predominan regionalmente.

Sin embargo, falta mucho por investigar, pues la meta es llegar al control de la enfermedad y esto sólo será posible con el apoyo de nuestros gobiernos y el idealismo por un mundo libre de enfermedades, que los investigadores extranjeros y nacionales sigan trabajando con el misticismo con que han venido haciéndolo.

Por el motivo antes expuesto, el Centro Nacional de Medicina Tropical de la Facultad de Ciencias Médicas de la Universidad de Guayaquil, se siente complacido de haber prestado todo su apoyo y colaboración en este trabajo y ofertar seguir haciéndolo hasta alcanzar el éxito deseado. Así como felicitar y estimular a todos y cada uno de los investigadores que han participado en este proyecto, en no desmayar en su empeño muy especialmente al Dr. Yoshihisa Hashiguchi, quién ha demostrado ser un buen investigador, planificador, administrador y querer al Ecuador como su segunda Patria.

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(6 March 1992; CENAMETROP)

Preface

Studies on leishmaniasis and its transmission have been carried out to date by the members of our scientific research project, which was first organized in 1986, and funded by the Ministry of Education, Science and Culture, Japan. Aided by this research grant, we made joint investigations on various aspects of leishmaniasis, aiming especially at understanding of the transmission mechanism(s) and the disease forms in Ecuador, and obtained a lot of information. Our research group has increased considerably in a few years, and investigators from different countries, Ecuador, Japan, the United Kingdom, United States, Brazil, Paraguay, Colombia and Peru, are currently working together, on diverse topics relating to leishmaniasis and its transmission.

I would like to emphasize that from the beginning of this study, we have tried to follow the principle that every parasitic disease, especially vector-borne diseases such as leishmaniasis, should be investigated from multi-disciplinary points of view even within such a small research project as ours. As Drs. Chang and Bray (1984) stated: "ultimate triumph over leishmaniases will have to depend on a further concerted effort through understanding and cooperation of all workers in every discipline; and it is no longer possible for any one specialist to grasp the complexity and diversity of the diseases, the causative agents and all the avenues of investigation". In accordance with this principle, our research group includes parasitologists, immunologists, medical entomologists, dermatologists, and molecular biologists.

The results of our investigations including information on *Leishmania* parasites, vector sandflies and clinical forms were summarized in Research Report Series Nos. 1 and 2, "Studies on leishmaniasis and its transmission, with particular reference to Ecuador", published in 1987 and 1990, respectively. The current report, "Research Report No. 3", mentions the results of our studies of leishmaniasis obtained mainly in Ecuador and Paraguay during 1990 and 1991. We detail results of trials to detect and identify parasites samples obtained from patients, reservoirs and insect vectors, collected in different endemic areas, using molecular techniques, such as polymerase chain reaction (PCR) and pulsed field electrophoresis. The results of preliminary studies on leishmaniasis in Paraguay are also described; works in that country have been done through the generous support of the Japan International Cooperation Agency (JICA) and the Paraguayan Government.

We could not have performed these investigations in Ecuador and Paraguay without the cooperation and support of our colleagues in Japan and elsewhere. On behalf of the research group, I would like to show my sincere thanks to all the persons involved at different phases of this study. Much of the materials and data collected on the field survey have yet to be examined and analyzed. The results will be published in detail elsewhere at a later date, under the authorship of all research workers involved in the study. Moreover, a further intensive study of leishmaniasis and its transmission will be continued from 1992 onwards, with the main intention of applying molecular techniques to elucidate pathophysiological features of leishmaniasis and its transmission in the New World.

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A part of the investigation in Paraguay was conducted in the project, "Paraguay and Japan Medical Cooperation Project on Chagas' disease and other infectious diseases" supported by the Paraguayan Government and the Japan International Cooperation Agency (JICA). We are extremely grateful to Dr. Ricardo Moreno A., Director, Instituto de Investigaciónes en Ciencias de la Salud (IICS), Asunción, Paraguay and Dr. Ruben D. Avila C., Ex-Director and Dr. Secilio Ortega, Director, Laboratorio Central e Instituto de Medicina Tropical (LACIMET), Asunción, Paraguay for their support. Thanks are also due to Dr. Carlos Levi Ruffinelli, Academia de Medicina del Paraguay, Drs. Richard Münz (Director), Juan A. Gimenez and all the members of Hospital Tavaí, Caazapá, Paraguay for their kind support and cooperation.

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Studies on New World Leishmaniasis and its Transmission, with Particular Reference to Ecuador

Introduction

Leishmaniasis, a zoonotic disease, is endemic in many tropical and subtropical regions in the Old and New World, and is classified as one of the six most important tropical diseases of the World Health Organization. The disease manifestations fall largely into three categories: a) a simple, often self-limiting form; b) a mucocutaneous form, involving the destruction of mucosal tissues of nasopharynges; and c) a visceral form, a systemic infection which is often fatal if not treated adequately. These clinical manifestations and prognosis of the disease depend in part on the infecting organisms of the causative agents, members of the genus *Leishmania*. The determination of *Leishmania* species in each endemic area is therefore very important for the suitable management of the patients or the follow up of the disease prognosis. In Ecuador, one of the smallest countries in South America, seven species of *Leishmania* have been recorded in different endemic areas of the Pacific coast, Amazon (Oriente) and Andean plateau regions, by our field survey to date (Mimori *et al.*, 1989; Hashiguchi and Gomez, 1990; Armijos *et al.*, 1990; Hashiguchi *et al.*, 1991).

The disease is transmitted by phlebotomine sandflies. Within these insects, the organisms develop as flagellated promastigote forms. In the vertebrate hosts the parasites develop almost exclusively in cells of the mononuclear phagocyte system, where they multiply as a non-flagellate form, the amastigote. Leishmaniasis is an important and very widespread disease in tropical and subtropical regions of the world. It is estimated that there are more than 12 million cases world-wide with about 400 thousand new cases recorded each year (Marinkelle, 1980). The control of leishmaniasis is complicated, especially in Central and South America, by the fact that many species of sandflies are potential vectors and that over 100 species of mammals may act as reservoir hosts.

In Ecuador, leishmaniasis occurs in many populations living in rural and mountainous areas on both sides of the Andes. Furthermore, we have discovered a form of the disease in persons living on the Andean plateau at altitudes from 2,300 to 2,700 meters. This disease form is very similar to "Peruvian uta" but the causative agent and vector are completely different (Takaoka *et al.*, 1990; Hashiguchi *et al.*, 1991; Gomez and Hashiguchi, 1991). Leishmaniasis is widespread in most provinces and is a considerable health problem in the country. However, little investigation has been done on the epidemiology and transmission mechanism(s) of the disease in endemic areas and no control measures have been applied to reduce or interrupt the risk of the disease. For future application of adequate control measures, the accumulations of epidemiological data at given endemic areas are urgently needed in the country.

During the period from 1982 to 1989, we have found a great number of cutaneous cases and a small number of mucocutaneous ones (Hashiguchi and Gomez, 1991). In 1990 we observed one rare case of diffuse cutaneous leishmaniasis through the coutesy of Ecuadorian physicians; the detailed result will be reported elsewhere. Hitherto, one case each has been recorded of visceral and diffuse cutaneous leishmaniasis; in both cases, however, no parasite isolation and/or identifications were done.

The present report deals with the results obtained from surveys performed in Pacific lowlands and Andean plateau of Ecuador, from various standpoints, including parasitology, vector entomology, immunology, dermatology, epidemiology and molecular biology. Special emphasis is given to the characterization of *Leishmania* parasites using several molecular techniques, and also given to clinico-pathological features of the disease forms in Ecuador. In addition, information on the epidemiological and entomological features of Paraguayan leishmaniasis has been added.

Yoshihisa Hashiguchi
Eduardo A. Gomez L.

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Chapter 1

A Review of Andean Leishmaniasis

ABSTRACT. Until 1986, the only form of leishmaniasis in the Andes was thought to be Peruvian uta caused by *Leishmania peruviana*. We have discovered another type of leishmaniasis in the Ecuadorian Andes which has a completely different species of causative agents and vectors from those of Peruvian uta. In this article, we briefly review Andean leishmaniasis including uta and revised a model to show how local conditions affect transmission in the Andes.

The finding of Andean leishmaniasis in 1986 in Ecuador has increased the known distribution of the disease (Hashiguchi *et al.*, 1987). Until that time, the only form of leishmaniasis in the Andes was thought to be Peruvian uta caused by *Leishmania peruviana**. We discovered another type of leishmaniasis in the Ecuadorian Andes; this disease form has completely different species of causative agents and vectors from those of Peruvian uta (Hashiguchi *et al.*, 1991). In this article, we briefly review Andean leishmaniasis including uta and revise our model of how the local conditions affect the transmission in the area.

Andean leishmaniasis, Peruvian uta, has been known from 1859 when a doctor (Villar) described the disease as being very like Aleppo button, an Oriental form of leishmaniasis (Lainson, 1983). Furthermore, a link between uta and its vector was known as far back as 1764 when Cosme Bueno made the first description of a disease transmitted by the bite of sandflies (Killick-Kendrick, 1986). Surprisingly, however, studies on Andean leishmaniasis have so far been limited mainly to the Peruvian regions but during 1986-1988 we found a few rare cases from the Andean highlands of Ecuador, and strongly suspected the presence of autochthonous leishmaniasis in this area. Our epidemiological surveys in 1986 in the same region resulted for the first time two parasitologically confirmed autochthonous cases, thereafter many more were recorded from Paute (elevation 2,300-2,500 m), Department of Azuay and Alausi (2,500 m), Department of Chimborazo, Ecuador (Hashiguchi *et al.*, 1987, 1991; Armijos *et al.*, 1990).

Leishmaniasis and its causative agents in the Andes

The clinical symptoms of Andean leishmaniasis in Ecuador are indistinguishable from case descriptions (Lumbreras and Guerra, 1985) of uta which is manifested as small circular lesions, generally seen on the face of children under 10 years of age; 56% were less than one year of age (Hashiguchi *et al.*, 1991). When *Leishmania* was isolated from humans at Paute and Alausi, Ecuador, both *L. mexicana* and *L. major*-like parasites were identified (Hashiguchi *et al.*, 1991). Recently, *L. mexicana* from patients living in two towns (Pueblo Rico and Samaniego, elevation ca. 1,550 m) situated in the western Andean cordillera of Colombia was reported (Corredor *et al.*, 1990). This

*Identifications of parasites in this text have followed the simplified nomenclature for the genus *Leishmania*, suggested by Saf'janova (1982) and Shaw and Lainson (1987) and used by the International Colloquium at Montpellier, France (Rioux, 1986).

range is contiguous with the mountains encompassing Paute and Alausi, Ecuador. The distance between these four towns suggests that *L. mexicana* may have a fairly wide geographic distribution in this part of northern South America. Because of its altitude and temperate climate, this region is ecologically quite distinct from the warm lowland areas where *L. mexicana* usually occurs in Central America and Mexico (Grimaldi *et al.*, 1989).

In Peru, uta is endemic on the western slopes of the Andes and in many inter-Andean valleys, and restricted to a zone 900-3,000 m above sea level (Romero *et al.*, 1987). This disease has been successfully controlled in the past by anti-bartonellosis campaigns involving the erradication of vector sandflies using DDT. However, in recent years there has been an increased number of cases and about 2,000 new cases of uta are reported annually to the Ministry of Health (Romero *et al.*, 1987). For a long time, this disease was generally thought to be caused by *L. peruviana*, based mainly on clinico-geographical grounds. However, recent molecular studies suggest that *L. peruviana* is not a distinct species but probably a variant of *L. braziliensis* (Romero *et al.*, 1987; Grimaldi *et al.*, 1987; Lopez *et al.*, 1988). More recently, however, Arana *et al.* (1990) reported that *L. braziliensis* and *L. peruviana*, could be distinguished by employing a reliable enzyme marker, mannose phosphate isomerase (MPI), by thin-layer starch gel electrophoresis. Thus, there remains some controversy as to the taxonomic status of these two species. The otherwise remarkable similarity between the two species contrast with the very diverse clinical manifestations of uta and espundia (Scorza *et al.*, 1979). Clinical, epidemiological and geographical evidence all support the assumption that classical uta from the Peruvian Andes is caused by an agent different from that responsible for the cutaneous and mucosal leishmaniasis (espundia) seen in the forested areas of the country (Arana *et al.*, 1990). Host-related factors cannot be ruled out as yet and deserve special consideration for future investigations (Romero *et al.*, 1987). Another parasite has been isolated from the Peruvian Andes that is neither the *L. braziliensis* nor the *L. mexicana* complexes but it has not been identified yet (Romero *et al.*, 1987). In the Venezuelan Andes, cutaneous leishmaniasis, caused by *L. garnhami*, has been reported from people living at altitudes of 800 to 1,600 m ; *Lutzomyia townsendi* is strongly suspected as its vector (Scorza *et al.*, 1979).

Vector sandflies and reservoir hosts in the Andes

Fewer than six phlebotomine species have been reported from the western slopes and valleys of the Peruvian Andes (Young and Lawyer, 1987). The vector of Peruvian uta was for a long time thought to be *Lu. verrucarum* or *Lu. peruensis* but there was no evidence of natural infections with the parasite. Recently, isolates obtained from *Lu. peruensis* and a sentinel hamster were identified as *L. peruviana* by molecular techniques (Perez *et al.*, 1991). Only two anthropophilic sandflies, *Lu. ayacuchensis* and *Lu. osornoi* have been found in the two foci we have studied in the Ecuadorian Andes (Paute), and natural infection with *L. mexicana* was always seen in the midgut of the former species (Takaoka *et al.*, 1990; Gomez and Hashiguchi, 1991). Surprisingly, neither *Lu. peruensis* and *Lu. verrucarum* have been found to date, in spite of our intensive search for these species. The only recorded reservoir host of uta in Peru has been the domestic dog, but recently an opossum, *Didelphis albiventris*, was added to the list of known reservoir hosts of *L. peruviana* in the country (Perez *et al.*, 1991). Dogs were also recorded as hosts of Andean leishmaniasis in Ecuador (Hashiguchi *et al.*, 1991) but in this case the infective agent was *L. mexicana*. This observation from Ecuadorian dogs and sandflies is very interesting, since both are new host records for *L. mexicana* and raises the posibility that dogs could be a domestic reservoir of *L. mexicana* in Andean regions.

A revised model for the disease transmission in the Ecuadorian Andes

We have recently made longitudinal studies on natural infections of *Lu. ayacuchensis* with *L. mexicana* in the Ecuadorian Andes (Gomez and Hashiguchi, 1991). Based on these data (Fig. 1.1) and ecological and epidemiological information on the area (Hashiguchi *et al.*, 1987, 1991), we have developed a model for *Leishmania* transmission in an endemic area (Paute) of the Andes (Gomez and Hashiguchi, 1990). The various ecological factors that influence transmission in this area and the complexity of their interactions are shown in Fig. 1.2. The model shows the change in niche overlap between domestic hosts (humans and dogs) and wild hosts, and the vectors under different environmental conditions. From October to December (C-1 in Fig. 1.2) the majority of transmission occurs in rural environments and largely between sandflies and wild mammals within rock crevices and burrows (habitat I in Fig. 1.2). During the rainy season from January to February (C-2 in Fig. 1.2), the sandfly population increases gradually. The new sandfly generations expand their biting activity towards periurban area (habitat II in Fig. 1.2) and transmission of *Leishmania* to humans intensifies in both types of habitat. During March to April there is a high density of infected sandflies (Fig. 1.1) and an increased population of wild rodents (rats, R1) living peridomestically in the periurban area (our unpublished data). Sandfly biting activity extends into urban area (habitat III in

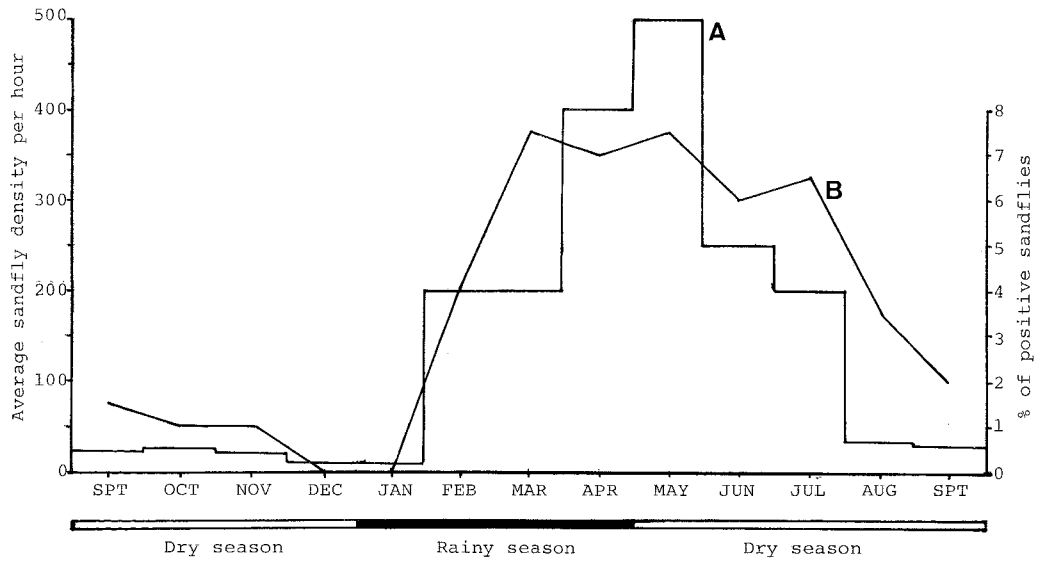

Figure 1.1. Relation between sandfly density (A) and natural infection (B) with *L. mexicana* in an Andean endemic area, Paute, Department of Azuay, Ecuador. Average density is expressed as the number of sandflies captured per hour in each collection, and percent of those positive for parasites, calculated from the rate of sandflies infected among the 200 dissected each month. Average temperature and humidity throughout the year ranged from 15°C to 18°C and from 50% to 80%, respectively. (Adapted from Gomez and Hashiguchi, 1991).

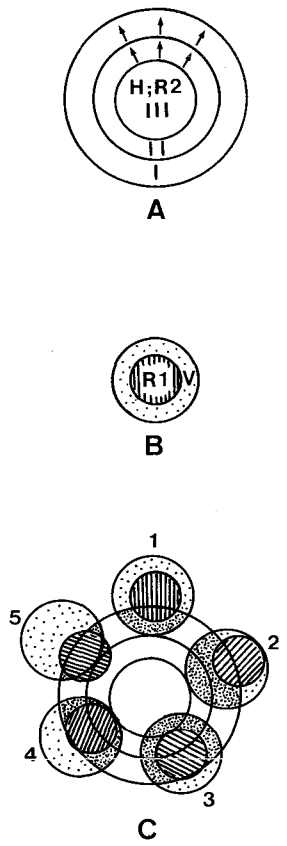

Figure 1.2. Hypothetical diagrams for the transmission of Andean leishmaniasis under different degrees of overlap of domestic and wild hosts and vectors. A, common habitat for humans (H) and dogs (secondary reservoirs, R2), where I is open field habitats, II, periurban area, and III, urban area; B, common habitat for vectors (V) and wild rodents (probably rats, principal reservoirs, R1), where zoonotic transmission occurs. C, represents the different degree (1 to 5) of overlap between A and B. The overlaped areas represent the place and magnitude of transmission to humans in each habitat (I, II and III) in different seasons (C-1, C-2, C-3, C-4 and C-5), showing the migration of wild hosts (R1) and vectors (V) from open field (higher land) into periurban and urban (Andean valley) areas. (Modified from Gomez and Hashiguchi, 1990).

Fig. 1.2; the town of Paute) with maximum overlap of domestic and wild hosts and vectors, and many new human leishmaniasis cases are observed (Hashiguchi *et al.*, 1991). During May to June (C-4 in Fig. 1.2), the end of the rainy season and the subsequent decrease in humidity causes a rapid decline in sandfly density (Fig. 1.1). Sandflies disappear from the town (III), though they are still observed in periurban (II) and open field (I) environments. The overlap of A and B in C-4 is somewhat similar to C-2, but the intensity of disease transmission in habitat II is markedly higher in C-4 owing to the larger number of infected sandflies (see Fig. 1.1). In July to September (C-5 in Fig. 1.2), because of the desiccating conditions in lower altitude terrain during the dry season, sandfly density falls to a minimum. Nevertheless, rock crevices and animal burrows in habitat I allow survival of the vectors even at this season.

Thus, it appears that *Leishmania* transmission in rural environments (habitat I) occurs throughout the year, although it is limited during the dry season because of the low density of vector sandflies. In periurban areas (habitat II) transmission can be prolonged, lasting about five or six months from January to May or June (Fig. 1.2; C-2, C-3 and C-4). In urban conditions (habitat III), the duration of transmission is very short, lasting only two months from March to April (Fig. 1.2; C-3) but shows a high incidence of disease.

In conclusion, transmission cycle of leishmaniasis in the Andes involves variable overlapping of two sets of biological entities, with the degree of overlap governed by micro- or macro-climatic conditions. The first set consists of three categories of habitat; open field, periurban and urban (Andean valley). Each of these habitats is occupied at same time by humans and domestic and wild animals. The second set consists of the relationship between sandflies and the principal reservoir hosts of *Leishmania*, presumed to be rats and other rodents. Changes in the incidence and frequency of human cases of leishmaniasis in the Andes are considered to be the result of migra-

tions of sandflies and reservoir hosts among the three habitat categories.

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Chapter 2

Molecular Parasitological Aspects

1. Karyotype Analysis of *Leishmania* Isolates from Ecuador by Pulsed Field Gel Electrophoresis

ABSTRACT. Karyotypes of *Leishmania mexicana*, *L. panamensis* and *L. major*-like parasites recently isolated in Ecuador were analyzed by pulsed field gel electrophoresis (PFGE). A total of 18-21 chromosomes from 200 kb to over 1,100 kb were resolved, depending on the isolates, using a turn-table type pulsed field gel electrophoresis apparatus. The PFGE revealed species-specific DNA karyotypes with three different *Leishmania* species. Karyotype of *L. mexicana* was almost identical among four isolates including three from patients and one from a canine at an Andean small village, further suggesting that domestic dogs play a role for reservoir hosts of *L. mexicana* in this region. Chromosomal DNA banding pattern of *L. major*-like parasites was very similar but not identical between two human isolates, one from an Andean highland and the other from the Pacific coastal lowland area. Karyotype of *L. panamensis* was also similar but exhibited minor chromosome size polymorphism among three isolates from different geographical regions of Ecuador. These karyotype variations among isolates from distinct regions appear to reflect the species diversity of *Leishmania* in the New World, which may be in part due to the variety of vectors and reservoir hosts.

The advent of pulsed field gel electrophoresis (PFGE) has facilitated the study of chromosomal organization of parasitic protozoa including *Leishmania*. The PFGE revealed the distinction of chromosomal DNA banding pattern or karyotype among different *Leishmania* species (Giannini *et al.*, 1986; Scholler, *et al.*, 1986). Intraspecific chromosome size polymorphism occurs among strains of distant geographical areas (Samaras and Spithill, 1987; Bishop and Miles, 1987) as well as among strains from the same endemic area (Pagès *et al.*, 1989), indicating a dynamic aspect of chromosome reorganization in *Leishmania*. Karyotype analysis by PFGE is, thus, a useful and convenient method for identification of unknown isolates and examination of the relationship among isolate populations.

In the present study, we analyzed karyotypes of new isolates from different regions of Ecuador using a turn-table type pulsed field gel electrophoresis apparatus.

Materials and Methods

Parasites

L. mexicana, *L. panamensis* and *L. major*-like parasites, which have been isolated in Ecuador in 1987 and 1988, were used in the present study (Table 2.1.1). Species identification for these isolates have been carried out by isoenzyme electrophoresis, kinetoplast DNA fingerprints and monoclonal antibodies (Mimori *et al.*, 1989, 1990; Hashiguchi *et al.*, 1991).

Pulsed field gradient gel electrophoresis (PFGE)

Agarose blocks for PFGE were prepared as before (Katakura and Chang, 1989). Promastigotes were cul-

Table 2.1.1. *Leishmania* isolates used in this study

Species	Designation*	Geographic origin
L. panamensis	MHOM/EC/87/G-05	Quininde, Esmeraldas
	MHOM/EC/87/G-06	Z. Grande, Esmeraldas
	MHOM/EC/87/G-07	S. Domingo, Pichincha
L. mexicana	MHOM/EC/88/Paute27	Paute, Azuay
	MHOM/EC/88/Paute29	Paute, Azuay
	MHOM/EC/88/Paute103	Paute, Azuay
	MCAN/EC/88/Paute Inu2	Paute, Azuay
L. major-like	MHOM/EC/87/G-09	Quininde, Esmeraldas
	MHOM/EC/88/Paute115	Paute, Azuay

* Designation code: Host (M for mammalia; HOM = Homo sapiens; CAN = Canis familiaris).

tured in Schneider's Drosophila medium (Gibco) with 20% heat-inactivated fetal calf serum at 25°C. Cells were harvested from the log phase, washed twice with Medium 199 (Gibco) and resuspended in Medium 199 at a concentration of 5×10^8/ml. The cell suspension was warmed to 37°C, and mixed with an equal volume of 1% low gelling temperature agarose (Sea Plaque, FMC) in 75 mM phosphate buffer, pH 8.0, containing 65 mM NaCl and 1% glucose. Fourty microlitter of the mixture containing 1×10^7 organisms were solidified into 5×5×1.5 mm blocks. The agarose blocks were lysed with 1% sarcosyl (Sigma) and 2 mg/ml proteinase K (Sigma) in 0.5 M EDTA, pH 8.0 at 50°C for 48 hr. Samples were washed with 1×TBE (TBE=90 mM Tris, 90 mM boric acid and 2 mM EDTA, pH 8.0) and stored in 0.5 M EDTA at 4°C until used. Electrophoresis was performed in 1.5% agarose at 180 V in 0.5×TBE at 10°C with a pulse interval of 60 s for 35 hr or 100 s for 38 hr using a turn-table type pulsed field gel electrophoresis apparatus (Cross Field Gel Electrophoresis, ATTO Corp. Tokyo, Japan). This apparatus was designed to make a circular gel plate of 20 cm in diameter rotate at an angle of 110° alternately with a pulsed time (Fig. 2.1.1). After the electrophoresis, the gel was stained with 0.1 mg/ml ethidium bromide in 0.5×TBE for 45 min, destained with 0.5×TBE for 30 min and photographed. Chromosomal DNAs of *Saccharomyces cerevisiae* (FMC) were used as the molecular standard.

Results and Discussion

In this study, chromosomal DNAs of *Leishmania* were nicely separated by pulsed field gel electrophoresis used. The PFGE revealed species-specific DNA karyotypes among three different *Leishmania* species isolated in Ecuador. Minor intraspecific chromosome size polymorphism was also showed by PFGE among isolates from distinct regions. The overall results of karyotype analysis of Ecuadorian *Leishmania* isolates were correlated well with those of zymodeme and schizodeme analysis as before

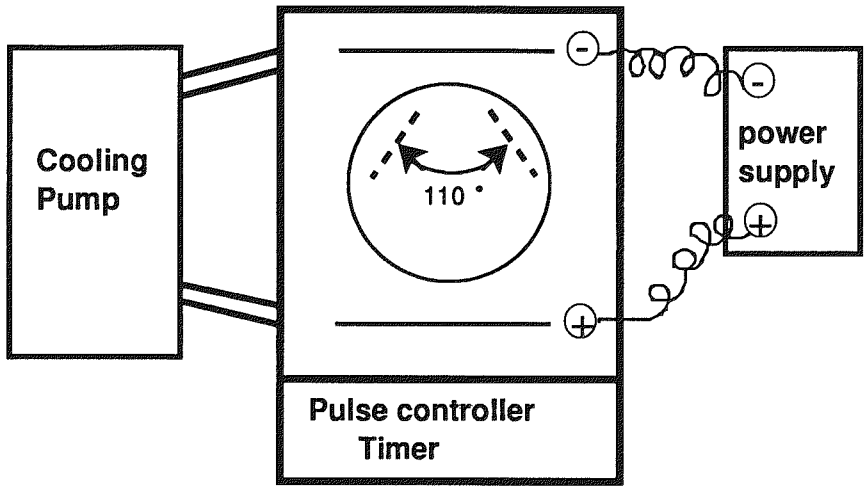

Figure 2.1.1. A schematic illustration of turn-table type pulsed field gel electrophoresis apparatus.

(Mimori *et al.*, 1989, 1990; Hashiguchi *et al.*, 1991).

L. mexicana isolates from patients, a canine and a sandfly at an Andean small village (Paute) exhibited identical kinetoplast DNA profiles by schizodeme analysis (Hashiguchi *et al.*, 1991). The present study by PFGE showed that promastigotes of *L. mexicana* isolates contained at least 19 chromosomal DNAs from 220 to 1,090 kb and more than two chromosomes over 1,100 kb (Fig. 2.1.2). Karyotypes of three isolates from humans (MHOM/EC/88/Paute27, MHOM/EC/88/Paute29 and MHOM/EC/88 Paute 103) and one from a domestic dog (MCAN/EC/88/ Paute Inu2) were also identical. These results indicate that mitochondria and genomic DNA organization of isolates of *L. mexicana* in Paute were very similar, further suggesting that domestic dogs in this region are important as the reservoir hosts.

It has been reported that some Brazilian *Leishmania* stocks revealed phenotypically similar to *L. major*. These stocks could be indistinguishable from *L.*

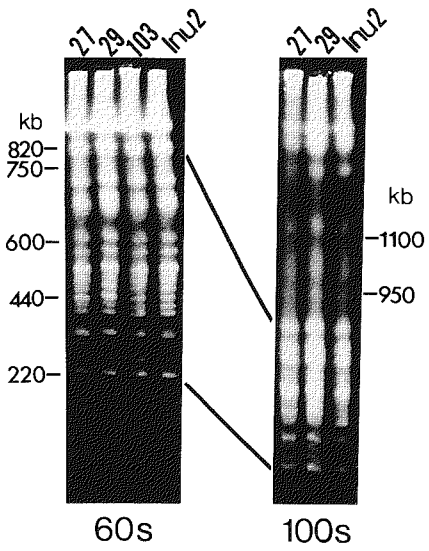

Figure 2.1.2. Karyotype analysis of *L. mexicana* isolated at a small village in an Andean region of Ecuador. PFGE was performed with a pulsed time of 60 or 100 s for three human isolates (27, 29 and 103) and one canine isolate (Inu2). Molecular sizes given were based on *Saccharomyces cerevisiae* DNAs.

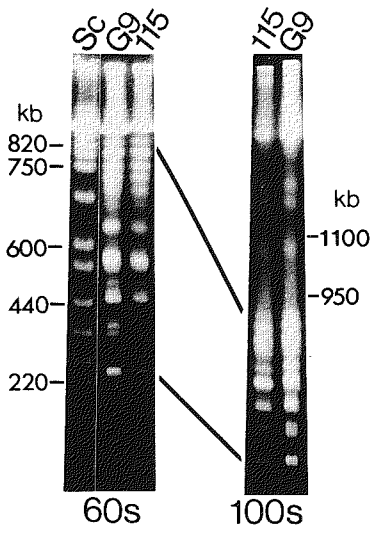

Figure 2.1.3. Karyotype analysis of *L. major*-like parasites isolated in Ecuador. PFGE was performed with a pulsed time of 60 or 100 s for two human isolates, one from an Andean highland village (115) and the other from the Pacific coastal lowland area (G9). Chromosomal DNAs of *Saccharomyces cerevisiae* (Sc) were used as the molecular marker.

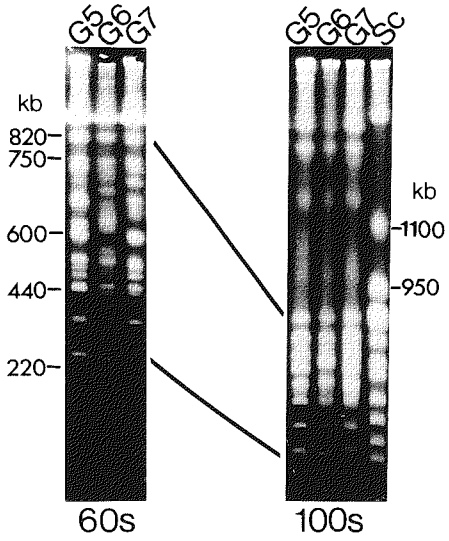

Figure 2.1.4. Karyotype analysis of *L. panamensis* isolated in Ecuador. PFGE was performed with a pulsed time of 60 or 100 s for three human isolates (G5, G6 and G7) from different lowland endemic regions. *Saccharomyces cerevisiae* (Sc) DNAs were used as the molecular standard.

major reference strains by zymodeme and serodeme analysis. A group of these stocks was, thus, designated as *L. major*-like although kinetoplast DNA profiles were different between *L. major*-like stocks and *L. major* reference strains (Momen *et al.*, 1985). *L. major*-like parasites have been also isolated from patients at two different regions of Ecuador (Hashiguchi *et al.*, 1991). Schizodemes of four isolates of *L. major*-like including the MHOM/EC/88/Paute115 isolated at an Andean highland village (Paute) and the MHOM/EC/87/G-09 at a Pacific coastal lowland place (Quininde) were very similar but not identical. Karyotype analysis in the present study also revealed similarity with minor differences between the MHOM/EC/88/Paute115 and MHOM/EC/87/G-09. At least 16 chromosomes and more than two chromosomal DNAs over 1,100 kb were separated, and differences in chromosome size between these isolates were apparent with respect to smaller chromosomes of 240-460 kb (Fig. 2.1.3).

The PFGE separated at least 16 chromosomes from 240 to 980 kb and more than two DNA bands over 1,100 kb for three human isolates (MHOM/EC/87/G-05, MHOM/EC/87/G-06 and MHOM/EC/87/G-07) of *L. panamensis* (Fig. 2.1.4). Karyotypes of these isolates from different endemic regions were similar but variable, indicating minor chromosome size polymorphism among *L. panamensis* isolates in Ecuador. Zymodeme analysis also indicated that these isolates were polymorphic for phosphogluconate dehydrogenase (6PGDH) (Mimori *et al.*, 1989).

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2. Detection of *Leishmania* Parasites by DNA Amplification using Polymerase Chain Reaction (PCR)

ABSTRACT. Polymerase chain reaction (PCR) techniques have been applied for DNA detection of *Leishmania* which had been isolated from human cutaneous lesions and biopsy materials from wild or domestic animals in Ecuador. Specificity and sensitivity of synthesized oligonucleotide primers which were derived from partial sequences of kinetoplast DNAs of parasites, *L. braziliensis*, have been tested for the PCR techniques using eight WHO reference strains, *L. braziliensis*, *L. panamensis*, *L. guyanensis*, *L. mexicana*, *L. amazonensis*, *L. pifanoi*, *L. garnhami* and *L. chagasi*, before application for Ecuadorian parasite DNAs. The results obtained were summarized as follows: (1) The oligonucleotide primers let to amplify only *L. braziliensis* complex DNA of the WHO reference strains. (2) Less than 10 parasites were enough numbers to amplify the specific DNA products. (3) Specificity of the primers was tested by a double blind test using already identified Ecuadorian *Leishmania* parasites. DNAs of *L. braziliensis*, *L. panamensis* and L. *guyanensis* identified by zymodeme analysis were amplified specifically. But DNAs of *L. mexicana* and *L. major*-like parasites were not amplified. (4) Fifty nine of unidentified Ecuadorian isolates at species level were examined by the PCR. DNA samples of the fifty two isolates were found to be amplified. Of the unamplified isolates, two from humans seemed to be *Leishmania mexicana* complex and five from human and *Rattus rattus* seemed to be *Trypanosoma* parasites. The current results indicate that the PCR techniques using the specific primers may differentiate *L. braziliensis* complex from others including *L. mexicana* complex.

An epidemiological survey of leishmaniasis has been done in Ecuador. In 1990, we had isolated and cultivated *Leishmania* parasites from humans and wild and domestic animals. Using these isolates, we tried to detect the parasite DNAs by PCR techniques with primers specific for *L. braziliensis* complex. In this study, we confirmed that *L. braziliensis* complex DNAs were amplified and detected by the specific primers.

Materials and Methods

Parasites used

Cultured parasites which were isolated from human cutaneous lesions and biopsy materials from domestic and wild animals in endemic areas of Ecuador were used to know amplification of *Leishmania* DNA products by PCR. Among these, one isolate was used for preliminary test to know the sensitivity of the PCR tests by limited serial dilution of parasites. Ecuadorian parasites which had been already identified by molecular techniques (Hashiguchi *et al.*, 1990; our unpublished data), were used to know the specificity of PCR by double blind test. Furthermore, in order to know the specificity of the oligonucleotide primers for PCR test, eight WHO reference strains, *L. braziliensis*, *L. panamensis*, *L. guyanensis*, *L. mexicana*, *L. amazonensis*, *L. pifanoi*, *L. garnhami*, and *L. chagasi* were examined.

Cultivation of parasites

The methods of isolation and cultivation of parasites from human cutaneous lesion and biopsy materials of domestic and wild animals were described previously (Gomez *et al.*, 1987; Hashiguchi *et al.*,

1990).

Preparation of parasite impregnated filter papers

Three MM papers (6 mm diameters, Whatman Company) which were impregnated appropriate numbers of cultivated parasites (approximately 10^4-10^6) were dried completely and used for further experiments. Triturated parasites (ranged from 2×10^4 to 2×10^{-1}) were impregnated in filter papers and dried completely. The parasite DNAs were extracted by using lysis buffer with proteinase K.

Pretreatment of the parasite impregnated filter papers

In a 1.5 ml micro-tube, a piece of the filter paper and 500 µl of lysis buffer (50 mM KCl, 10 mM Tris-Cl pH 8.3, 2.5 mM $MgCl_2$, 1 mg/ml gelatin, 0.45% NP-40, 0.45% Tween 20, 60 µg of proteinase K) were mixed and heated at 56°C for 1 hr and then at 96°C for 20 min.

Oligonucleotide primers

Sequences for specific DNA primers were derived from kinetoplast DNA of *L. braziliensis*. Oligonucleotide primers, 19 mer and 20 mer (primer 1 and primer 2, respectively) were synthesized respectively by using Cyclone Plus (Milligen Company).

Condition for the PCR test

DNA thermal cycler (Perkin Elmer Cetus Company) was used for the test. Thirty cycles of the reaction were repeated, as one cycle: denaturation condition at 94°C for one min, hybridization condition at 54°C for 2 min, and extension condition at 72°C for 3 min (Fig. 2.2.1).

The amplified DNA products were checked by 1% agarose gel electrophoresis. And also, to check the specificity of the amplified products against the *L. braziliensis* complex, digoxigenin labeled DNA probes were prepared by PCR techniques (Eshita *et al.*, 1990; Eshita and Fukuma, 1992). The southern blot hybridization was done to check the specificity of the amplified DNA.

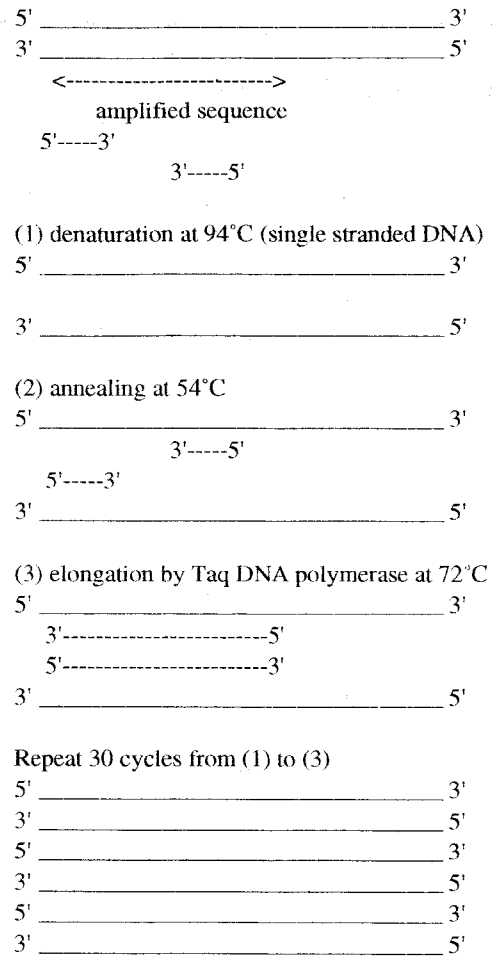

Figure 2.2.1. Parasite DNA amplification using PCR techniques.

Results and Discussion

Oligonucleotide primers amplified specifically only the parasite DNAs of *L. braziliensis* complex,

such as *L. braziliensis*, *L. guyanensis* and *L. panamensis* of WHO reference strains (Table 2.2.1). However other *Leishmania* species, *L. mexicana* complex and *L. donovani* complex were not amplified. These results suggested that the primers used for the present PCR test amplified specifically the DNAs of *L. braziliensis* complex parasites.

Diluted solution of the parasites (2×10^4, 2×10^3, 2×10^2, 2×10^1, 2×10^0, 2×10^{-1}) was prepared to know the limitation of DNA amplification for the PCR test. At least less than 10 parasites seemed to be enough numbers for the amplification and detection of the DNA (Table 2.2.2).

In this study, PCR techniques using primers specific for the *L. braziliensis* complex have been applied to Ecuadorian parasites. Double blind tests were done for PCR experiments, using 72 Ecuadorian isolates most of which had not been identified at species level yet. As the results, DNAs from parasites which have been identified as *L. braziliensis* complex, *L. panamensis* or *L. guyanensis* were amplified. However, those which have been identified as *L. mexicana* or *L. major*-like species were not amplified (Table 2.2.3). The results support that the primers used are specific for the amplification of *L. braziliensis* complex DNAs.

A total of 52 of the 59 isolates were amplified specifically using *L. braziliensis* complex primers. Out of seven negative isolates, two were found to be *L. mexicana* complex by molecular techniques and the remaining five were morphologically *Trypanosoma* parasites (Table 2,2.4). From the results obtained it may be possible to separate *L. braziliensis* complex from others including *L. mexicana* complex. Development of oligonucleotide primers for the detection of *L. mexicana* is in progress.

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Table 2.2.1. DNA amplification of 8 WHO reference strains by PCR test

WHO reference strain		PCR product*
L. braziliensis complex:		
L. braziliensis	(MHOM/BR/75/M2904)	+
L. guyanensis	(MHOM/BR/75/M4147)	+
L. panamensis	(MHOM/PA/71/LS94)	+
L. mexicana complex:		
L. mexicana	(MHOM/BZ/82/Bel21)	–
L. amazonensis	(MHOM/BR/73/M2269)	–
L. pifanoi	(MHOM/VE/57/LL1)	–
L. garnhami	(MHOM/VE/76/JAP78)	–
L. donovani complex:		
L. chagasi	(MHOM/BR/74/M2682)	–

※+, parasite DNA amplified ; –, no PCR product.

Table2.2.2. PCR amplification of the parasite DNA by limited serial dilution of *Leishmania* promastigotes

No. of parasites* (# PE-130)	PCR product**
2×10^4	+
2×10^3	+
2×10^2	+
2×10^1	+
2×10^0	+
2×10^{-1}	–
No parasite (NaCl only)	–

* No. of *Leishmania* parasites per micro-tube before amplification by PCR reaction.
**+, Parasite DNA amplified; –, no PCR product.

Table 2.2.3. Results of the double blind test of DNA amplification by PCR and zymodeme analysis

Designation code	PCR product*	Identification by zymodeme **
MHOM/EC/88/Paute103	–	*L. mexicana*
MHOM/EC/88/Paute115	–	*L. major*-like
MHOM/EC/89/INH3	+	*L. braziliensis*
MHOM/EC/89/INH10	+	*L. guyanensis*
MHOM/EC/89/INH13	+	*L. guyanensis*
MHOM/EC/89/INH36	+	*L. panamensis* or *L. guyanensis*
MHOM/EC/89/INH38	+	*L. panamensis* or *L. guyanensis*
MHOM/EC/88/Paute1	–	*L. mexicana*
MHOM/EC/88/Paute6	–	*L. mexicana*
MHOM/EC/88/Paute29	–	*L. mexicana*
MHOM/EC/87/GO5	+	*L. panamensis*
MHOM/EC/87/GO7	+	*L. panamensis*
MHOM/EC/87/GO9	–	*L. major*-like

* +, Parasite DNA amplified; –, no PCR product.

** Hashiguchi *et al.*, 1991; our unpublished data.

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Table 2.2.4. Parasite complex suspected by PCR tests, using *Leishmania* isolates from Ecuador in 1990

Designation code	Origin	PCR product*	*Leishmania* complex suspected**
MHOM/EC/90/VC98	human	+	*L. braziliensis* complex
MHOM/EC/90/SG14	human	+	*L. braziliensis* complex
MHOM/EC/90/PE130	human	+	*L. braziliensis* complex
MHOM/EC/90/DD35	human	–	*Leishmania* sp.1
MHOM/EC/90/VC96	human	+	*L. braziliensis* complex
MHOM/EC/90/VC127	human	+	*L. braziliensis* complex
MHOM/EC/90/PV1	human	+	*L. braziliensis* complex
MHOM/EC/90/PV156	human	+	*L. braziliensis* complex
MHOM/EC/90/PV158	human	+	*L. braziliensis* complex
MHOM/EC/90/PV159	human	+	*L. braziliensis* complex
MHOM/EC/90/PV160	human	+	*L. braziliensis* complex
MHOM/EC/90/PV162	human	+	*L. braziliensis* complex
MHOM/EC/90/SG40	human	–	*Trypanosoma* sp.
MHOM/EC/90/INH785	human	+	*L. braziliensis* complex
MHOM/EC/90/PV10	human	+	*L. braziliensis* complex
MHOM/EC/90/SG33	human	+	*L. braziliensis* complex
MHOM/EC/90/SG34	human	+	*L. braziliensis* complex
MRAT/EC/90/ASG223	rat***	–	*Trypanosoma* sp.
MRAT/EC/90/ASG224	rat***	–	*Trypanosoma* sp.
MHOM/EC/90/EM52	human	+	*L. braziliensis* complex
MHOM/EC/90/EM53	human	+	*L. braziliensis* complex
MHOM/EC/90/EM54	human	+	*L. braziliensis* complex
MHOM/EC/80/EM58	human	+	*L. braziliensis* complex
MHOM/EC/90/EM59	human	+	*L. braziliensis* complex
MHOM/EC/90/PQ91	human	+	*L. braziliensis* complex
MHOM/EC/90/PR88	human	+	*L. braziliensis* complex
MHOM/EC/90/EC50	human	+	*L. braziliensis* complex
MHOM/EC/90/LC47	human	+	*L. braziliensis* complex
MHOM/EC/90/INH932	human	+	*L. braziliensis* complex
Subtotal 29	(+) 25 (–) 4 (2: from humans, 2: from rats)		

* +, Parasite DNA amplified; –, no PCR product.

** *L. braziliensis* complex: *braziliensis*, *guyanensis*, *panamensis*; *Leishmania* sp.1: probably *L. mexicana* or *L. major*-like from its locality (Andes: Paute-Dug Dug); *Trypanosoma* sp.: identified morphologically.

*** *Rattus rattus*.

Table 2.2.4. (contd.)

Designation code	Origin	PCR product*	*Leishmania* complex suspected**
MHOM/EC/90/PV2	human	+	*L. braziliensis* complex
MHOM/EC/90/PV4	human	+	*L. braziliensis* complex
MHOM/EC/90/PV6	human	+	*L. braziliensis* complex
MHOM/EC/90/PV12	human	+	*L. braziliensis* complex
MHOM/EC/90/PV18	human	+	*L. braziliensis* complex
MHOM/EC/90/PV19	human	+	*L. braziliensis* complex
MHOM/EC/90/PV157	human	+	*L. braziliensis* complex
MHOM/EC/90/PV161	human	+	*L. braziliensis* complex
MHOM/EC/90/SG4	human	+	*L. braziliensis* complex
MHOM/EC/90/SG13	human	+	*L. braziliensis* complex
MHOM/EC/90/SG16	human	+	*L. braziliensis* complex
MHOM/EC/90/SG17	human	+	*L. braziliensis* complex
MHOM/EC/90/SG23	human	+	*L. braziliensis* complex
MHOM/EC/90/SG24	human	+	*L. braziliensis* complex
MHOM/EC/90/SG26	human	+	*L. braziliensis* complex
MHOM/EC/90/SG27	human	+	*L. braziliensis* complex
MHOM/EC/90/SG35	human	+	*L. braziliensis* complex
MHOM/EC/90/SG37	human	+	*L. braziliensis* complex
MHOM/EC/90/SG39	human	+	*L. braziliensis* complex
MHOM/EC/90/SG44	human	+	*L. braziliensis* complex
MHOM/EC/90/SG45	human	+	*L. braziliensis* complex
MHOM/EC/90/SG46	human	+	*L. braziliensis* complex
MHOM/EC/90/EM49	human	–	*Trypanosoma* sp.
MHOM/EC/90/EM55	human	+	*L. braziliensis* complex
MHOM/EC/90/EM57	human	+	*L. braziliensis* complex
MHOM/EC/90/EM60	human	+	*L. braziliensis* complex
MHOM/EC/90/EM64	human	+	*L. braziliensis* complex
MHOM/EC/90/VC97	human	+	*L. braziliensis* complex
MHOM/EC/90/INH690	human	–	*Leishmania* sp.2
MRAT/EC/90/ASG222	rat	–	*Trypanosoma* sp.
Subtotal 30		(+) 27 (–) 3 (2: from humans, 1: from rat)	
Total 59		(+) 52 (–) 7 (4: from humans, 3: from rats)	

* +, Parasite DNA amplified; –, no PCR product.

** *L. braziliensis* complex: *braziliensis, guyanensis* and *panamensis*; *Leishmania* sp.2: *L. mexicana* by zymodeme analysis (unpublished data); *Trypanosoma* sp.: identified morphologically.

Chapter 3

Vector Entomological Aspects

1. Biting Activity and *Leishmania* Infections of Man-biting Species of Sandflies, *Lutzomyia* spp.

ABSTRACT. Man-biting activity and *Leishmania* infections of sandflies, *Lutzomyia* spp. have been studied during July and August 1990 in Ecuador. Sandflies were collected by four methods, human bait, illuminated Shannon traps, direct aspiration from diurnal resting site and sticky traps at six localities. Total numbers of the sandflies collected were 2,401 belonging to 12 species. *Leishmania* promastigotes were demonstrated in *Lutzomyia trapidoi* collected at two sites, Pt. Quito and Ocaña, and in *Lu. hartmanni* at the latter site. Most of man-biting sandflies came to human bait within two hrs after dark and formed one peak through dusk to dawn. The number of sandflies collected varied by climatic factors as well as darkness. The sandflies collected during/after dusk (18:00 to 20:00) were greater in numbers than those collected before/during dawn (04:00 to 06:00). Infection rates with *Leishmania* of sandflies caught before/during dawn were slightly higher than those of sandflies collected during/after dusk. Parous rates of the sandflies collected before/during dawn were higher than those of flies during dusk (55.2% *vs* 33.3% in *Lu. hartmanni* and 40.9% *vs* 20.0% in *Lu. trapidoi*). The higher parous rates brought the higher rates of *Leishmania* infection. Sandflies caught during/after dawn had a tendency to have more suck-like ovarian follicles than those during/after dusk.

Leishmaniasis is one of the six important tropical diseases (World Health Organization, 1987). The New World leishmaniasis is transmitted by blood-sucking sandflies, *Lutzomyia* spp. (Diptera: Psychodidae) and many species of *Leishmania* parasites cause serious manifestations in humans. The infected sandfly vector possesses *Leishmania* promastigotes in the gut.

Recently, many information on Ecuadorian leishmaniasis, especially on the vector has been reported by Hashiguchi and his co-workers (Hashiguchi *et al.*, 1985, 1987, 1990, unpublished data; Armijos *et al.*, 1990; Takaoka *et al.*, 1990a,b; Gomez *et al.*, 1990; Alexander *et al.*, 1990). In Ecuador, they have confirmed the presence of seven *Leishmania* species, *i.e., L. panamensis, L. braziliensis, L. guyanensis. L. amazonensis, L. equatorensis, L. mexicana* and *L. major*-like, and also incriminated three species of the sandfly vectors, *Lu. trapidoi, Lu. hartmanni* and *Lu. gomezi* as possible vectors of the disease. In 1990, a further entomological investigation on *Lutzomyia* spp. has been done in Ecuador. In the present survey, the biting activity and *Leishmania* infections of sandflies have been studied during July and August 1990. Natural biting activities of sandflies began at dusk and continued until dawn, with the hematophagous characteristics varying from species to species.

Hashiguchi *et al.* (1985a) reported that biting pattern of *Lu. trapidoi* during the period formed two peaks, higher peak at evening and lower peak at early morning, while *Lu. hartmanni* formed only one peak at midnight. In this study, we tried to analyze these

phenomena by comparisons of the infection rates and follicular development of sandflies. We found that the parous rates of sandflies collected before/during dawn were slightly higher than those of sandflies during/after dusk, and higher parous rates caused proportionally higher infection rates of sandflies. Furthermore, as a preliminary trial, polymerase chain reaction (PCR) was attempted to characterize parasites derived from the gut of a promastigotes-positive sandfly.

Materials and Methods

Sandfly collection and rearing for a short period

Sandflies were collected by four methods, human bait, illuminated Shannon trap, direct aspiration from diurnal resting site and sticky traps (Alexander *et al.*, 1990) at six different localities in Ecuador during July and August 1990 (Table 3.1.1). Sandflies collected were maintained in a glass bottle at a highly humid condition and a low temperature until dissected.

Identification and dissection of sandflies

Fresh (live) sandflies were dissected and their spermathecae were examined for the identification under binocular microscope.

Isolation of parasites

The parasites isolated from the gut of sandflies were aspirated by a syringe with sterile saline, and inoculated into the nose and footpads of golden hamsters. Amastigotes were recovered from the animals about 4 weeks later and then inoculated into culture medium (Gomez *et al.*, 1987; Hashiguchi *et al.*, 1990) for further characterization. Multiplicated amastigotes were transformed into promastigotes in Schneider's drosophila medium (Hendricks *et al.*, 1978), supplemented with 15% heat-inactivated fetal calf serum (Flow Laboratories, Rockville, MD). The promastigotes obtained were used for characterization by polymerase chain reaction (PCR) test (Eshita *et al.*, 1990; Eshita and Fukuma, 1992; see Chapter 2.2 in this text).

Results and Discussion

A total of 2,401 sandflies were collected at six localities in this study (Table 3.1.1). They were 12 species in total belonging to the genus *Lutzomyia*. Most of the man-biting sandflies came to human bait within two hrs after dark, and formed one peak (Table 3.1.2). The number of sandflies varied by climatic factors as well as darkness. Man-hour index was quite variable at each locality (Table 3.1.3).

Three sandfly species, *Lu. trapidoi*, *Lu. hartmanni* and *Lu. gomezi* are suspected to be important vectors of leishmaniasis in Ecuador. Promastigotes of *Leishmania* parasites have been demonstrated in *Lu. trapidoi* which were collected at Pt. Quito, and *Lu. trapidoi* and *Lu. hartmanni* at Ocaña during our survey in 1990 (Table 3.1.4). Infection rates of the sandflies collected during early morning were slightly higher than those of the flies during evening (Table 3.1.4).

The parous rates of sandflies collected in early morning were higher than those of the flies caught during dusk (59.2% *vs* 33.3% in *Lu. hartmanni* and 40.9% *vs* 20.0% in *Lu. trapidoi*) (Table 3.1.5). The higher parous rates of sandflies caused the higher *Leishmania* infection rates. Parous flies with suck-like ovarian follicles were caught more frequently during dawn. However the current data could not explain why the flies collected in the early morning had higher parous rates than those in the evening. The higher infection rate during dawn may reflect gonotrophic cycle of sandflies in the present study site of tropical regions. In order to clarify the difference of parous rates between dawn and dusk, it will be necessary to accumulate more information on the oviposition activity and other reproductive behaviors of sandflies in the field.

As mentioned previously (see Chapter 2.2), ol-

Table 3.1.1. Detectin of *Leishmania* parasites from sandflies in Ecuador (27 July - 6 September 1990)

Locality	Type of collection	No. of sandflies		
		collected	dissected	infected (%)
Paute	HBC*	2	not done	
	RSC**	0		
	OPT***	6	not done	
	ST****	0		
Zumba	HBC	174	30 (*Lu. nevesi*)	0
			6 (*Lu. tortura*)	0
			4 (*Lu. serrana*)	0
	RSC	58	not done	
	OPT	0		
Pt. Quito	HBC	728	3 (*Lu. gomezi*)	0
			56 (*Lu. panamensis*)	0
			142 (*Lu. trapidoi*)	3 (2.1)
			29 (*Lu. hartmanni*)	0
			1 (*Lu. shannoni*)	0
	RSC	109	not done	
	ST	510	not done	
Esmeraldas	HBC	3	not done	
	RSC	45	3 (*Lu. triacantha*)	0
			2 (*Lu. hartmanni*)	0
			10 (*Lu. trapidoi*)	0
			8 (*Lu. shanonii*)	0
			3 (*Lu. tortura*)	0
Ocaña	HBC	315	75 (*Lu. hartmanni*)	2 (2.6)
			4 (*Lu. trapidoi*)	0
	HBC/ST*****	78	24 (*Lu. hartmanni*)	0 (12.5)
			23 (*Lu. trapidoi*)	1 (4.3)
	ST	238	7 (*Lu. hartmanni*)	0
	HBC/ST	46	not done	
	RSC	5	1 (*Lu. hartmanni*)	0
			4 (*Lu. trapidoi*)	0
Manabi	HBC	35	30 (*Lu. hartmanni*)	0
			2 (*Lu. gomezi*)	0
			2 (*Lu. serrana*)	0
			1 (*Lu. oswaldoi?*)	0
	ST	49	8 (*Lu. hartmanni*)	0
			12 (*Lu. dysponeta*)	0
			13 (*Lu. trapidoi*)	0
			13 (*Lu. serrana*)	0
			2 (*Lu. gomezi*)	0
			1 (*Lu. shannoni*)	0

*, HBC: Human bait collection during night (18:00-20:00); **, RSC: Resting site collection during daytime. ***, OPT: Oil-impregnated sticky paper trap during overnight; ****, ST :Illuminated Shannon trap during night (18:00-20:00); *****, HBC/ST: Human bait collection (HB) or illuminated Shannon trap (ST) during early morning (04:00-06:00).

Table 3.1.2. Collection of sandflies by human bait and illuminated Shannon traps, respectively, at 24 de Mayo, Pt. Quito in 11 August 1990

Time	Temp. (°C)	No. of sandflies collected by human-baited	No. of sandflies collected by Shannon trap
18:00-18:10	28.0	0	0
18:10-18:20	27.7	0	0
18:20-18:30	26.6	0	0
18:30-18:40	26.1	0	2
18:40-18:50	24.9	0	4
18:50-19:00	25.0	0	10
19:00-19:10	24.2	1	6
19:10-19:20	24.2	10	50
19:20-19:30	23.9	11	30
19:30-19:40	24.3	2	10
19:40-19:50	24.0	1	3
19:50-20:00	24.3	0	0
Total		25	125
Man-hour index*		7.7	60

* No. of sandflies collected per man per hour.

igonucleotide primers used were found to be specific for amplification of DNAs of *L. braziliensis* complex, *viz., L. braziliensis, L. guyanensis* and *L. panamensis,* but not for *L. mexicana* complex, *viz., L. mexicana, L. amazonensis, L. pifanoi and L. garnhami,* and *L. donovani* complex, *viz., L. chagasi,* of the WHO reference strains. Based on these results, we tried to use the primers against the parasites (promastigotes) derived from an infected sandfly in PCR experiments. However, the primers specific for *L. braziliensis* complex did not lead amplification of the promastiogte DNA. As less than 10 parasites were enough numbers for the amplification and detection of the DNA (see Chapter 2.2), PCR methods may be directly applicable for amplification of the parasite DNA of infected sandflies. Further such an examination is required using more sandfly samples infected with *Leishmania.*

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Table 3.1.3. Man-hour index of man-biting sandflies collected at different localities in Ecuador, 1990

Locality and date	Latitude (km)	Time (hr)	Temp. (°C)	No. of sandflies collected	No.of human bait	Man-hour index*
Paute (Mt. Yumacay), 27 July 1990	2.0-2.3	18:30-19:30 (1 hr)	16-16	1	3	0.3
Pt. Quito (El Pite), 1 August 1990	2.3	19:00-20:00 (1 hr)	22-19	11	9	1.2
Zumba (El Pite), 1 August 1990	1.1	11:00-12:00 (1 hr)	25-25	13	3	4.3
Zumba (Zapotal), 3 August 1990	0.9-0.95	19:00-21:00 (2 hrs)	24-19	164	9	9.1
	0.95			100	3	16.6
	0.93			50	3	8.3
	0.9			14	3	3.5

* No. of sandflies collected per man per hour.

Table 3.1.4. Natural infection with *Leishmania* promastigotes of sandflies, *Lutzomyia* collectd by human bait at Pt. Quito and Ocaña in Ecuador, 1990

Locality	Date and time	No. infected / no. dissected (%)	
		Lu. hartmanni	*Lu. trapidoi*
Pt. Quito	11 Aug.1990 (18:00-20:00)	0	3/124 (2.1%)
Ocaña	4 Sept.1990 (18:00-20:00)	2/75 (2.6%)	0/4 (0.0%)
Ocaña	5 Sept.1990 (04:00-06:00)	3/24 (12.5%)	1/23 (4.3%)

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2. Transmission of *Leishmania panamensis* to Man by the Sandflies *Lutzomyia hartmanni* and *Lu. trapidoi* (Diptera: Psychodidae) in Ecuador

ABSTRACT. Transmission of *Leishmania panamensis* to man by the bite of infected sandflies *Lutzomyia hartmanni* (Fairchild & Hertig) or *Lu. trapidoi* (F. & H.) in Ecuador is reported. This finding confirms the importance of these species, both of which were known previously to be highly anthropophilic and in which natural infections with *Leishmania* had already been observed, in the epidemiology of leishmaniasis in the Department of Cañar.

Previous studies of leishmaniasis in Ecuador have revealed the presence of the disease in all three geographical regions of the country, with foci described from 14 of the 21 Departments. Human cases due to *Leishmania braziliensis, L. panamensis, L. guyanensis, L. amazonesis* and *L. major*-like have been reported from the coastal plain, *L. mexicana* and *L. major*-like from the Andean plateau and *L. braziliensis* from the Oriente or Amazon basin region (Mimori *et al.*,1989; Armijos *et al.*,1990; Hashiguchi *et al.*, 1991). Some sandfly species known from Ecuador has been incriminated or suspected as a vector of *Leishmania* in that country (Hashiguchi *et al.*, 1985, 1991; Takaoka *et al.*, 1990; Gomez and Hashiguchi, 1990). Among the known Ecuadorian species, several are proven or suspected vectors in other parts of their range. These include *Lutzomyia carrerai carrerai* in Bolivia (Le Pont *et al.*, 1988); *Lu. yuilli, Lu. hirsuta hirsuta, Lu. amazonensis, Lu. ubiquitalis, Lu. ayrozai, Lu. davisi and Lu. flaviscutellata* in Brazil; *Lu. trapidoi, Lu. flaviscutellata* and *Lu. hartmanni* in Colombia; *Lu. panamensis* and *Lu. trapidoi* in Honduras; *Lu. gomezi, Lu. trapidoi* and *Lu. panamensis* in Panama; *Lu. flaviscutellata* in Surinam and Trinidad and Tobago; and *Lu. gomezi, Lu. lichyi, Lu. olmeca bicolor, Lu. flaviscutellata* and *Lu. panamensis* in Venezuela (Grimaldi *et al.*, 1989). To date 60 species of phlebotomines, including 55 belonging to the genus *Lutzomyia*, have been reported from Ecuador (Alexander *et al.*, 1992). Natural infections with *Leishmania* species have been recorded from four Ecuadorian sandfly species, *i.e., Lu. trapidoi, Lu. hartmanni* (Hashiguchi *et al.*, 1985a,b) *Lu. gomezi* and *Lu. ayacuchensis* (Hashiguchi *et al.*, 1985a,b, 1987, 1991; Gomez and Hashiguchi, 1991).

The Department of Cañar in southwest Ecuador contains territory pertaining to two of the geographical regions of the country, with the coastal plain occupying the western half and giving way to the Andean foothills in the east. La Troncal (2°30'S, 79°0'E) is a town of approximately 30,000 inhabitants lying at the boundary of the two regions. Studies made in the vicinity by Hashiguchi *et al.* (1985a, 1987) and Alexander *et al.* (1992) have revealed that the sandfly fauna consists of at least four species, *i.e., Lu. aclydifera* (Fairchild & Hertig), *Lu. shannoni* (Dyar), *Lu. trapidoi* (F. & H.) and *Lu. hartmanni* (F. & H.), with only the latter two species apparently attracted to human bait in the area.

This paper reports the results of a sandfly collecting trip that resulted in the infection of the senior author by *Leishmania* following the bite of an infected sandfly. The date and place at which infection is thought to have occurred were recorded and both the parasite and the anthropophilic species were identified. We suggest that either *Lu. hartmanni* or *Lu.*

trapidoi was the species that transmitted the infection and that these are the main vectors of *Leishmania* to man in the La Troncal area.

Materials and Methods

Sandfly collections were made using an illuminated Shannon trap and as the insects came to bite protected human volunteers in secondary forest above the Balneario "El Chorro" near La Troncal at kilometer nine of the main Guayaquil-Cuenca highway, at an altitude of 3,500 metres above sea level. Two collections were made during the night of 4-5 September 1990, from 17:30-19:20 and from 04:00-05:45. Three volunteers collected sandflies as they came to bite while the senior author (a 33 year-old white male from the U.K. who had not been infected previously with *Leishmania*) caught the insects as they alit on the walls of the Shannon trap, during the course of which he was bitten 15-20 times on the neck, forearms and hands. Each of the biting flies was caught with an aspirator (Endris *et al.*, 1982) and dissected shortly after capture or preserved in liquid nitrogen (Young *et al.*, 1987) and dissected at a later date.

Parasite identification followed aspiration from the (non-ulcerated) lesion and culturing in Senekjie's medium. The sample, identified by the code 1563, was subjected to monoclonal antibody analysis using an indirect immunofluorescent antibody assay. Promastigotes were acetone-fixed to glass slides then incubated with a monoclonal panel at a 1:1000 dilution of the ascites-derived reagents. Monoclonal antibodies were supplied by Dr. Diane McMahon-Pratt (Department of Epidemiology and Public Health, Yale University). Serial two-fold dilutions of serum beginning at 1:8 were incubated with amastigote antigen prepared from a WHO reference srain of *L. amazonensis* (VEC/BR/1967/PH8) using the procedure described by Shaw and Lainson (1977). Fluorescein-conjugated rabbit antibody to mouse immunoglobulin (Accurate Chemicals, Westbury, NY) at a 1:20 dilution was used to demonstrate reactivity with the monoclonal antibodies. Positive sera were assigned a titre of >1:16 on negative controls.

Results and Discussion

A total of 677 sandflies were caught as they came to protected human bait and to the illuminated Shannon trap during the two sampling periods (Table 3.2.1). Of 133 flies dissected in 0.7% saline solution shortly after collection 106 (79.7%) were female *Lu. hartmanni* and the remainder *Lu. trapidoi*. Only seven flies were identified from the Shannon trap collections, and all of these were the former species. A difference in the relative proportions of the two species was seen between the two collections; *Lu. hartmanni* made up 75 (94.9%) of the evening collection but only 51.1% of those captured before dawn. This appears to be consistent with the findings of Hashiguchi *et al.* (1985b), who noted that *Lu. trapidoi* showed its second nightly peak of biting activity in the early hours of the morning while *Lu. hartmanni* bit consistently through the night.

Natural infections with flagellates were seen in the hindguts of 5 (5.0%) of the 99 *Lu. hartmanni* dissected from protected human bait collections and none of the seven from the Shannon trap. A single *Lu. trapidoi* (4.3%) was infected of the 23 females of this species dissected. Identification of these parasites has not been completed but based on their morphology and position in the sandfly gut they are assumed to have been *Leishmania* of the L. *braziliensis* species group, which includes *L. panamensis*.

During the last week of September 1990, a reddish, slightly raised swelling of approximately 25 mm diameter appeared on the outer edge of the senior author's right hand. A Montenegro test applied the following week gave a positive result, manifest as a reddish circular blemish on the forearm of diameter 20 mm. Samples aspirated from the plaque on October 15th reacted with the monoclonal antibodies B2

Table 3.2.1. Sandflies collected by illuminated Shannon trap and on protected human bait near La Troncal, Department of Cañar, Ecuador, September 4-5, 1990

Time of collection	Type of collection	No. of flies collected	*Lutzomyia* spp.	No. of flies (% infected)
18:00-20:00	Shannon trap	238	*Lu. trapidoi*	–
			Lu. hartmanni	7 (0.0%)
" "	Human bait	315	*Lu. trapidoi*	4 (0.0%)
			Lu. hartmanni	75 (2.8%)
04:00-06:00	Shannon trap	46	*Lu. trapidoi*	0
			Lu. hartmanni	0
" "	Human bait	78	*Lu. trapidoi*	23 (4.3%)
			Lu. hartmanni	24 (12.5%)

(VI-4B9-D10) and B11 (VII-5G3-F3) (both *L. panamensis*), B21 (VII-6H9-G10) (*L. braziliensis* / *L. panamensis*), as well as B4 (VI-2A5-A4) and B7 (VI-2A4-E3) (both *L. panamensis*). The titre was positive for total *Leishmania*-specific serum antibody at a dilution of 1:32.

Within the two months prior to the field trip to La Troncal, the senior author had visited several other areas of Ecuador endemic for *Leishmania*, including Paute (Department of Azuay), Zumba (Zamora Chinchipe), Puerto Quito (Pichincha), Muisne (Esmeraldas) and Portoviejo (Manabi). The possibility also exists that the infected fly that bit the author did not belong to either of the two known man-biting species, and that it escaped after feeding from the Shannon trap in which the author was working. Infected sandflies (*Lu. trapidoi*) were collected at one other site (Puerto Quito) during a field trip made from 10-14th August and it is possible that infection occurred there rather than at La Troncal. However, based on the large number of bites the senior author received on the night of 4/5 September, and the unusually high percentage of infected flies collected on that night we believe that infection occurred here and that either *Lu. hartmanni* or *Lu. trapidoi* was the species involved.

Although *Lu. hartmanni* was known to be the major man-biting species in the La Troncal-Ocaña area and natural infections with *Leishmania*-like parasites had already been noted in wild-caught specimens (Hashiguchi *et al.*, 1985b, 1987) the observations reported here provide further evidence that this species may be a vector of *Leishmania* to man. The biting activities of *Lu. hartmanni* and *Lu. trapidoi* in the Ocaña area were reviewed by Hashiguchi *et al.* (1985a), who found that the former species was active throughout the night while the latter bit more frequently during two distinct peaks. The geographical distribution of *Lu. hartmanni* extends from Panama to southern Ecuador, and it appears to be an important man-biter throughout its range (Young, 1979). *Lu. trapidoi* is a proven vector of *L. panamensis* in Costa Rica, Panama and Colombia (WHO, 1990).

The known distribution of *L. panamensis* extends from Central America to Ecuador (Grimaldi *et al.*, 1989). Prior to the present study positive identifications of the parasite from Ecuador had been made

only from the Department of Esmeraldas and Pichincha, both further north than Cañar, although specific typing of a number of *Leishmania* isolates from human cases in several Ecuadorian provinces is at present being performed (Hashiguchi and Gomez, 1990). The proven reservoirs of *L. panamensis* include the sloths *Bradypus griseus* and *Choloepus hoffmanni* (Zeledon *et al.*, 1975), although the domestic dog *Canis familiaris* and several wild mammal species including rodents, carnivores and monkeys may be implicated in certain areas (Grimaldi *et al.*, 1989). Hashiguchi *et al.* (1985c) failed to isolate *Leishmania* from wild mammals of three species in Ocaña and the host of *L. panamensis* in Ecuador remains unknown.

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3. The Phlebotomine Sandfly Fauna (Diptera: Psychodidae) of Nine *Leishmania* Endemic Sites in Ecuador

ABSTRACT. The phlebotomine sandfly fauna of each of nine sites endemic for *Leishmania* in Ecuador was sampled using a variety of methods. A total of 30 species were collected and three *Lutzomyia* species, *i.e., Lu. saulensis, Lu. furcata* and *Lu. strictivilla,* recorded for the first time in Ecuador. The genus *Warileya* was also recorded in the country for the first time, represented by *W. phlebotomanica* Hertig. The first collections of sandflies from the Department of Loja were made. The known ranges of 23 species were increased by 36 new province records.

Prior to the present (1990) study, the phlebotomine sandfly fauna of Ecuador was known to consist of at least 56 species, following the most recent survey by Alexander *et al.* (1992). Previous information on the Ecuadorian sandfly fauna has been published by Young and Rogers (1984), Arzube (1960) and Rodriguez (1950, 1953, 1956). At least 15 of these species are known to be anthropophilic in Ecuador or in other parts of their ranges and may therefore be involved in the transmission of *Leishmania* to man. Four species among the Ecuadorian sandfly fauna are proven or suspected vectors of *Leishmania* elsewhere (Hashiguchi *et al.*, 1985a,b; Gomez and Hashiguchi, 1987; Grimaldi *et al.*, 1989) and there is at least one substantiated case of *L. panamensis* to man by the bite of an infected *Lu. hartmanni* or *Lu. trapidoi* (see Chapter 3.2 in this text).

This article reports the results of a survey of the sandfly of nine areas endemic for human *Leishmania* in the three geographical regions of Ecuador, *i.e.*, the coastal plain, Andean plateau and the "Oriente" or Amazon basin. The survey represents a continuation of work carried out between July and October 1988 and published in our previous report (Hashiguchi *et at.*, 1990).

The survey was carried out between July and October 1990 and *Leishmania*-endemic case sites visited in eight of the 21 Departments of Ecuador, including the previously unsurveyed Department of Loja. Trapping methods used were as described in our previous report (Hashiguchi *et al.*, 1990). The nine *Leishmania*-endemic sites were visited.

Study sites and Collection methods

Coastal plain

Esmeraldas. Canton Muisne (Map ref. O°56'N, 80°00'W; August 16-17, 1990). Protected human bait collection at Muisne, resting site collections at Recintos "El Mamey" and "La Correntada".

Pichincha. Puerto Quito (Map ref. 0°10'N, 79°16'W; August 10-15, 1990). Protected human bait, Shannon trap and resting site collections near Puerto Quito and at Recintos "Paraiso Escondido", "El Kaoni", "Las Delicias" and "24 de Mayo".

Manabi. Portoviejo (Map ref. 1°90'S, 80°45'W; August 27-28, September 14-16 and October 3-4, 1990). Castor oil trap collections at Pueblito Rocafuerte and protected human bait and Shannon trap collections at Recintos "El Progreso" and "San Sebastián".

Manabi. Junin (Map ref. 1°90'S, 80°18'W; August 27-28 and October 2-4, 1990). Protected human bait, Shannon trap and resting site collections at Recintos "El Toro" and "La Tablada de Mendoza".

Cañar. Ocaña (Map ref. 2°47'S, 79°04'W; September 3-5, 1990). Protected human bait, Shannon trap

and resting site collections near Balneario "El Chorro", Km 9 of Guayaquil-Cuenca highway.

El Oro. Zaruma (Map ref. 3°72'S, 79°63'W; September 13-15, 1990). Protected human bait, Shannon trap and resting site collections made near Zaruma, and at Balneario "El Recreo" near La Avanzada. Single castor oil trap collection made above Rio Pindo on El Oro-Loja border.

Andean plateau

Azuay. Canton Paute (Map ref. 2°52'S, 78°45'W; July 28-31 and August 4, 1990). Protected human bait, castor oil trap and Shannon trap collections made near town of Paute.

Loja. Parroquias Zambi and Guayquichuma (Map ref. 3°90'S, 79°54'W; September 13-15, 1990). Resting site collection made in scrub forest near highway in Parroquia Zambi. Protected human bait and Shannon trap collections made in coffee plantation at El Prado, Parroquia Guayquichuma.

Oriente

Zamora Chinchipe. Canton Zumba (Map ref. 4°90'S, 79°09'W; August 1-3, 1990). Protected human bait, Shannon trap and resting site collections made at Recintos "La Chonta", "El Pite", "Izimanchi" and "El Zapotal".

Results and Discussion

The complete list of 30 species collected in Ecuador between July and October 1990 is presented in Table 3.3.1. Four of these represent new records for the country and a total of 36 new province records were made among 23 species. The genus *Warileya*, represented by *W. phlebotomanica*, is reported in Ecuador for the first time. These four new species records are discussed in detail below, together with others of note.

***Warileya phlebotomanica* Hertig**. Two females of this species were collected from a tree hole in scrub forest near the Catamayo highway in the parish of Zambi, Loja province on the afternoon of 14. ix. 1990. No males were collected on this or a second attempt made the following day. This represents the first record of the genus *Warileya* in this province and in Ecuador as a whole, and the first time *W. phlebotomanica* has been collected outside Peru.

***Lutzomyia saulensis* (Floch & Abonnenc)**. A female of this species was collected in a Shannon trap at Paraiso Escondido, near Puerto Quito on 14. viii. 1990. This is the first record of *Lu. saulensis* from Ecuador, although Young (1979) reported it as occurring in both Peru and Colombia and its presence in Pichincha is to be expected.

***Lutzomyia furcata* (Mangabeira)**. Three males and one female of this species collected off a tree trunk at Recinto 24 de Mayo, near Puerto Quito, Pichincha on 11. viii. 1990 represent the first examples of *Lu. furcata* recorded from Ecuador. This species was already known from the neighbouring countries of Colombia and Peru, as well as Brazil, French Guiana and Venezuela (Young, 1979) so that its presence in northern Ecuador is not surprising.

***Lu. sallesi* (Galvao & Coutinho)**. This species was first recorded from Guayas by Arzube (1960) but no examples were collected from this or any other province during the 1988 survey. It was however present in tree trunk and Shannon trap collections made in two coffee plantations at Recintos "La Tablada de Mendoza" and "El Toro", near Junin, Manabi on 28. viii, 2. x and 3. x. 1990, and was collected by the same methods at Campozano, also in Manabi, the following day.

***Lu. strictivilla* Young**. This was one of three species taken in human bait collections in the vicinity of Zumba, Zamora Chinchipe on 1-3. viii. 1990. A single male was taken in these collections and no specimens were found on tree trunks or in Shannon trap samples. This species was first described by Young (1979) from Antioquia, northern Colombia, and the present record therefore considerably increases its known range.

Table 3.3.1. Species of phlebotomine sandfly collected in *Leishmania*-endemic areas of Ecuador July-October 1990, together with methods of capture, diurnal resting sites and distribution. Departments for which no records of a particular species prior to the present survey are shown in capital letters, and new species records for Ecuador marked with an asterisk. Methods of capture abbreviated as follows: DA - direct aspiration (from diurnal resting sites); HB - protected human bait; ST - Shannon trap; LT - (CDC) light trap; COT - castor oil trap

Species	Methods of capture	Resting site(s)	Distribution by Department
Brumptomyia leopoldoi (Rodriguez)	DA	tree trunks	Cañar, El Oro, Esmeraldas, Guayas, Los Rios, Manabi, Pichincha
*Warileya phlebotomanica** Hertig	DA	tree holes	LOJA
Lutzomyia gomezi (Nitzulescu)	DA, HB, ST, LT	tree trunks, animal burrows	Azuay, Bolivar, Cañar, EL ORO, Esmeraldas, Guayas, LOJA, Manabi, Morona Santiago, Pichincha, Sucumbios
Lu. serrana (Damasceno & Arouck)	DA, HB, ST	tree trunks, animal burrows	Bolivar, Cañar, EL ORO, ESMERALDAS, Guayas, LOJA, Los Rios, Manabi, Pichincha, Sucumbios, ZAMORA CHINCHIPE
Lu. nevesi (Damasceno & Arouck)	DA, HB, ST	tree trunks	Guayas, Morona Santiago, Sucumbios, ZAMORA CHINCHIPE
Lu. vespertilionis (Fairchild & Hertig)	DA, ST, LT	tree trunks	Cañar, Guayas, Pichincha
Lu. dysponeta (Fairchild & Hertig)	DA, ST, LT	tree trunks, animal burrows, leaf litter, under rocks	AZUAY, Bolivar, Cañar, EL ORO, Esmeraldas, Guayas, Los Rios, MANABI, PICHINCHA
Lu. camposi (Rodriguez)	DA	tree trunks, animal burrows	Bolivar, Cañar, Los Rios, Pichincha
*Lu. furcata** (Mangabeira)	DA	tree trunks	PICHINCHA

Table 3.3.1 (contd.). List of species collected in *Leishmania*-endemic sites in Ecuador, July-October 1990

Species	Methods of capture	Resting site(s)	Distribution by Department
Lu. gorbitzi (Blancas)	DA, COT	tree trunks tree holes	Guayas, ESMERALDAS, MANABI
Lu. sallesi (Galvao & Coutinho)	DA, ST	tree trunks	Guayas, MANABI
Lu. shannoni (Dyar)	DA, HB, ST	tree trunks	Bolivar, Cañar, EL ORO, ESMERALDAS, Guayas, Los Rios, Manabi, Morona Santiago, Pichincha, Sucumbios
Lu. abonnenci (Floch & Chassignet)	DA, ST	tree trunks	EL ORO, ESMERALDAS, Guayas, Los Rios, Manabi
Lu. dasymera (Fairchild & Hertig)	DA	tree trunks	Cañar, Manabi, PICHINCHA
Lu. undulata (Fairchild & Hertig)	DA, ST	tree trunks	Cañar, ESMERALDAS, Manabi, Pichincha
Lu. triramula (Fairchild & Hertig)	DA, LT	tree trunks, animal burrows	Cañar, PICHINCHA
Lu. aragaoi (Costa Lima)	DA, ST, LT	tree trunks, animal burrows	Cañar, PICHINCHA
Lu. barrettoi majuscula Young	DA, ST, LT	tree trunks, animal burrows	Bolivar, Cañar, Guayas, Los Rios, Manabi, Pichincha
Lu. aclydifera (Fairchild & Hertig)	DA, LT	tree trunks	Cañar, Pichincha
Lu. reburra (Fairchild & Hertig)	DA, ST	tree trunks, animal burrows	Cañar, El Oro, Pichincha
Lu. trapidoi (Fairchild & Hertig)	DA, HB, ST, LT	tree trunks, animal burrows, leaf litter	Bolivar, Cañar, El Oro, ESMERALDAS, Guayas, Los Rios, Pichincha
Lu. flaviscutellata (Mangabeira)	ST	not known	PICHINCHA, Sucumbios

Table 3.3.1 (contd.). List of species collected in *Leishmania*-endemic sites in Ecuador July-October 1990

Species	Methods of capture	Resting site(s)	Distribution by Department
Lu. panamensis (Shannon)	HB, ST, LT	not known	Bolivar, Cañar, Manabi, Pichincha
Lu. hartmanni (Fairchild & Hertig)	DA, HB, ST, LT	tree trunks, animal burrows	Bolivar, Cañar, El Oro, ESMERALDAS, Guayas, LOJA, Pichincha
Lu. ayacuchensis (Caceres & B. Galati)	DA, HB, ST, LT	rock crevices	Azuay
Lu. osornoi (Ristorcelli & Van Ty)	HB, ST , COT	rock crevices	Azuay
*Lu. strictivilla** Young	HB	not known	ZAMORA CHINCHIPE
Lu. cayennensis (Floch & Abonnenc)	DA, LT, COT	tree trunks, tree holes	EL ORO, Esmeraldas, Guayas, LOJA, Los Rios, Manabi
Lu. micropyga (Mangabeira)	DA, ST	tree trunks	EL ORO, Guayas, Los Rios, Manabi, PICHINCHA, Sucumbios
Lu. trinidadensis (Newstead)	DA	tree trunks	ESMERALDAS, Pichincha, Sucumbios
*Lu. saulensis** (Floch & Abonnenc)	ST	not known	PICHINCHA

***Lu. dysponeta* (Fairchild & Hertig).** This species was found to be abundant in animal burrow collections made at Echeandia (Bolivar) and Zhucay (Cañar) during the 1988 survey and its distribution in Ecuador assumed to be restricted to the coastal plain. For this reason, single females caught at Taisha, Morona Santiago in 1988 and at Izimanchi, Zamora Chinchipe in August 1990 were thought to belong to the morphologically indistinguishable species *Lu. tri-acantha* (Mangabeira). However the discovery of two males of *Lu. dysponeta* under rocks near Paute, Azuay, at an altitude of 2,300 metres suggests that the range of this species may extend across the Andean plateau and into the Oriente.

Nine areas endemic for *Leishmania* were visited during the present survey, of which six were surveyed for the first time and three (Ocaña, Puerto Quito and Paute) had been surveyed in 1988.

Muisne. This area was visited only briefly and sampling limited to a single human bait collection near the town of Muisne and two resting site collections in coffee plantations bordering the river at La Correntada and El Mamey. Three females of *Lu. gomezi* were taken off human bait in scrub forest approximately 300 meters from the beach at Muisne. This widespread species is a suspected vector of *Leishmania panamensis* in much of its range, including Nicaragua, Venezuela and Colombia as well as Ecuador (Grimaldi *et al.*, 1989). A single afternoon collection at La Correntada yielded nine species, including the anthropophilic species *Lu. trapidoi, Lu. hartmanni*, and *Lu. serrana* as well as *Lu. shannoni, Lu. abonnenci, Lu. dysponeta, Lu. trinidadensis, Lu. gorbitzi* and *Brumptomyia leopoldoi*. The collection from a similar habitat at El Mamey, approximately 3 km upriver from La Correntada, also contained three females of *Lu. undulata*.

Puerto Quito. This area was surveyed in 1988, and the sandfly fauna found to consist of at least eight species. A more prolonged study in 1990 collected 18 species, not including 2 (the man-biter *Lu. carrerai thula* and *Lu. cayennensis*) found on the previous visit. The fauna of the Puerto Quito area therefore contains at least five anthropophilic sandfleis, the above plus *Lu. panamensis, Lu. hartmanni, Lu. trapidoi* and *Lu. nevesi*. Two other occasional man-biters, *Lu. shannoni* and *Lu. flaviscutellata*, have been implicated in the transmission of *Leishmania* in other parts of their ranges, and *Lu. saulensis* is also somewhat anthropophilic. Two other *Lu. shannoni* group species are present, *i. e., Lu. dasymera* and *Lu. undulata*, and the remainder of the fauna consists of *Lu. vespertilionis*, known to feed on bats; the reptile-biting *Lu. trinidadensis* and *Lu. micropyga*; and six other species the feeding habits of which are unknown, *i.e., Lu. aragaoi, Lu. barrettoi majuscula, Lu. triramula, Lu. dysponeta, Lu. camposi* and *Lu. furcata*. The last of these had never been recorded in Ecuador prior to the present survey.

Portoviejo. Collections were made at three sites near the city of Portoviejo, *i.e.*, in coffee plantations at the Recintos of San Sebastián and El Progreso and in pastureland adjoining the road to Crucita, near Pueblito Rocafuerte. Samples at the first two sites were made using protected human bait and Shannon trap, and consisted of four anthropophilic species, *i.e., Lu. gomezi, Lu. serrana, Lu. hartmanni* and *Lu. trapidoi*, together with *Lu. shannoni, Lu. abonnenci* and *Lu. dysponeta*. Castor oil trap collections from between the roots of kapok (*Ceiba pentandra*) trees were dominated by the reptile-biting sandfly *Lu. cayennensis*, although a single female of *Lu. gorbitzi*, the feeding habits of which are unknown, was also taken.

Junin. The sandfly fauna of Recintos "El Toro" and "La Tablada de Mendoza" near Junin consisted of the anthropophilic species *Lu. serrana* and *Lu. gomezi*, as well as *Lu. shannoni, Lu. abonnenci, Lu. dasymera, Lu. undulata, Lu. dysponeta, Lu. gorbitzi, Lu. sallesi* and *Lu. cayennensis*, all of which were taken in resting site collections from tree trunks. A single Shannon trap collection at La Tablada de Mendoza on 3. x. 1990 yielded 311 males and 24 females of *Lu. serrana* in an hour (18:20-19:20).

Ocaña. This area has been surveyed several times over the past eight years (Hashiguchi *et al.*, 1985a,b; 1987; Alexander *et al.*, 1992) and the sandfly fauna in the vicinity of Balneario "El Chorro" found to consist of two strongly anthropophilic species. *i.e., Lu. hartmanni* and *Lu. trapidoi* together with the occasional man-biter *Lu. shannoni*, which predominates in tree trunk collections. During the course of the most recent visit to the area (September 3-6 1990) the senior author was infected with *Leishmania panamensis* by the bite of an infected *Lu. hartmanni*, the first positive incrimination of this species as a vector of *Leishmania* in Ecuador. Resting site collections revealed the presence of a fourth member of the sandfly fauna, *Lu. aclydifera*, the feeding habits of which are unknown.

Zaruma. Once again, the predominant man-biting species in this area appear to be *Lu. serrana* and *Lu. gomezi*, with a single female of *Lu. hartmanni* also

taken in a human bait collection near the town of Zaruma, and one male and six female *Lu. shannoni* collected at Balneario "El Recreo" near La Avanzada. Other species collected from tree trunks and with the Shannon trap were *Lu. dysponeta, Lu. abonnenci* (the females of which are indistinguishable from *Lu. shannoni* and may have also featured in the human bait collection), the reptile-feeder *Lu. micropyga* and *Brumptomyia leopoldoi*.

Collections made at El Prado (Parroquia Guayquichuma) in the neighbouring province of Loja included a fauna similar to that of the above, with *Lu. serrana* predominant in human bait and Shannon trap collections and *Lu. gomezi* and *Lu. hartmanni* also featured. A single female of *Lu. nevesi* was also collected off human bait. The remaining collections in Loja comprised species unlikely to be involved in the epidemiology of leishmaniasis, *i.e., Warileya phlebotomanica* (Zambi) and *Lu. cayennensis* (Rio Pindo).

Paute. This was the only endemic focus of leishmaniasis to be visited in the Andean plateau. A previous survey of the area revealed that the sandfly fauna consisted of at least two species, *Lu. ayacuchensis* and *Lu. osornoi*, both of which are anthropophilic and may be implicated in the transmission of *Leishmania* in the area (Alexander *et al.*, 1992; Takaoka *et al.*, 1990). Small numbers of the former species and a single example of the latter were collected in July and August 1990. Two male *Lu. dysponeta* were collected as they alit on a collector engaged in a protected human bait collection at 17:00 on 28.vii.1990, and had presumably rested during the day under rocks situated nearby. This species is widespread on the coastal plain of Ecuador, but its presence in the Paute area, at an altitude of 2,300 meters was completely unexpected and may indicate a much more extensive distribution than assumed previously, perhaps extending into the Oriente.

Zumba. The sandfly fauna of the Zumba region in Zamora Chinchipe was surprisingly depauperate, with only four species recorded, three of them anthropophilic. Two of these species, *i.e., Lu. serrana* and *Lu. nevesi* belong to the *Lu. verrucarum* species group, which contains at least one probable vector of *Leishmania, i.e., Lu. spinicrassa* Morales *et al.* (Young *et al.*, 1987). The other man-biting species, *Lu. strictivilla*, belongs to the *Lu. vexator* species group, which contains a number of suspected vectors of *Leishmania*, including *Lu. hartmanni* (Alexander *et al.*, 1992), *Lu. peruensis* (Shannon) (Herrer, 1982) and *Lu. ayacuchensis* (Takaoka *et al.*, 1990). A single female sandfly of the subgenus *Pressatia* was also collected at Izimanchi; this may have been either *Lu. dysponeta* or *Lu. triacantha* (Mangabeira), neither of which is known to be anthropophilic. It is unclear why there are so few species in this region although it has been deforested to a considerable extent and may have supported a much more diverse sandfly fauna in the recent past.

The results of the present study confirm our finding of that 1988 survey that the most widespread anthropophilic species in Ecuador, at least in the coastal plain, are *Lu. trapidoi, Lu. hartmanni, Lu. serrana, Lu. gomezi* and *Lu. panamensis*. Other anthropophilic species in this region include *Lu. carrerai thula* and *Lu. nevesi*, and occasional man-biters include *Lu. shannoni, Lu. saulensis, Lu. flaviscutellata* and *Lu. olmeca bicolor*. With the exception of the two *Lu. verrucarum* group species, all are proven or suspected vectors of *Leishmania* in Ecuador or other parts of their ranges.

Little additional information was accumulated during the 1990 survey on the sandfly faunae of the other two regions of Ecuador. Although *Lu. dysponeta* was collected at Paute for the first time this species is not known to be anthropophilic and there is no evidence that it is involved in *Leishmania* transmission in the area. The fauna of Zumba resembles that of the southern coastal plain in that the two major man-biting species are *Lu. serrana* and *Lu. nevesi*, although the latter has been recorded in two other provinces of the Oriente, Sucumbios and Morona Santiago. The third species, *Lu. strictivilla*, had not been

recorded previously in Ecuador but appears to be a close relative of *Lu. ayacuchensis* and *Lu. osornoi*, both of which may be involved in *Leishmania* transmission in the Andean plateau..

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Chapter 4

Seroepidemiological Aspects

1. An Analysis of *Leishmania* Skin Test Antigen

ABSTRACT. Concanavalin A-binding glycoconjugates (CABG) were purified from *Leishmania panamensis* (MHOM/PA/71/LS94) promastigote. By SDS-PAGE analysis, 3 major bands migrating at approximately 30, 16 and 12 kDa were confirmed in the CABG preparation. Antigenicity of the CABG against a specific delayed-type hypersensitivity were examined in active cutaneous leishmaniasis patients. The responsiveness of the CABG antigen was extremely low in comparison with those of soluble-extract preparation (CA) and whole parasites preparation (LST).

The intradermal skin test is a useful tool for both presumptive diagnosis and epidemiological studies in endemic areas of New World cutaneous and visceral leishmaniasis. Although the leishmanin skin test (LST) preparation prepared from whole promastigotes is used normally, a little information on other preparations prepared from promastigotes-extract has been also reported (Furtado and Pellegrino, 1956; La Placa *et al.*, 1975; Shaw and Lainson, 1975; Reed *et al.*, 1986; Weigle *et al*, 1991). We have also demonstrated that a crude soluble extract prepared from *Leishmania panamensis* and partially purified preparations of the soluble extract are highly sensitive for a specific delayed-type hypersensitivity (DTH) in active cutaneous leishmaniasis patients. It was also reported that five proteins, approximately 66, 55, 45, 28 and 26 kDa, were common components among our useful skin testing preparations (Furuya *et al.*, 1989, 1991).

In order to gain a further information on *Leishmania* parasite antigens related to the DTH response in cutaneous leishmaniasis, the responsiveness of the concanavalin A (Con A)-binding glycoconjugates of *L. panamensis* to active cutaneous leishmaniasis patients were examined in this study.

Materials and Methods

Intradermal skin test and the antigen preparations

L. panamensis (MHOM/PA/71/LS94) obtained from Dr. Desjeux, PDP, WHO (formerly Instituto Boliviano de Biologia de Altura, Bolivia) was cultured with Schneider's medium supplemented with 15% inactivated fetal bovine serum. After washing of parasites with balanced salt solution, the harvested promastigotes were ruptured by a freeze-thawing procedure and centrifuged at 10,000 g for 30 min at 4°C (Furuya *et al.*, 1991). The supernatant was adjusted to 100 μg protein concentration per ml after filtration with 0.45 μ sterile filter (Millipore Co., USA) and lyophilyzed as crude solube extract (CA) preparation.

Con A-binding glycoconjugates (CABG) preparation

Con A Sepharose (Pharmacia, Sweden) column was equilibrated with 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM $MnCl_2$, $CaCl_2$ and $MgSO_4$. The harvested parasites were ruptured with 10 mM Tris-HCl buffer (pH 7.5) containing 2 mM phenylmethylsulfonyl fluoride. The supernatant was obtained by centrifugation at 150,000 g for 60 min, and was applied to column. Bound material was eluted from

the column by using 10 mM Tris-HCl buffer (pH 7.5) containing 100 mM methyl α-mannoside, 150 mM NaCl, 1 mM $MnCl_2$, $CaCl_2$ and $MgSO_4$. After dialyzing against distilled water and filtration by Millipore filter, the CABG was adjusted to 100 µg protein concentration per ml and lyophilized .

LST preparation and skin test

5×10^7 whole promastigotes per ml in sterile saline containing 0.5% phenol was used. Intradermal skin test was made on 25 patients. The preparations were injected intradermally in 0.1 ml in flexor surface of the forearm. After 48 hours, induration size of more than 5 mm (mean vale of length and breadth) at the injection site was considered to be a positive reaction.

Analysis of components of CABG

SDS-PAGE analysis of the CABG was carried out according to the method of Laemmli (1970).

Results

SDS-PAGE plofile of CABG

Three bands were stained heavily by 0.25% Coomassie brilliant blue R 250. These major bands were migrated at the region approximately 30, 16 and 12 kDa. Four bands migrating at approximately 53, 28, 27 and 20 kDa were stained very weakly.

Skin test

Intradermal skin test using CA, CABG and LST preparations was carried out against 25 patients, 9 females and 16 males. Most of these patients had one active cutaneous lesion infected at three to six months ago. Parasite was confirmed by microscopical observation with smear specimen prepared from the lesions.

All of the patients examined reacted to CA (25/25) and LST (17/17) preparations. The mean induration size against CA and LST preparations were 19.7 and 14.7 mm, respectively. On the other hand, the positive ratio against CABG was 28% (7/25). The mean induration size was also smaller than those of CA and LA. The mean value was 6.5 mm (Table 4.1.1). There was significant difference in positive reatio of skin test response between CA and CABG ($p<0.001$). Intensity of induration size of skin test was shown in Table 4.1.2. Among the negative reactant of CABG, no response was observed in 10 patients, the rest was shown 3 or 4 mm. Mean induration size of the positive patients against CABG was 8.2 mm. The induration size was extremely small compared with those of other two preparations ($p<0.001$).

Table 4.1.1. Intradermal skin test responses to leishmanin skin test (LST), crude soluble extract (CA) and con A-binding glycoconjugates (CABG) antigens prepared from *Leishmania panamensis* in active cutaneous leishmanisis paticnts

	LST	CA	CABG
Positive ratio	100 (17/17)	100 (25/25)	28.0 (7/25)
Mean induration size (± s.d.)	14.7 ± 2.6	19.7 ± 3.9	6.5 ± 2.5

Table 4.1.2. Frequency distribution of induration size of leishmanial skin test using LST, CA and CABG preparations in active cutaneous leishmaniasis patients

Induration size	LST (mm)	CA	CABG
<5			18
5 - 10	1		6
11 - 15	9	5	1
16 - 20	7	10	
21 - 25		7	

Discussion

In this experiments, three major glycoconjugates were purified with Con A resin and the molecular weight of those conjugates were also determined. Constituent glycoconjugates of promastigotes of 14 different *Leishmania* strains from six different species has been fully investigated by Rossell *et al.* (1990). According their results, CABG of *L. panamensis* LS94 strain was 11, 16 and 62 kDa. Among our conjugates purified by Con A-affinity chromatography, two conjugates, 12 and 16 kDa, might be same ones of their conjugates, 11 and 16 kDa. Although Con A-binding 30 kDa glycoconjugate was confirmed in *L. braziliensis* and *L. chagasi* (Rossell *et al.*, 1990), it was new in *L. panamensis*. However, Con A-binding 62 kDa glycoconjugate reported by Rossell *et al.* (1990) in several strains, and by Wilson and Hardin (1988, 1990) in *L. chagasi*, and 53 kDa conjugates by Nagakura *et al.* (1988) in *L. braziliensis* were not isolated from our curde soluble extract.

It has been reported that a purified glycoprotein fraction from *L. amazonesis* was able to induce a specific DHT response to mice (Rodrigues *et al.*, 1986, 1988). They also demonstrated that the glycoprotein fraction contained two major polypeptide components, 11 and 17 kDa, and the both components were products of the same 17 kDa protein (1988). In this study, however, the ability of the present CABG preparation to induce a specific DTH response in active cutaneous leishmaniasis patients was extremely lower than that of CA preparation. The CABG preparation contained 12, 16 and 30 kDa proteins. Whether or not the present CABG preparation contains the 17 kDa protein reported by Rodrigues *et al.* (1988) is unknown. Almost nothing is known regarding the DTH response of patients with active lesions to a purified antigen, especially glycoconjugates. For the moment it can be only be said with certainty that the present Con A-binding glycoconjugates, 12, 16 and 30 kDa, may does not play an impotant role in a specific DTH response of active cutaneous leishmanisis patients.

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2. A Seroepidemiological Survey of Canines in an Area Endemic for Andean Leishmaniasis in Ecuador

ABSTRACT. Domestic dogs are considered to be an important reservoir host of Andean leishmaniasis as well as wild rodents in Ecuador. Fifty eight domestic dogs in Alausi, a town of Andean highland, of Ecuador were examined using serological and parasitological methods. Nineteen (32.8 %) of the 58 dogs showed a high ELISA value compared with that of controls. However, no *Leishmania* parasite was isolated from any culture of liver aspirates from these dogs.

New World leishmaniases are widely distributed in Ecuador, where they present a considerable public health problem. Since the first human leishmaniasis was described in this country in 1920, many additional cases of the disease have been reported from bilateral lowlands of the Andes Mountain, Pacific coast and Amazonian regions. Recently, we also found endemic focus of leishmaniasis in the Andean highlands of Ecuador (Hashiguchi *et al.*, 1991).

Leishmania parasites have been isolated from a great variety of mammalian species in endemic areas of Central and South America. In Ecuador, we performed examinations of reservoir hosts of leishmaniasis since 1982. A total of 260 wild animals of 24 species belonging to several genera, and 134 domestic dogs had been examined, and three species of *Leishmania* parasites were isolated from 10 animals mentioned above (Hashiguchi *et al.*, 1985; Gomez *et al.*, 1987; Mimori *et al.*, 1989; Hashiguchi *et al.*, 1990). In this paper, we deal the result of the epidemiological survey of domestic dogs performed in an area endemic for Andean human leishmaniasis using parasitologic and serodiagnositic methods.

Materials and Methods

Description of the study area

The investigation was carried out Canton Alausi in the Department of Chimborazo, which is situated at the mountainous region of the Andes, 2,400 m above sea level, 2°12'S and 78°52'W. In this town, a total of 58 domestic dogs from two sites were examined (Fig. 4.2.1), Aypan Grande (old community) and Aypan Chico (newly established community).

ELISA

Promastigotes of *Leishmania panamensis* (MHOM /PA/71/LS94) were cultured in Schneider's medium with 10% fetal bovine serum. The parasites were washed three times with physiological saline and were ruptured by ultrasonic treatment in 0.05 M carbonate bicarbonate buffer (CBB), pH 9.6, containing 0.02% sodium azide. The homogenate was centrifuged at 10,000 r.p.m. for 30 minutes at 4°C. The supernatant was used as antigen. The ELISA procedure was done according to the slightly modified method described by Mimori *et al.* (1987). Microtitration plates were coated overnight at 4°C with 100 µl of the 40 µg/µl antigen solution. The plates were washed three times with 0.05% Tween 20 in saline. 100 µl of test sera diluted to 1:25 in 0.05% Tween 20 in 0.02 M phosphate buffer saline, were added to each well and the plates were incubated at 37°C for 30 minutes. After washing, 100 µl of conjugate diluted to 1:250 in 0.05 M PBS-T were added. The conjugate was peroxidase-goat anti-dog heavy and light chain IgG. The substrate was 0.1 mg of orthophenylendiamine per ml and 150 µl of 0.03% of H_2O_2 in 0.05 M acetate buffer, pH 4.5 were added to each

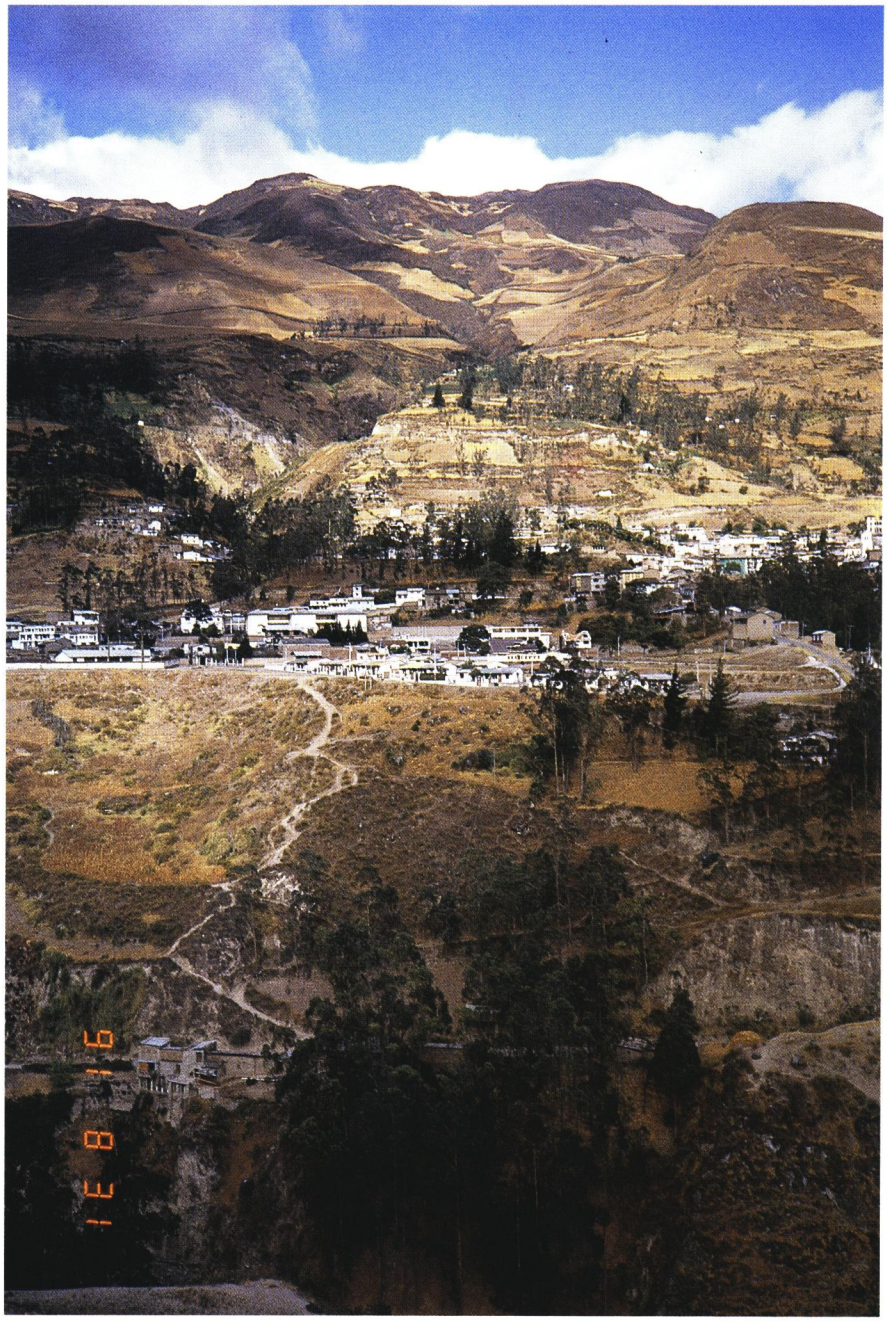

Figure 4.2.1. Landscape of a newly found Andean leishmaniasis-endemic area, Alausi, Department of Chimborazo, Ecuador, showing study sites of two communities, Aypan Grande and Aypan Chico, located at the north of the town.

well and the plates were incubated at 25°C in the dark. The enzyme reaction was stopped by the addition of 50 µl of 4N H_2SO_4. The absorbance was read by a spectrophotometer at 492 nm. Positive ELISA values were defined as 2 SD above the mean of the eight healthy Japanese dog's sera (absorbance; 0.275 ±0.056) according to the criteria by Evans *et al.* (1990).

Isolation of parasites

Materials were collected by syringe aspiration from the liver of dogs, and were cultured in the blood agar slant medium with 20% defibrinated rabbit blood at room temperature. Each culture was checked for the presence of promastiogtes under microscope, every three or four days during 30 days.

Results

As a preliminary epidemiological survey of canine leishmaniasis, an evaluation was made on both serodiagnosis and *Leishmania* isolation *in vitro* culture of materials from domestic dogs in Alausi, Andean highlands, of Ecuador.

Sera from 58 dogs were examined by ELISA and 19 (32.8%) were found to have an absorbance 2 SD above the controls. In the 19 positives, seven dogs showed 2-3 SD in ELISA values; two, 3-4 SD; three, 4-5 SD; and the remainder, > 5 SD. There was no significant difference in the age of dogs between ELISA-positives (2.8 years ±1.6) and ELISA-negatives (2.4 years ±1.3) ($t = 0.79$, $0.4 < P > 0.5$). No difference was recognized in the ELISA-positive rate between male (32.3%) and female (36.4%).

The ELISA-positive rate of dogs (38.1%, 16 positives per 42) in Aypan Grande (an old community) was twice of that (18.8%, 3 positive per 16) in Aypan Chico (a newly established community).

In the present examination, no *Leishmania* parasite was isolated from culture *in vitro* of liver aspirates of 58 dogs.

Discussion

Since 1986, epidemiological studies of Andean leishmaniasis were performed in two towns of Andean highlands of Ecuador, Paute (Department of Azuay) and Alausi (Department of Chimborazo). The Andean form of Ecuadorian leishmaniasis is clinically very similar to the Peruvian uta due to *L. peruviana*. However, the causative agents and vectors are completely different each other. In Ecuador, the parasites are *L. mexicana* and *L. major*-like, and the vector is *Lutzomyia ayacuchensis* (Hashiguchi *et al.*, 1991).

In the Andean regions, there may be not so many wild mammals acting as reservoir hosts of leishmaniasis compared to lowland regions on the Pacific cost and Amazonian areas. However, many domestic dogs, which are very important reservoir of several *Leishmania* species, are found in the areas endemic for Andean leishmaniasis. Moreover, *L. mexicana* parasites have been isolated from dogs in the town of Paute of the Ecuadorian Andes (Hashiguchi *et al.*, 1991). From these findings, in the Andean highlands, it was thought that the main reservoir host would be domestic dogs (Gomez and Hashiguchi, 1990). The current serological examination indicated that the positive rate of ELISA was relatively high, showing 32.8% (19 positives per 58 dogs). The ELISA-positive rates obtained showed a difference between the two human communities examined; the rate in Aypan Grande (an old community) was twice that in Aypan Chico (a newly established community). This finding might correlate with human infection with *Leishmania* in the areas. In Aypan Grande, there were higher rates of the human infections with active or cured lesions (data not shown).

In this survey it was not possible to isolate *Leishmania* prasites from any dog. Further such a trial, however, should be done employing more sensitive methods to isolate *Leishmania* parasites, especially in ELISA-positive dogs.

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Chapter 5

Epidemiological Aspects

1. A Preliminary Study of Andean Leishmaniasis in Alausi and Huigra, Department of Chimborazo, Ecuador

ABSTRACT. Autochthonous Andean leishmaniasis is reported from the towns of Alausi and Huigra, 2,300 m and 1,300 m above sea level respectively in the Department of Chimborazo, Ecuador. Four patients with active lesions positive for *Leishmania* parasites were examined during the periods July - September 1991 and January - February 1992. These patients had not visited other areas endemic for *Leishmania*. The parasites isolated from their lesions were inoculated into hamsters and culture medium, and characterization is still in progress; however *L. mexicana* has already been reported from Alausi and is considered to be the species involved. Promastigotes were also isolated from a female sandfly (*Lutzomyia ayacuchensis*). Twenty-three (18.9%) of 122 schoolchildren examined in Alausi had scars suggesting previous infection with leishmaniasis and showed positive for Montenegro skin tests.

Cutaneous leishmaniasis was reported from the Ecuadorian Andes for the first time in 1986 (Hashiguchi *et al.*, 1986). Although the disease is similar clinically to Peruvian uta, the causative organisms and vectors appear to be completely different. In Peru the parasite is *L. peruviana*, and the suspected vectors *Lu. peruensis* and *Lu. verrucarum*, while in Ecuador it appears that two parasite *Leishmania* species are involved (*L. mexicana* and *L. major*-like), with *Lu. ayacuchensis* the probable sandfly vector. Recently several cases of cutaneous leishmaniasis have been reported from the town of Alausi in the Department of Chimborazo (Hashiguchi *et al.*, 1990, 1991; Armijos *et al.*, 1991) and we continued to study this focus, collecting data from hospitals and health centers in the area. We also visited the town of Huigra, also in Chimborazo, from which several leishmaniasis cases were confirmed. These cases are reported in detail in this article, together with a description of the ecological conditions at Huigra and Alausi, and the results of dissections of sandflies collected on protected human bait and Shannon trap.

Materials and Methods

The study area (Fig. 5.1.1)

Canton Alausi (2°15'S: 78°50'W) has a population of ca. 4,000 and lies at 2,300 - 2,500 m above sea level (Fig. 5.1.2A). Canton Huigra (2°20'S: 78°58'W) has a population of ca. 2,000 and an altitude of 1,300 - 1,500 m a.s.l. (Fig. 5.1.2B). Vegitation in both areas is sparse, and consisted of a typical alpine flora. There are scattered human dwellings and cultivated fields on the outskirts of both towns.

Sandfly collections and dissections

Sandfly collections were made by protected human bait and Shannon trap in the outskirts of Alausi and Huigra, as well as in the centre of the former during 18:00 and 20:00 in the periods July - September 1991 and January - February 1991. A total of

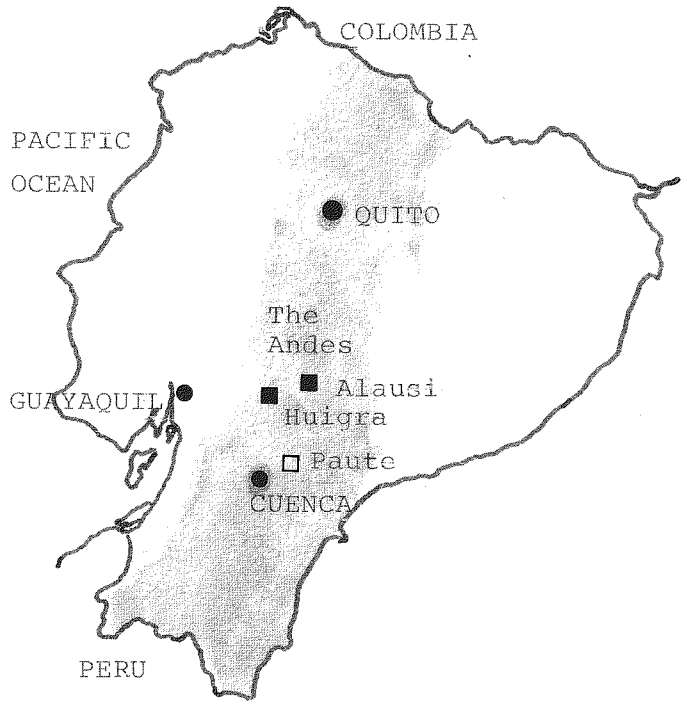

Figure 5.1.1. Outline map of Ecuador, showing two study sites, Huigra (1,300 m above sea level) and Alausi (2,300 - 2,500 m a.s.l.), Department of Chimborazo and other related sites. Shaded area shows 1,000 m or more above sea level.

174 flies were collected, dissected and examined microscopically for the presence of *Leishmania* in the gut.

Human subjects and parasite isolations

Children in kindergartens and schools from both towns were examined for the presence of cutaneous lesions and scars during July - September 1991 (dry season) and January - February 1992 (wet season) (Fig. 5.1.3A, B). House to house visits were also made in both areas in order to detect leishmaniasis cases, particularly among young children with preschool age.

Skin tests were also performed in schoolchildren of Alausi (Escuela "Ines Jimenez") using *Leishmania* promastigote antigens (Furuya *et al.*, 1991) (Fig. 5.1.4A). The antigen was injected intradermally (0.05 ml) in the flexor surface of the forearm, and the skin test area observed for erythema and induration at 48 hrs. A positive result was indicated by an induration diameter of 5 mm or more. In addition, when the subjects showed active lesions, thin smears were made on slides for microscopic examination (Fig. 5.1.4B).

Leishmania isolates were made by aspiration from the margins of active lesions (Gomez *et al.*, 1986). The aspirated material was then inoculated into culture media and golden hamsters. Some of these isolates from Alausi have already been identified as *L. mexicana* (Hashiguchi *et al.*, 1991) but categorization of the majority of samples obtained from humans and sandflies is still in progress. Detailed characterization of these isolates will be published elsewhere.

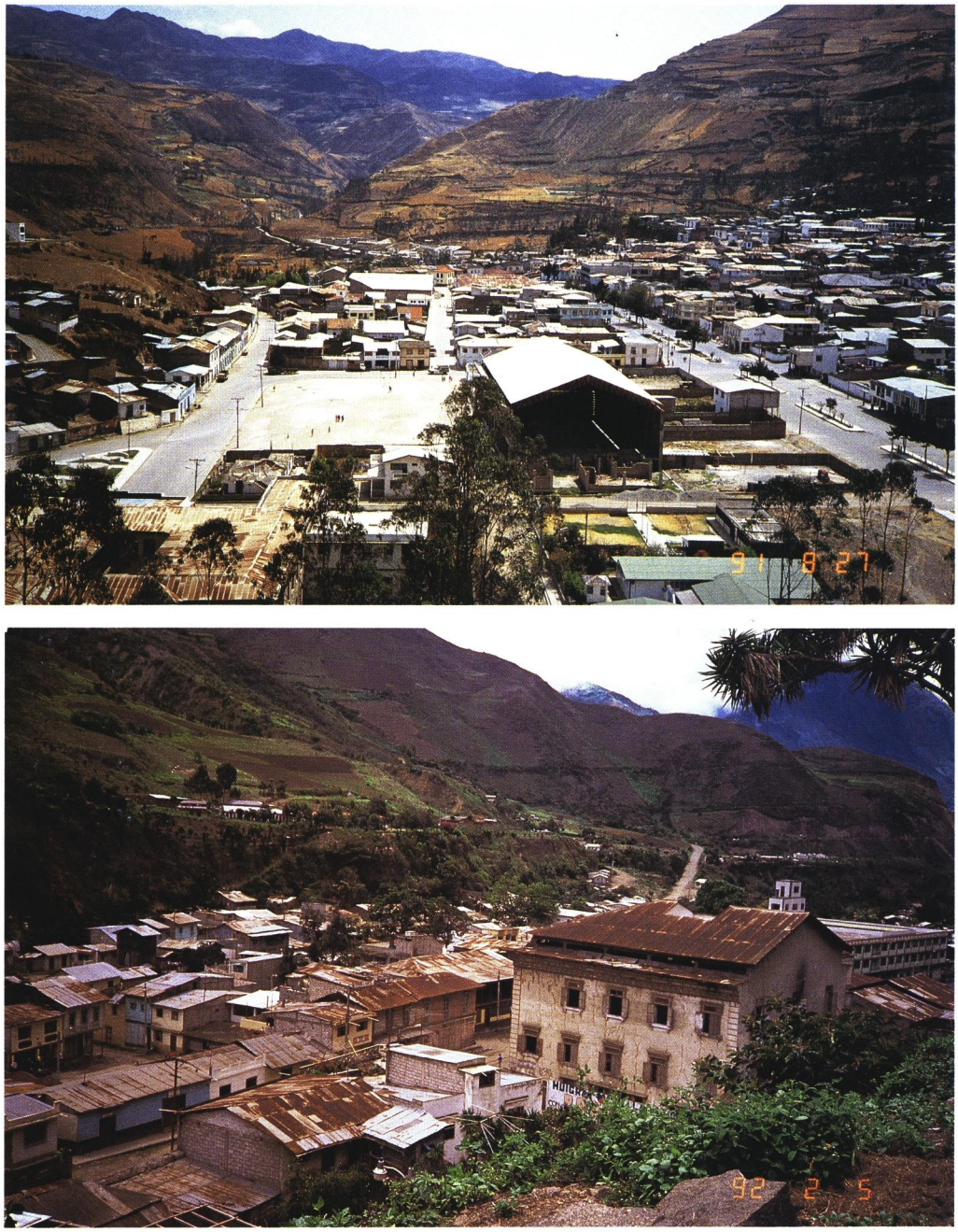

Figure 5.1.2. A view of center and outscarts of two newly found areas endemic for Andean leishmaniasis. A (above), Alausi (2,300 - 2,500 m above sea level), B (below), Huigra (1,200 - 1,300 m a.s.l.).

Figure 5.1.3. Children of primary (A: above) and secondary (B: below) schools in an endemic area (Huigra) of Andean leishmaniasis; suspective leishmaniasis scars were observed in some of them.

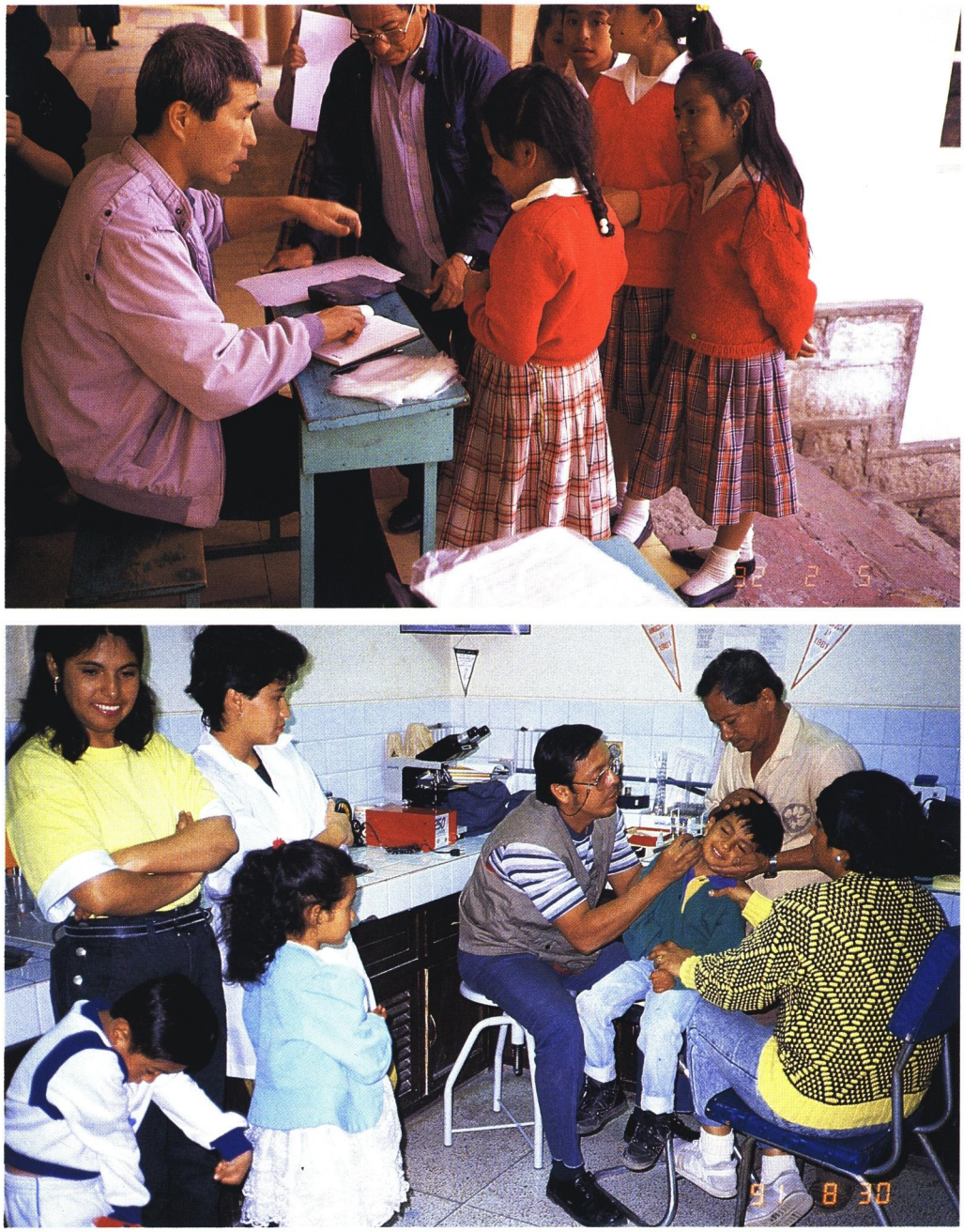

Figure 5.1.4. A preliminary survey of Andean leishmaniasis patients at a primary school, Escuela " Inez Jimenez,' (A: above) and a medical center "Hospital Civil de Alausi" (B: below), performing parasitological, dermatologica and immunological (skin test) examinations.

Results

Parasitologically proven active cases from Alausi and Huigra

Following a program of house to house visits, the following subjects with dermal lesions were found to be positive for *Leishmania.*

Case 1. A 3 year-old boy (J.L.S.C.), living in Bua, Alausi. Scar of dimensions 25 mm × 15 mm and three small (active) papules of diameters 5 mm, 3 mm and 5 mm on the right cheek, showing lymphadenopathy. The scar was the result of a lesion that had appeared 2 years previously as a small papule, gradually increasing in size over the next year. The other papules appeared before the original one had healed completely. This patient showed a positive skin test result and his smear sample had abundant amastigotes. Material aspirated from the edges of the three active lesions was inoculated into the noses of hamsters. This patient had never visited other *Leishmania*-endemic areas, and had received no prior treatment. Three of his brothers had possible leishmaniasis scars on their faces and gave positive skin test results.

Case 2. A 5 year-old boy (E.M.N.) living in the newly established community of Aypan Chico, Alausi. Papule on the right cheek of dimensions 5 × 3 mm not showing lymphadenopathy. This patient had been bitten by a sandfly three months before and a lesion developed gradually at this site. This patient had received no specific treatment prior to the study. He gave a positive skin test result, and his smear sample showed abundant amastigotes. Aspirates from his lesion were inoculated into the nose of a hamster. This patient had never visited other *Leishmania*-endemic areas.

Case 3. A 1 year-old girl (K.N.O.), living in Los Violetos, Huigra. This patient had two erythematous plaques of diameter 10 mm and 5 mm on her right cheek, not showing lymphadenopathy (Fig. 5.1.5A). The lesions had appeared nine months before as small papules and had gradually increased in size. This patient gave a positive skin test result, and material cultured *in vitro* showed *Leishmania* promastigotes after one week of inoculation, suggesting that they belong to the *L. mexicana* complex. This patient had been bitten by an insect and the lesions had evolved gradually from these bites.

Case 4. A 2 year-old girl (M.P.B.) living in Barrio Azuay, Huigra. This patient had three papules of diameter 3 mm, 1 mm and 1 mm on the left cheek, not showing lymphadenopathy (Fig. 5.1.5B). Material aspirated from the margin of lesions was inoculated into culture media and the nose of hamsters; culture medium isolates showed *Leishmania* promastigotes one week after inoculation, again suggesting that the parasites belonged to the *L. mexicana* complex.

Examination of schoolchildren in Alausi and Huigra

Andean cutaneous leishmaniasis lesions normally leave a characteristic depressed scar with radial striations. We observed large numbers of schoolchildren in Alausi and Huigra with this type of scar. Pupils of the Escuela "Inez Jimenez" were skin tested and 26 (21.3%) of the 122 examined gave positive results. All but three of these (88.5%) had facial scars typical of leishmaniasis. None of the pupils giving positive results had visited other *Leishmania*-endemic areas. No active lesions were observed in any of the schoolchildren.

Sandfly collections and Leishmania *infection*

In Alausi (Fig.5.1.6A), a total of 67 sandflies, 49 *Lu. ayacuchensis* and 16 *Lu. hartmanni*, were collected and dissected. Three (3/49, 6.12%) of the former species were positive for *Leishmania* promastigotes, showing 4.48% (3/67) of natural infection rates in the area. In Huigra, all the fly collection was made around the housing area of one of the present patients (Case 3). In the site (Fig. 5.1.6B), 107 flies belonged to three species of *Lutzomyia, viz.*, 105 *Lu. ayacuchensis*, one *Lu. nevesi* and one *Lu. gomezi*,

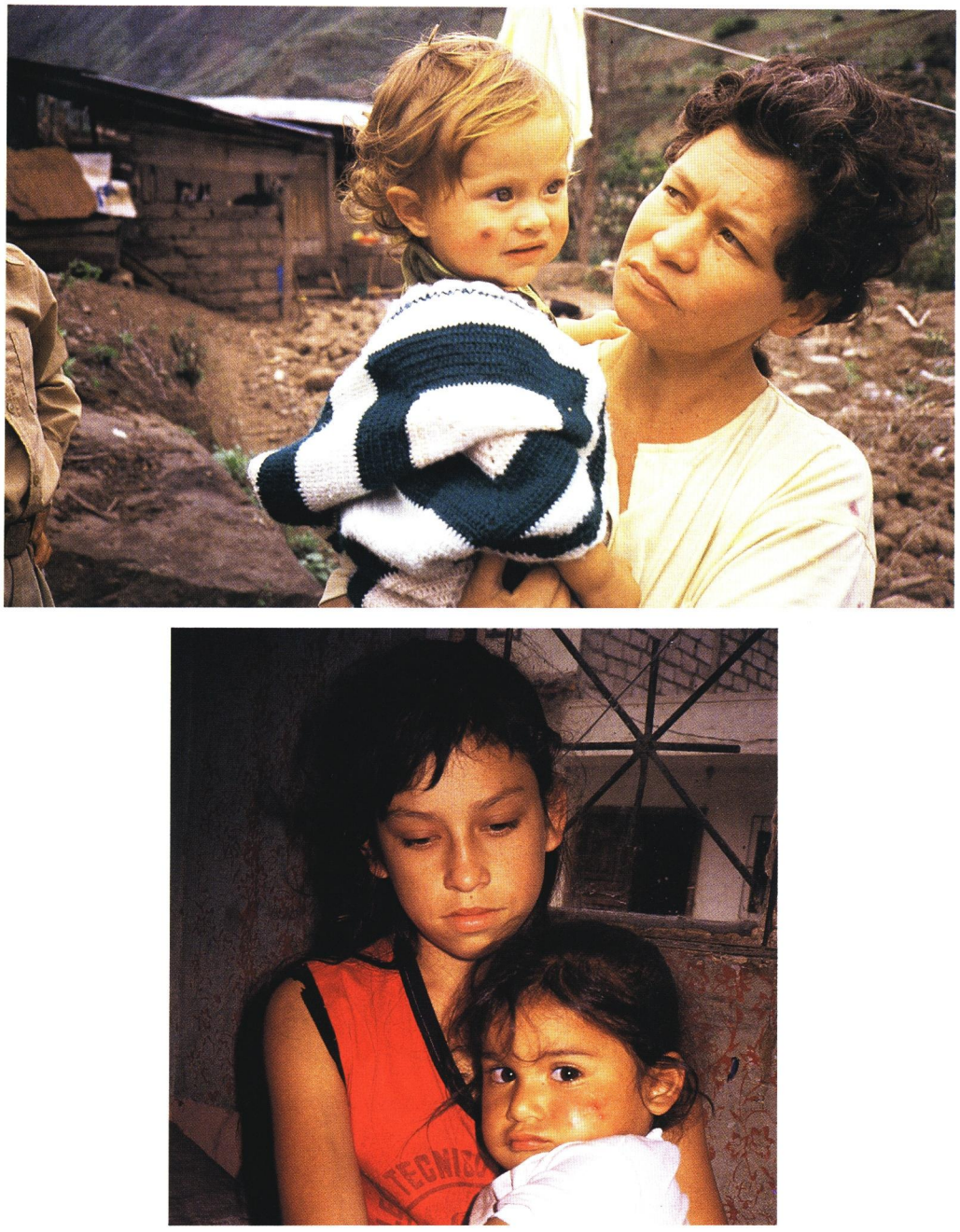

Figure 5.1.5. Parasitologically proven (both smear and culture positive) patients with active lesions of Andean leishmaniasis. A (above), a 1 year-old female with 2 small lesions on the right cheek; B (below), a 2 year-old female with 3 small lesions on the left cheek.

Figure 5.1.6. Two study sites (arrows) in which positive sandflies were collected. A (left), a slightly remote area of Alausi; B (right), Huigra, arrows show both the fly collecting site and the housing area of patient (Case 3).

were collected and dissected. Only one of *Lu. ayacuchensis* revealed positive for *Leishmania* parasites, demonstrating 0.95% of infection rates in the area; Huigra (1,300 m a.s.l.) is a relatively temperate Andean area compared to Alausi (2,300-2,500 m a.s.l.). Thus, at both study sites, only *Lu. ayacuchensis* was positive for *Leishmania* promastigotes and was highly dominant. In all the positive flies, midgut localizations with promastigotes were observed, suggesting a *L. mexicana* complex infection. Sandflies were collected during both the dry (July - September) and wet seasons (January - February). Although a considerable number were captured in the outskirts of both towns during the rainy season, it was noteworthy that none were collected in the center of Alausi during this period.

Comments

This article reports four parasitologically proven cases of autochthonous cutaneous leishmaniasis from

the Ecuadorian Andes. A further 23 subjects were positive for both Montenegro skin test and possible leishmaniasis scars. These figures seem to indicate a rather low endemicity of the disease in the region, comparable with that in Paute, Department of Azuay (Hashiguchi *et al.*, 1991). By contrast, in Peru 2,000 cases of uta are reported annually to the Ministry of Health, a figure that is probably a considerable underestimate of the actual number (Lumbreras and Guerra, 1985). Uta is endemic in the western slopes and in many intermontane valleys of the Peruvian Andes (Herrer, 1957) and the number of cases has increased in recent years (Herrer *et al.*, 1980).

With regard to *Leishmania* species in Alausi, Armijo *et al.* (1990) and Hashiguchi *et al.* (1991) reported *L. mexicana* as the causative agent, and we suspect that the same species is responsible for cutaneous leishmaniasis in Alausi and Huigra. Midgut infections seen in female sandflies (*Lu. ayacuchensis*) from Alausi and Huigra support this theory. This species has already been suggested as the vector of *L. mexicana* in Paute, Department of Azuay, Ecuador (Takaoka *et al.*, 1990; Gomez and Hashiguchi, 1991). The results of parasite identification from the present isolates by zymodeme, serodeme and schizodeme analysis will be reported elsewhere.

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2. Studies on Leishmaniasis in an Endemic Focus of *Leishmania* on the Pacific Coast of Ecuador

ABSTRACT. In the current study, we reviewed 1,296 leishmaniasis cases diagnosed at the outpatient facility of the national laboratory (NIHTM-Portoviejo, Manabi) of Ecuador between 1984 and 1990. All the cases were from rural areas of the Department of Manabi, the Pacific coastal region endemic for cutaneous leishmaniasis. Of these cases, 1,027 (79.2%) were positive for *Leishmania* amastigotes in impression smears. The majority of leishmaniasis cases occurred between 1989 and 1990. A markedly high rate of occurrence (onset time) was found in the period from August to October, just before the beginning of rainy season. The period was estimated as the main time of transmission of the disease in the Pacific coastal region of Ecuador.

Cutaneous leishmaniasis constitutes a considerable threat to human health in most rural areas of Ecuador. In the Department of Manabi on the Pacific coastal region, many cutaneous forms of the disease have recently been diagnosed. Little information, however, is currently available on the transmission of *Leishmania* in this endemic region. In order to obtain some information on the disease in the Department, we tried to review leishmaniasis cases diagnosed at the outpatient facility of the National Institute of Health and Tropical Medicine in Portoviejo, Manabi. Based on this information we offer some suggestions on the distribution and seasonal occurrence of the disease in the given endemic area, that may be useful in future transmission studies in the area.

Materials and Methods

Geographical and climatic situation

The Department of Manabi is situated in the Pacific coastal region of north-western Ecuador, between 0°20'N latitude, 80°03'W longitude and 1°45'S latitude, 80°10'W longitude (Fig. 5.2.1). It is divided into two geographical regions, *i.e.*, the Pacific coastal dry area (Fig. 5.2.2A; elevation: less than 300 m) and the cordilleran wet area (Fig. 5.2.2B; elevation: 300-600 m). Ecological features such as temperature, relative humidity, annual precipitation, vegetation and fauna, are quite variable within these areas and are dependent mainly on the elevation above sea level. The climate of the Pacific coastal region is divided into two seasons, the hot wet season (January to April) and cool dry season (May to December). In the dry season, the climate of the area resembles that of the Peruvian desert some distance to the south.

Subjects

All the subjects examined came from different endemic foci within the Department of Manabi; the majority was from the cordilleran wet areas. All received differential diagnosis of leishmaniasis at the outpatient facility in a laboratory branch of the National Institute of Hygiene and Tropical Medicine, Portoviejo (NIHTM-Portoviejo). The laboratory is located in the city of Portoviejo (population, ca. 50,000), the provincial capital. One of the main activities of the laboratory is to provide differential diagnosis of various parasitological, bacterial, viral and fungal infections. In general, when physicians in the city or rural health centers preliminary diagnose patients with dermal or mucosal lesions as having leishmaniasis, the examinees are recommended for differ-

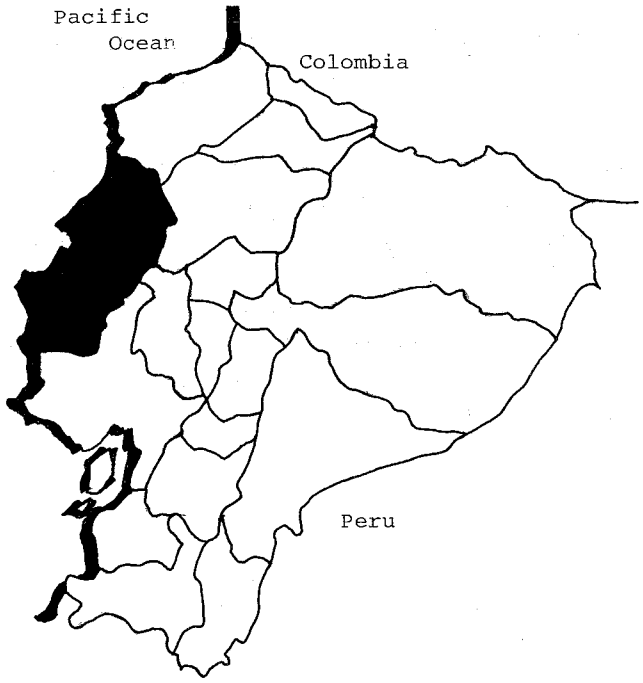

Figure 5.2.1. Outline map of the Republic of Ecuador, showing the Department of Manabi (solid area) where the National Institutute of Health and Tropical Medicine, Portoviejo (NIHTM-Portoviejo) is located and the present study cases originate.

ential diagnosis at this laboratory. Thus suspected leishmaniasis-cases came from almost all the endemic areas of Manabi to the laboratory in Portoviejo. In our studies, made at the outpatient facility of the laboratory, questionnaires were prepared to record the residence and occupation of the patients, history of the disease and leishmanial lesions, treatment, and other features.

Microscopical examinations of smear materials

Materials were taken from the margins of ulcerous or nodular lesions using a surgical knife by an experienced technician (A.E.M.C.). They were then smeared onto a slide glass to make a thin film. After drying the materials at room temperature, they were stained with Giemsa and then examined under a microscope at magnifications of x 400 or x 1,000. We reviewed 1,296 such cases seen at the outpatient facility of the laboratory between 1984 and 1990. We excluded those cases in which *Leishmania* amastigotes were not demonstrated microscopically, unless otherwise specified.

Results

Leishmaniasis-cases diagnosed between 1984 and 1990

Between 1984 and 1990, a total of 1,296 persons with dermal or mucosal lesions visited the outpatient facility of the laboratory in Portoviejo, 1,027 (79.2%) of which were positive for *Leishmania* amastigotes in impression smears (Table 5.2.1). The ratios of the amastigote-positive cases per total persons examined dermatologically in NIHMT-Portoviejo ranged from 35.0% to 91.2% during seven years. It is noticeable

Figure 5.2.2. A view of two ecologically different areas of the Department of Manabi, Ecuador. **Above (A)**, the Pacific coastal dry area less than 300 m of elevation; **below (B)**, the cordilleran wet and mountainous area with 300 - 600 m of elevation.

Table 5.2.1. Yearly leishmaniasis cases diagnosed in National Institute of Health and Tropical Medicine (NIHTM-Portoviejo), Department of Manabi, Ecuador

Year	1984	1985	1986	1987	1988	1989	1990	Total
Smear +*	114	123	35	7	50	166	532	1,027
Smear −*	11	29	26	13	19	58	113	269
Total	125	152	61	20	69	224	616	1,296

* Smears are positive or negative for *Leishmania* amastigotes.

that the majority of *Leishmania* positive cases occurred between 1989 and 1990, *i.e.*, 698 (68.0%) out of 1,027 cases. A remarkably low number of cases (7-50 per year) was found between 1986 and 1988.

Geographical distribution of the cases

Based on 698 *Leishmania*-positive cases diagnosed during 1989 and 1990 in NIHMT-Portoviejo, the distribution of patients was geographically analyzed as shown in Fig. 5.2.3 and Table 5.2.2. Of those positives which were possible to identify the locality of subjects, the majority came from mountainous areas, such as San Placido, 16.7%; Calceta, 15.4%, San Gabriel, 10.0%; Progreso, 8.7%; San Sebastián, 8.1%; and Junin, 6.7% (Fig. 5.2.3). A few cases were also recorded from the coast in towns, such as Manta and San Vicente. Of course, these figures do not indicate the actual incidence or prevalence of the disease, but they seem to show some tendency of the disease occurrence in each endemic focus. Furthermore, the low number of recorded cases from the northern parts of the province might be due to greater distance from Portoviejo (14 in Fig. 5.2.3).

Seasonal occurrence of the cases

Based on 698 *Leishmania*-positive cases diagnosed during 1989 and 1990 in NIHTM-Portoviejo (Table 5.2.3), seasonal occurrence of leishmaniasis cases in the Department of Manabi is depicted in Fig. 5.2.4. The data give a rough idea of the seasonal occurrence of leishmaniasis in this endemic area. In these cases the exact onset time of the disease was not known, but according to the interview records it was estimated that almost all of the cases were diagnosed within three months (Fig. 5.2.5) with most infections apparently occurring from August to October, just before the beginning of rainy season (Fig. 5.2.6).

Age and sex distribution of the cases

Frequency distribution of leishmaniasis cases diagnosed in NIHTM-Portoviejo is arranged by age and sex of the subjects in Table 5.2.4 and Fig. 5.2.7. The results showed that the greatest number (356 out of 417, 85.4%, in males; 234 out of 281, 83.3%, in females) of cases occurred among people of less than 30 years old, with a pronounced peak between 16 and 20 in males and between 0 and 5 in females. A considerable number of cases (23.0% in males and 38.4% in females) was recognized in children under 10 years old. This suggests the occurrence of peridomiciliary infection of leishmaniasis in the area. The number of patients aged 30 or over was relatively lower than expected.

Anatomical localization of the lesions

The patients examined in this study from whom *Leishmania* amastigotes were demonstrated had de-

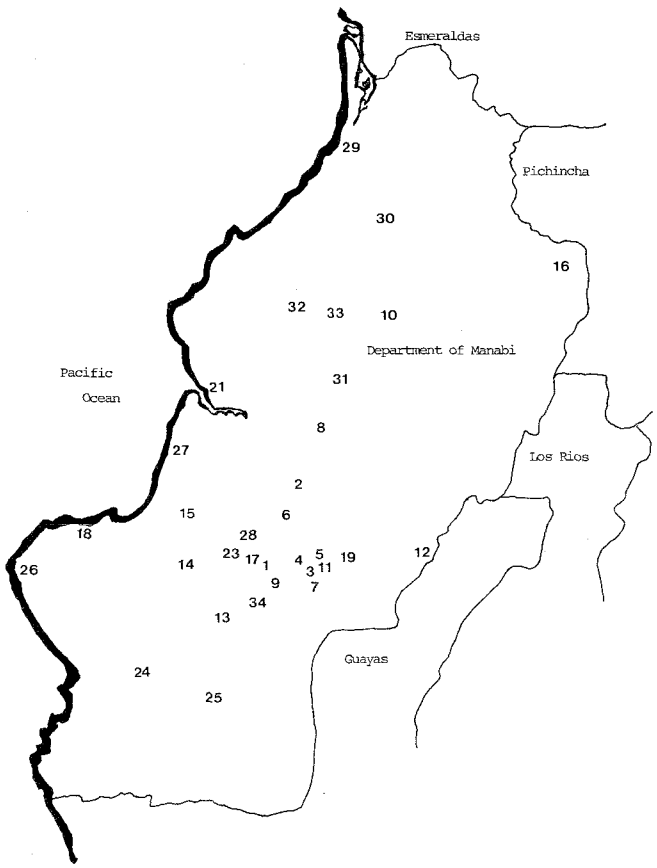

Figure 5.2.3. Location of the sites in the Department of Manabi where the present 698 *Leishmania*-positive cases come from. Each number corresponds with the locality number shown in Table 2.2.2. 1. San Placido, 2. Calceta, 3. San Gabriel, 4, Progreso, 5. San Sebastián, 6. Junin, 7. Poza Honda, 8. Chone, 9. H. Vasquez, 10. Flavio Alfaro, 11. El Moral, 12. Pichincha, 13. Sta. Ana, 14. Portoviejo, 15. Rocafuerte, 16. El Carmen, 17. Alhajuela, 18. Manta, 19. La Chontilla, 20. 24 de Mayo, 21. San Vicente, 22. 10 de Agosto, 23. Calderon, 24. Jipijapa, 25. Noboa, 26. San Lorenzo, 27. San Clemente, 28. Puebro Nuevo, 29. Pedernales, 30. Piedras, 31. Ricaurte, 32. San Isidro, 33. Eloy Alfaro, 34. Ayacucho.

veloped either single or multiple cutaneous lesions. The majority (70.6%) of the lesions observed were found in the upper (34.3%) or lower (36.3%) extremities, with fewer on the head (20.6%), and fewer still (8.8%) on the trunk. The main affected areas on the head were the cheek (9.3%) and nose (4.9%) but not mucous areas; some subjects had lesions on the ears (3.4%).

Discussion

Among 1,296 subjects with dermal lesions, who visited NIHTM-Portoviejo, for differential diagnosis of leishmaniasis between 1984 and 1990, a total of 1,027 (79.2%) revealed a positive diagnosis for *Leishmania* parasites. These numbers were considered to be rather lower than the actual rates of infection,

Table 5.2.2. Leishmaniasis cases, 698 in total, positive for *Leishmania* parasites diagnosed during 1989 and 1990 in NIHTM-Portoviejo from different areas of the Department of Manabi, Ecuador

Locality*	No. of cases	Locality	No. of cases
1. San Placido	115	18. Manta	6
2. Calceta	106	19. La Chontilla	5
3. San Gabriel	69	20. 24 de Mayo	5
4. Progreso	60	21. San Vicente	5
5. San Sebastián	56	22. 10 de Agosto	3
6. Junin	46	23. Calderon	3
7. Poza Honda	38	24. Jipijapa	2
8. Chone	31	25. Noboa	2
9. H. Vasquez	30	26. San Lorenzo	2
10. Flavio Alfaro	22	27. San Clemente	2
11. El Moral	20	28. Puebro Nuevo	2
12. Pichincha	13	29. Pedernales	1
13. Sta. Ana	12	30. Piedras	1
14. Portoviejo	9	31. Ricaurte	1
15. Rocafuerte	8	32. San Isidro	1
16. El Carmen	7	33. Eloy Alfaro	1
17. Alhajuela	7	34. Ayacucho	1

* Locality numbers are same as those shown in Fig. 5.2.3.

since impression smears alone had been employed as a diagnostic tool. This method is not sensitive enough to detect all positive leishmaniasis cases and detection could be improved by the use of more sophisticated diagnostic methods, such as immunological or molecular techniques.

In a review of the positive cases diagnosed at NIHTM-Portoviejo, about 68.0% of the total had occurred during the past two years, 1989 and 1990. This abnormally high occurrence might have been caused by some unknown ecological or climatic factor in the endemic regions, perhaps favouring increases in vector populations. No marked change of migration or activities of the human inhabitants was discerned. In order to understand the mechanism(s) of transmission in the present endemic area, we per-

Table 5.2.3. Monthly occurrence of 698 *Leishmania*-positive cases diagnosed during 1989 and 1990 in NIHTM-Portoviejo, Department of Manabi, Ecuador

	No. of *Leishmania*-positive cases												Total
Month:	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SPT	OCT	NOV	DIC	
1989	16	15	9	9	6	3	4	3	15	17	19	50	166
1990	33	11	37	17	17	10	12	30	105	100	123	37	532
Total	49	26	46	26	23	13	16	33	120	117	142	87	698
%	7.0	3.7	6.6	3.7	3.3	1.9	2.3	4.7	17.2	16.8	20.3	12.5	100.0

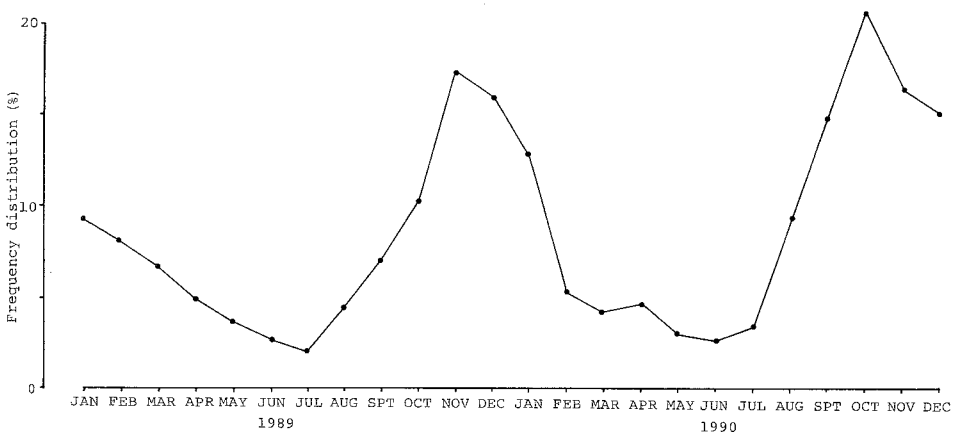

Figure 5.2.4. Frequency distribution of 698 *Leishmania*-positive cases diagnosed in NIHTM-Portoviejo, expressed as % of the triple running average of the occurrence during 1989 and 1990.

formed investigations of the ecology of sandflies and their natural infections with *Leishmania* parasites (data not shown). However, clear-cut answer was not obtained on the transmission which caused an abnormally high rate of infection in the area during 1989 and 1990.

With respect to the geographical distribution of the cases in the province, more than 50% of the total

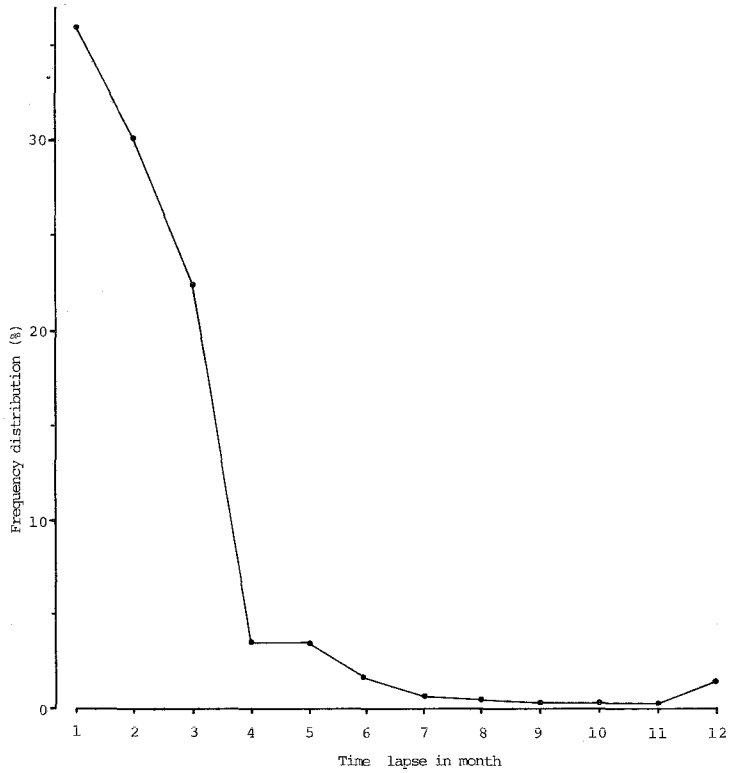

Figure 5.2.5. Time lapse in months from the onset of leishmanial lesions to diagnosis in NIHTM-Portoviejo, depicted based on the data obtained from 698 subjects with parasitologically positive results.

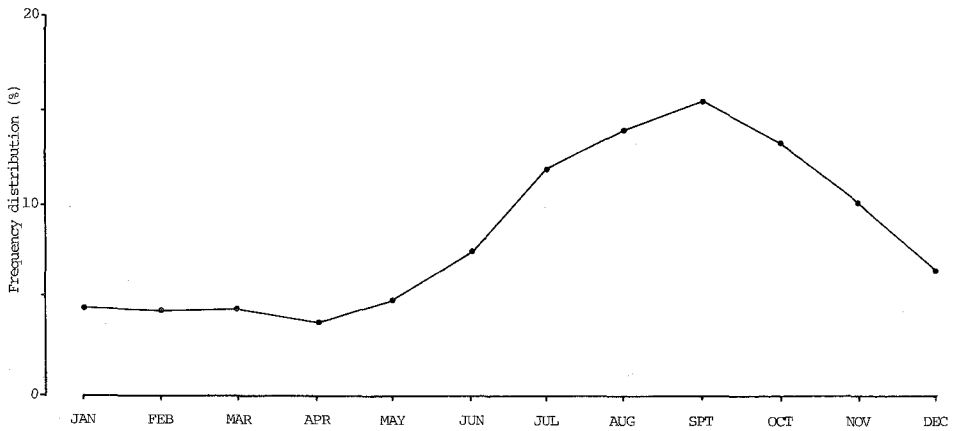

Figure 5.2.6. Seasonal fluctuation of the onset time of 698 *Leishmania*-positive cases, expressed as % of the triple running average.

Table 5.2.4. Leishmaniasis cases diagnosed in NIHTM-Portoviejo, Department of Manabi, Ecuador, arranged by age and sex

Age	Total	Male		Female	
		No.	%	No.	%
0 - 5	112	54	12.9	58	20.6
6 -10	92	42	10.1	50	17.8
11-15	102	56	13.4	46	16.4
16-20	131	96	23.0	35	12.5
21-25	87	65	15.6	25	8.9
26-30	63	43	10.3	20	7.1
31-35	22	16	3.8	6	2.1
36-40	26	15	3.6	11	3.9
41-45	20	10	2.4	10	3.6
46-50	11	5	1.2	6	2.1
51-55	11	4	1.0	7	2.5
56-60	9	5	1.2	4	1.4
61-65	2	2	0.5	0	0.0
66-70	4	2	0.5	2	0.7
71-	3	2	0.5	1	0.4
Total (%)	698	417	(59.7)	281	(40.3)

were found in the south-eastern mountainous region (Fig. 5.2.2) bordering the Department of Guayas. Thirty-seven of the leishmaniasis cases diagnosed at NIHTM-Guayaquil between 1975-1986 were reported from Manabi, most (24 cases, 64.9%) of them from the central (Ricaurte) of the province (Coronel *et al.*, 1987). The affected areas appear to be located largely in the warm, humid mountainous areas of Manabi.

The Ecuadorian Andes rise to altitudes of 5,000 meters above sea level and divide the country into three large natural regions: the Pacific coastal region, a lowland extending westward the Pacific Ocean, where the present study sites are located; the Andean plateau and the Oriente, a lowland forested area contiguous with the Amazonian basin (Teran, 1984). Within this diversity of ecological situations of Ecuador, several of the causative agents of cutaneous or mucocutaneous leishmaniasis occur. To date, seven *Leishmania* species, *viz.*, *L. braziliensis, L. panamensis, L. guyanensis, L. amazonensis, L. equatorensis, L. mexicana* and *L. major*-like, have been reported from the country based on zymodeme, serodeme and schizodeme analysis of samples from humans, animals and sandflies (Mimori *et al.*, 1989; Hashiguchi *et al.*, 1990, Armijos *et al.*, 1990). The seven species mentioned above have been recorded from the Department of Esmeraldas, Pichincha, Los Rios and

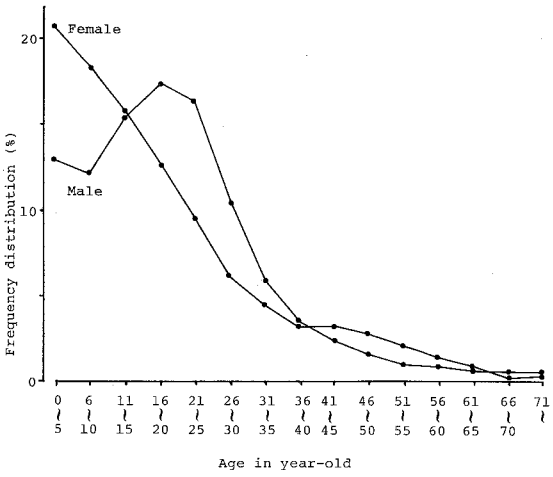

Figure 5.2.7. Age and sex distribution of 698 *Leishmania*-positive cases diagnosed in NIHTM-Portoviejo, expressed as % of the triple running average.

Guayas, which border Manabi. Most of the earliest case reports of leishmaniasis in Ecuador were recorded from the Pacific coastal region (Heinert, 1924; Valenzuela, 1931; Leon, 1951; Carrera, 1953; Rodriguez and Aviles, 1953; Rodriguez, 1969; Calero and Coronel, 1981; Hashiguchi *et al.*, 1984) with very few from the Oriente (Carrera, 1945; Amunarriz, 1982) where communications and the medical care system are generally inadequate. Many cases of cutaneous leishmaniasis have recently been reported from the Andean plateau region (Hashiguchi *et al.*, 1991).

Age and sex distribution of leishmaniasis cases diagnosed in NIHTM-Portoviejo revealed a pronounced difference between male and female. The peak occurrence of cases was found between 16 and 20 years of age in the former, and between 0 and 5 years of age in the latter. From this high rate of infection among the lower age groups especially in females, it is suggested that transmission of leishmaniasis in the area might be occurring within or around houses in the study area. Almost all of the houses observed during our surveys were located among or around a dense forest. Hashiguchi *et al.* (1984) reported that no marked age and sex difference with respect to *Leishmania* infection rates in a newly established settlement in Naranjal, Department of Guayas, Ecuador was found, and similar conclusions were drawn in a study of a *Leishmania* focus in Panama (Herrer and Christensen, 1976). Pessoa (1961) pointed out that American leishmaniases could attack persons regardless of age, sex and race, and that its prevalence depended on the occupation of subjects as well as on the distance between human habitations and forest areas. A low rate of leishmaniasis cases in examinees aged 31 or over might indicate the acquisition of total lasting immunity in the susceptible population. Recovery from any leishmanial skin lesion has generally been thought to impart a firm and life-long immunity to reinfection (Lainson and Shaw, 1978). In the present study, therefore the low rate of infection in higher age groups might be explained by the fact that only subjects with active lesions visited the laboratory, not those who had been infected years before and those lesions had healed.

In the current study, monthly leishmaniasis cases recorded were highest between November and January in 1989 and between September and November in 1990. Taking in consideration the time lapse from onset to diagnosis of these cases, the main transmission time of leishmaniasis in the Department of Manabi was therefore estimated as the period from August to October, just before the beginning of rainy season. This finding would be important in the timing of future preventative or control measures in the endemic regions.

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Chapter 6

Experimental Leishmaniasis

1. Histological Observations of Golden Hamsters Infected with an Ecuadorian Isolate of *Leishmania mexicana*

ABSTRACT. An experimental study was performed to investigate the *Leishmania mexicana* infection in golden hamsters. The animals were infected with *L. mexicana* from Ecuador. At the autopsy 6 months after inoculation, the inoculated sites were shallow ulcerative covered with thick crusts. No cutaneous metastasis was observed on other exposed parts of the body. Histologically, specimens of both the nose and footpads showed large numbers of amastigotes with extensive infiltration of histiocytes, lymphocytes and some extent of neutrophils, eosinophils and plasma cells. A number of mast cells was prominant in the upper and lower dermis of granulomatous lesions. Amastigotes were found in the macrophages inside the large parasitophorous vacuoles, mostly at the central part of the lesion. Amastigotes were also observed in the liver and spleen by electron microscope but the number was fewer in visceral sections than cutaneous ones. Regular destruction of parasites was observed within macrophages in all the cutaneous and visceral sections indicating the phagocytizing role of these cells against parasites.

American cutaneous leishmaniases, caused by *Leishmania mexicana* and *L. braziliensis* complexes, are widely endemic in Central and South America. Among many species of the New World *Leishmania*, *L. mexicana* (= *L. mexicana mexicana*) is well known to be responsible for a variety of disease forms showing from a localized simple and mild lesion to generalized diffuse type lesions, with a wide range of distributions from lowlands to Andean highlands. In Ecuador, the species, *L. mexicana*, was isolated from both Andean highland and Pacific lowland patients (Armijos *et al.*, 1990; Hashiguchi *et al.*, 1991). However, the clinical manifestations cuased by *L. mexicana* had a tendency to vary depending on several unknown factor(s), such as the geographical distributions and the immunological or physiological conditions of human hosts (Hashiguchi *et al.*, 1991; our unpublished data). In order to investigate the factor(s) involved, we commenced a comparative study of Ecuadorian leishmaniasis between lowlands and highlands, carrying out histopathological and electromicroscopical observations on the biopsy materials from humans and experimental animals. In the current study, as the first step, an attempt was made to obtain some baseline data (information) on the pathology of experimental (animal) leishmaniasis casused by *L. mexicana* isolated from Pacific lowlands of Ecuador. The information reported here, will be useful for an understanding of leishmaniasis in Ecuador, together with the ultrastructural study of the Ecuadorian forms reported previously (Bhutto *et al.*, 1992). The present work describes mainly on the results of histopathological findings of the dermal lesions (ulcers) of hamsters infected experimentally and the visceral organs invaded by the parasites, *L. mexicana*.

Materials and Methods

Hamsters

Male golden hamsters, 100 to 150g and 7 weeks of age, were used. The animals were fed a standard pellet diet and given water *ad libitum*.

Parasites and mode of infection

The parasites used were isolated from an Ecuadorian leishmaniasis patient and maintained *in vitro* for several months before animal inoculation. Using isoenzyme electrophoresis they were idenified as *L. mexicana* (MHOM/EC/90/INH690) by Dr. T. Agatsuma, Department of Parasitology, Kochi Medical School, Japan. The promastigotes of log phase were inoculated to the nose and hind footpads of 10 hamsters. Each hamster received 1×10^7 promastigotes. The evolution of lesions was observed and recorded periodically during infection.

Histopathology

At about 6 months after post infection the animals were sacrificed and specimens were taken from central and peripheral parts of the lesions. Specimens were also taken from liver and spleen and divided into two parts. One part of material was fixed in 10% formalin, from which paraffin sections were made and 5 μm sections were stained with hematoxylin-eosin. For the differentiation of mast cells, the sections were stained with toluidine blue (pH 5.0).

Electron microscopy

The other part of autopsy material was cut into 2-3 mm and fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4). The tissues were then washed with 0.1M cacodylate buffer and post-fixed in 2% osmium oxide for 2 hrs. After dehydration in different cocentrations of alcohol, specimens were embedded in epon 812. One micron semi-thin sections were cut with a glass knife on an LKB ultrotome and stained with toluidine blue. Ultra-thin sections were cut with a diamond knife, stained with lead citrate and uranyl acetate, and examined under a JEM 1200 EX electron microscope (JEOL, Japan).

Results

Almost all the hamsters inoculated with *L. mexicana* promastigotes developed erythematous lesions at the site of inoculation. The lesions gradually changed into nodules, and later ultimately resulted in ulcers. At the time of autopsy, the inoculated sites of the nose and both hind footpads were observed as swallen and large ulcerations (Fig. 6.1.1 A, B). The ulcers were covered with thick crusts. There was no any metastasis or nodule formation on the other exposed body surface.

Light microscopic findings

No significant epidermal changes were seen in both the nasal and footpad sections. The epidermis was intact and thin at the center of the lesion, and crusts and parakeratosis were present at some parts of the specimens. In dermis, the histiocytes were predominant in the sections and most of them contained numerous amastigotes in large vacuoles, particularly at the central part of the lesions (Fig. 6.1.2A, B). The huge numbers of lymphocytes were accumulated surrounding the parasitized macrophages mostly at the peripheral parts of the lesion (Fig. 6.1.3A, B). Neutrophils, eosinophils and plasma cells were also seen in the whole specimens. Many mast cells were observed in the upper dermis in both the nasal and footpad sections (Fig. 6.1.4A, B). Mast cells were located only at the intact sites of the dermis from parasites, but absent from the site where macrophages were occupied with parasites. Focal necrosis was also recognized in deep dermis.

In sections of liver and spleen, it was considerably difficult to find the parasites, because of few in number.

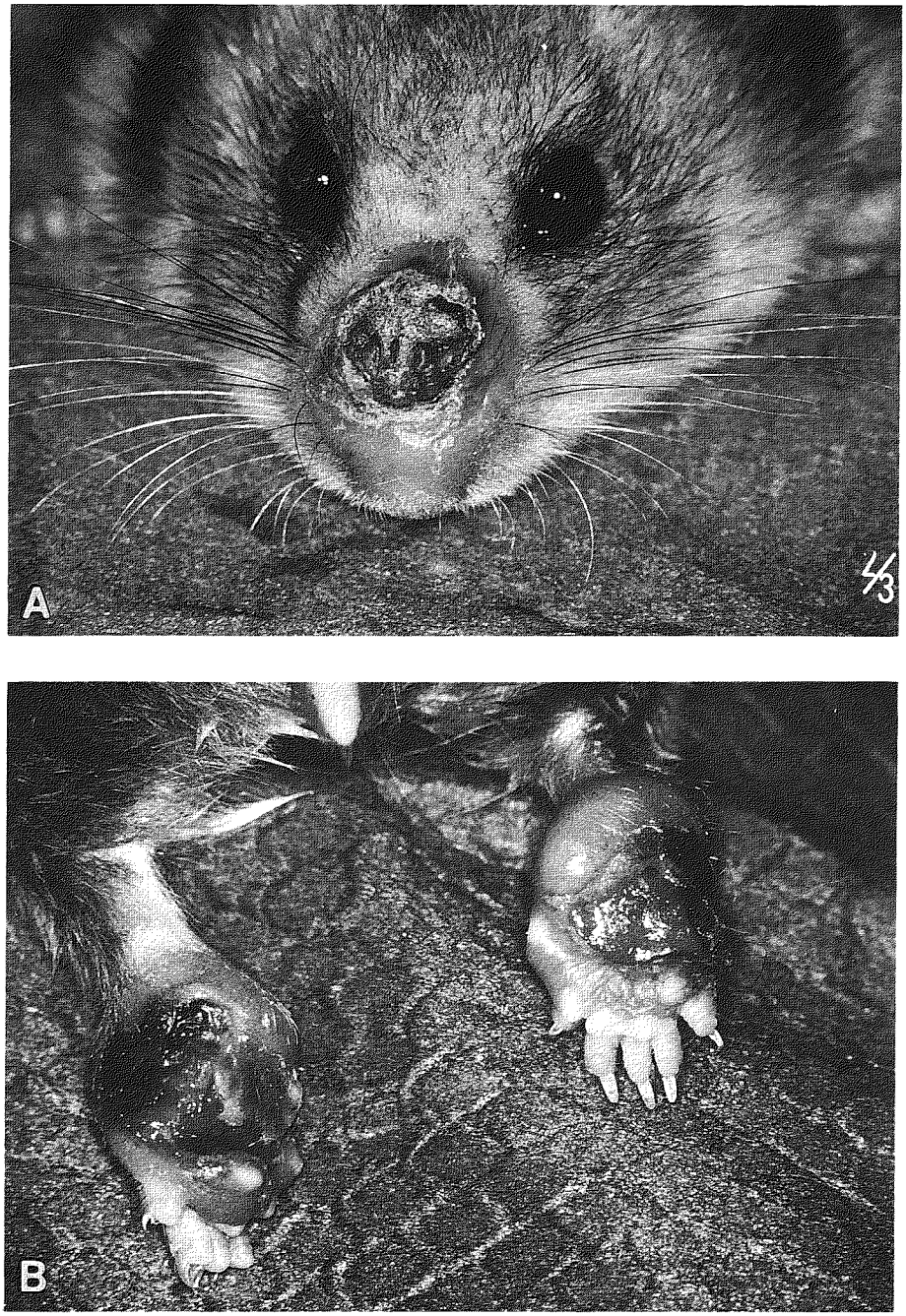

Figure 6.1.1. Picture of the inoculated sites of the golden hamsters infected with *L. mexicana* isolated from Ecuadorian patients. Shallow ulcers are visible on the nose (A) and both hindfootpads (B).

Electron microscopic findings

The epidermis of inoculated sites was free from the invasion of parasites. In the dermis, parasites were found both intracellularly and extracellularly. Amastigotes in the cytoplasm of macrophages were located either inside or outside vacuoles (Figs. 6.1.5 and 6). More than one vacuole were seen in most of the macrophages and various numbers of amastigotes were present in most of the vacuoles. Some parasite-free vacuoles were also seen, and they connected with parasitophorous vacuoles (Fig. 6.1.7). Parasites were neither inside the mast cells nor in the eosinophils or neutrophils.

Parasites were also found in the liver and spleen of hamsters infected experimentally with *L. mexicana* from Ecuador. They were located within the macrophages but very few in number. Regular degeneration of parasites was noted within macrophages inside or outside the vacuoles in all the cutaneous and visceral sections (Figs. 6.1.8, 9, 10 and 11). Morphologically, the normal amastigotes were rounded or elongated, surrounded by two layers of membranes and contained a rounded nucleus with a small nucleolus. The flagellum, flagellar pocket, kinetoplast, mitochondria, golgi apparatus, lysosomes, vacuoles and electron-dense granules in vacuoles could be distinguished (Figs. 6.1.12, 13 and 14).

Discussion

At the autopsy of hamsters, 6 months of inoculation with *L. mexicana*, we observed shallow ulcers covered by thick crusts over the nose and both hind footpads in all the animals, confirming the finding that hamster is a good animal model for *L. mexinana* infection (Wilson, 1979; Bretana *et al.*, 1983).

The knowledge of relation between *L. mexicana* and host's (Ecuadorian patient's) cells has a considerable importance to understand the pathogenesis of the disease in the country. In our specimens it was observed that once the parasite is phagocytized by macrophage it is enclosed by phagolysosome. Then, the phagolysosome starts to distend and inside it multiplication of parasites takes place (Berman *et al.*, 1979).

With regard to the evolution of experimental cutaneous lesions, Bretana *et al.* (1983) proposed that 1) toxic factors from the amastigote inhibit the action of lysosomal enzymes; 2) this causes the phagolysosome to become enlarged and new vacuole is ready to invade the parasites; 3) extensive enlargement of the vacuoles and certain other factors cause the rupture of cell membranes, this repeatedly results in ulceration at the sites. From the observations of many stained specimens in this study, a similar process of ulceration in hamsters infected with *L. mexicana* from Ecuador was estimated.

In the present light microscopic sections, large numbers of *L. mexicana* amastigotes inside the parasitophorous vacuoles were observed in the nose and footpad sections but it was difficult to find the amastigotes in the liver and spleen sections. The amastigotes in the liver and spleen were confirmed in ultrathin sections, however the number was limited. This indicates the well-multiplication of *L. mexicana* (dermatophilic) parasites in the inoculated sites (nose and footpads) but not in the liver and spleen. These findings suggest that although *L. mexicana* inoculated to the nose and footpads of hamsters can disseminate from the cutaneous sites to the liver and spleen, they may not replicate in these organs.

Similar results have also been presented by Hill (1988), who showed the possibility of dissemination of *L. amazonensis* to the liver and spleen in two different strains of mice. However, the metastasis (visceral dissemination) was not observed in C3H mice infected with *L. mexicana* (Grimaldi *et al.*, 1980). When the five different strains of mice were infected with *L. mexicana*, different responses were observed including the appearance of metastatic lesions in the tail of BALB/c mice (Perez *et al.*, 1979). Recently, *L. amazonensis*, well known as a causative agent of diffuse cutaneous leishmaniasis, was isolated from

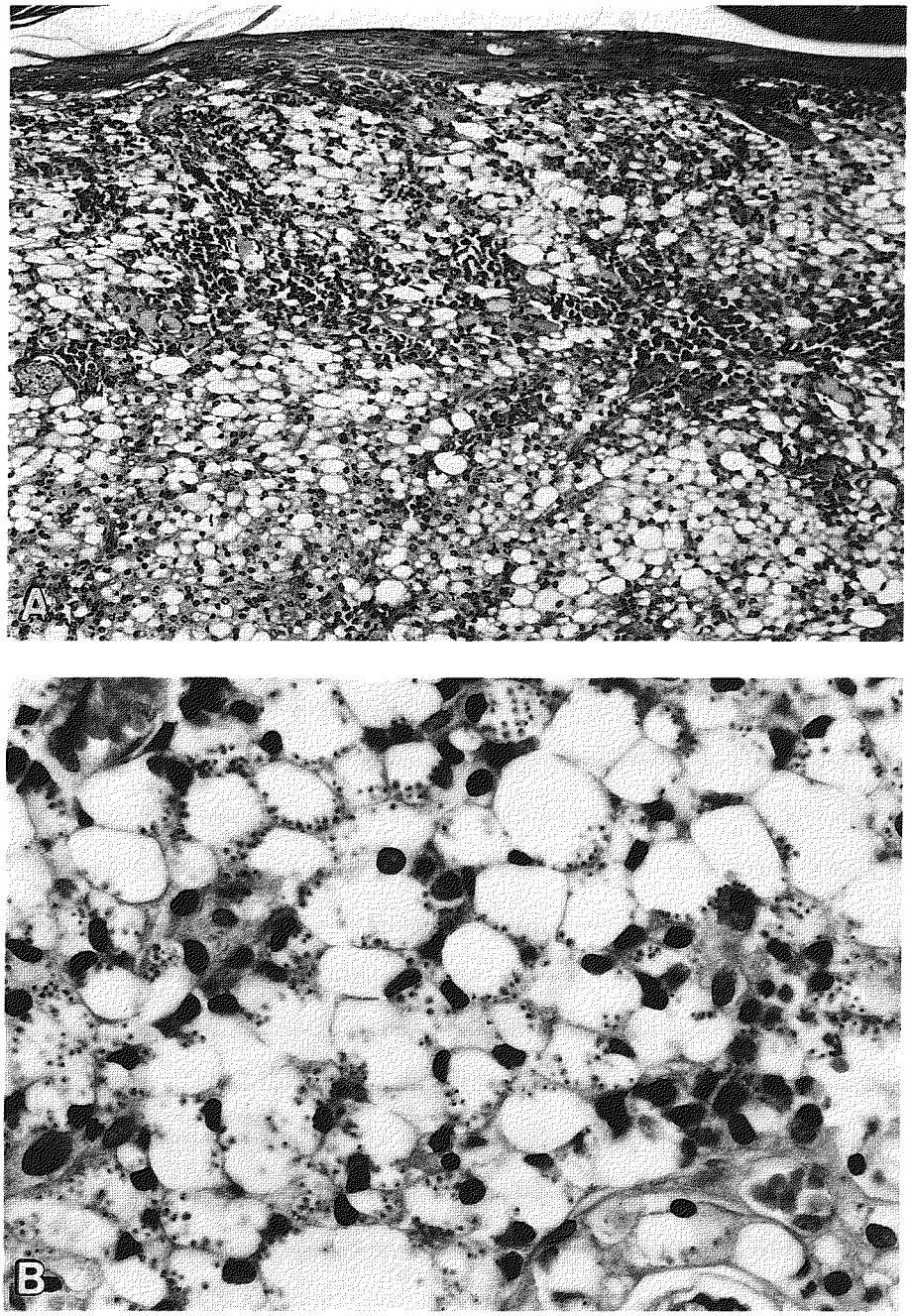

Figure 6.1.2. A, Light photomicrograph of formalin embedded section from nose of the golden hamster infected with *L. mexicana*, showing the extensive infiltration of histiocytes and the formation of parasitophorous vacuoles. B, Many parasites invaded by macrophages are visible inside the parasitophorous vacuoles. (H & E stain x40, x160)

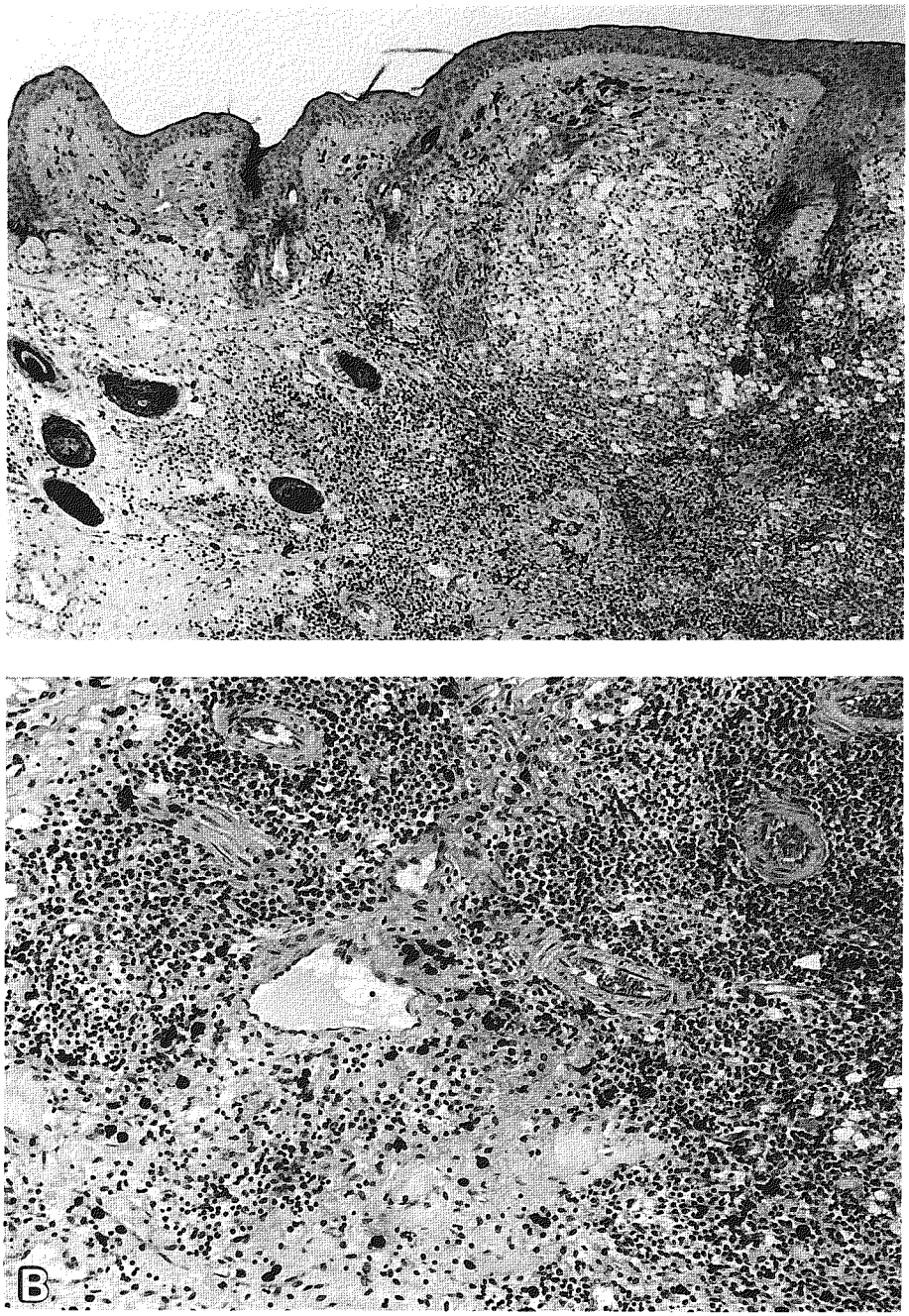

Figure 6.1.3. A, Footpad section, showing the presence of large number of lymphocytes and the parasitized macrophages. B, high magnification of 3A, showing the perivascular infiltration of lymphocytes. (H & E stain x16, x40)

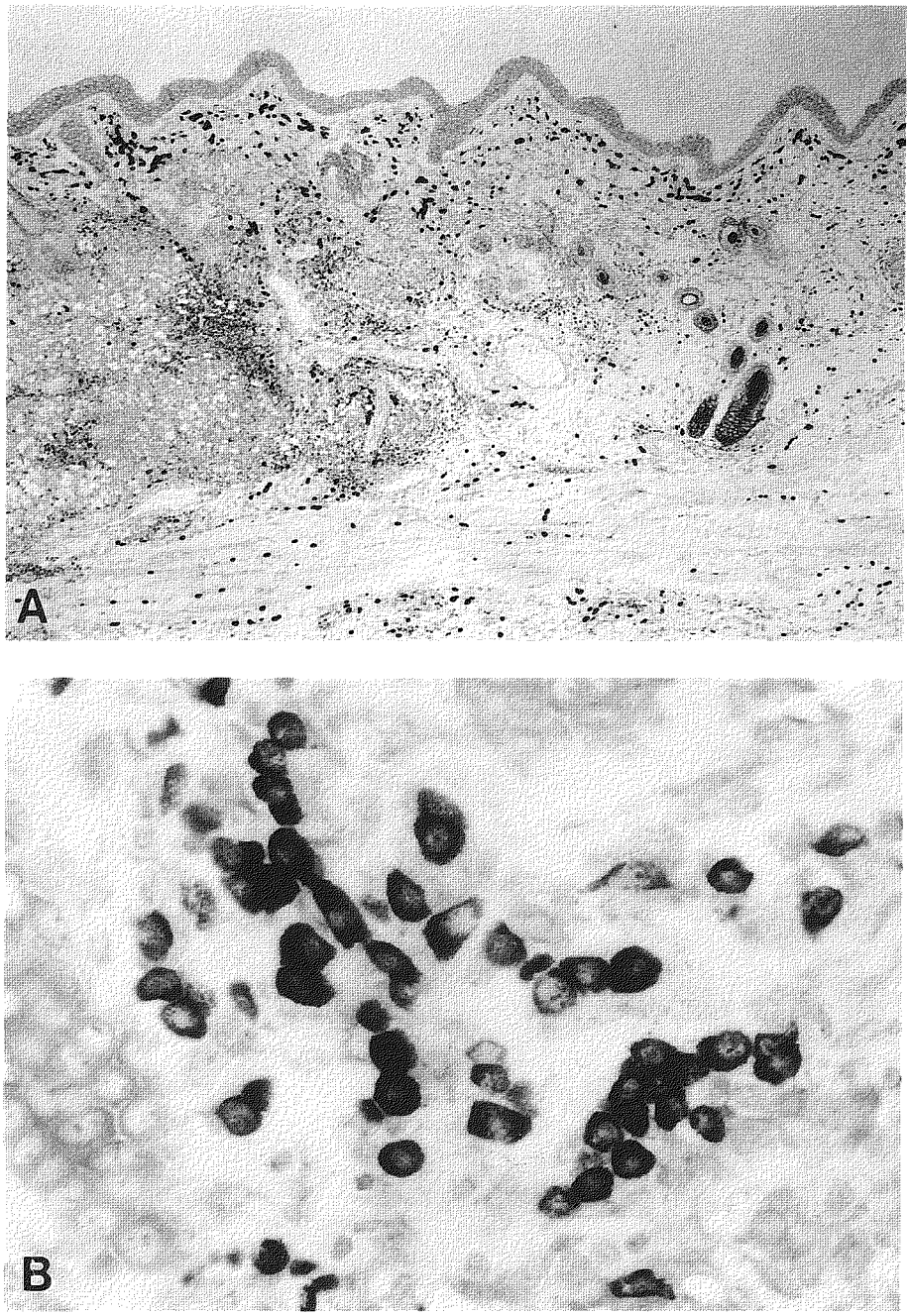

Figure 6.1.4. A, Huge number of mast cells in the upper and lower dermis. B, high magnification of 4A. (toluidine blue stain x16, x160)

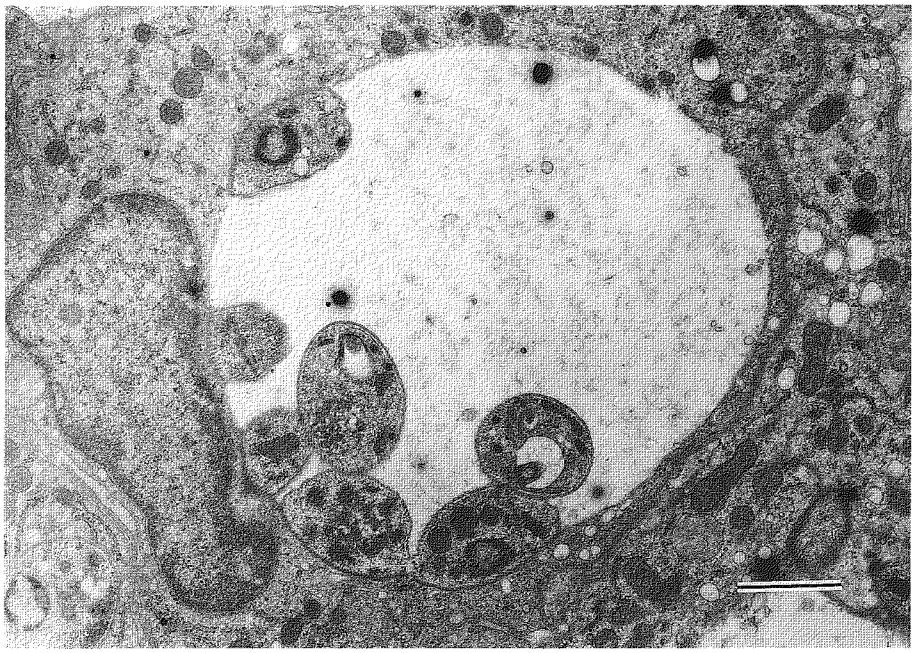

Figure 6.1.5. Electron micrograph, a group of *L. mexicana* parasites inside the parasitophorous vacuole of macrophage. bar = 2 μm.

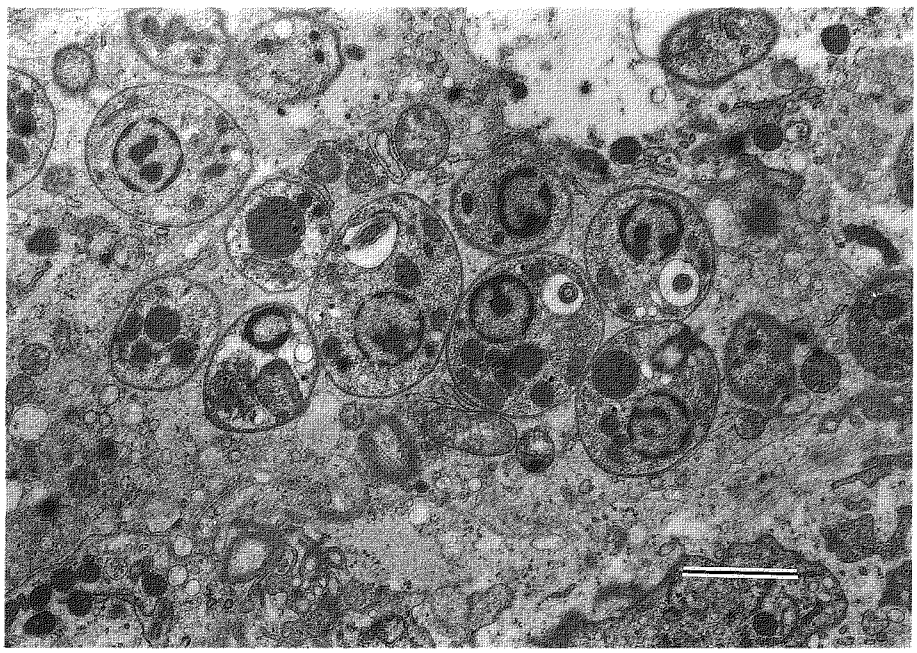

Figure 6.1.6. Figure showing the parasites inside the cytoplasm of macrophage without formation of vacuole. Mitochondria of the macrophage are also visible. bar = 2 μm.

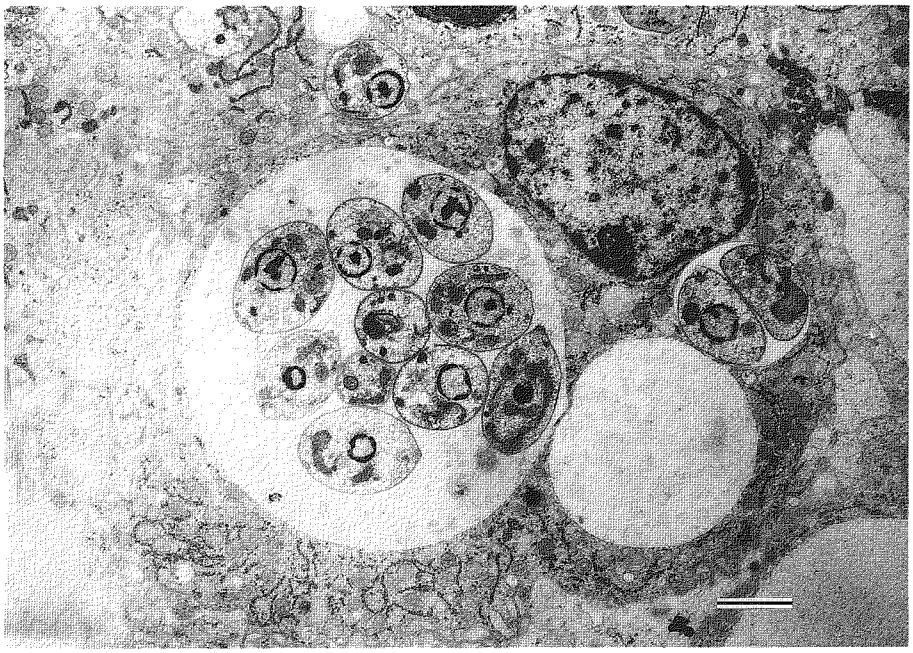

Figure 6.1.7. Various number of parasites with and without vacuoles. A small vacuole which is empty from parasites is also visible near the large parasitophorous vacuole. bar = 2 μm.

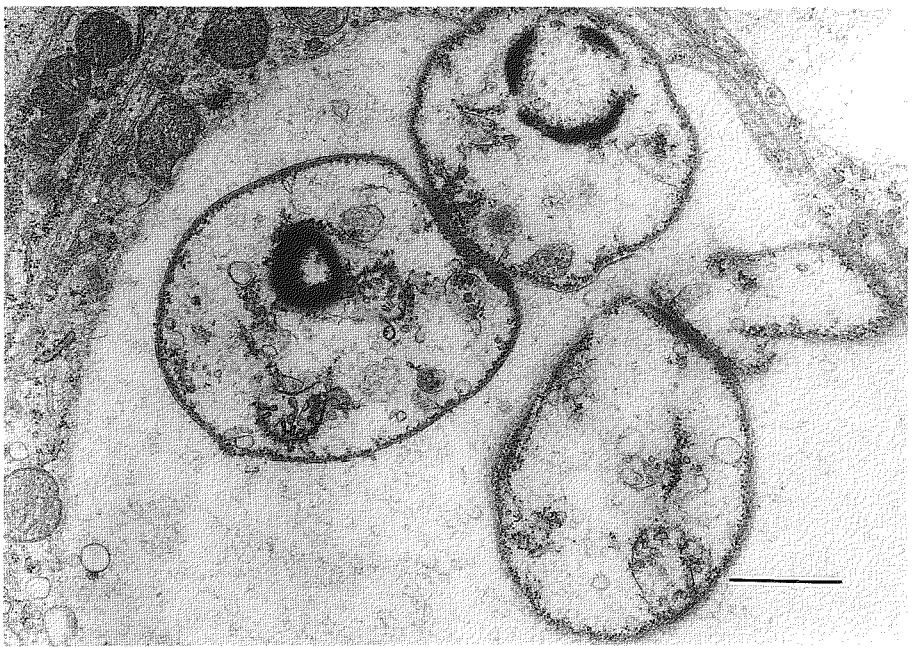

Figure 6.1.8. The degeneration of some *L. mexicana* parasites inside the macrophage of the liver of golden hamsters. bar = 1 μm.

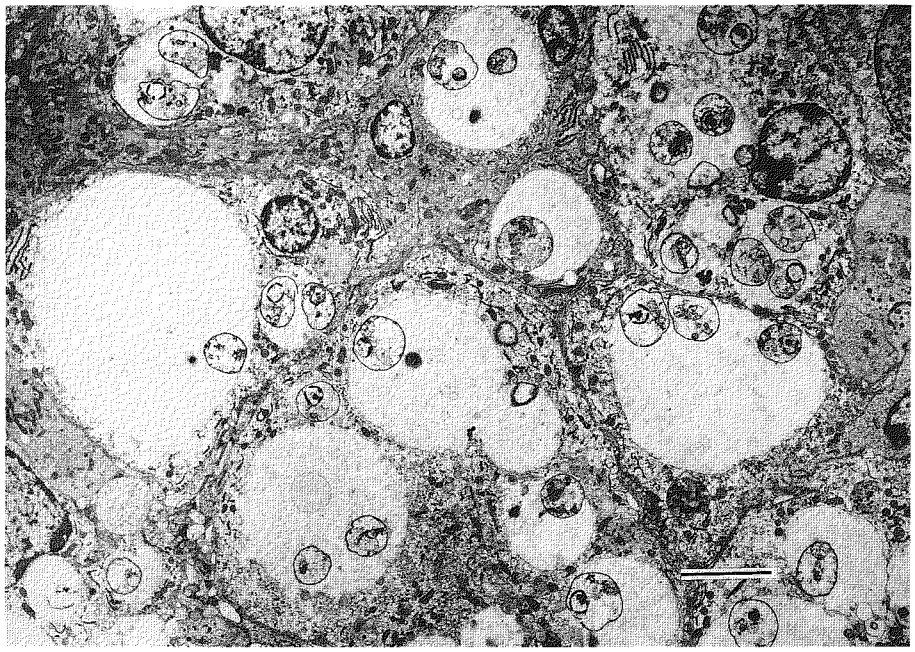

Figure 6.1.9. Electron micrograph of the section from nose. Parasites are under the various stages of degeneration bar = 5 μm.

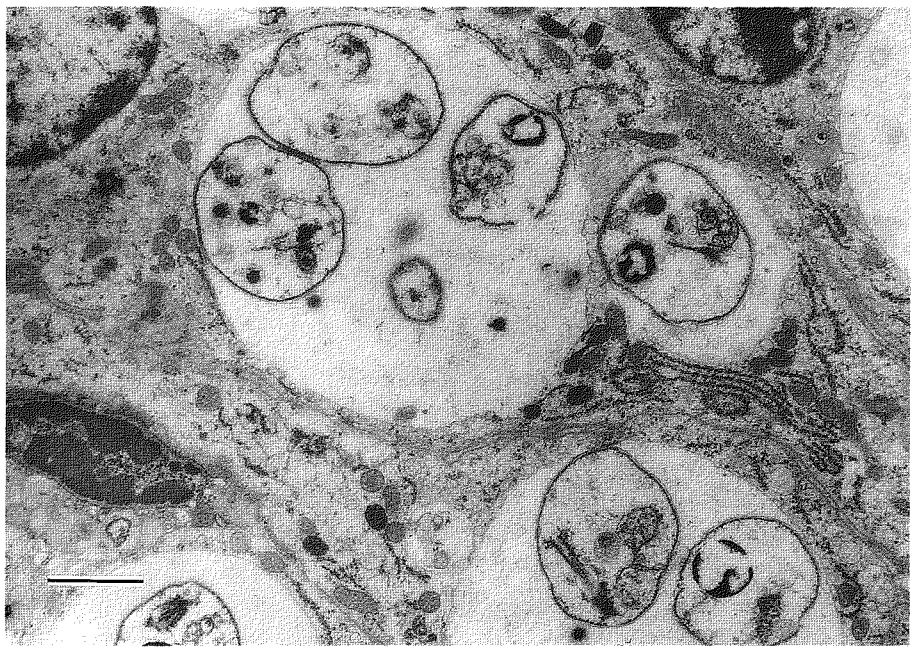

Figure 6.1.10. Degenerating parasites inside the parasitophorous vacuole of macrophage in the right footpad. bar = 2μm.

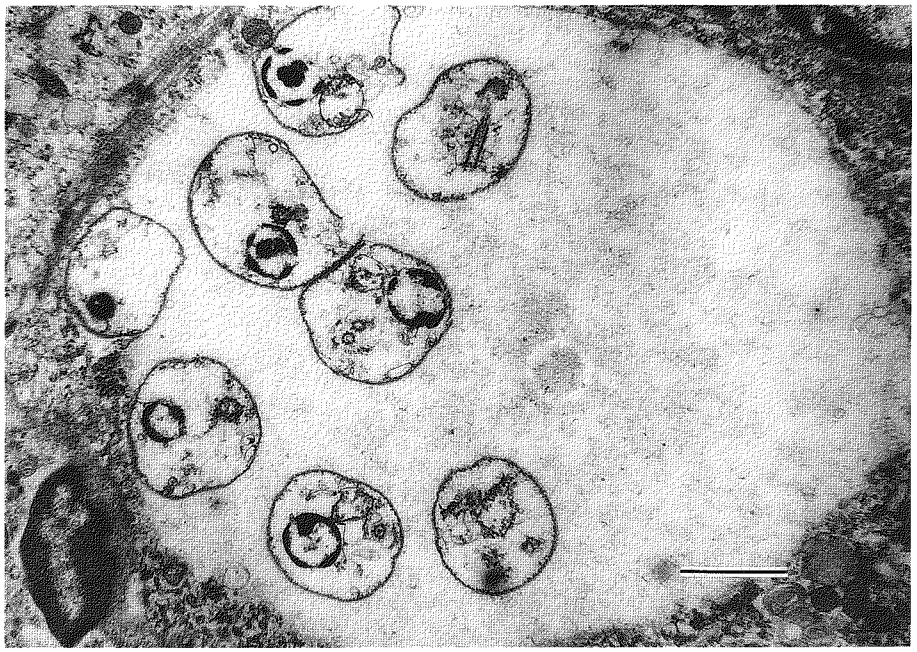

Figure 6.1.11. Section from left footpad. All the parasites are in the various phase of degeneration inside the vacuole of macrophage. bar = 2 μm.

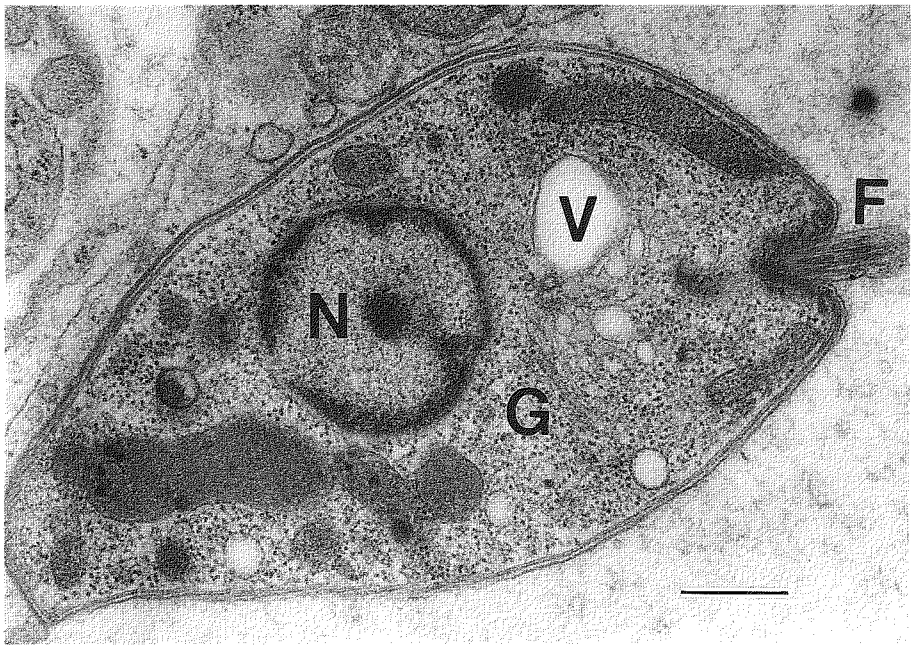

Figure 6.1.12. Ultrastructure of the *L. mexicana* parasite. F, flagellum; N, nucleus with small nucleolus; G, golgi apparatus;V, vacuoles. bar = 500 nm.

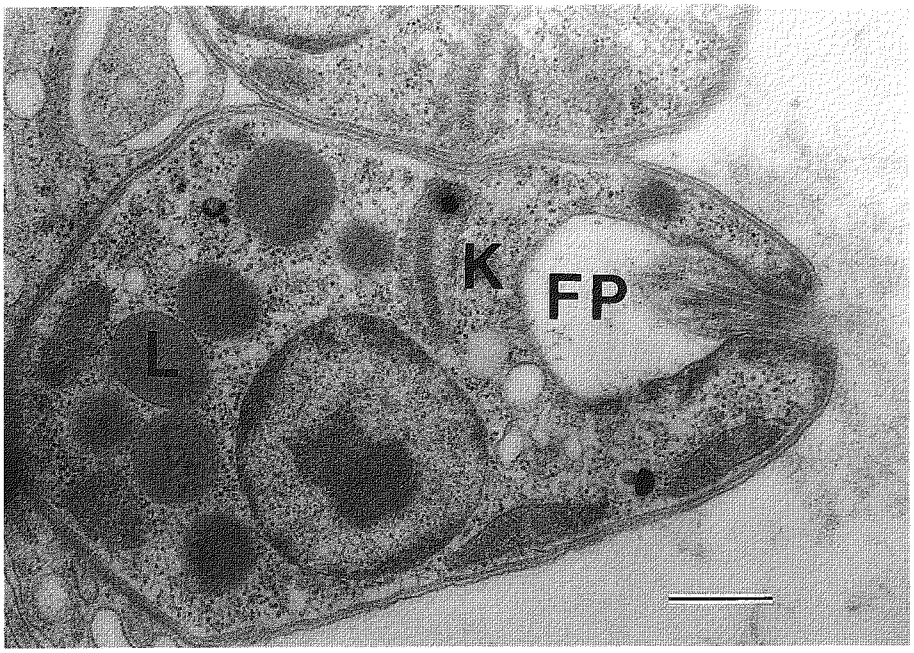

Figure 6.1.13. Ultrastructure of the *L. mexicana* parasite. FP, flagellar pocket; K, kinetoplast; L, lysosomes. bar = 500 nm.

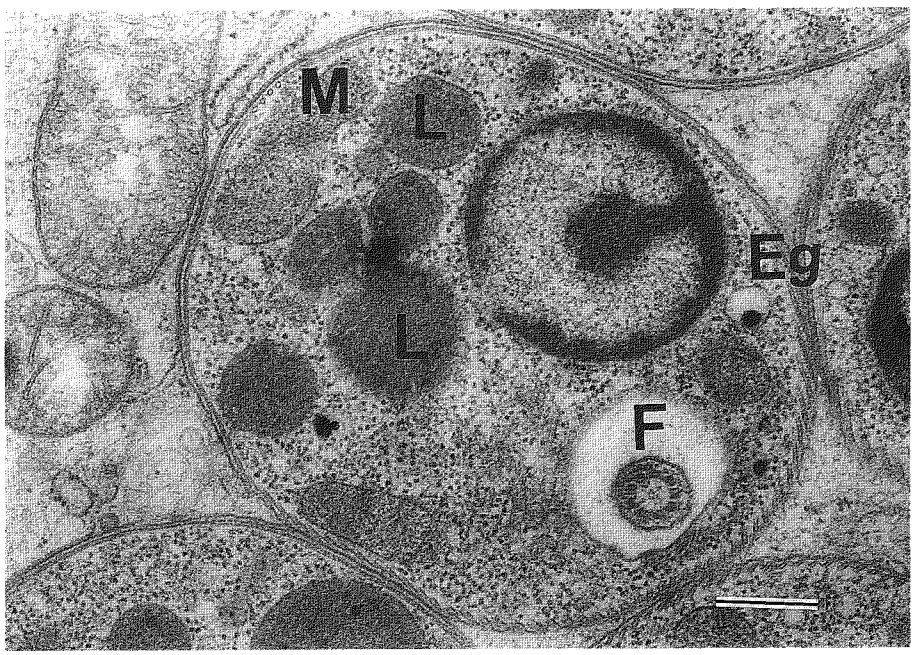

Figure 6.1.14. Ultrastructure of the *L. mexicana* parasite. F, flagellum; M, mitochondria; L, lysosomes; Eg, electron-dense granules inside the vacuole. bar = 500 nm.

bone marrow of a Brazilian patient with visceral leishmaniasis (Barral *et al.*, 1986). In *L. mexicana* infection, therefore, such a visceral dissemination in humans may be observed in future examination, as well as the result found in the present animal (hamster) model.

There have been controversial opinions about the role of the macrophage in killing the parasite intracellularly. In previous human specimens (Bhutto *et al.*, 1992) we found the degeneration of parasites inside the macrophages. In the present study, the large numbers of parasites also showed degeneration within macrophages inside or outside the vacuoles in all of the cutaneous and visceral specimens. This indicates the active role of macrophages in the destruction of parasites intracellularly, although the destruction mechanism is unknown. Such an intracellular degeneration of parasites inside macrophages has also been reported in other human cases (Sandbank, 1976) and experimental works (Mauel *et al.*, 1978). However, Bretana *et al.* (1983) showed the incapability of macrophages to destroy the parasites intracellulaly.

By immunocytochemical and electron microscopic studies in animals, it has been suggested that T-cells provide lymphokines (cytokines) that can activate the host macrophage to destroy the parasites intracellularly or that T-cells play a cytotoxic role, killing the infected macrophages and helping to destroy the liberated extracellular parasites (McElrath *et al.*, 1987). Sypek *et al.* (1984) believed that intracellular destruction of *L. tropica* can take place only by the direct cell contact mechanism between lymphocyte and macrophage. Moreover, Murray *et al.* (1982) have shown the killing of *L. donovani* amastigotes inside macrophages by oxygen dependent mechanism and concluded that non-activcated phagocytes may display effective microbicidal activity against intracellular parasites utilizing oxygen dependent mechanism.

From the present hamster and *L. mexicana* model, we propose that some unknown factor/factors (as discussed above) under host immune mechanism(s), would play an effective role and would promote the destruction of parasites inside the macrophages, and thus, effective killing of parasites finally might result in healing of the lesions. However, the precise conditions and mechanisms of the destruction of parasites were not elucidated in the present study. In our specimens, severe degeneration of amastigotes in large numbers of macrophages may show a forward step towards the healing process, that may allow us to suggest the possible mechanism in the self-healing of the disease; though, we did not observe the complete healing of the lesions. Our experimental study, using hamsters infected with *L. mexicana* isolated from an Ecuadorian patient, represents a model of classic self-healing of cutaneous form, which helps the understanding of the mechanism of pathogenesis of Ecuadorian leishmaniasis. On the basis of the results obtained, again, we conclude that macrophage can destroy the parasites intracellularly under certain mechanism(s) and play an effective and major role in the spontaneous healing of the disease.

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2. Evaluation of Antileishmanial Activity of Paromomycin, Meglumine Antimonate and Mercury Chrome *in vitro* and *in vivo* for Their Topical Applications to American Cutaneous Leishmaniasis

ABSTRACT. Leishmanicidal activity of paromomycin, meglumine antimonate and mercury chrome was evaluated *in vitro* and *in vivo* for the purpose of their topical applications to American cutaneous leishmaniasis. Paromomycin inhibited *in vitro* growth of promastigotes of different species of New World *Leishmania* at relatively low concentrations. Mercury chrome solution (mercurochrome) was highly effective against leishmanial promastigotes *in vitro*. Topical treatment with mercury chrome against the skin ulcers of BALB/c mice infected with *L. amazonensis* revealed a slight delay of development of the lesion. These results suggest that paromomycin and mercury chrome are potent chemotherapeutic agents for American cutaneous leishmaniasis. In this study, however, no obvious synergistic inhibitory effect of meglumine antimonate on the promastigote proliferation *in vitro* was observed.

Pentostam® (sodium stibogluconate) and Glucantime® (meglumine antimonate) have long been used for treatment of cutaneous leishmaniasis (Berman, 1985). However, the treatment with pentavalent antimonials by intravenous, intramuscular or intralesional administration (Kellum, 1986) is highly toxic, painful and expensive. The pentavalent antimony is also insufficiently supplied in the endemic countries, and does not always produce complete healing (Berman, 1985; Jacobson *et al.*, 1990). Under such a circumstance, development of suitable topical chemotherapy appears to be a rational approach for treatment of American cutaneous leishmaniasis, since local treatment reduces quantities of drug and risk of systemic side effect. In addition, topical treatment has an advantage that the patients could treat their own skin lesions by themselves. Thus, we started a new trial of topical treatment against limited cutaneous leishmaniasis in Ecuador with paromomycin ointment or Glucantime® lotion in combination with mercury chrome, and found that these treatments appeared to be promising (see Chapter 7.3). In the present study, we evaluated antileishmanial activity of these compounds *in vitro* and *in vivo*.

Materials and Methods

Parasites

Two WHO reference strains, *i.e.*, *L. amazonensis* (MHOM/BR/73/ M2269) and *L. mexicana* (MHOM/BZ/82/BEL21) and an isolate (a new species, *L. equatorensis*) from *Choloepus hoffmanni* in Ecuador (Grimaldi *et al.*, 1992) were used for *in vitro* drug susceptibility tests. Promastigotes of these species were cultured in Schneider's Drosophila medium (Gibco) containing 20% heat-inactivated fetal calf serum (HIFCS) and 100 µg/ml gentamycin. Virulent phenotype of *L. amazonensis* LV78 strain (Kink and Chang, 1988) was used for *in vivo* chemotherapeutic study. The promastigotes were cultured in Medium 199 (Gibco) with 10% HIFCS.

Reagent preparations

Paromomycin sulfate (Humatin, Parke-Davis & Comp., Ecuador, 250 mg/tablet) was dissolved in distilled water at a concentration of 10 mg/ml, and steriled through 0.22 µm millipore filter.

Mercury chrome solution (50% mercurochrome) was prepared by mixing one volume of distilled ster-

ile saline and one volume of mercury chrome (Mercuriocromo, Elaborado por Labolatorio Weir, Ecuador). Mercury chrome with Glucantime® solution was prepared by mixing one volume of Glucantime® (Specia, France), three volume of sterile saline and four volume of mercury chrome. This solution, thus, contained 12.5% Glucantime® and 50% mercury chrome. Glucantime® contained 300 mg/ml meglumine antimonate, and meglumine antimonate contained 28.3% antimony according to the description.

Procedure of drug susceptibility test in vitro

Promastigotes were harvested from the late-log phase and resuspended in the fresh medium at a concentration of 2×10^5/ml. The organisms (4×10^4) in a volume of 200 ml were added into each well of 96-well flat-bottomed microplates (Falcon). Eight or 10 microlitters of two-fold dilutions of each drug solution were added into duplicate rows of the plate. Two wells were controls without drug. The plates were sealed with a tape and incubated at 24°C for four days until the control culture reaches to the maximum growth. Ten microlitters of 20% glutaraldehyde were added into each well to fix the cells, and the number of parasites in the wells was counted on a hemocytometer. The initial cell density was subtracted from the final cell density and the resultant difference was expressed as a percentage of the control growth. Effective concentration of drugs which inhibited parasite growth by 50% (EC_{50}) was then determined.

Treatment of experimental cutaneous leishmaniasis in mice

Promastigotes of *L. amazonensis* LV78 were harvested from the late-log phase, washed three times with PBS (pH 7.2) and resuspended in PBS at a concentration of 2×10^8/ml. Fifty microlitters of the suspension (1×10^7 cells) were intradermally inoculated at the tail base of 14 male BALB/c mice of six weeks of age. After the infection, the lesion diameter at the inoculation site was measured weekly by a scale. Five mice, having ulcerous lesions of 4.0-5.8 mm in diameter at 28 days of infection, were topically treated with 50% mercury chrome alone twice a day for three weeks. Five of them, having ulcerous lesions of 5.3-6.7 mm in diameter at 42 days of infection, were topically treated twice a day with a lotion containing 12.5% Glucantime® and 50% mercury chrome for 10 days. The remaining four mice were untreated as a control group.

Results

Drug susceptibility test of Leishmania *promastigote* in vitro

EC_{50} values of paromomycin were 25, 46 and 55 mg/ml for *L. mexicana, L. amazonensis* and *L. equatorensis*, respectively, in the present study (Fig. 6.2.1A). The values were, however, variable when a different culture medium or a defined paromomycin from a different source was used (unpublished data).

Mercury chrome showed a strong inhibitory effect on leishmanial promastigote growth. Promastigotes of all of three species tested were completely killed in the presence of 0.15% mercury chrome (Fig. 6.2.1B) although *L. equatorensis* was relatively resistant to the drug than the other two species. No obvious synergistic inhibitory effect on the promastigote proliferation was observed when Glucantime® was mixed to the mercury chrome solution (Fig. 6.2.1C). This may be due to an insufficient concentration of Glucantime® in the solution.

A trial of topical treatment of experimental cutaneous leishmaniasis in mice with mercury chrome in combination with Glucantime®

As an animal model for topical treatment of cutaneous leishmaniasis, BALB/c mice were intradermally inoculated with *L. amazonensis* at the tail base and the ulcerous lesions at the inoculation sites were topically treated with mercury chrome alone or in combination with Glucantime®(Fig. 6.2.2). We applied 50% mercury chrome to the ulcerous lesions of

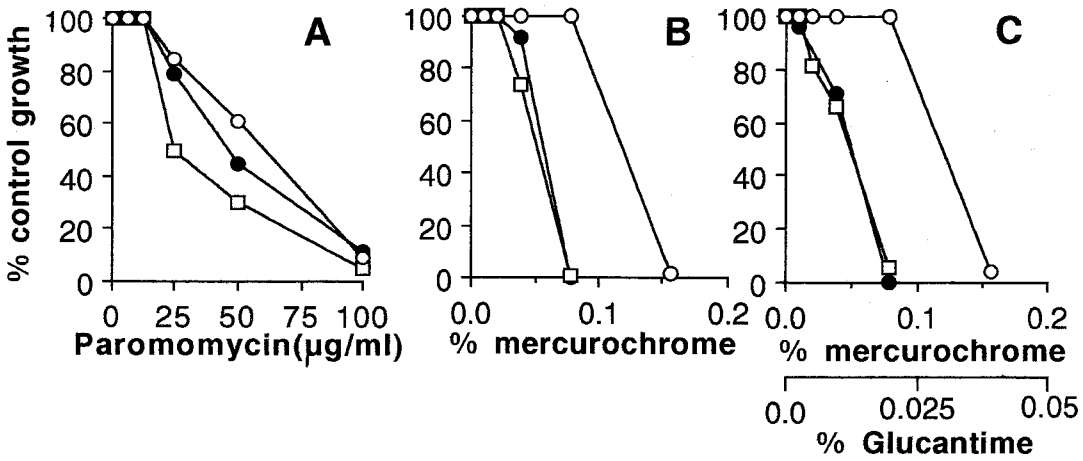

Figure 6.2.1. Susceptibility of *Leishmania* promastigotes to paromomycin, mercury chrome and mercury chrome plus Glucantime® *in vitro*. Promastigotes of *L. mexicana* BEL21 (open square), *L. amazonensis* M2269 (closed circle) and *L. equatorensis* (open circle) were cultured at 24°C for 4 days in the presence of various concentrations of paromomycin (A), mercury chrome (B), or mercury chrome plus Glucantime® (C). Inhibitory effect of each drug solution on promastigote growth was determined as described in Materials and Methods.

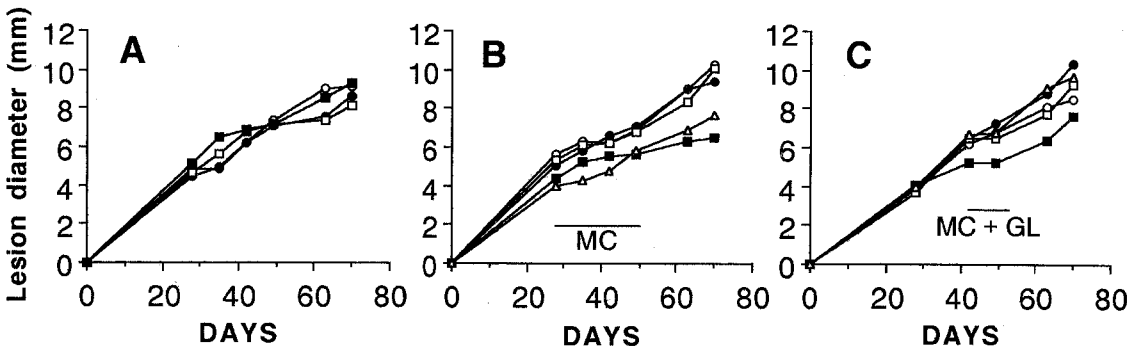

Figure 6.2.2. A trial of topical treatment of experimental cutaneous leishmaniasis in mice with mercury chrome or mercury chrome plus Glucantime®. BALB/c mice were inoculated with 1×10^7 promastigotes of *L. amazonensis* LV 78 at the tail base intradermally. Development of the tail base lesion was monitored; (A) four mice as an untreated control group, (B) five mice in which the lesions were topically treated with 50% mercury chrome (MC) twice a day from 28 days after the infection for three weeks, and (C) five mice topically treated with 50% mercury chrome plus 12.5% Glucantime® (MC + GL) twice a day from 42 days after the infection for 10 days.

4.0-5.7 mm in diameter for five mice twice a day for three weeks. Two of five mice showed a delay of development of the lesion, although effect of the treatment against this group was not statistically significant compared to the untreated control group (Fig. 6.2.2A and 2B). Ulcers were dried within several days after the mercury chrome treatment, but mercury chrome seemed not to be absorbed through the surface of the granuloma. When mice with lesions of 5.7-6.7 mm were treated with a lotion containing 50% mercury chrome plus 12.5% Glucantime® twice a day for 10 days, only one of five mice exhibited a slight delay of lesion progression at no significant level (Fig. 6.2.2A and 2C).

Discussion

Paromomycin is an aminolycosidic antibiotic. An ointment containing paromomycin and methylbenzethonium chloride have recently been revealed to be effective against cutaneous leishmaniasis in patients infected with *L. major, L. tropica* or *L. aethiopica* in the Old World (El-On *et al.*, 1988). In experimental cutaneous leishmaniasis, topical application of the ointment to the skin lesions resulted in total elimination of the parasites and healing of the lesions in mice infected with *L. major* or *L. mexicana*, and in guinea-pigs infected with *L. enriettii* (El-On *et al.*, 1988). However, there have been no such reports so far with respect to clinical application of paromomycin to American cutaneous leishmaniasis.

The present study showed that paromomycin was effective *in vitro* against promastigotes of different *Leishmania* species isolated in the New World. This result suggested a chemotherapeutic effect of paromomycin against American cutaneous leishmaniasis. In fact, our preliminary trial revealed that improvement or cure of the limited leishmanial skin lesions was observed in some patients at an endemic region of Ecuador by local treatment with paromomycin ointment (see Chapter 7.3). This is the first trial of topical treatment of American cutaneous leishmaniasis with pentavalent antimony and mercury chrome lotion.

The present study also showed that mercury chrome was markedly effective against leishmanial promastigotes *in vitro* (Fig. 6.2.1). Mercury chrome has been widely used for prevention of skin wounds from bacterial and fungal infections. The mercury chrome solution is topically applied to the skin since it is highly toxic when administered intravenously or intramuscularly. Mercury chrome is known to bind to SH-residues of a variety of molecules and impair their functions.

Despite of strong antileishmanial activity of mercury chrome *in vitro*, topical application of mercury chrome alone or mercury chrome plus Glucantime® exhibited no substantial effect against experimental cutaneous leishmaniasis in BALB/c mice caused by *L. amazonensis* (Fig. 6.2.2). It may be required more frequent treatments with the drug for improvement of the lesions. Alternatively, this animal model may not be suitable for chemotherapeutic study on limited cutaneous leishmaniasis since *L. amazonensis* LV78 strain is highly virulent and causes severe infection with metastasis in genetically susceptible BALB/c mice (Kink and Chang, 1988). A more suitable animal model must be considered for further parasitological and pathological studies.

Although cure of the lesion was not observed in *L. amazonensis*-infected mice in the present experimental condition used, some mice topically treated with mercury chrome showed a tendance of delay of the lesion progression. This is encouraging us that this treatment is useful for cutaneous leishmaniasis. Indeed, we observed definitive improvement of the eruption and cure of the leishmanial skin lesions in some patients by treatment with a lotion of mercury chrome plus Glucantime® (see Chapter 7.3). It is possible that mercury chrome promoted self-cure of the lesions by elimination of bacterial and fungal contaminations from the ulcers. Moreover, mercury chrome is extremely cheep and easy to obtain in Latin

American countries. Thus, mercury chrome is a potent topical chemotherapeutic agent for treatment of cutaneous leishmaniasis in combination with other antileishmanial drugs. Further clinical investigations are required to ensure this feasibility.

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Chapter 7

Clinical and Pathological Aspects

1. Clinical Survey of Cutaneous Leishmaniasis in an Area, San Sebastián, Manabi, Ecuador

ABSTRACT. In this study, dermatological and parasitological examinations and skin test were performed. The study was conducted in the village of San Sebastián (Ciento Tres), Department of Manabi, Ecuador between January and March, 1991. Each patient was thoroughly examined clinically and parasitologically. The total subjects in this study were 143, 74 males and 69 females. Young persons less than 20 year-old occupied more than half of all the subjects examined. The most frequent duration time of lesions was three months as of the present examination. Approximately 25% of lesions persisted for more than five months. The mean number of cutaneous lesions was 2.7 in both sexes. The subjects with single lesion were most frequent, and 40%, possessed of multiple lesions. There were three cases, one male and two females, with more than 10 cutaneous lesions. The most frequent sites of lesions were extremities. There was no site difference in frequency between upper and lower extremities. Trunk was the least frequent site of lesions. Most popular cutaneous change was ulcer, but non-ulcered cutaneous changes such as papules, plaques and nodules were also seen frequently. The lesions less than 400 mm^2 were frequent. There were 21 cases with lesions more than 900 mm^2, and all of them were ulcerations. Lymphnode swellings were also seen in half of the patients examined, showing more frequent occurrence in males than in females.

Cutaneous leishmaniasis is a disease caused by a protozoan parasite, *Leishmania* spp. It is considered that there is a difference of clinical symptoms between Old and New World types of leishmaniasis. We had a chance to survey on cutaneous leishmaniasis in the west Pacific coast area of Ecuador (Nonaka *et al.*, 1990, Hashiguchi *et al.*, 1991). In this study, dermatological and parasitological surveys including skin test were performed.

Materials and Methods

A total of 143 cutaneous leishmaniasis patients living in the village of San Sebastián (Ciento Tres), Department of Manabi, Ecuador, was recruited for this study (Table 7.1.1). The study area is located on the Pacific coast of Ecuador, and the population was approximately 2,000. The patients were observed between January and March, 1991. Each patient was thoroughly examined clinically and parasitologically. Smears were taken from the edge of ulcers and stained with Giemsa and then examined microscopically using oil emulsion. Cutaneous changes of leishmaniasis patients were thoroughly examined for characteristics, size, site and number of lesions. Skin test was also performed as an immunological examination, by using *Leishmania* promastigote antigens prepared by Reed's method (Furuya *et al.*, 1991).

The severity of lymphnode-swelling was graded as

0, 1 and 2, that is, 0, not palpable, 1, less than pea-sized lymphnodes and 2, more than small finger tip-sized lymphnodes.

Results

The results obtained are summarized as shown in Tables 7.1.1 to 7.1.8. The total patients in this survey were 143, 74 males and 69 females (Table 7.1.1). The mean age of patients was 18.6 year-old (±1.63 s.d.) in males, 18.4 year-old (±1.81 s.d.) in females, and 18.5 year-old (±1.21 s.d.) in total. Young aged patients less than 20 year-old occupied more than half of all the subjects examined. The mean duration period of cutaneous lesion was 3.9 months (±0.27 s.d.) in males, 4.4 months (±0.30 s.d.) in females and 4.2 (±0.27 s.d.) months in total. The most frequent duration time was three months as of the present examination, and approximately 25% of lesions persisted for more than five months. The mean number of cutaneous lesions was 2.7 in both sexes. The subjects with single lesion were most frequent, followed by those with multiple lesions (more than 40%). Three cases, one male and two females, were possessed of more than 10 lesions. The most frequent sites of lesions were extremities, showing no difference between the upper and lower sites. Trunk was the least frequent site of lesions.

Most popular cutaneous change was ulcer. Typical ulcers with demarcated margin were diagnostically valuable (Figs. 7.1.1 and 2). Non-ulcered cutaneous changes such as papules, plaques and nodules were also seen frequently (Figs. 7.1.3 and 4). Sometimes erythematous plaques or papules located around the margin of the scar were recognized (Fig. 7.1.5). Some of them had a small ulcer with crust on the center (Figs. 7.1.6 and 7). The lesions less than 400 mm^2 were frequent. There were 21 cases with lesions more than 900 mm^2, and all of them were ulcerations. Lymphnode swellings were also seen in half of patients, showing more frequent presence in males than in females.

Discussion

In this survey, mean age of patients was almost similar to our previous study (Nonaka *et al.*, 1990). Between the two studies, in the present case the duration of lesions was shorter than the previous one; the number of cutaneous lesions, less; the frequency of ulcer, lower; and the size of cutaneous lesions, smaller. Such a finding shows that the present cutaneous leishmaniasis lesions may be rather mild than those found in the previous study site (Zhucay, Cañar). In the current study, only one place was selected for the examination of inhabitants, while several sites were surveyed in the previous one. These different situations might cause the discrepancy of clinical symptoms between the two observations.

There have been many reports on the severity of cutaneous lesions of leishmaniasis in the world. Cuba Cuba *et al.* (1985) reported 16 cases of cutaneous leishmaniasis in Brazil which included eight single lesions and eight multiple lesions. Christensen *et al.* (1983) reported on cutaneous leishmaniasis in Panama. Llanos-Cuentas *et al.* (1984) compared the skin lesions between patients with and without mucocutaneous manifestations with respect to the number, site and size. Their data showed that the frequency of single lesions was 68.1%, the lesions were distributed more frequently below the belt than above the belt, and the lesions bigger than 900 mm^2 were 60 out of 145 cases. On the contrary, in our case the frequency of single lesions was lower; the distribution of lesions, different; and the size of lesions, smaller than their case. Weigle *et al.* (1986) reported the results of epidemiological study in Colombia. Their data showed a lower infection rate of children, similar occurrence of single lesion, higher incidence of lesions on the trunk, and higher incidence of ulcers (95.6%), compared with the present ones. These dif-

Table 7.1.1. Summary of patients with cutaneous leishmaniasis in San Sebastián (Ciento Tres), Manabi, Ecuador

Age	Male	Female	Total
0 - 9	23 (31.1%)	21 (30.5%)	44 (30.8%)
10 - 19	22 (29.7%)	25 (36.3%)	47 (32.8%)
20 - 29	14 (18.9%)	11 (15.9%)	25 (17.5%)
30 - 39	8 (10.8%)	3 (4.3%)	11 (7.7%)
40 - 49	4 (5.4%)	4 (5.8%)	8 (5.6%)
50 - 59	0 (0.0%)	3 (4.3%)	3 (2.1%)
60 -	3 (4.1%)	2 (2.9%)	5 (3.5%)
Total	74 (100.0%)	69 (100.0%)	143 (100.0%)
Mean	18.6	18.4	18.5
± s.d.	1.63	1.81	1.21

Table 7.1.2. The duration time of cutaneous leishmaniasis lesions

Duration of lesions (months)	Male	Female	Total
1	5 (6.8%)	6 (8.7%)	11 (7.7%)
2	13 (17.5%)	9 (13.0%)	22 (15.4%)
3	20 (26.9%)	14 (20.4%)	34 (23.7%)
4	13 (17.5%)	11 (15.9%)	24 (16.8%)
5	9 (12.2%)	8 (11.6%)	17 (11.9%)
6	6 (8.1%)	5 (7.2%)	11 (7.7%)
7	2 (2.7%)	6 (8.7%)	8 (5.6%)
8	1 (1.4%)	6 (8.7%)	7 (4.9%)
9	1 (1.4%)	0 (0.0%)	1 (0.7%)
10<	3 (4.1%)	2 (2.9%)	5 (3.5%)
Unknown	1 (1.4%)	2 (2.9%)	3 (2.1%)
Total	74 (100.0%)	69 (100.0%)	143 (100.0%)
Mean	3.9	4.4	4.2
±s.d.	0.27	0.30	0.20

ferences might be induced by differences of *Leishmania* species, vectors and environmental states between the two countries, Colombia and Ecuador.

In Old World, Nadim *et al.* (1968) reported 179 active cases of leishmaniasis in Iran. The age of their cases was less than 11 year-old. Bienzle *et al.* (1978)

Table 7.1.3. The numbers of cutaneous leishmaniasis lesions

No. of lesions	Male	Female	Total
1	37 (49.9%)	26 (37.8%)	63 (44.0%)
2	11 (14.8%)	17 (24.6%)	28 (19.6%)
3	12 (16.2%)	9 (13.0%)	21 (14.7%)
4	3 (4.1%)	6 (8.7%)	9 (6.3%)
5	2 (2.7%)	6 (8.7%)	8 (5.6%)
6	1 (1.4%)	0 (0.0%)	1 (0.7%)
7	3 (4.1%)	1 (1.4%)	4 (2.8%)
8	2 (2.7%)	2 (2.9%)	4 (2.8%)
9	2 (2.7%)	0 (0.0%)	2 (1.4%)
10<	1 (1.4%)	2 (2.9%)	3 (2.1%)
Total	74 (100.0%)	69 (100.0%)	143 (100.0%)
Mean	2.7	2.7	2.7
±s.d.	0.34	0.29	0.23

Table 7.1.4. The site of cutaneous leishmaniasis lesions

Site of lesions	Male	Female	Total
Face	40 (20.2%)	30 (15.9%)	70 (18.1%)
Trunk	21 (10.6%)	9 (4.8%)	30 (7.8%)
Upper extremities	71 (35.9%)	77 (40.7%)	148 (38.2%)
Lower extremities	66 (33.3%)	73 (38.6%)	139 (35.9%)
Total	198 (100.0%)	189 (100.0%)	387 (100.0%)

reported on epidemiological and clinical situations of the disease in Saudi Arabia. A total of 47 out of their 87 subjects showed a sign of past or present infection with *Leishmania*. Children cases under 10 year-old were less in their study than those in ours. There were 260 skin lesions in the 47 patients demonstrating a mean number of 5.5 skin lesions. The mean number of lesions in their study (Bienzle *et al.*, 1978) was higher than that in ours. It is interesting that there is a difference of distribution of skin lesions

Table 7.1.5. The clinical forms of cutaneous leishmaniasis lesions

Forms of lesions	Male	Female	Total
Ulcer	68 (34.4%)	74 (39.2%)	142 (36.7%)
Papule	37 (18.7%)	52 (27.5%)	89 (23.0%)
Plaque	48 (24.2%)	47 (24.9%)	95 (24.5%)
Scar	13 (6.6%)	11 (5.8%)	24 (6.2%)
Induration	1 (0.5%)	0 (0.0%)	1 (0.3%)
Nodule	23 (11.6%)	5 (2.6%)	28 (7.2%)
Unclassified	8 (4.0%)	0 (0.0%)	8 (2.1%)
Total	198 (100.0%)	189 (100.0%)	387 (100.0%)

Table 7.1.6. The size of cutaneous leishmaniasis lesions

Size of lesions (mm^2)	Male	Female	Total
>100	80 (40.4%)	71 (37.6%)	151 (39.0%)
100 - 399	83 (41.9%)	71 (37.6%)	154 (39.9%)
400 - 899	16 (8.1%)	34 (17.9%)	50 (12.9%)
900<	10 (5.1%)	11 (5.8%)	21 (5.4%)
Uncalculated	9 (4.5%)	2 (1.1%)	11 (2.8%)
Total	198 (100.0%)	189 (100.0%)	387 (100.0%)

between the both studies. Only 1.9% of skin lesions were existed on the face in their cases (Bienzle *et al.*, 1978). Our cases, however, showed that 18.1% of cutaneous lesions were seen on the face. The difference of distribution of skin lesions may be related to a difference of blood-sucking behaviours of vector sandflies.

The differences mentioned above may be caused by a difference of the *Leishmania* species, vectors, and environmental factors in given endemic areas. The *Leishmania* species in the present area was identified as *L. panamensis* or *L. guyanensis*. The climatic situation in the area showed high temperature and humidity. The majority of the present subjects examined were farmers. The most difficult problem for inhabitants in the area was a lack of protection methods against sandfly biting. Such conditions should be taken in mind in future planning of control measures, not only from technical maneuver but also from economical situations in the area. Thus, from

Table 7.1.7. The lymphnode swellings of 78 leishmaniasis patients palpated

Lymphnode swelling (grade*)	Male	Female	Total
0	13 (37.2%)	25 (58.2%)	38 (48.8%)
1	11 (31.4%)	9 (20.9%)	20 (25.6%)
2	11 (31.4%)	9 (20.9%)	20 (25.6%)
Total	35 (100.0%)	43 (100.0%)	78 (100.0%)

* 0, not palpable; 1, less than pea-sized lymphnodes; 2, more than small finger tip-sized lymphnodes.

Table 7.1.8. Summary of the lymphnode swelling of leishmaniasis patients with the lesion sizes more than 400 mm^2

Lymphnode swelling (grade*)	Male	Female	Total
0	5 (41.7%)	15 (60.0%)	20 (54.1%)
1	1 (8.3%)	3 (12.0%)	4 (10.8%)
2	6 (50.0%)	7 (28.0%)	13 (35.1%)
Total	12 (100.0%)	25 (100.0%)	37 (100.0%)

* see Table 7.1.7.

the result obtained in this survey it seemed to be very important to improve the economical basis of the inhabitants, in order to erradicate the disease in the area, besides, medical care and entomological maneuvers.

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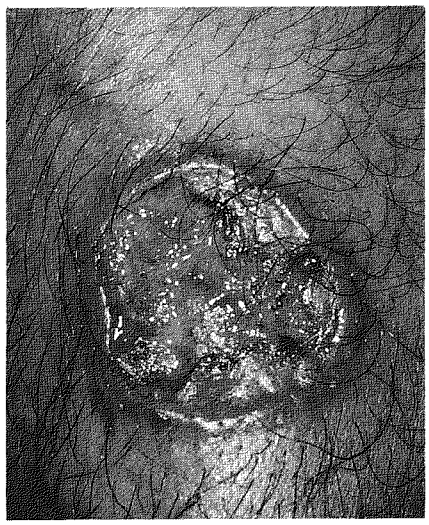


Figure 7.1.1 A 14 year-old boy had a typical ulcer with sharp demarcated bank on the left lower leg. The size of ulcer was 30×30 mm.

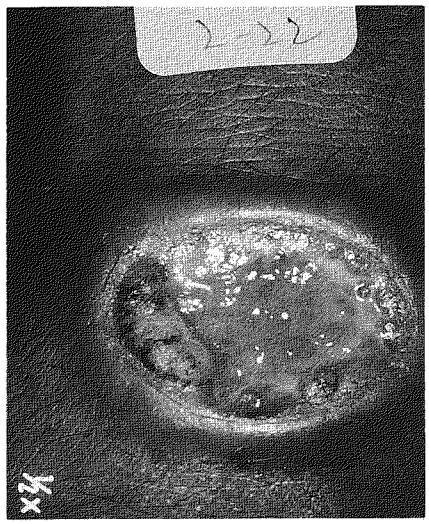


Figure 7.1.2. A 19 year-old man had a typical ulcer with more sharply demarcated bank on the right hand. The size of ulcer was 40×30 mm.

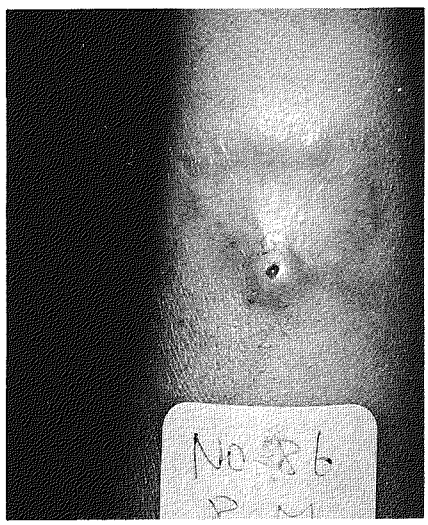


Figure 7.1.3. A 8 year-old boy had an ulcer on the left forearm for 7 months. The lesion was healed with a scar, but two nodules have still remained on the margin of scar.

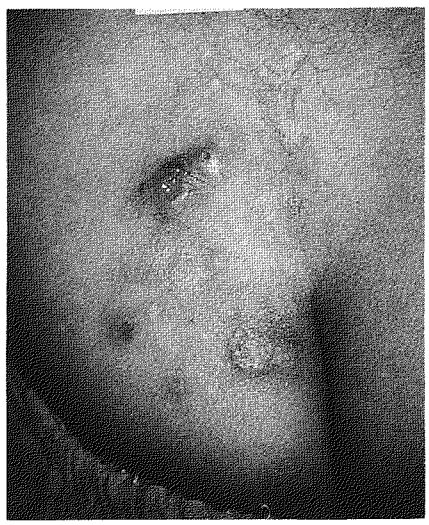
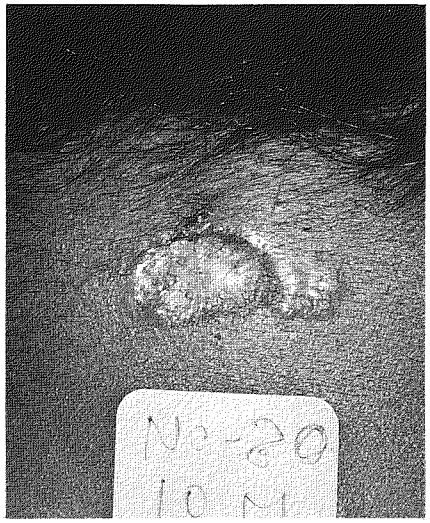


Figure 7.1.4. A 3 year-old boy had six papules and nodule on the buttock. Some of them were accompanied with a slight ulcer and crusts.



Figur 7.1.5. A 10 year-old boy had an erythematous plaque around the margin of scar on the forehead.

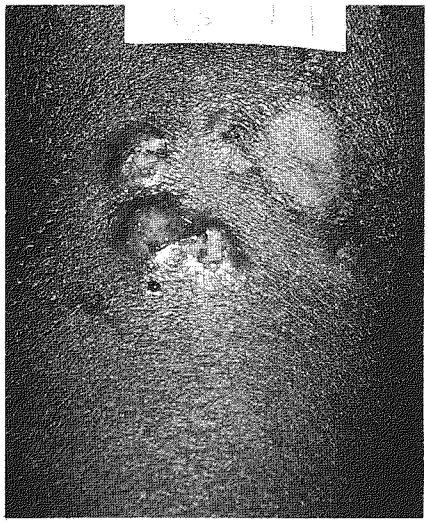


Figure 7.1.6. A 13 year-old boy had papules sorrounding the scar like a satellite. These papules were accompanied with an ulcer on the top.

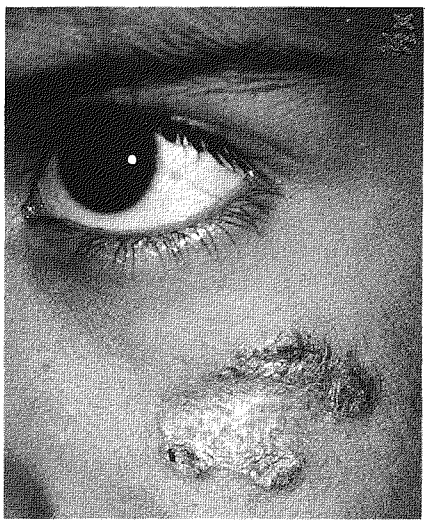


Figure 7.1.7. A 11 year-old boy had papules around the scar on the left cheek. These papules were accompanied with shallow ulcer.

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2. Electron Microscopic Studies of Cutaneous Leishmaniasis in Ecuador

ABSTRACT. Ultrastructural observations on the cutaneous lesions of three patients with cutaneous leishmaniasis in Ecuador were done. Parasites located intracellularly or extracellularly were either inside the vacuoles or free from the vacuoles. The mean diameter of the parasites was 2.62 μm (±0.17 s.d) and 2.18 μm (±0.28 s.d) length and width, respectively. Parasites showed degeneration in the vacuoles presented either inside the host macrophage or outside the cell. Lymphocytes were in close contact with parasitized macrophage as well as directly attached with the parasites. Furthermore, amastigotes were confirmed in the epidermis where lymphocytes and other mononuclear cells were present near the amastigotes. Mononuclear cells contained melanin granules showed amastigotes in their cytoplasm. Amastigotes were also observed in and between the keratinocytes, and were attached with lymphocytes. The parasite-macrophage relationship, the role of T-cells against the parasite in this disease and the fate of parasite in the host are discussed.

Cutaneous leishmaniases are widely distributed in many parts of both Old and New World. It is, therefore, a considerable dermatological problem, especially in the two continents, Africa and South America. The disease is largely classified into two forms, Old World forms mainly caused by *Leishmania tropica* complex and New World ones caused by *L. braziliensis* and *L. mexicana* complexes (Lainson and Shaw, 1987; Pearson *et al.*, 1985). In order to obtain information on the clinical, epidemiological and immunological features of the latter forms of the disease, we have performed detailed investigations at different endemic areas of Ecuador. The results obtained have already been reported by Nonaka *et al.* (1990 a, b) and Hashiguchi *et al.* (1990, 1991).

In Ecuador cutaneous leishmaniasis is prevalent all over the country and classified as regionally. To date, six species of the genus *Leishmania, i.e., L. panamenesis, L. guyanensis, L. braziliensis, L. amazonensis, L. mexicana* and *L. major*-like from humans and mammals have been identified in the country based on zymodeme, serodeme and schizodeme analysis (Mimori *et al.*, 1989; Hashiguchi *et al.*, 1990; 1991; Armijos *et al.*, 1990). Morphology and ultrastructure also have, at some extent, an importance in the differentiation of *Leishmania* species, but such an information is not fully available.

For the better understanding of the morphology and/or nature of Ecuadorian parasite (New World *Leishmania*) and the relationship between host (cell) and parasite, we have performed the ultrastructural study of the dermal lesions of patients with leishmaniasis.

Materials and Methods

Patients

The Ecuadorian patients with cutaneous leishmaniasis, being different age and sex, were diagnosed on the basis of clinical features and history. Detailed clinical and histopathological aspects of three patients, I-02 (*L. guyanensis* infection, unpublished data), I-04 (*L. panamensis* infection, unpublished data) and I-25 (*Leishmania* sp., not thoroughly characterized yet), were described in previous paper (Nonaka *et al.*, 1990 a, b). None of these patients had received any kind of treatment before the biopsies were taken.

Processing of biopsy materials

Four-millimeter-punch biopsies were taken from

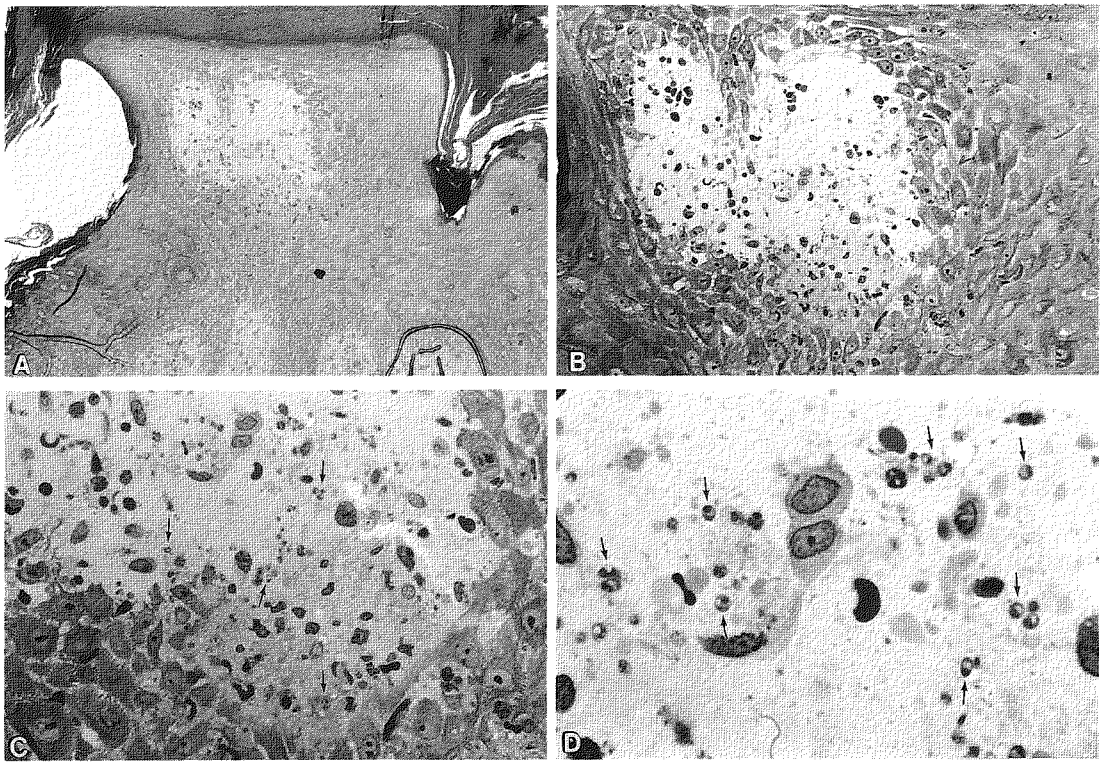

Figure 7.2.1. Light micrographs of epon-embedded semi-thin sections of cutaneous leishmaniasis, stained with toluidine blue, showing spongiotic vesicle in the epidermis. Arrows indicate the amastigotes in the vesicle. A, ×40; B, ×100; C, ×200; D, ×400.

the edge of ulcers or nodules under local anaesthesia and fixed in different fixatives. The specimens were divided into two parts. One part of biopsy material was fixed in 10% formalin and then embedded with paraffin. Finally the five micra sections were cut and stained with haematoxylin and eosin. Another part of the biopsy was cut into small pieces and fixed in cold 2% paraformaldehyde, 2% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.4. The tissues were then washed with 0.1M cacodylate buffer, pH 7.4, and post-fixed in 2% osmium tetraoxide for two hours. After the dehydration in different concentrations of alcohol the specimens were embedded in Epon 812. One micron semi-thin sections were cut with glass knife on an LKB ultrotome and stained with toluidine blue. Ultra-thin sections were cut with diamond knife and stained with lead citrate and uranyl acetate, and then examined under JEM, 1200 EX; JEOl Japan, electron microscope.

Results

Light microscopic findings

In epidermis, along with other minor alterations, an intraepidermal spongiotic vesicle was observed in one of the three patients' specimens (Fig. 7.2.1A, B). Amastigotes and mononuclear cells were observed inside the epidermal vesicle and between the keratinocytes (Fig. 7.2.1C, D). The dermis showed a great number of amastigotes with cellular infiltration.

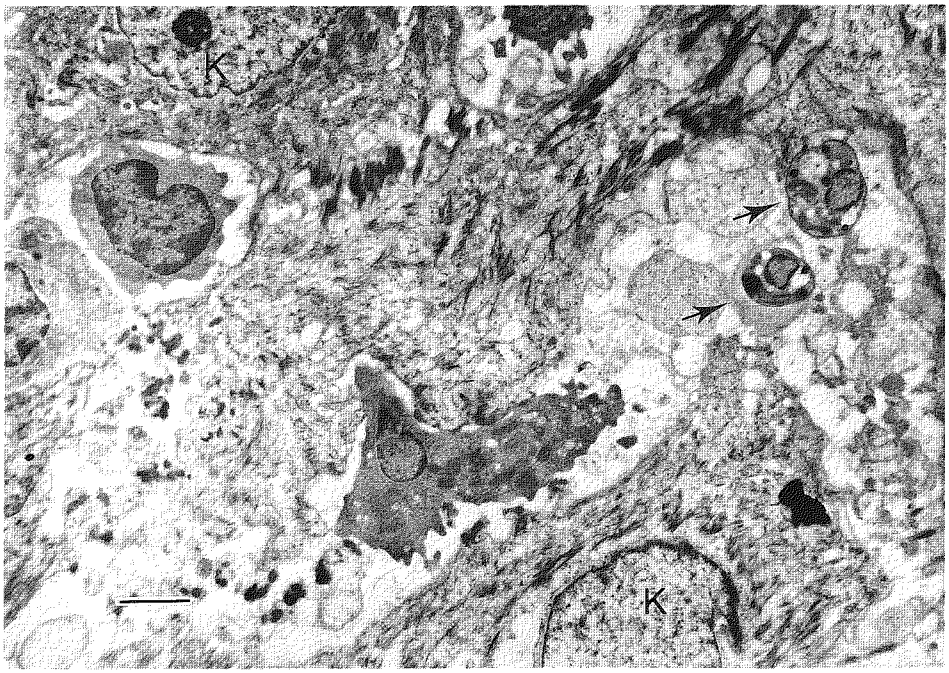

Figure 7.2.2. Electron micrograph showing the amastigotes (arrows) in the epidermis. Mononuclear cells are also visible. bar = 2 μm.

Electron microscopic findings Epidermis

Leishmania parasites inside the epidermal vesicle were confirmed in ultra-thin sections, where lymphocytes and other mononuclear cells were present near the parasites (Fig. 7.2.2). In the epidermis, a mononuclear cell contained melanin granules showed *Leishmania* parasite in its cytoplasm (Fig. 7.2.3A, B). Parasites were also observed in and between the keratinocytes, and were attached with lymphocytes (Fig. 7.2.4A, B) or lying free in the micro-vesicle abscesses (Fig. 7.2.5A, B).

Dermis

Parasites were found intracellularly and extracellularly. Amastigotes in the cytoplasm of the macrophage were located either inside the vacuole (Fig. 7.2.6) or free from vacuole (Fig. 7.2.7). Extracellular amastigotes were also seen inside the vacuoles or outside the vacuoles. Many host macrophages were seen activated against the parasites by showing the dilatation and enlargement of the cytoplasmic reticulum surrounding the cell (Fig. 7.2.8A, B). Electron dense granules were observed frequently in the parasitophorous vacuoles inside or outside the cell. Specific lymphocytes were seen active in the specimens, in which some of them were in a close contact with parasitized-macrophage (Fig. 7.2.9) and some were directly attached with the parasites (Fig. 7.2.10A, B). Intracellular degeneration of parasites was observed in the vacuole of host macrophage where host cell was also under parasitic attack and was near to destroy (Fig. 7.2.11A, B). Parasitic degeneration was also noted in the extracellular vacuoles. Parasites were located in the cytoplasm of the eosinophil (Fig. 7.2.12) and neutrophil (Fig. 7.2.13) without noticing any intracellular vacuole.

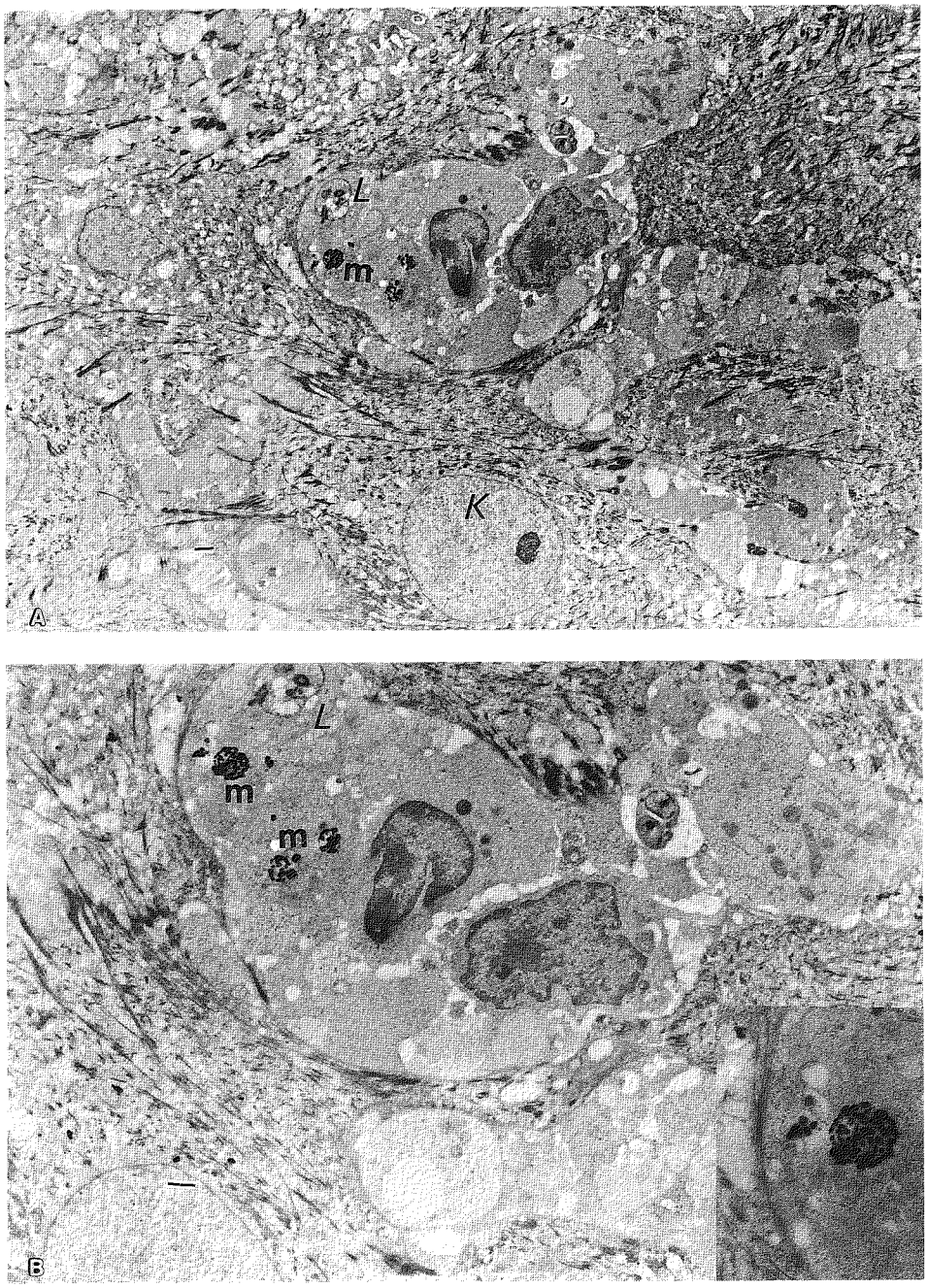

Figure 7.2.3. In the epidermis, a mononuclear cell with melanin granules (m) containing one amastigote (L) in its cytoplasm. A, low magnification; B, high magnification; bars = 1 μm. Inset indicates the melanin granules.

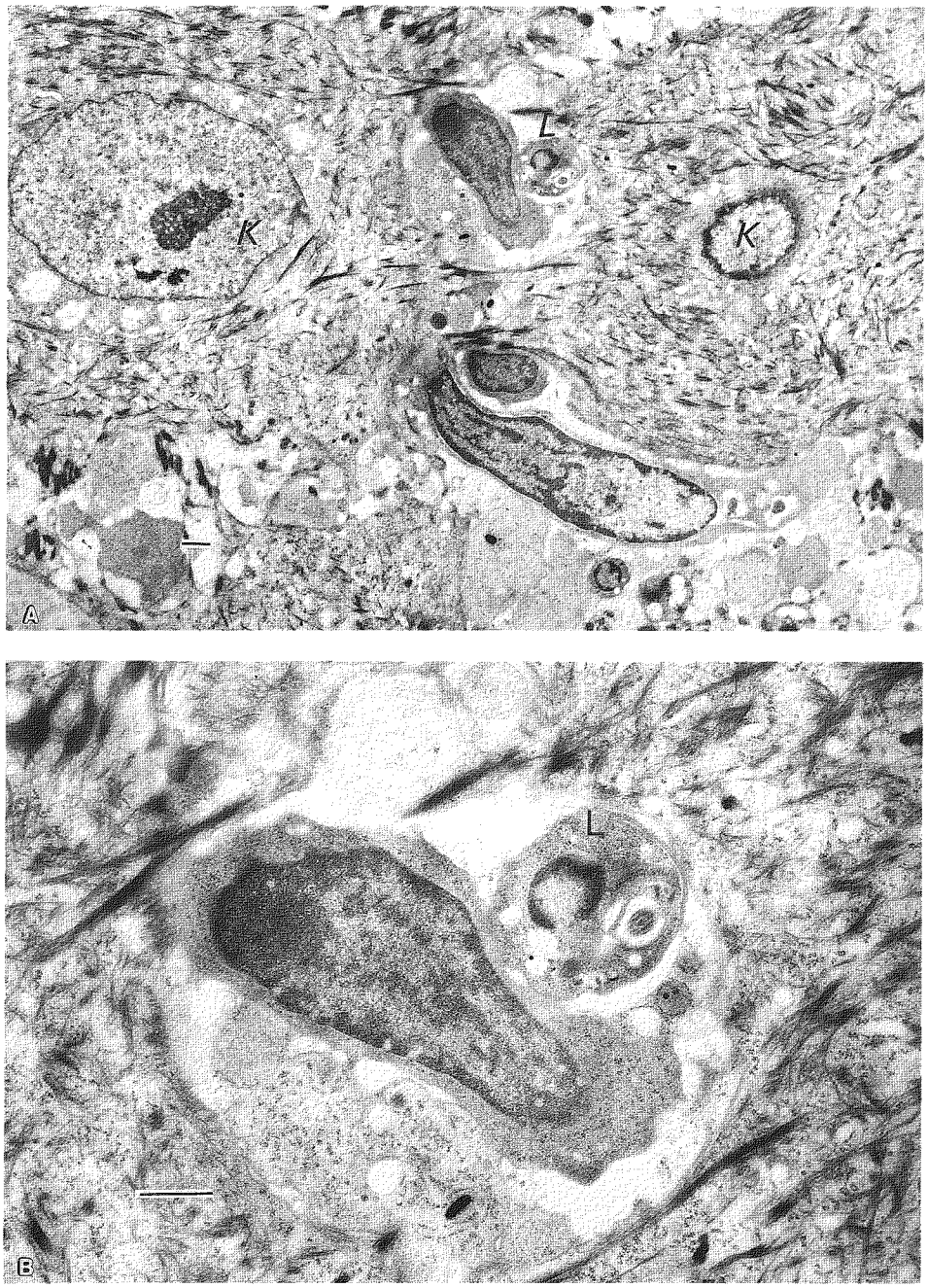

Figure 7.2.4. A lymphocyte (ly) is in close contact with *Leishmania* parasite (L) between the keratinocytes (K). A low magnification; B, high magnification; bar = 1 μm.

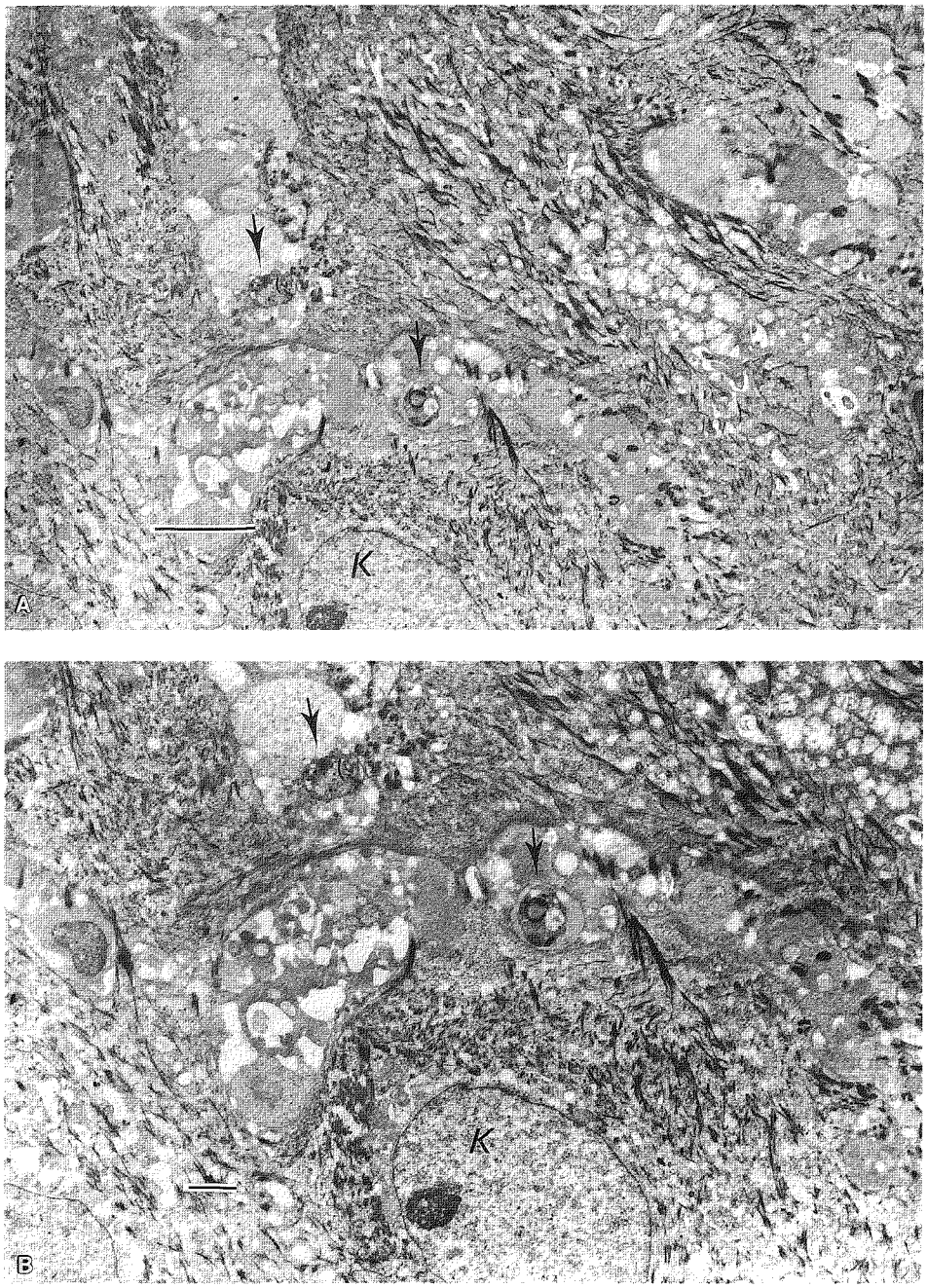

Figure 7.2.5. Two *Leishmania* parasites (arrows) lying free in the intra-epidermal abscess. A, low magnification bar = 5 μm; B, high magnification, bar = 2 μm.

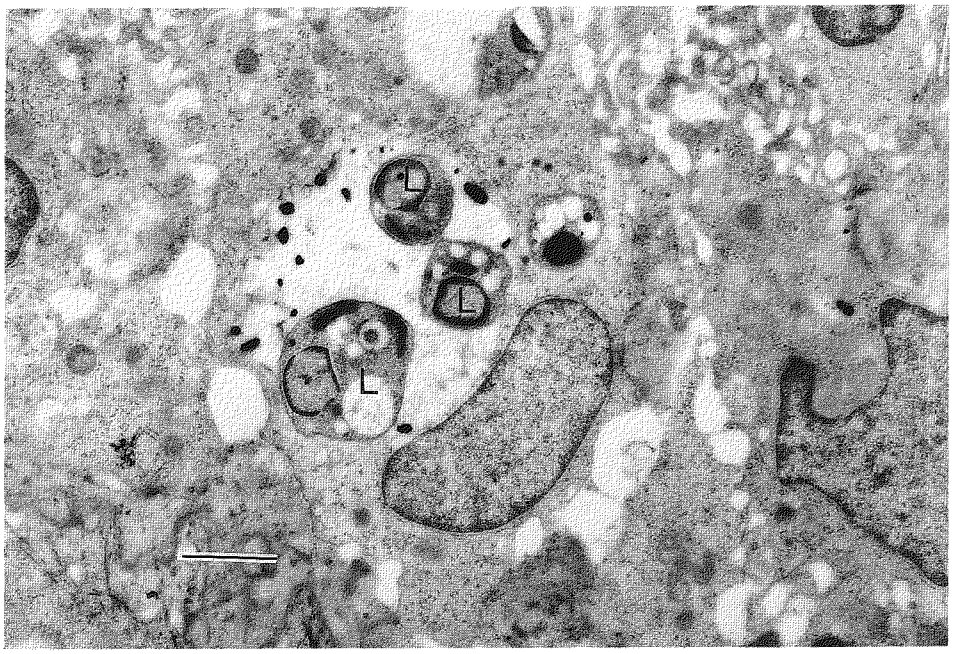

Figure 7.2.6. A parasitized macrophage showing *Leishmania* amastigotes (L) in the intra-cytoplasmic vacuole. bar = 2 μm.

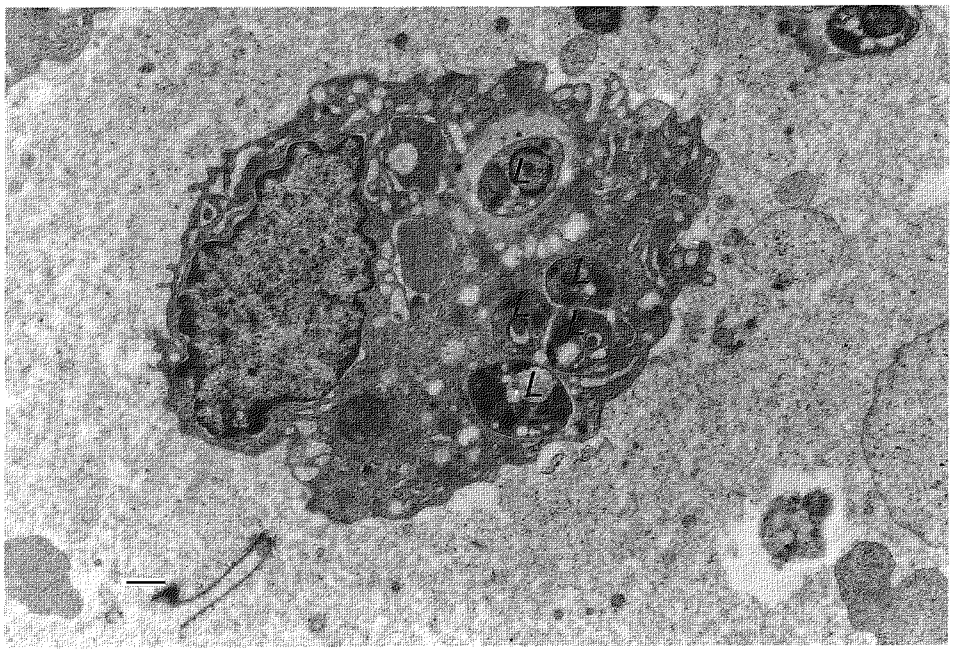

Figure 7.2.7. A parasitized activated macrophage showing many *Leishmania* parasites (L) inside the cytoplasm. No vacuole is visible in this host cell. bar = 1 μm.

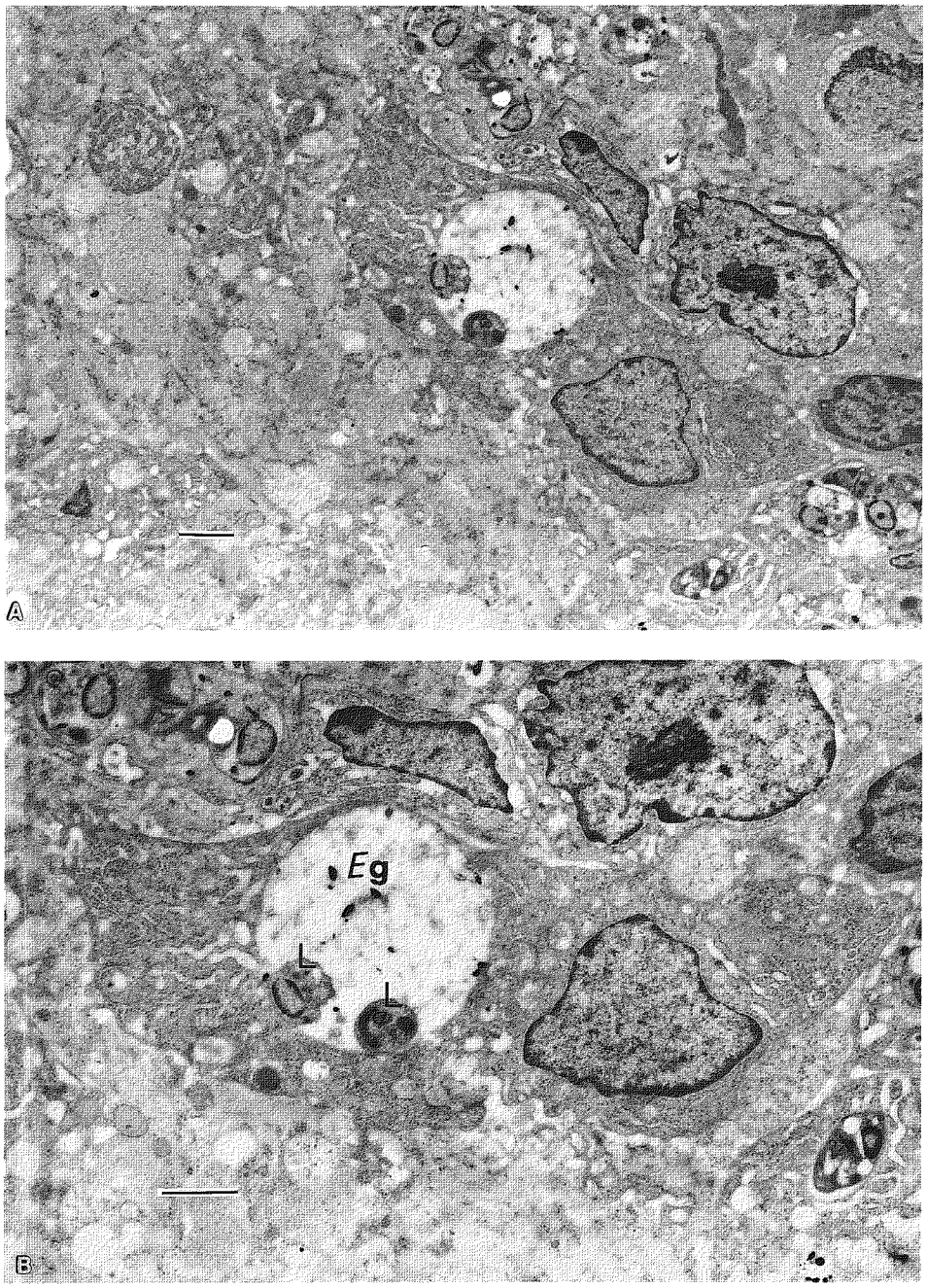

Figure 7.2.8. A, an activated host macrophage with parasitophorous vacuole showing the dilatation and enlargement of endoplasmic reticulum. B, in high magnification, electron dense granules (Eg) are visible inside the vacuole. Amastigote looks still intact. bars = 2 μm.

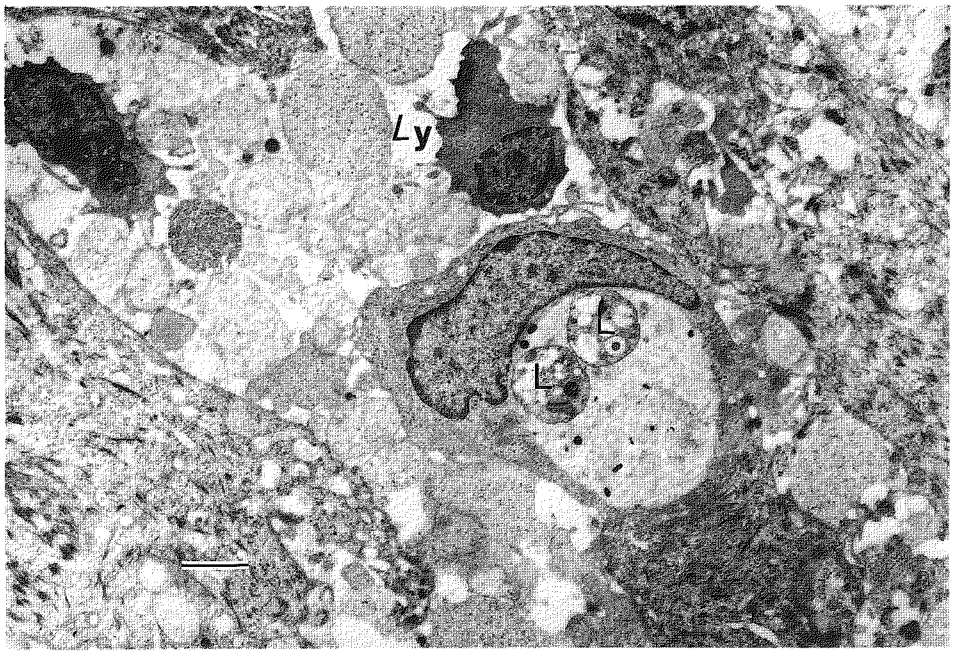

Figure 7.2.9. A lymphocyte (Ly) is in close contact with host macrophage showing two intact parasites (L) inside the cytoplasmic vacuole. bar = 2 μm.

Ultrastructure of amastigote

The shape of the amastigotes observed was rounded or oval, with little differences in the size. The mean diameter was 2.76 μm (±0.17 s.d) in length and 2.18 μm (±0.28 s.d) in width. The amastigotes were surrounded by two layer membranes. They contained one rounded nucleus with small nucleolus and cytoplasm with certain structures mainly flagellum, flagellar pocket, kinetoplast, vacuoles and electron dense granules (Figs. 7.2.14, 15 and 16).

Discussion

Various changes in the epidermis have been reported in the lesions in New World and Old World leishmaniasis (Zaar *et al.*, 1982; Grimaldi *et al.*, 1980). We observed the spongiotic vesicle in the mid epidermis where leishmania bodies (amastigotes), lymphocytes and other mononuclear cells were recognized. The lymphocytes, antigen presenting cells, were seen in close contact with *Leishmania* parasite which shows the capacity of lymphocytes to recognize the parasite and make it able for further process of destruction. In the epidermis, we also found melanin granules in host cell (macrophage) having one parasite in its cytoplasm. The nature of this melanin-contained macrophage is unknown. Leukocytes carrying melanin granules have been reported in blood of normal humans, amphibians and reptilians (Wassermann *et al.*, 1965). Melanin-containing macrophages are prominent within the epithelium of certain fish groups. In histological findings, Kurban *et al.* (1966) have reported the presence of

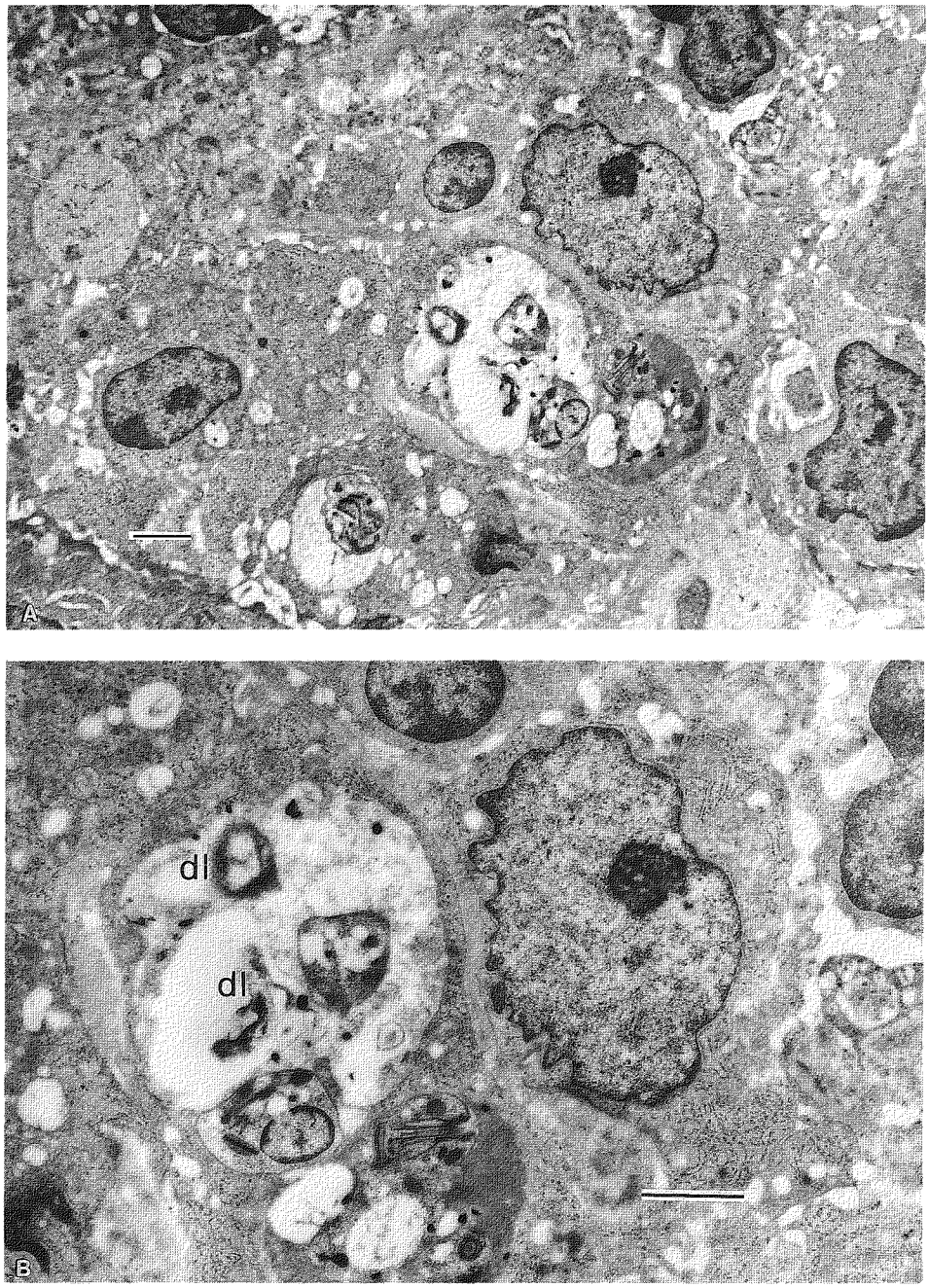

Figure 7.2.10. Degeneration of *Leishmania* parasites (dl) are visible inside the intracellular vacuole. Same time host cell looks under parasitic attack by showing the destruction of nucleus of host cell. A, low magnification; B, high magnification. bars = 2 μm.

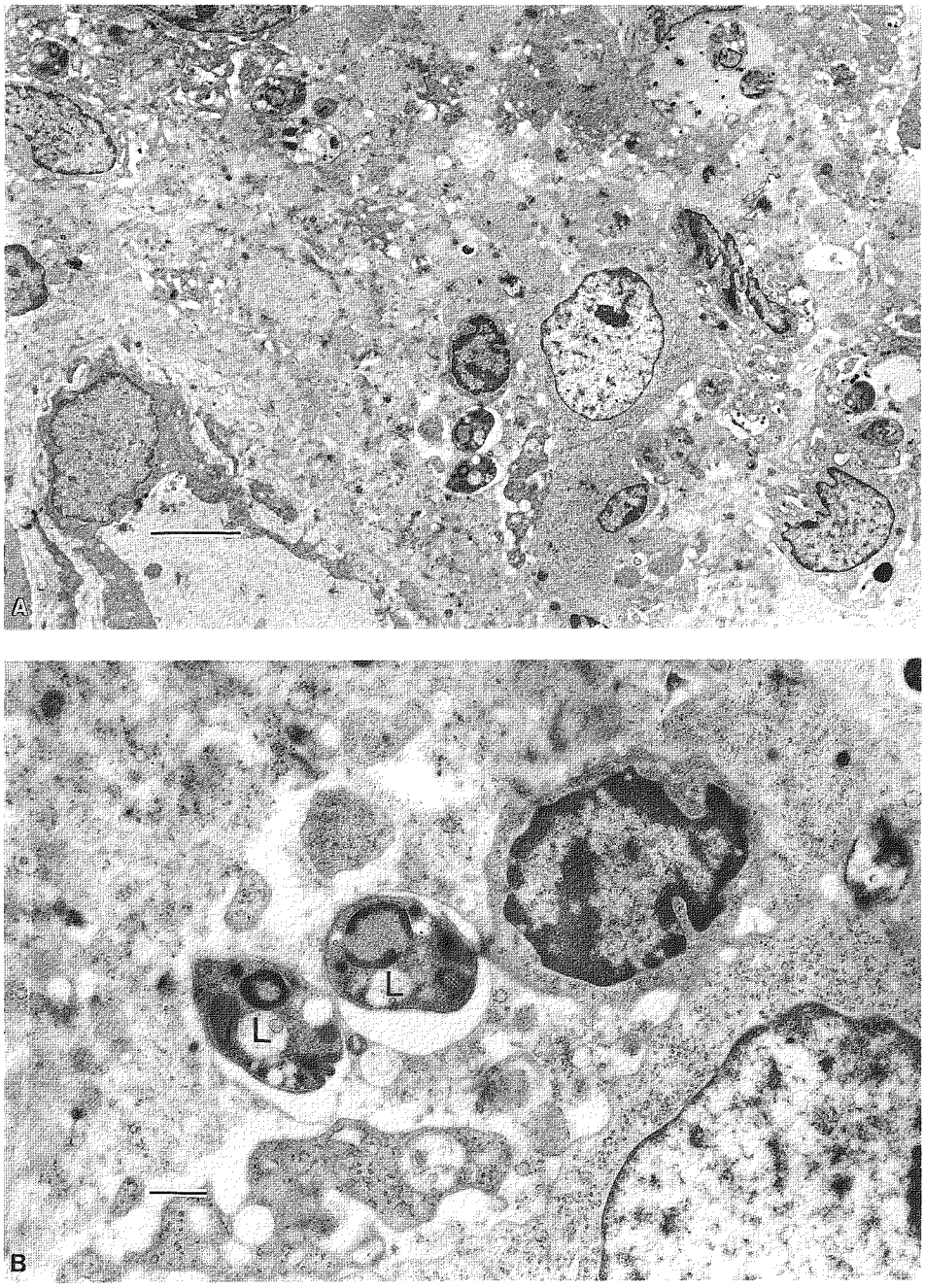

Figure 7.2.11. A lymphocyte showing direct attachment with *Leishmania* (L). A, low magnification, bar = 5 μm; B high magnification, bar = 1 μm.

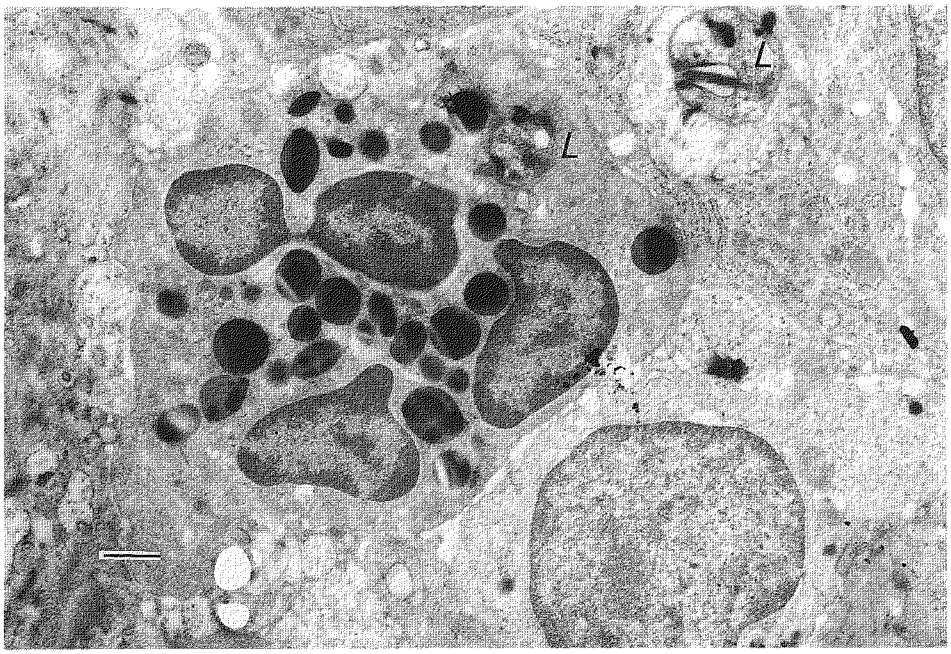

Figure 7.2.12. *Leishmania* parasites (L) showing inside and outside the eosinophil. bar = 1 μm.

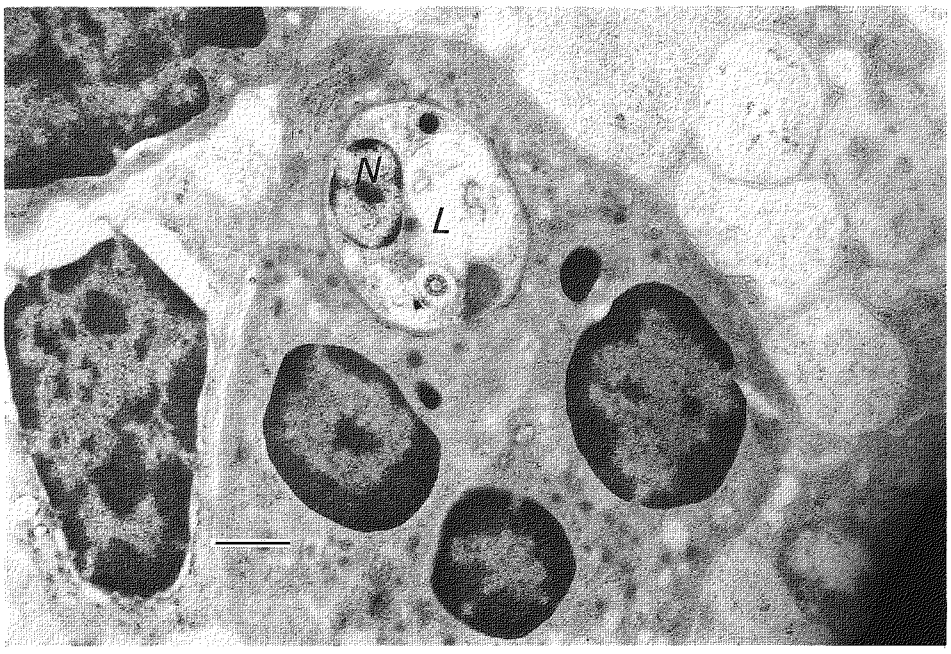

Figure 7.2.13. *Leishmania* parasite (L) is present inside the cytoplasm of neutrophil. bar = 1 μm.

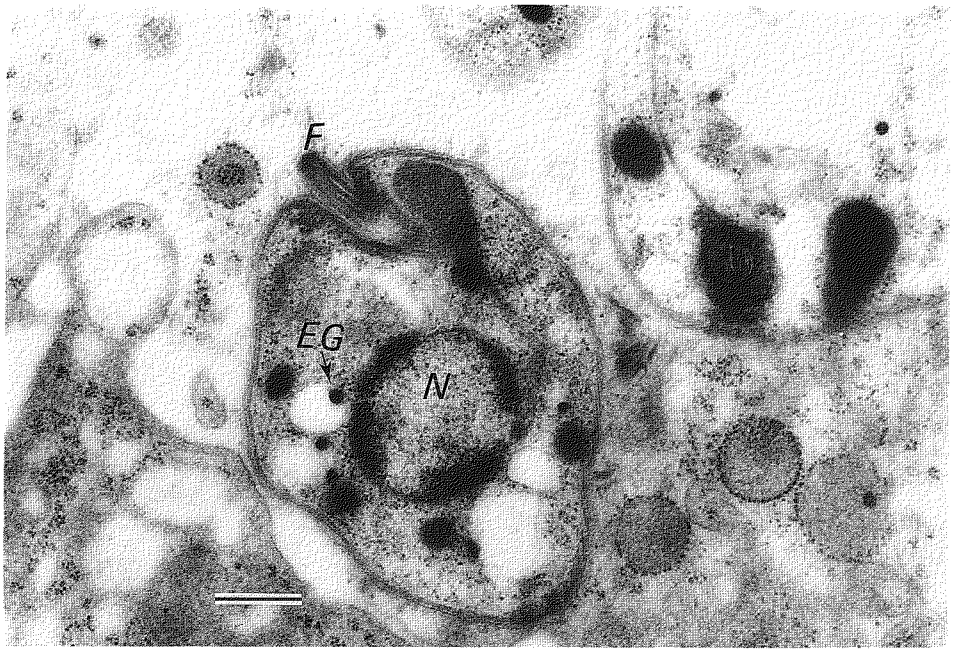

Figure 7.2.14. Ultrastructure of *Leishmania* amastigotes. N, nucleus; Eg, electron dense granule inside the vacuole.

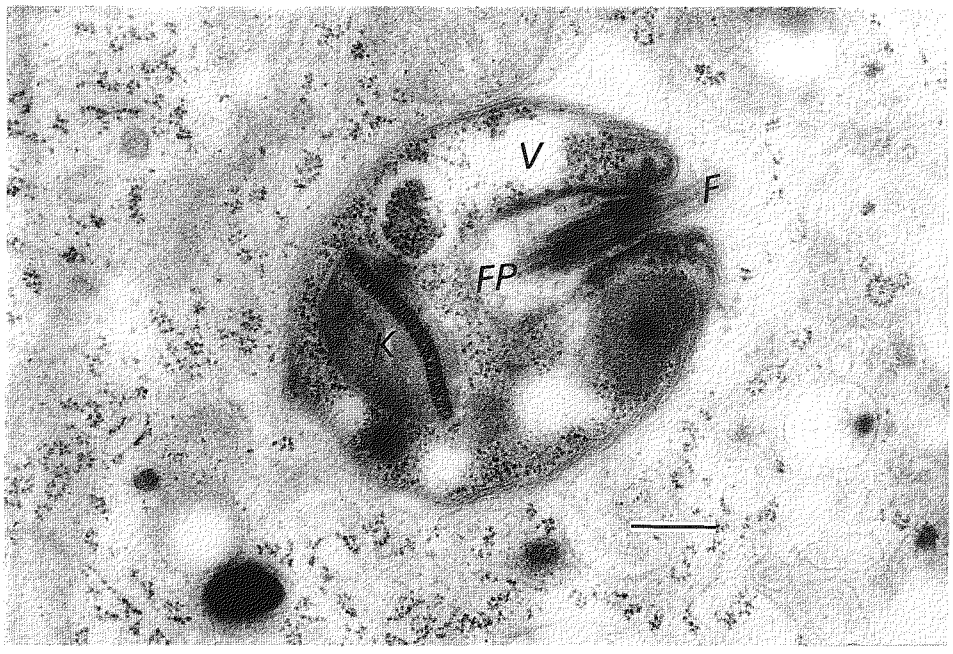

Figure 7.2.15. Ultrastructure of *Leishmania* amastigotes. F, flagellum; FP, flagellar pocket; V, vacuole; K, kinetoplast.

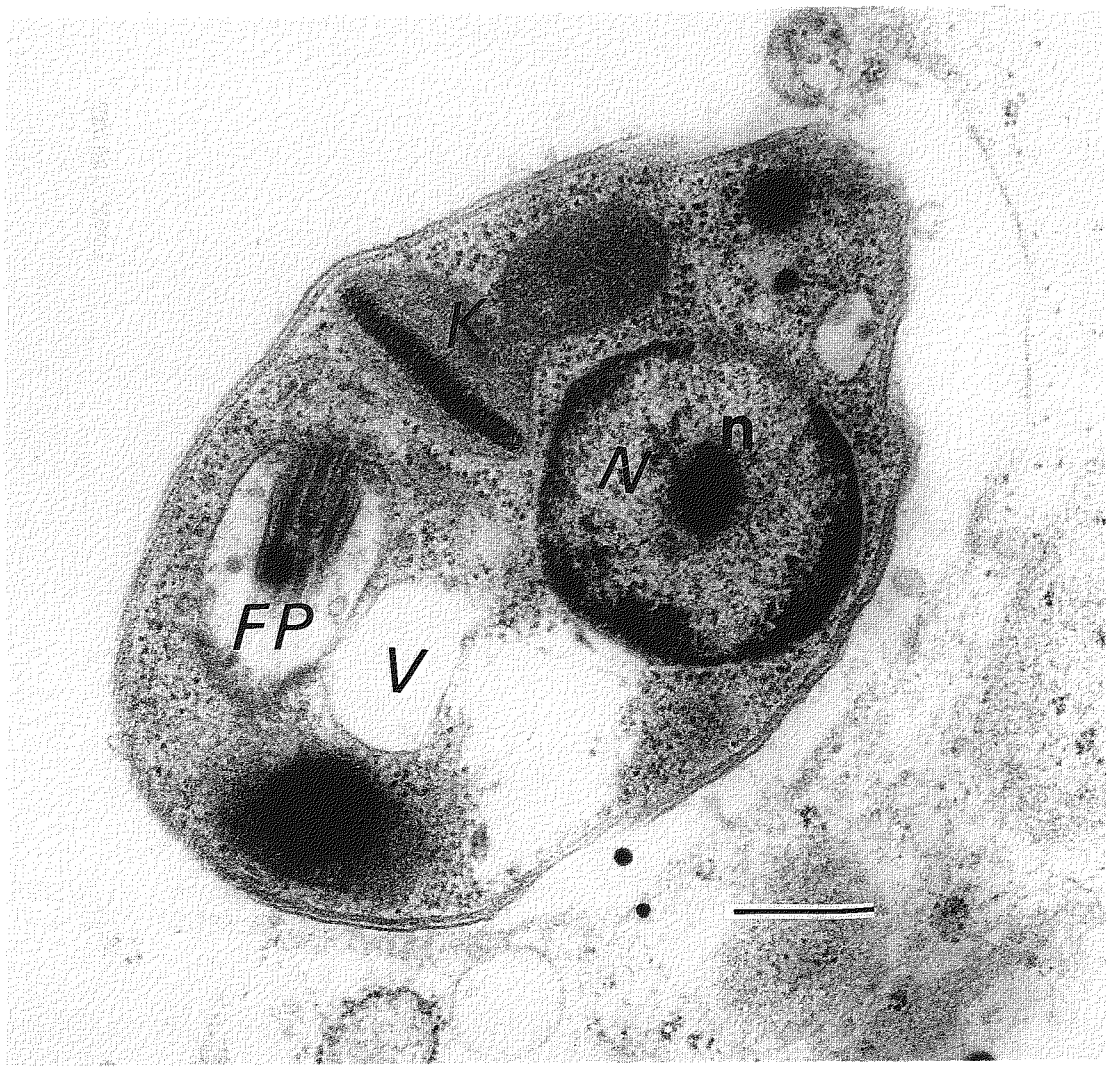

Figure 7.2.16. Ultrastructure of *Leishmania* amastigotes. N, nucleus; n, nucleolus; FP, flagellar pocket; V, vacuole.

leishmania bodies in the prickle cell layer along with the intra-epidermal abscesses in three of the 27 cases. Our finding indicates that like Old World *Leishmania* species, New World ones can hazard the epidermis and also that from the findings described above keratinocytes seemed to play an active role against parasites.

Morphology of the amastigote was almost similar as described in other species of *Leishmania*. Furthermore, no marked difference was found between the two biopsy materials parasitized by *L. guyanensis* (I-02) and *L. panamensis* (I-04). We observed the small electron dense granules, rounded or rod shaped structures inside the parasitized vacuoles in host macrophage or outside the cell. Similar finding was observed by Schurr *et al.* (1987) and considered that electron dense granules originate either from the macrophage or amastigotes. We suggest that these electron dense dots were probably discharged from the parasites because these structures were found regularly in the extracellular parasitized vacuoles; however, the function of these granules is unknown. Lysosomes were observed inside the parasites of *L. tropica* by Lin *et al.* (1986). Liposomes contained wax-like material in the amastigote of *L. garnhami*, were noted by Scorza *et al.* (1979). Furthermore, virus-like particles in promastigotes of *L. hertigi* have been described by Molyneux *et al.* (1974). In the present specimens, no such findings, lysosomes, wax-like materials and virus-like particles, were observed.

To understand the fate of parasite inside the host body, various theories have been reported. Generally it is agreed that parasites grow and multiply inside the macrophage (Berman *et al.*, 1979). Many researchers (Schurr *et al.*, 1987; Farah *et al.*, 1975; Bretana *et al.*, 1983) believe that *Leishmania* can live a safe life inside the host macrophage and the parasite may be destroyed only outside the cell. While on the other hand, it has been shown that parasites also may be destroyed inside the cell (Sandbank, 1976; Buchmuller *et al.*, 1979). Our data showed that amastigotes located in the cytoplasm of host macrophage were either inside the vacuoles or free from vacuoles. Here, the cytoplasm of the cell was seen in active phase which indicates the active role of host cell against parasite. From this, it remained unclear that does the cell successed in killing the parasite or the parasite safely escaped from the host cell.

In our specimens the lymphocytes were seen in close contact with parasitized macrophages. This macrophage-lymphocyte combination represents the co-operation of these two cells against the parasites (Skamene *et al.*, 1983). Here the function of lymphocyte should be assumed by two means: 1) to recognize the parasitized macrophage that may finally result in the destruction of host macrophage (Schurr *et al.*, 1987); or 2) helping the macrophage to kill the parasite intracellularly (Mauel *et al.*, 1978). Lymphocyte attached with parasites that may be probably cytotoxic T-cells indicates the specificity of lymphocytes to attack the parasites directly. Amastigotes inside the macrophage were seen degenerated where host cell was also under parasitic attack. From this finding, we can conclude that parasite may be destroyed intracellularly by host macrophage and in unknown conditions parasite may kill the host cell. In this study, the conditions and mechanism(s) of the destruction of parasite is unknown. The observation of amastigotes inside the cytoplasm of eosinophil and neutrophil, indicates the phagocyting role of these cells against the parasites. Parasites inside the eosinophils have already been reported in the experimental leishmaniasis studies in BALB/c mice (Barral-Netto *et al.*, 1987; McElrath *et al.*, 1987).

From the results mentioned above, it is suggested that parasites may be destroyed both inside and outside the host macrophage and T-cells have a major role against the parasites in this disease.

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3. Topical Treatment for Cutaneous Leishmaniasis in Ecuador

ABSTRACT. In this study, we undertook an evaluation of topical treatment for cutaneous leishmaniasis. A total of 132 cutaneous leishmaniasis patients living in the village of San Sebastián (Ciento Tres), Department of Manabi, Ecuador, were recruited for the study. Paromomycin ointment was prepared in two types of concentrations, 10% and 2%. Meglumine antimonate solution was prepared at the concentration of 3.75% with mercury chrome solution. Among 20 patients treated with 10% paromomycin ointment, two showed marked improvement; 10, a good reaction; four, a slight reaction; and four, no reaction. On the other hand, the treatment with 2% paromomycin ointment revealed a marked improvement in five patients, a good result in 11, slight improvement in 18 and no reaction in 10. Some patients with large ulcerative lesions complained of a burning sensation during the application, while the patients with plaque or nodulous type of lesion did not. We, therefore, selected a low concentration (2%) for large ulcerative lesions and a high concentration (10%) for dry and nodulous and/or plaque-type of lesions. From the result obtained, paromocycin ointment may be useful for ulcerative lesions, but not so effective against non-ulcerative lesions. Meglumine antimonate solution was topically given to 30 patients with cutaneous leishmaniasis. This solution was combined with mercury chrome to enhance the reduction of secondary infections in the lesions. Among these 30, a complete cure was found in 16; a slight improvement, in 11; and no effect, in three. The dryness of lesions was more remarkable when meglumine antimonate solution was used, compared with paromomycin ointment application. Meglumine antimonate solution plus mercury chrome would also be useful for the treatment of ulcerative dermal leishmaniasis.

There are many forms of treatments for cutaneous leishmaniasis. In the New World, drugs of the first choice are antimonate chemicals and sodium stibogluconate (Person *et al.*, 1985). However, there are either lacking in potency or producing various side effects in these drugs. Therefore, an urgent development of more effective treatments without side effects is awaited. Recently, topical chemotherapy for cutaneous leishmaniasis has been reported, using paromomycin ointment in combination with methylbenzethonium (El-On *et al.*, 1988). In Ecuador, there is a report on topical treatment for cutaneous leishmaniasis, using the same preparation of paromomycin ointment (Krause *et al.*, 1991).

Topical treatment of leishmaniasis will be rational, when the lesions are completely limited to the skin without metastasis to mucosae or viscera. In the current study, therefore, we undertook an evaluation of topical treatment for cutaneous leishmaniasis, employing two types of medications, meglumine antimonate plus mercury chrome solution and paromomycin ointment. The study was conducted in an area endemic for cutaneous leishmaniasis caused by *Leishmania panamensis* or *L. guyanensis*; in the area no distribution of *L. braziliensis* and *L. chagasi* was ascertained and no mucosal or visceral cases were reported hitherto.

Materials and Methods

Subjects

A total of 132 cutaneous leishmaniasis patients living in the village of San Sebastián (Ciento Tres), De-

partment of Manabi, Ecuador, who agreed with informed consent were recruited for the present study (Table 7.3.1). All the subjects continued their daily activity during the treatment without hospitalization. Follow-up of the treatment was performed between January and March, 1991. Seventy patients were treated with 2% paromomycin ointment (Table 7.3.2), 31 were treated with 10% paromomycin ointment (Table 7.3.3) and 61 were treated with meglumine antimonate plus mercury chrome solution (Table 7.3.4). The diagnosis was made based on clinical features of the disease, smears or cultures from the lesions and leishmanin skin test. Clinical manifestations in detail were described in this text (see Chapter 7.1).

Preparation for topical ointment and solution

Paromomycin ointments were prepared in two concentrations, 10% and 2%. Meglumine antimonate solution was prepared as follows: 250 ml of 30% meglumine antimonate, 750 ml physiological saline and 1,000 ml of mercury chrome solution. This mixed solution was then devided into 25 ml each in small bottles.

Applicating procedure of topical ointment and solution

The patients were given a guide to apply the ointment or solution two or three times a day using a cotten apllicator. The applications were completely done by the patients themselves or their family members in the houses. Before the topical application, cleaning of the lesions using a soap and water was recommended.

Efficacy criteria for the topical treatments

The effect of topical application was judged primarily by the clinical features. Photographs of lesions were taken at each ten days, and the sizes and conditions of lesions were carefully observed and recorded. Especially, ulcer sizes and indurations were measured at each clinical observation. The effects were graded into four criteria as follows: 1) -, no change of the eruption during treatment; 2) +, slight improvement of the eruptions; 3) ++, deffinite improvement of the eruptions; and 4) +++, marked improvement or complete cure of the lesions. In this study, final evaluation was made at seven weeks after the treatment with 2% and 10% paromomycin ointments, and it was done at five weeks after, in the treated groups with meglumine antimonate solution. Some of patients were observed at July, August and September, 1991 (seven to nine months from the beginning of the present treatment) and at January and February, 1992 (one year later). In this article, the result of early evaluation will be reported mainly, including a part of the data of later evaluation. More detailed results will be reported elsewhere.

Results

The results are summarized in Tables 7.3.5 to 7.3.7. The number of patients treated with 2% paromomycin ointment was 70; 44 (21 males and 23 females) of these, were followed up to the final evaluation (Table 7.3.5). Among the 44 subjects, the mean age was 14.9 year-old; the mean duration time of eruption, 3.8 months; and the mean number of lesions, 2.6.

The treatment with 2% paromomycin ointment produced a marked improvement in five patients, a good reaction in 11, a slight reaction in 18 and no reaction in 10. When 2% paromomycin ointment was applied to the skin, ulcers started to dry but the induration tended to persist. The number of patients treated with 10% paromomycin ointment was 31; 20 (10 each of both sexes) of these were followed up to the final evaluation (Table 7.3.6). The mean age of the 20 patients treated with 10% paromomycin ointment was 26.3 year-old; the mean duration time of eruption, 4.8 months; and the mean number of lesions, 3.3. In this group, two patients showed deffinite improvement; 10, marked reaction; four, slight

Table 7.3.1. Summary of cutaneous leishmaniasis patients treated topically in San Sebastián (Ciento Tres), Manabi, Ecuador

Age	Male	Female	Total
0 - 9	21 (30.9%)	21 (32.8%)	42 (31.8%)
10 - 19	20 (29.4%)	22 (34.4%)	42 (31.8%)
20 - 29	13 (19.1%)	10 (15.6%)	23 (17.4%)
30 - 39	7 (10.3%)	3 (4.7%)	10 (7.6%)
40 - 49	4 (5.9%)	4 (6.3%)	8 (6.1%)
50 -	3 (4.4%)	4 (6.3%)	7 (5.3%)
Total	68 (100.0%)	64 (100.0%)	132 (100.0%)

Table 7.3.2. A trial of treatment of cutaneous leishmaniasis patients with 2% paromomycin ointment

Age	Male	Female	Total
0 - 9	14 (42.4%)	15 (40.5%)	29 (41.4%)
10 - 19	10 (30.3%)	14 (37.9%)	24 (34.3%)
20 - 29	6 (18.2%)	3 (8.1%)	9 (12.8%)
30 - 39	2 (6.1%)	2 (5.4%)	4 (5.7%)
40 - 49	1 (3.0%)	1 (2.7%)	2 (2.9%)
50 -	0 (0.0%)	2 (5.4%)	2 (2.9%)
Total	33 (100.0%)	37 (100.0%)	70 (100.0%)

reaction; and four, no reaction. Some patients with large ulcerative lesions complained of a burning sensation during the application of 10% paromomycin, while those with plaque or nodulous type of lesion did not. The effect of high concentration (10%) was better than that of low one (2%).

Meglumine antimonate solution was applied to 61 patients with cutaneous leishmaniasis. The numbers of subjects followed up to the final evaluation were 30, 15 males and 15 females (Table 7.3.7). The mean age of patients was 15.8 year-old; the mean duration time of eruption, 4.8 months; and the mean number of lesions, 2.6. Among these, 16 showed a marked improvement of lesions and 11 demonstrated a slight improvement. The solution was not effective in three patients. The dryness of lesions was more remark-

Table 7.3.3. A trial of treatment of cutaneous leishmaniasis patients with 10% paromomycin ointment

Age	Male	Female	Total
0 - 9	0 (0.0%)	2 (14.3%)	2 (6.4%)
10 - 19	5 (29.4%)	1 (7.1%)	6 (19.4%)
20 - 29	6 (35.3%)	6 (42.9%)	12 (38.7%)
30 - 39	2 (11.8%)	1 (7.1%)	3 (9.7%)
40 - 49	3 (17.6%)	2 (14.3%)	5 (16.1%)
50 -	1 (5.9%)	2 (14.3%)	3 (9.7%)
Total	17 (100.0%)	14 (100.0%)	31 (100.0%)

Table 7.3.4. A trial of treatment of cutaneous leishmaniasis patients with meglumine antimonate plus mercury chrome solution

Age	Male	Female	Total
0 - 9	9 (31.0%)	11 (34.4%)	20 (32.8%)
10 - 19	9 (31.0%)	12 (37.5%)	21 (34.4%)
20 - 29	6 (20.7%)	5 (15.6%)	11 (18.0%)
30 - 39	3 (10.4%)	1 (3.1%)	4 (6.6%)
40 - 49	0 (0.0%)	2 (6.3%)	2 (3.3%)
50 -	2 (6.9%)	1 (3.1%)	3 (4.9%)
Total	29 (100.0%)	32 (100.0%)	61 (100.0%)

able after the application of meglumine antimonate solution than after paromomycin ointment application.

The details of clinical course of four patients treated with topical ointment are described as follows:

Case 25 a 3 year-old male

The lesion, which showed ulcer, was located on the right cheek (Fig. 7.3.1A) and suffered for three months. For this patient 2% paromomycin ointment was given and the lesion was markedly improved after 40 days. Fig. 7.3.1B shows complete cure after six months.

Case 31 a 24 year-old male

The patient was suffered two demarcated ulcers on his lower legs for two months (Fig. 7.3.2A). Initially, 10% paromomycin ointment was given to him, but he could not continue this ointment because of

Table 7.3.5. Effects of 2% paromomycin ointment for the treatment of cutaneous leishmaniasis

Grade of effects*	Male	Female	Total
(−)	4 (19.0%)	6 (26.1%)	10 (22.7%)
(+)	6 (28.6%)	12 (52.2%)	18 (40.9%)
(++)	8 (38.1%)	3 (13.0%)	11 (25.0%)
(+++)	3 (14.3%)	2 (8.7%)	5 (11.4%)
Subtotal	21 (100.0%)	23 (100.0%)	44 (100.0%)
Unknown**	12	14	26
Total	33	37	70
Mean age	12.7 (±9.3)	16.8 (±13.4)	14.9 (±11.8)
Mean duration time of lesions	3.7 (±1.7)	4.0 (± 1.9)	3.8 (± 1.8)
Mean number of lesions	2.1 (±1.5)	3.1 (± 3.2)	2.6 (± 2.6)

* −, no change of the eruption; +, slight improvement; ++, deffinite improvement; +++, complete cure.

** Unable to follow-up at the final evaluation; but according to the information of neighbours the majority of these subjects seemed to be cured completely.

Table 7.3.6. Effects of 10% paromomycin ointment for the treatment of cutaneous leishmaniasis

Grade of effects*	Male	Female	Total
(−)	1 (10.0%)	3 (30.0%)	4 (20.0%)
(+)	3 (30.0%)	1 (10.0%)	4 (20.0%)
(++)	4 (40.0%)	6 (60.0%)	10 (50.0%)
(+++)	2 (20.0%)	0 (0.0%)	2 (10.0%)
Subtotal	10 (100.0%)	10 (100.0%)	20 (100.0%)
Unknown**	7	4	11
Total	17	14	31
Mean age	28.5 (±15.2)	24.0 (±14.6)	26.3 (±15.1)
Mean duration time of lesion	4.3 (± 2.9)	5.3 (± 2.7)	4.8 (± 2.8)
Mean number of lesions	3.3 (± 2.5)	3.2 (± 1.8)	3.3 (± 2.2)

*, **, see Table 7.3.5.

Table 7.3.7. Effects of meglumine antimonate plus mercury chrome solution for the treatment of cutaneous leishmaniasis

Grade of effects*	Male	Female	Total
(−)	1 (6.7%)	2 (13.3%)	3 (10.0%)
(+)	7 (46.7%)	4 (26.7%)	11 (36.7%)
(++)	7 (46.6%)	9 (60.0%)	16 (53.3%)
(+++)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Subtotal	15 (100.0%)	15 (100.0%)	30 (100.0%)
Unknown**	14	17	31
Total	29	32	61
Mean age	18.3 (± 16.0)	16.1 (± 14.9)	17.2 (± 16.0)
Mean duration time of lesions	4.5 (± 2.5)	5.4 (± 2.7)	4.9 (± 2.6)
Mean number of lesions	2.9 (± 2.7)	1.9 (± 1.7)	2.4 (±

pain. Then, 2% paromomycin ointment was applied to him and the topical treatment was continued for two months. Fig. 7.3.2B shows a good result of treatment, demonstrating only scar after six months.

Case 55 a 13 year-old male

The patient was suffered a giant ulcer sized 70×45 mm on the right lower leg for two months (Fig. 7.3.3A). For the patient 2% paromomycin ointment and mercury chrome were given and the eruption was gradually improved. Then the patient was changed to 10% paromomycin ointment. Fig. 7.3.3B shows complete cure after three months.

Case 63 a 11 year-old female

The patient had two ulcers on the right lower leg for five months. Fig. 7.3.4A shows an ulcer sized 35 x 30 mm. Two satellite lesions were seen around the main ulcer. In this case, 2% paromomycin ointment was initially given, and the lesions showed a good improvement. Fig. 7.3.4B shows almost cured lesion after two months.

Discussion

The present topical applications to the lesions of cutaneous leishmaniasis produced an excellent result. In particular, almost complete healing of the lesions was obtained in several patients by topical treatment with paromomycin ointment alone. There have already been few reports on the topical use of paromomycin ointment (El-On *et al.*, 1988). Paromomycin is usually used topically in high concentrations such as 12.5%. Furthermore, it is used as an ointment in combination with methylbenzethonium chloride. Krause *et al.* (1991) tried topical treatment with same paromomycin-methylbenzethonium cream in Ecuador (Krause *et al.*, 1991). They gave the topical treatment to 52 patients with cutaneous leishmaniasis and obtained cure in 90% to 98% of patients after 100 days. However, their cure rates decreased after 360 days of treatment. We compared the effects between high (10%) and low (2%) concentra-

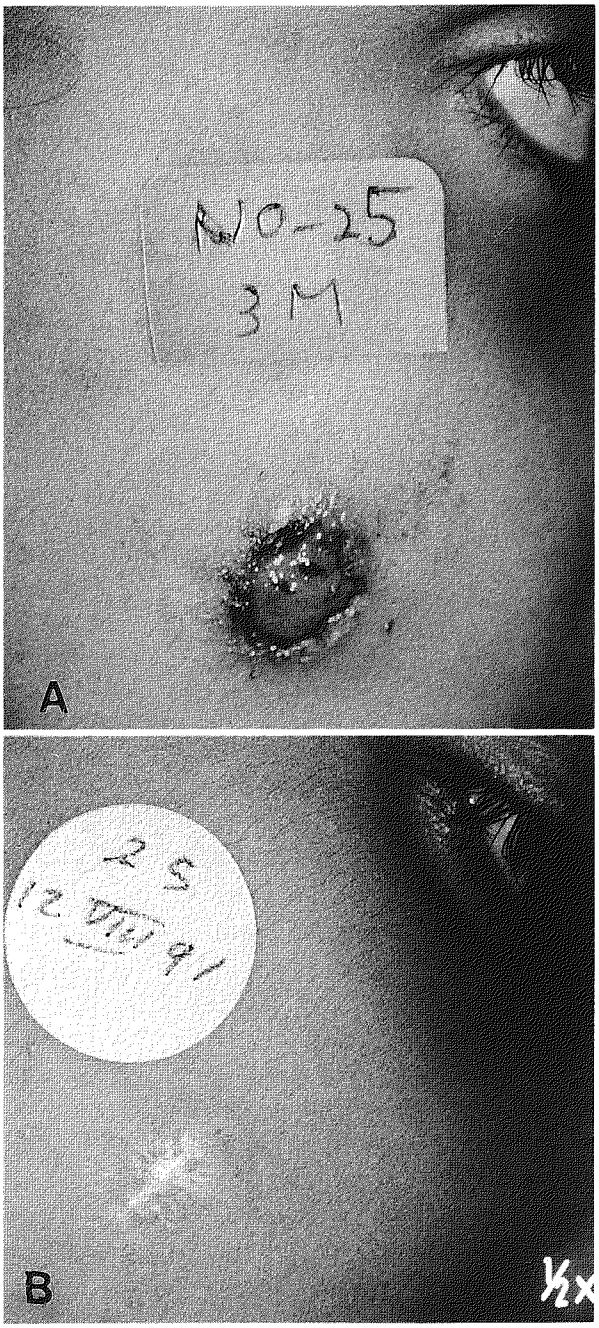
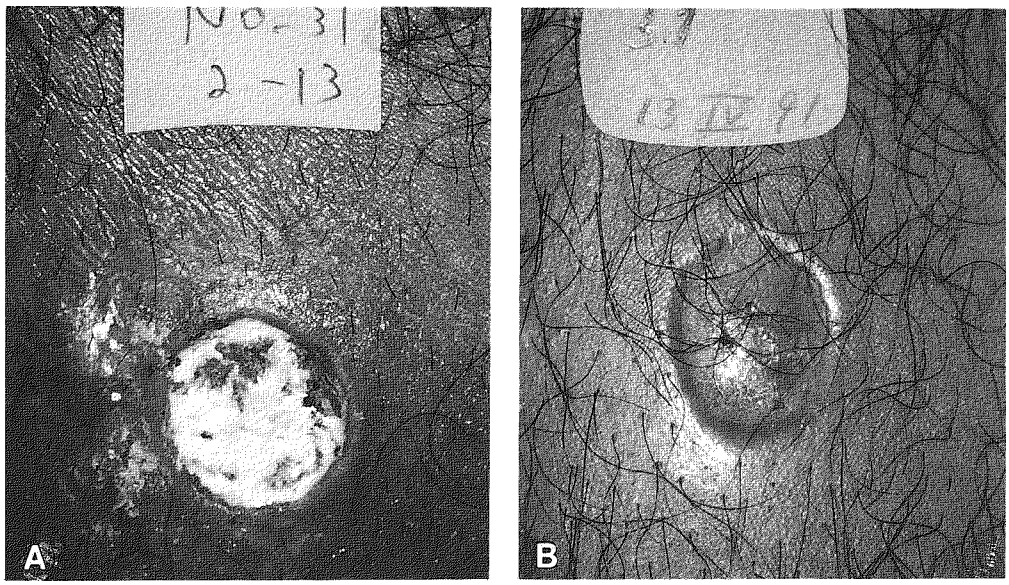


Figure 7.3.1. A, an ulcer of Case 25. The ulcer sized 10 x 13 mm located on the right cheek, before treatment with 2% paromomycin ointment. B, the same lesion (scar), showing complete cure, after six months of treatment.



Figuer 7.3.2. A, an ulcer of Case 31. The ulcer sized 14 x 15 mm located on the left lower leg, before treatment with 10% and 2% paromomycin ointment. B, demarcated scar of the same lesion, after six months of treatment.

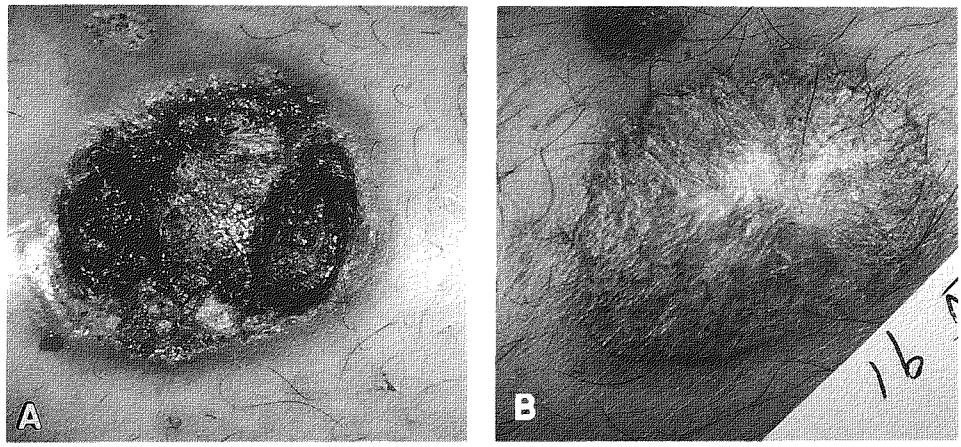


Figure 7.3.3. A, an ulcer of Case 55. The large ulcer sized 35 x 30 mm seen on the right lower leg, before treatment with 2% paromomycin ointment. Two satellite lesions located around the large ulcer. B, the cured lesion including satellite ones, after two months of treatment.

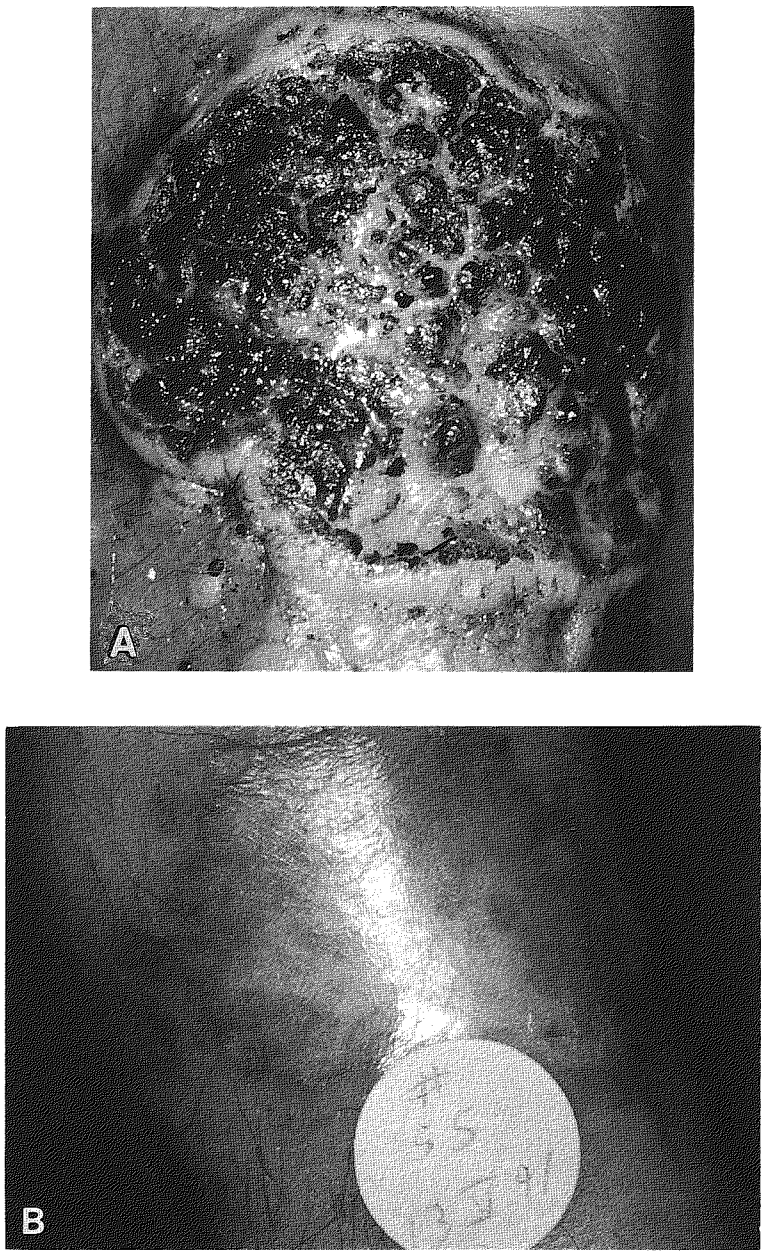


Figure 7.3.4. A, an ulcer of Case 63. The large lesion located on the right lower leg, before treatment with 2% paromomycin. B, the lesion improved rapidly and cured after three months of treatment.

tion of paromomycin. In our preliminary study, some patients with large ulcerative lesions rejected to use our ointment because of burning sensation at the application of high concentration of the ointment. In a few case low concentration also caused a slight burning sensation as well as high one. However, the degree was so mild that the patients could continue to use the ointment. Therefore, we selected a low concentration for large ulcerative lesions and a high concentration for dry, nodulous or plaque-type lesions. Futhermore, the children less than five year-old were selected to use low concentration. The present trials using paromomycin ointment were still not enough to establish a treatment of cutaneous leishmaniasis, because of a short evaluation period. However, paromomycin ointment was found to be quite useful for ulcerative lesions but not so effective against non-ulcerative lesions.

Meglumine antimonate is generally used by injections. Therefore, there is a problem on side effects and some cases show uneffective results. If patients with cutaneous leishmaniasis do not have a potential to metastasize into mucosae or viscera, it is not necessary to treat patients systemically. In the present study site, only *L. panamensis* or *L. guyanensis* has been identified as the causative agents, and no mucocutaneous cases are reported (see Chapter 5.2). From these reasons, we decided to use the topical treatment using meglumine antimonate solution. The solution was combined with mercury chrome solution to enhance the reduction of secondary infections of lesions. The concentration of meglumine antimonate was 3.75%. There was no irritation at this concentration. However, more adequate concentration should be investigated in future.

An evaluation of topical treatment for cutaneous leishmaniasis is not easy. There are several problems to be solved in the treatment of American cutaneous leishmaniasis. The first problem is a natural healing. Guderian *et al.* (1991) confirmed untreated cures on nine patients during their therapeutic study for cutaneous leishmaniasis in Ecuador. The second is a reactivation of the lesions. The third is a possibility to transfer to mucocutaneous or visceral forms. Furthermore, applications of the medications may be considerably different in each patient, although our colleagues explained in detail how to use our medication. Such a standardization will also be necessary in future. Although the topical treatment should not be selected as a first choice for patients with cutaneous leishmaniasis at the moment, it may be useful for the patients who are not able to obtain any other treatment.

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4. Bacterial Flora in Suspected *Leishmania* Ulcers of Patients from an Endemic Focus on the Pacific Coast of Ecuador

ABSTRACT. Bacterial infection was examined in suspected leishmaniasis lesions of the subjects living in the tropical dense and humid forest of two endemic areas on the Pacific coast of Ecuador. Of the 82 subjects examined, 80 (97.6%) were positive for bacterial infections. The most prevalent bacteria were staphylococci, *Staphylococcus aureus* and *S. epidermidis*, followed by micrococci and Gram positive bacilli. The importance of such a study in the New World leishmaniasis-endemic areas was discussed briefly.

Leishmaniasis is a zoonosis of international interest of public health, and is considered one of the six most important tropical diseases of the World Health Organization. The disease is widely distributed in Ecuador, and at the current time, studies on epidemiology, entomology, parasitology, immunology etc. of the disease have been acomplished in the country.

The purpose of the present study is to determine the bacterial flora and their influence on the evolution or clinical features of leishmanial ulcers (cutaneous lesions). In Ecuador Kawabata *et al.* (1987) preliminary reported bacterial flora found in two types of leishmaniasis ulcers (dry and wet types) from the Andean highlands and the Pacific coast lowlands. On the other hand, more recently, in Iran Edrissian *et al.* (1990) reported bacterial infections in clinically diagnosed cutaneous lesions of the Old World leishmaniasis patients; and they recommended that bacterial infections should be considered in diagnosing and treating suspected cutaneous leishmaniasis, particularly in areas where there is no facility for carrying out bacteriological examinations. Such an information also seems to be very important from the point of view of both the management of patients and prognosis of the disease. However, a few investigations have been done in the world to date, as pointed out by Edrissian *et al.* (1990).

As a preliminary study, this article deals with the result of examinations on the bacterial infection in cutaneous leishmaniasis lesions of patients from lowland (Pacific coast) endemic area of Ecuador.

Materials and Methods

A total of 82 scraping samples were taken from suspected cutaneous leishmaniasis patients from two endemic sites, San Sebastián and San Gabriel, Department of Manabi, Ecuador; 24 from the former site and 58 from the latter. The two sites are located on the Pacific coastal cordillera of the country. In the areas, almost all the houses are surrounded by wet tropical dense forest and the persons cultivated coffee, cacao and yucca.

Samples were taken from the following subjects: 0-10 of age, 34 (19 males and 15 females); 11-20, 26 (15 males and 11 females); 21-30, 9 (4 males and 5 females); 31-40, 2 (2 males); 41-50, 3 (1 male and 2 females); and 50 or over, 8 (4 males and 4 females). The samples were scratched from the cleaned lesions with cotton wool moistened with alcohol, using a flamed sterilized vaccinostyle, and then inoculated on to nutrient blood agar medium for cultivation. The materials were identified by their reaction to Gram stain, size, form, and colony characteristics on the culture medium.

Results

Of the 82 samples taken from the suspected lesions, 80 (97.6%) were positive for bacterial infections. Thirty six of these samples consisted of two or more micro-organisms, resulting 130 isolates of bacteria in total; 109 Gram positive bacteria and 21 Gram negative ones. Gram positive bacteria obtained were: 44 isolates of staphylococci, 40.4%, 33 of which were coagulase-positive staphylococci (*Staphylococcus aureus*) and 11 were coagulase-negative staphylococci (*Staphylococcus epidermidis*); 34 isolates of micrococci, 31.2%; 28 isolates of bacilli, 25.7%; 2 isolates of streptococci, 1.8%; and 1 isolate of beta-hemolytic streptococci, 0.9%. Gram negative bacteria obtained were: *Proteus mirabilis*, 5 (23.8%); *Citrobacter diversus*, 3 (14.3%); *Klebsiella pneumoniae*, 3 (14.3%); *Klebsiella oxytoca*, 2 (9.5%); *Escherichia coli*, 2 (9.5%); *Enterobacter cloacae*, 2 (9.5%); *Enterobacter aerogenes*, 2 (9.5%); *Citrobacter freunii*, 1 (4.8%); and *Proteus vulgaris*, 1 (4.8%).

Comments

Among the bacterial infections found in the present subjects with suspected cutaneous leishmaniasis lesions from endemic areas of Ecuador, staphylococci were the most prevalent, followed by micrococci and Gram positive bacilli. According to Edrissian *et al.* (1990), in Iran microscopic examination of skin scrapings from 2,202 individuals with clinically diagnosed cutaneous leishmaniasis lesions revealed bacterial infections in 788, showing 35.7%. On the contrary, the current examination in Ecuador showed a markedly high rate (97.6%) of bacterial infections. Comparing the infection rates of bacteria from the Old and New World, it was estimated that the great difference might be caused by the difference of bacterial flora and environmental conditions between the two continents. In this study no analysis on the relation between *Leishmania* and bacterial infections was made; the result will be published elsewhere. Thus, with regard to the bacterial flora of American cutaneous leishmaniasis lesions a few studies have been performed. Further investigation, therefore, is required in given endemic areas of the disease, especially in the tropical dense forest of the New World where the environmental condition seems to be quite favorable for bacterial infections in cutaneous leishmaniasis lesions.

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5. Fungi Isolated from Suspected *Leishmania* Ulcers of Patients from an Endemic Focus on the Pacific Coast of Ecuador

ABSTRACT. The current study was prompted to know the role of fungi on the establishment and course of cutaneous leishmaniasis lesions. Of the 52 subjects 47 (90.4%) were positive for one or more fungi, while five remained negative. Thus, in total 72 fungous strains were isolated from the suspected leishmaniasis lesions of the subjects living in the village of San Sebastián (Ciento Tres), Department of Manabi, Ecuador. Among these, 26 unidentified strains were included. At generic level, most prevalent fungi are *Aspergillus* spp. and *Penicillium* spp. followed by *Cladosporium* spp. Five strains of yeast, including *Candida* spp. were isolated from the lesions. However, no marked correlation between causative fungi and the evolution of leishmaniasis lesions was found in this preliminary study.

For the adequate control and treatment of cutaneous leishmaniasis, it is worthwhile to know the species and the population of microorganisms other than *Leishmania* parasite which exist in the lesions. Fungi are known to be an important member of microorganisms in the environment to contaminate the ulcerative lesions, and after made invasion, to infulence the natural course of cutaneous diseases. This study was prompted to know the role of fungi on the establishment and course of cutaneous leishmaniasis lesions.

Materials and Methods

A total of 52 suspected cutaneous leishmaniasis patients living in the village of San Sebastián (Ciento Tres), Department of Manabi, Ecuador were recruited for the present examination. The clinical state of patients with cutaneous leishmaniasis was reported in this text (see Chapter 7.1). The suspected lesions of leishmaniasis were examined clinically and parasitologically, and a part of the lesions was also examined histopathologically.

The specimens for the mycological inoculation were obtained from the surface of the lesions by using a platinum needle. They were inoculated on the surface of Sabouraud's dextrose plate and incubated at room temperature. When a sufficient growth of fungous colonies was observed, they were identified macro- and microscopically, and if necessary, they were transplanted onto adequate media for further examinations. Identification of the fungi was made at generic level.

Results

A total of 47 out of 52 subjects with suspected leishmaniasis lesions were positive for one or more fungi, demonstrating 72 fungous strains, while five subjects remained negative for the microorganisms (Table 7.5.1). From some patients more than two genera of fungi were isolated. Among these, 26 unidentified strains (11 hyaline = mycelia sterilia and 15 dematiaceous fungi) were included. At generic level, most prevalent fungi are *Aspergillus* spp. and *Penicillium* spp. (13 strains each) followed by *Cladosporium* spp. (9 strains). Yeasts, including *Candida* spp. were isolated only from five patients (5 strains). In this preliminary study, there seemed no marked correlation between the fungi observed and the duration, localization, clinical form and size of the lesions of cutaneous leishmaniasis patients.

Table 7.5.1. Results of mycological cultures from suspected cutaneous leishmaniasis lesions of 47 subjects* living in a lowland endemic area, San Sebastián, Manabi, Ecuador

Fungous infection	No. of strains isolated
Aspergillus spp.	13
Penicillium spp.	13
Geotrichum spp.	3
Mycelia sterilia	11
Others	3
Cladosporium spp.	9
Other dematiaceous fungi	15
Candida spp.	3
Other yeasts	2
Total	72

* Fourty-seven (90.4%) of the 52 subjects were positive for one or more fungi.

Comments

By the current study, it was clarified that the cutaneous lesions of leishmaniasis patients in the endemic area were quite frequently contaminated by fungi. The microorganisms isolated in the present examination were representatives of those found commonly in the environment, except some species of *Candida* which are also commensal members in the human body.

The majority of the microorganisms observed, except several species, are classified into the fungi with a low pathogenic potential. They have been rarely encountered as the causative pathogens of cutaneous infections, especially in immune compromised hosts. Since detailed histological examinations were not performed in this study, the pathological role of the fungi observed remained obscure. Edrissian *et al.* (1990) reported the active role of bacteria isolated from the suspected cutaneous leishmaniasis lesions of the Old World. In their study, however, no examination of fungous infections was done. The present results suggested that the fungi isolated from the New World (Ecuadorian) leishmaniasis patients belong to a group with a extremely low pathogenecity. They, therefore, seemed to have no active role on the establishment and course of leishmaniasis lesions. However, in order to elucidate a more detailed role of these fungi on the lesions, further investigation is required.

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Chapter 8

Leishmaniasis in Paraguay

1. An Epidemiological Survey of Leishmaniasis in an Endemic Area of Paraguay

ABSTRACT. A study was performed of the epidemiology of leishmaniasis in a newly established community in south-eastern Paraguay. A total of 149 persons, out of 172 inhabitants, were examined by clinical, parasitological and immunological (skin test) methods. Eighty-eight of the examinees (59.1%) were clinically positive for dermal and nasal (mucosal) lesions or dermal scars, while 74 (49.7%) were positive by leishmanin skin test. Of the 88 persons, 66 (75.0%) showed positive for both leishmanial (dermal and nasal) signs and skin test; in this study, therefore, these subjects were considered to be leishmaniasis patients. Most of the patients exhibited a single dermal lesion (60.8%). Serious mucosal (nasal septum) lesions were observed in the following 41 subjects including two with loss of nasal septum; eight with ulceration; and 31 with erythema. In this community the persons who had dermal and/or nasal problems had been treated with Glucantime®, without precise diagnosis, by parasitological or immunological examinations. The socioeconomical and sociomedical aspects of *Leishmania* infection in the community is discussed.

Both American cutaneous and mucocutaneous leishmaniases are widely endemic in many parts of the Republic of Paraguay. It is, therefore, a considerable public health problem, especially in the south-eastern part of the country. Little information, however, has been available on epidemiological features, such as infection rates of humans, sandflies and wild and domestic mammals with *Leishmania* parasites in different endemic areas. According to cases reported in the country, the clinical manifestations of the disease differ markedly, from single localized cutaneous lesions to disfiguring and non-healing mucocutaneous ones, as well as the visceral form of the disease, a systemic infection that often causes a death if untreated (Boggino and Insaurralde, 1945; Boggino and Maas, 1945; Gonzalez and Arce, 1955a, b; Arias *et al.*, 1984; Ruffinelli *et al.*, 1986; Walton, 1987; Münz and Fricke, 1989). These divergences of clinical forms may be due to the *Leishmania* species involved, though the degree of susceptibility of the human host may be equally important (Blackwell and Alexander, 1986).

The diverse nature of leishmaniasis clearly requires a diverse control program with specific targets for each focus of the disease. For this purpose, more extensive collection of baseline data will be needed with regard to the *Leishmania* species infecting humans, the sandfly vectors involved and the mammalian reservoirs (Hashiguchi and Gomez, 1987, 1990; Wirth *et al.*, 1989). A clinical decision must be made whether or not to treat with drugs of varying toxicity, depending on which *Leishmania* is present (Barker *et al.*, 1986). Thus, identification of the causative agent (*Leishmania* spp.) is very important with regard to treatment regimens and/or control programs of the disease in each endemic area.

In Paraguay, a total of 483 cases were recorded in 1981 and over 1,600 cases were reported in 1982 (Walton, 1987). Both cutaneous and mucocutaneous forms are common and seem to be associated with *L. braziliensis* (Grimaldi *et al.*, 1990). Diffuse cutaneous leishmaniasis, probably due to *L. amazonensis* has also been reported (Grimaldi *et al.*, 1990). The first case of American visceral leishmaniasis known from Asuncion, Paraguay (Migone, 1913), but the infection was probably acquired in Brazil. Later, however, Boggino and Maas (1945) reported the first case of autochthonous visceral leishmaniasis in the country based on observation of amastigotes in liver and spleen samples from a Paraguayan patient.

In the present paper, we report the results of a study in which we examined subjects living in a newly established community in a dense tropical forest near the Brazilian border.

Materials and Methods

Study area.

The current study was carried out during July and September 1989 in Limóy (24°50'S; 54°50'W), a newly established community of 172 people, which is situated at 450 m above sea level in the Department of Alto Parana in south-eastern Paraguay, near the Brazilian border (Fig. 8.1.1). In this region, the population engorged in agriculture is dense, because of the fertile terrain. Almost all of the houses in the area are surrounded by dense primary tropical forest (Fig. 8.1.2). The houses are wooden structures that present no barrier to the entry of sandflies or other bloodsucking insects. The community was founded in 1986 by immigrants from different regions of Paraguay and Brazil. The local people cultivate cassava (yucca) and maize after felling the surrounding primary forest. Their livestocks consists of dogs, horses, cattle, pigs and chickens.

Subjects.

In the examination of inhabitants, questionnaires were prepared to record period of residence, origin and occupation of each person, history of the disease and leishmanial dermal and mucosal lesions (location, size, type, number and onset), treatment etc. A total of 149 persons (59 males and 90 females) were examined in the present study. The majority (70.5%) of persons examined had immigrated into the community two years previously. Their age composition was as follows: less than 10 years old, 55 (23 males and 32 females); 11-20, 50 (22 males and 28 females); 21-30, 24 (10 males and 14 females); 31-40, 12 (5 males and 7 females); and 41 or over, 8 (3 males and 5 females).

In this community, one person who had received short-term paramedical training was acting as a health volunteer to treat leishmaniasis patients. When someone with dermal or nasal (mucosal) problems came for treatment, he gave intramuscular injection of Glucantime® (meglumine antimonate) without performing any precise diagnosis by parasitological or immunological methods. Thus, almost all the subjects examined in the present study had been treated with Glucantime®.

Skin test.

Leishmania panamensis (MHOM/PA/71/LS94 provided by Dr. P. Desjeux, PDP, WHO) was cultured with Pan's medium (Pan, 1984). A soluble antigen used for the skin test in this study was prepared by the method of Reed *et al.* (1986) and slightly modified (Furuya *et al.*, 1989). *Leishmania* promastigotes were harvested and washed (1,000 x g, 10 min, 4°C) five times with phosphate buffered saline (PBS), pH 7.2. After the final wash the parasite pellet was resuspended in five volumes of distilled water, immersed in acetone-dry ice, and thawed in tepid water. The freeze-thaw procedure was repeated 10 times. The disrupted parasites were diluted in approximately 10 volumes of PBS, centrifuged at 10,000 x g for 30 min at 4°C. The supernatant was adjusted to 250 µg protein concentration per ml after filtration through a sterile 0.45 µ millipore filter (Millipore Co., Mass., USA), and

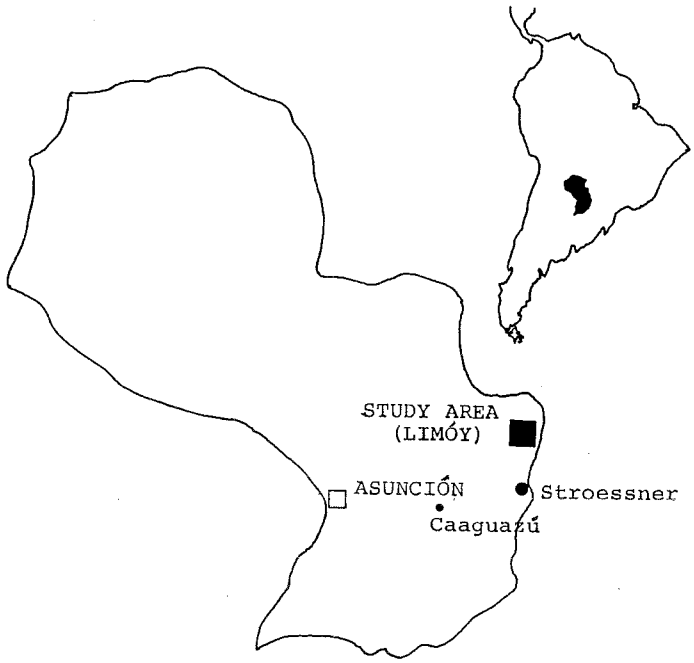

Figure 8.1.1. Outline map of the Republic of Paraguay, showing study area, Limóy, Department of Alto Parana, and other related localities.

lyophilized as skin test antigen. The lyophilized filtrate was adjusted to the desired concentration with sterile PBS. The antigen was injected intradermally in 0.1 ml (25 µg protein concentration) in the flexor surface of the forearm. Induration size of more than 5 mm at the site 48 hours after injection was considered to be a positive reaction based on the criteria employed by Reed *et al.* (1986) and Furuya *et al.* (1989).

Smear and culture materials from active lesions.

Smear samples were taken from the margins of active dermal lesions, and then stained with Giemsa. Saline aspirates using 27-guage needles from these lesions were cultured *in vitro* (Hendricks and Wright, 1979). No such trials were made on active nasal lesions, in order to avoid discomforts to the patients. The *in vitro* culture medium used was slightly modified from that reported by Walton *et al.* (1977). This was prepared from 40 g Difico Blood Agar Base (Code B45, Difico Laboratories, Detroit, Michigan, USA) per 1,000 ml distilled water with 20% defibrinated rabbit blood. Two ml of melted media were poured into each tube, and the tubes sealed with rubber caps. The blood agar slants were left at room temperature for several hours to allow formation of condensation fluid, and then stored at 4°C until used. When cool, an overlay of sterile saline (0.9%) was added to each tube. Two drops of 20% gentamycin were also added to combat microbial contamination.

Results

As shown in Table 8.1.1, of a total of 172 inhabitants 86.7% (149: 63 males and 86 females) were examined clinically, parasitologically and immunologically (by performing leishmanin skin-tests).

Figure 8.1.2. An area, Limóy, Department of Alto Parana, Paraguay, very close to the Brazilian border, highly endemic for both cutaneous and mucocutaneous leishmaniasis. A (above), houses newly constructed after felling a dense primary tropical forest, and its surroundings. B (below), persons came to the examination performed at a small health center.

Eighty-eight (59.1%) had active lesions (dermal and nasal signs) or dermal scars, indicative of cutaneous and mucocutaneous leishmaniasis. All of these subjects had been treated with Glucantime ®; 74 (49.7%) were positive for skin test (Table 8.1.2). There was a slight (age- and sex-related) difference in the presence of active lesions or scars and in positive skin test results; infection increased with age and more males were infected than females, which suggests that exposure is related to occupation. The correlation between leishmanin skin-test results and the presence of active lesions or scars is shown in Table 8.1.3. Sixty-six of 88 subjects (75.0%) with active lesions or scars gave a positive result to the skin test, although only 2 (0.3%) had active dermal lesions, but no *Leishmania* amastigotes were seen in stained impression smears, or promastigotes demonstrated in *in vitro* cultures. Eight of 74 people (10.8%) with positive skin tests lacked demonstrable active lesions or scars. In this study, therefore only the 66 subjects positive for both leishmanial signs and skin tests, were considered to be leishmaniasis patients.

Most of these 66 people (59.2%) had become infected within 12 months of their arrival in the present community (Limóy); 26% within 13-24 months of birth or immigration; and 14.3% after 25 months or more. All of these subjects had been treated with Glucantime® and had dermal scars when examined (July and September, 1989: dry season), suggesting a relatively short period of ulceration (mean: 9 months; range 2-24 months) from the onset. Four cases (7.3%) had had two years of dermal ulceration, although 28 (50.9%) had less than six months. The patients had from one to seven active lesions or scars (single, 60.3%; 2, 16.2%; 3, 11.8%; 4, 4.4%; 5, 2.9%; 6, 2.9%; and 7, 1.5%). The lesions observed were cutaneous or mucocutaneous types. Two patients (a married couple) with positive skin test had completely lost their nasal septa (Table 8.1.4) and their lesions were clinically still active at the time of examination. The husband (36 years old) had suffered cutaneous leishmaniasis 18 years ago, while his wife (30 years old) had been infected 22 years previously in Caaguazú, Paraguay considered a hyper-endemic area for *Leishmania*; both of them had received Glucantime® treatment before.

In dermatological examination, the leishmanial lesion seemed to appear as a small, painless papule at the onset, which then ulcerated and was healed with Glucantime® treatment. Later, however, these subjects with scar seemed to reveal ulcers and erythema of the nasal septum (Table 8.1.4). In Table 8.1.4, it should be emphasized that out of 75 subjects with negative skin tests, 40 (53.3%) revealed erythema of nasal septum and all the subjects had received intramuscular injection of Glucantime®.

The location of lesions observed is depicted in Fig. 8.1.3. The active lesions or scars of the 66 subjects positive for skin tests were mainly located on the exposed body surface (lower extremities, 54.8%; upper extremities, 23.8%; face, 10.3%), although a few (11.1%) were found on the trunk of the male subjects. This result suggests that the vector sandflies in the area prefer to bite lower parts of the body exposed. A preliminary sandfly collection using human bait and Shannon trap in the area revealed the presence of seven species, *i.e., Lutzomyia shannoni, Lu. whitmani, Lu. intermedia, Lu. migonei, Lu. cortelezzii, Lu. walkeri* and *Lu. longispina*; the first two species were predominant (see Chapter 8.2).

The size of 126 dermal lesions (leishmanial scars and active lesions) observed in 66 patients (positive for both dermal lesions and skin test) ranged from 5 x 5 mm to 35 x 35 mm; namely, 5 x 5 mm - 10 x 10 mm, 33.7%; 11 x 11 mm - 20 x 20 mm, 42.4%; 21 x 21 mm - 30 x 30 mm, 17.4%; and 31 x 31 mm - 35 x 35 mm, 6.5%.

Discussion

This article documents a high rate of leishmanial infection among inhabitants of a community established in a dense primary tropical forest of south-

Table 8.1.1. Dermatological examinations and skin tests (ST) of 149 subjects in a leishmaniasis-endemic area, Limóy, Department of Alto Parana, Paraguay

Age	No. examined			No.(%) + for signs			No.(%) + for ST		
	total	male	female	total	male	female	total	male	female
0-10	55	23	32	23 (42)	15	8	21 (38)	10	11
11-20	50	22	28	31 (62)	11	20	23 (46)	11	12
21-30	24	10	14	17 (71)	9	8	17 (71)	8	9
31-	20	8	12	17 (85)	8	9	13 (65)	6	7
Total	149	63	86	88 (59)	43	45	74 (50)	35	39

Table 8.1.2. Results of skin test (ST) in 149 subjects in a leishmaniasis-endemic area, Limóy, Department of Alto Parana, Paraguay

Age	No. tested	No. + for ST (%)	Male +/tested (%)	Female +/tested (%)
0-10	55	21 (38)	10/23 (44)	11/32 (34)
11-20	50	23 (46)	11/22 (50)	12/28 (43)
21-30	24	17 (71)	8/10 (80)	9/14 (64)
31-	20	13 (65)	6/ 8 (75)	7/12 (58)
Total	149	74 (50)	35/63 (56)	39/86 (45)

eastern Paraguay. it is assumed that the transmission of *Leishmania* was maintained between the vector and wild reservoir host before the community was founded. In the community, almost all the houses are surrounded by dense primary tropical forest, suitable habitat for both the vector and the reservoir host of *Leishmania*. Adults engage in felling and clearing of surrounding forest with their children, in order to cultivate crops. Local inhabitants are therefore exposed to frequent biting of sandflies in the area.

The causative organism in this region seems to be *L. braziliensis*; recently Grimaldi *et al.* (1990) typed the species from these regions.

In the present examination, of the 66 subjects who showed positive for both active lesions or scars and skin tests, and who had been treated with Glucan-

Table 8.1.3 Summary of the results of physical and immunological (skin test) examinations of 149 subjects, in a leishmaniasis-endemic area, Limóy, Department of Alto Parana, Paraguay

		Dermal lesions		Total
		+	−	
Skin	+	66	8	74
test	−	22	53	75
Total		88	61	149

Table 8.1.4 Clinical signs of nasal septum and skin test reactivity of 149 subjects in a leishmaniasis-endemic area, Limóy, Department of Alto Parana, Paraguay

Clinical signs	No. among skin test + (%)	No. among skin test − (%)
Loss of nasal septum	2/74 (3)	0/75
Ulceration of nasal septum	8/74 (11)	0/75
Erythema of nasal septum	31/74 (42)	40/75 (53)*

* All the 40 subjects had received Glucantime® injection for several days; these erythema seemed to be caused by other infectious diseases.

time®, 64 had dermal scars, suggesting past infection with *Leishmania*. The remaining two subjects had active dermal lesions, but they were negative for the parasite in smear specimens and in *in vitro* culture. These negative results would be caused mainly by the treatment of patients with Glucantime®. The secondary infections also highly affect the results of microscopical examinations of clinically suspected cutaneous leishmaniasis lesions, causing negative results of 45.6% in such cases (Edrissian *et al.*, 1990).

Among eight (10.8%), 4 males and 4 females, out of 74 subjects positive for skin test, no dermal lesions or scars were found. A proportion of these negative results may be due to mis-diagnosis and the overlooking of dermal lesions covered by clothing, since the sensitivity and specificity of the present *Leishmania* antigen is very high (Reed *et al.*, 1986; Furuya *et al.*, 1989). On the other hand, 14 of the 88 (15.9%) persons with apparent active dermal lesions or scars were negative for leishmanin skin test, suggesting that these were due to other causes such as trauma, burns and other infectious diseases (bacterial and fungal infections etc.).

In the subjects examined, more than a half of leishmanial lesions were located in the lower parts of the body exposed, suggesting a preferable biting site of vector sandflies in the area. The biting sites of vector sandflies are different from endemic-area to endemic-area or from species to species. For example, in coastal regions of Ecuador the sandfly biting rate was 26.9% (Hashiguchi *et al.*, 1984) or 34.2% (Rodriguez and Aviles, 1953) in lower extremities, while in Amazonian regions of the same country it was 60.0% (Ammunarriz, 1982). In French Guiana, the distribution of 636 cutaneous lesions on the body surface of 201 patients were 31.9% on the lower extremities, 39.3% on the upper extremities, 15.4% on the trunk and 13.4% on the face and neck (Dedet *et al.*, 1989). Information on the location of dermal lesions would be useful for reducing a risk of infection of the disease in given endemic areas. The localization of the leishmanial lesions would also be influenced by the type of clothing worn (Hashiguchi *et al.*, 1984). The patterns of clothing and localization of lesions of patients showed a perfect correlation (Dedet *et al.*, 1987). By analizing the location of dermal lesions on the body it would be possible to reduce a risk of infection of the disease in given endemic areas at least to some extent.

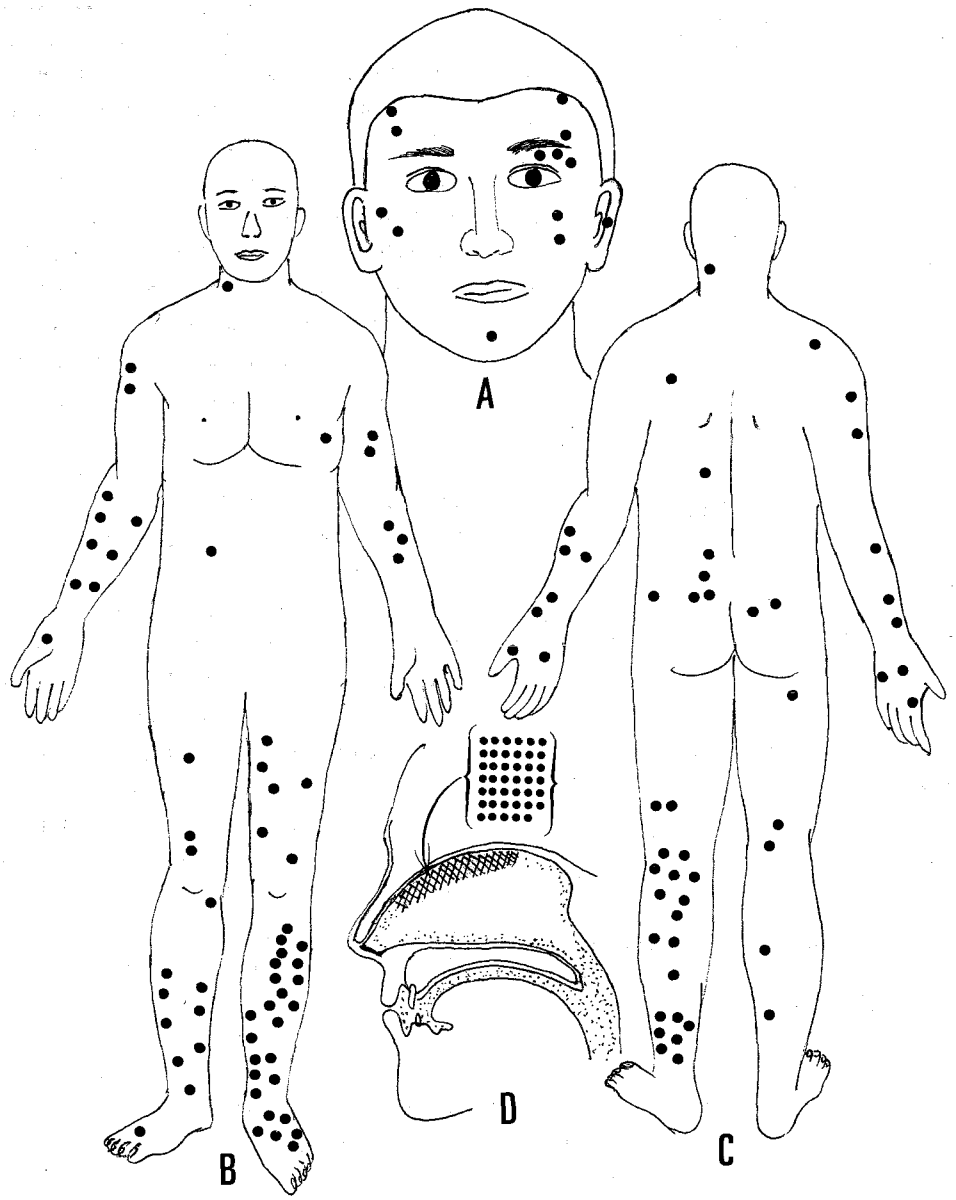

Figure 8.1.3. Distribution of cutaneous leishmaniasis lesions, scars and ulcers, on the exposed body surface (A, B and C). D shows 41 mucocutaneous (nasal) lesions: 2, loss of nasal septum; 8, ulceration; and 31, erythema.

In the present study area, the persons who had any dermal or nasal problems had received intramuscular injections of Glucantime® over several days without having precise diagnosis. Leishmaniasis is a great problem from the socioeconomical and sociomedical standpoints, compounded by difficulties such as misdiagnosis, inavailability of drugs (Glucantime® is very scarce and expensive in Paraguay, especially in rural areas), loss of work times (patients may have to travel long distance for treatment). In order to eliminate these problems, adequate medical care systems should be urgently established in each endemic area of the country, by employing sensitive and field-applicable diagnostic tools.

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2. Anthropophilic Sandflies and *Leishmania* Infection in Paraguay

ABSTRACT. Nine species of sandflies, *viz., Lutzomyia whitmani, Lu. intermedia, Lu. shannoni, Lu. migonei, Lu. fischeri, Lu. pessoai, Lu. cortelezzii, Lu. walkeri* and *Lu. longispina,* were caught, by protected human bait and Shannon trap, in four areas hyper-endemic for *Leishmania* in Paraguay; the first two species were predominant. Hind-gut infections with flagellates resembling *Leishmania* promastigotes were observed only in one specimen of *Lu. whitmani* with a low infection rate of 0.38% (1 out of 266 flies). Overall infection rate was 0.16% (1 out of 615 flies of all species dissected), suggesting a low rate of natural infection in *Leishmania* endemic areas of Paraguay.

American tegumentary leishmaniases are widely endemic in Paraguay, especially in the southeast, where the human population is very high because of the fertile terrain. Leishmaniasis is a considerable public health problem in the country, although very little is known about the details of disease transmission, other than sporadic reports of clinical cases. A total of 483 cases were recorded in 1981 and over 1,600 cases were reported in 1982 (Walton, 1987). Both cutaneous and mucocutaneous forms are common and seem to be associated with *Leishmania braziliensis,* though a few cases of diffuse cutaneous forms, probably due to *L. amazonensis,* have also been recorded (Grimaldi *et al.*, 1990). Growing concern among the inhabitants of endemic areas as well as health officials in Paraguay prompted the assessment of the sandfly fauna and their natural infections with *Leishmania* reported here.

Materials and Methods

Phlebotomine sandflies were collected from the following four areas (Fig. 8.2.1) of southeast Paraguay where cutaneous and/or mucocutaneous leishmaniasis is highly endemic: 1) Brazilero Cué, Department of Caaguazú, 2) Cantera Boca, Department of Caaguazú, 3) Limóy, Department of Alto Parana, 4) Tava-í, Department of Caazapá. All collections were made between June and September 1989. Two sampling methods were used to collect sandflies at each of the sites surveyed. These were as follows: 1) Illuminated Shannon trap (Shannon, 1939). In this sampling method, a large white tent of dimensions 2.0 m x 1.5 m x 1.5 m was suspended from four saplings or tree branches and illuminated from within by a fluorescent lamp. Sandflies were aspirated as they landed on the outer and inner walls of the trap. These collections were made between 18:00-22:00. 2) Protected human bait collections. Anthropophilic sandflies were collected as they landed on human volunteers after dark (18:00-22:00) preparatory to biting. During each collection the volunteers used flashlights to illuminate the insects just before they were aspirated. The majority of the sandflies collected were preserved at low temperature (less than 4°C) ready for identification and/or dissection, and the remainder were dissected immediately after collection.

In order to search for *Leishmania* promastigotes, dissections of female sandflies were made by the method of Johnson *et al.* (1963) with a slight modification (Hashiguchi *et al.*, 1985). The internal organs were covered by glass cover slips and then examined microscopically at x 400. Sandfly species identifications were made at the time of dissection by observ-

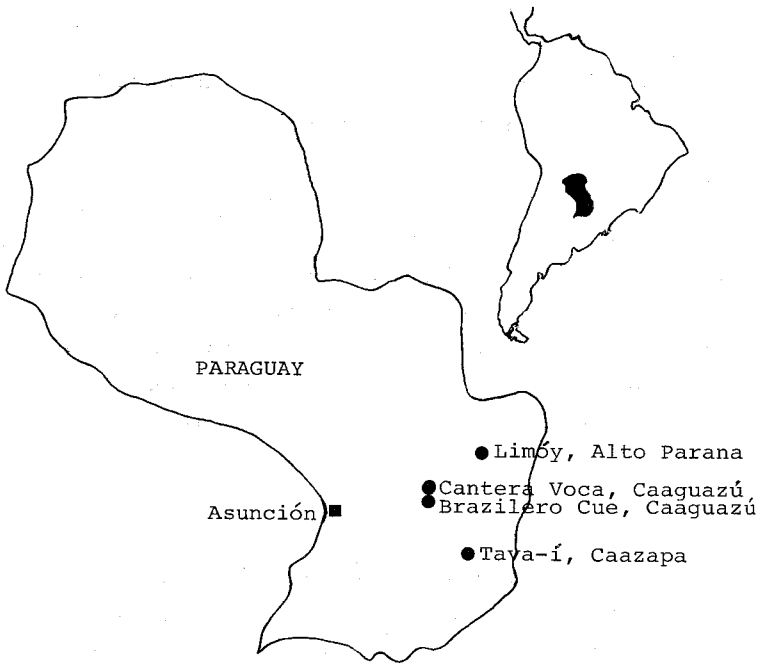

Figure 8.2.I. Map of Paraguay showing 4 sandfly collecting sites and other related localities: Brazilero Cué, Department of Caaguazú; Cantera Boca, Department of Caaguazú; Limóy, Department of Alto Parana; and Tavaí, Department of Caazapá.

ing the spermathecae and cibarial armatures. For the purpose of taxonomical examinations, furthermore, certain specimens (both males and females) were preserved dry and then boiled for three minutes in a 5% solution of NaOH or KOH and placed in 10% phenol for identification under the microscope. Cleared and identified specimens were mounted permanently on glass slides in Canada balsam.

Results

A total of 1,393 sandflies were collected from the four study sites, of which 778 were used for taxonomical examinations, while the remaining 615 were processed to dissections in order to detect *Leishmania* promastigotes. As shown in Table 8.2.1, five species of the genus *Lutzomyia, viz., Lu. whitmani* (Antunes & Coutinho), *Lu. intermedia* (Lutz & Neiva), *Lu. shannoni* (Dyar), *Lu. migonei* (Franca) and *Lu. fischeri* (Pinto), were found to be man-biters in areas endemic for *Leishmania* in Paraguay. These species were caught at the following frequencies: in human bait collections, *Lu. whitmani*, 78.3%; *Lu. fischeri*, 6.7%; *Lu. intermedia*, 5.1%; *Lu. shannoni*, 5.1%; *Lu. migonei*, 4.8%; and in Shannon trap collections, *Lu. intermedia*, 53.2%; *Lu. whitmani*, 24.4%; *Lu. shannoni*, 15.1%; *Lu. migonei*, 5.5%; and *Lu. fischeri*, 1.8%. In addition, small numbers of four other *Lutzomyia* species, *i. e., Lu. pessoai* (Coutinho & Barretto), *Lu. cortelezzii* (Brethes), *Lu. walkeri* (Newstead) and *Lu. longispina* (Mangabeira), were also caught, for a total of nine anthropophilic species. Of the 615 female sandflies dissected (424 from protected human bait and 91 from Shannon trap), only one specimen (0.16%) was positive for leishmanial promastigotes as shown in Table 8.2.2. This positive sandfly was observed among 266 *Lu. whitmani* col-

lected from human bait in a hyper-endemic area (Brazilero Cué, Department of Caaguazú) of the disease (0.38% of those collected). The parasites were mainly localized on the hind-gut. Unfortunately, *in vitro* cultivation of these parasites failed because of contamination. However, the promastigotes found in *Lu. whitmani* were morphologically similar to *Leishmania*. Nevertheless the findings of the current study suggest that natural infections of Paraguayan sandflies with *Leishmania* occur at a very low rate even in areas hyper-endemic for *Leishmania* spp. infecting man.

Discussion

In the current study, nine *Lutzomyia* species, *i. e., Lu. whitmani, Lu. intermedia, Lu. shannoni, Lu. migonei, Lu. fischeri, Lu. pessoai, Lu. cortelezzii, Lu. walkeri* and *Lu. longispina*, were caught by human bait and/or Shannon trap collections; all of these species were found to be anthropophilic. In a previous survey of the Paraguayan sandfly fauna, Vianna Martins *et al.* (1978) listed eight species, *viz., Lu. aragaoi* (Costa Lima) from Aca-Poi, *Lu. intermedia* from Caaguazú, Chaco, Villa Rica, *Lu. longipalpis* (Lutz & Neiva) from Villa Rica, *Lu. migonei* from Asunción, Chaco, *Lu. pessoai* from Chaco, Caaguazú, *Lu. shannoni* (locality not mentioned), *Lu. walkeri* from Cerro Pero and *Lu. whitmani* from Caaguazú. Furthermore, in this country one other species, *Lu. monticola* (Costa Lima), had been recorded from *Leishmania*-endemic areas (Gonzalez and Queirolo, 1955). Recently, a preliminary study was performed on man-biting sandflies in an area endemic for *Leishmania, i. e.,* Tavapy II, Department of Alto Parana, near the Parana river, and three species, *i. e., Lu. intermedia, Lu. whitmani* and *Lu. migonei*, were caught (Arias and Ayala, pers. commun.). Thus, the sandfly fauna of Paraguay seems to be very poor as compared with other South American countries. In the present fly collection, three species, *Lu. fischeri, Lu. cortelezzii* and *Lu. longispina*, were recorded for the first time in Paraguay.

As to natural infections of sandflies with leishmanial promastigotes, only one of the *Lu. whitmani* females dissected was positive, showing a very low rate (0.16%) in total. This sandfly species has already been incriminated as a vector of *Leishmania* in Brazil (Arias and De Freitas, 1978; Lainson and Shaw, 1979; Young and Lawyer, 1987). Because of contamination of the sample from this positive fly, no characterization of the parasite was possible, although Grimaldi *et al.* (1990) recently typed *L. braziliensis* isolated from patients residing in these endemic regions of the southeast Paraguay. More detailed investigations should be performed in the future, in order to incriminate the vectors of *Leishmania* in the areas of Paraguay endemic for the parasite.

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Masato Kawabata
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Table 8.2.1. Phlebotomine sandfly collection from 4 study sites endemic for leishmaniasis in Paraguay

Locality*		Fly collection					
		Human bait			Shannon trap		
	Lutzomyia spp.**	male	female	total	male	female	total
Brazilero Cué	*Lu. whitmani*	8	323	331	18	58	76
	Lu. intermedia	3	18	21	68	85	153
	Lu. shannoni	0	14	14	42	5	47
	Lu. migonei	15	4	19	7	10	17
	Lu. fischeri	0	29	29	4	2	6
	Sub-total	26	388	414	139	160	299
Cantera Boca	*Lu. whitmani*	2	7	9	1	6	7
	Lu. intermedia	0	1	1	15	4	9
	Lu. shannoni	4	4	8	3	0	3
	Sub-total	6	12	18	19	10	19
Limóy	*Lu. whitmani*	0	0	0	1	0	1
	Lu. intermedia	0	0	0	1	0	1
	Lu. shannoni	0	0	0	2	0	2
	Lu. migonei	0	2	2	0	0	0
	Sub-total	0	2	2	4	0	4
Tava-í	*Lu. intermedia*	0	0	0	1	19	20
	Lu. migonei	0	0	0	2	0	2
	Sub-total	0	0	0	3	19	22
	Total	32	402	434	165	189	344

* Number of capture (during 18:00 and 22:00) in each locality: 4 in Brazilero Cué; 2 in Cantera Boca; 1 in Limóy; and 1 in Tava-í.

** The sandflies were identified based on 778 specimens treated with NaOH or KOH and phenol.

Table 8.2.2. Results of the sandfly dissection to search for *Leishmania* promastigotes infections

Locality	Species	Type of collection	No. examined	No. infected (%)
Tava-í	*Lu. shannoni*	Human bait*	55	0
		Light trap**	0	0
	Lu. intermedia	Human bait	25	0
		Light trap	0	0
	Lu. whitmani	Human bait	27	0
		Light trap	0	0
	Lu. fischeri	Human bait	1	0
		Light trap	0	0
Brazilero Cué	*Lu. shannoni*	Human bait	30	0
		Light trap	0	0
	Lu. intermedia	Human bait	27	0
		Light trap	38	0
	Lu. whitmani	Human bait	266	1(0.38%)
		Light trap	20	0
	Lu. fischeri	Human bait	13	0
		Light trap	1	0
	Lu. migonei	Human bait	6	0
		Light trap	3	0
Cantera Voca	*Lu. shannoni*	Human bait	4	0
		Light trap	5	0
	Lu. intermedia	Human bait	0	0
		Light trap	1	0
	Lu. whitmani	Human bait	6	0
		Light trap	0	0
	Lu. fischeri	Human bait	1	0
		Light trap	0	0
	Lu. pessoai	Human bait	1	0
		Light trap	1	0
Limóy	*Lu. shannoni*	Human bait	21	0
		Light trap	8	0
	Lu. intermedia	Human bait	0	0
		Light trap	2	0
	Lu. whitmani	Human bait	35	0
		Light trap	11	0
	Lu. migonei	Human bait	0	0
		Light trap	1	0
	Lu. cortelezzii	Human bait	1	0
		Light trap	0	0
	Lu. walkeri	Human bait	1	0
		Light trap	0	0
	Lu. longispina	Human bait	4	0
		Light trap	0	0
Total			615	1(0.16%)

* Protected human bait collection; ** Shannon trap collection.

infections with promastigotes in man-biting species of sand flies in leishmaniasis-endemic areas of Ecuador. Am. J. Trop. Med. Hyg., 34, 440-446.

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Summary

The current text dealt with the results of field and laboratory studies derived from surveys conducted during 1990 and 1991 in the Pacific lowlands and Andean highlands of Ecuador. All the data and materials obtained were analyzed from the view of parasitological, entomological, immunological, molecular biological, clinical and pathological points. In addition, information on the epidemiological and entomological features of Paraguayan leishmaniasis has been given briefly. The results mentioned are summarized as follows.

Findings on Andean leishmaniasis and its ecology

Until more recently, the only form of leishmaniasis in the Andes was considered to be Peruvian uta caused by *Leishmania peruviana*. However, in 1986, we have discovered another type of leishmaniasis in the Ecuadorian Andes which has a completely different species of causative agents and vectors from those of Peruvian uta. In this text, we briefly reviewed Andean leishmaniasis including uta and revised an ecological model of the disease in the Andean plateau. Furthermore, in this text autochthonous Andean leishmaniasis cases were reported from two regions of Ecuador, Huigra (1,300 m above sea level) and Alausi (2,300 - 2,500 m a.s.l.), Department of Chimborazo. In the areas schoolchildren, domestic dogs as reservoir host and vector sandflies were examined: 18.9% of the 122 children from Alausi showed positive for both leishmanin skin test and dermal scars; 32.8% of the 58 dogs from the same site revealed a high ELISA value; and *Leishmania* parasites were isolated from *Lutzomyia ayacuchensis* caught in both sites, Alausi and Huigra. The parasites were also isolated from two children (one and two year-old females) living in Huigra.
(see Chapters 1, 4.1 and 5.1).

Molecular biological findings

Karyotypes of *L. mexicana, L. panamensis* and *L. major*-like parasites from Ecuador were analyzed by a turn-table type pulsed field gel electrophoresis (PFGE) apparatus. A total of 18-21 chromosomes from 200 kb to over 1,100 kb were resolved, depending on the *Leishmania* isolates. The PFGE revealed species-specific DNA karyotypes. The observed karyotype variations among isolates from distinct regions appear to reflect the species diversity of *Leishmania* in the New World. Polymerase chain reaction (PCR) techniques have been applied for detection of *Leishmania* DNA, using synthesized oligonucleotide primers derived from *L. braziliensis*. The primers used differentiated *L. braziliensis* complex from *L. mexicana* complex or *Trypanosoma* spp.
(see Chapter 2.1 and 2.2).

Vector entomological findings

Biting activity and *Leishmania* infection of sandfly, *Lutzomyia* spp. collected by four different methods were examined, especially in relation to parous rates. The higher parous rates produced the higher *Leishmania* infection rates. Sandflies caught during/after dawn tended to possess more suck-like ovarian follicles than those collected during/after dusk. A strong possibility of transmission of *L. panamensis* to man by the bite of *Lu. hartmanni* or *Lu. trapidoi* was discussed, based on the infection of one (J.B.A.) of our research members during a sandfly collecting trip. The sandfly fauna of each of nine sites endemic for *Leishmania* was sampled using a variety of collection methods. A total of 30 species were collected and three of them, recorded for the first time in the country. The genus *Warileya* was also recorded in the country for the first time, represented *Wa. phlebotomanica*. The known ranges of 23 species were increased by 36 new province records.
(see Chapter 3.1, 3.2 and 3.3)

Clinico-epidemiological findings on the disease of lowlands

A total of 1,296 leishmaniasis cases diagnosed at the

outpatient facility of the national institute were thoroughly reviewed. All the cases were from rural areas of the Department of Manabi, the Pacific coastal region endemic for cutaneous leishmaniasis. The majority of cases occurred between 1989 and 1990. A markedly high rate of onset time was found in the period from August to October, just before the beginning of rainy season; the period was estimated as the main time of transmission of the disease in the area. An epidemiological and clinical study was conducted in a leishmaniasis-endemic area, San Sebastián (Ciento Tres), Department of Manabi. Clinical forms of the disease in the area were described in detail; lymphnode swellings were seen in half of the 143 subjects examined, showing a more frequent occurrence in male than in female. Bacterial and fungal floras in suspected *Leishmania* ulcers of patients from the endemic area were also studied preliminary, in order to evaluate their influence against the natural course of cutaneous leishmaniasis.
(see Chapters 5.2, 7.1, 7.4 and 7.5).

Light and electron microscopical findings

Specimens of both the nose and footpads of golden hamsters infected experimentally with *L. mexicana* from Ecuador showed large numbers of amastigotes with extensive infiltration of histiocytes, lymphocytes and some extent of neutrophils, eosinophils and plasma cells. A number of mast cells were prominent in the upper and lower dermis of granulomatous lesions. Amastigotes were found in the macrophages inside the large parasitophorous vacuoles, mostly at the central part of the lesion. Regular destruction of parasites was observed within macrophages in all the cutaneous and visceral sections indicating the phagocytizing role of these cells against the *Leishmania* parasites. Ultrastructural observations on the cutaneous lesions of three patients with leishmaniasis were also performed. Lymphocytes were in close contact with parasitized macrophage as well as directly attached with the parasites. Amastigotes were confirmed in the epidermis where lymphocytes and other mononuclear cells were present near the parasites. Amastigotes were also observed in and between the keratinocytes, and were attached with lymphocytes.
(see Chapters 6.1 and 7.2)

Findings on the treatment of cutaneous leishmaniasis

Leishmanicidal activity of paromomycin, meglumine antimonate and mercury chrome was evaluated *in vitro* and *in vivo* for the purpose of the topical applications to American cutaneous leishmaniasis. The result obtained showed that paromomycin and mercury chrome are potent chemotherapeutic agents for the disease. However, in this experiment no obvious synergistic inhibitory effect of meglumine antimonate on the promastigote proliferation *in vitro* was observed. In San Sebastián (Ciento Tres), Department of Manabi, Ecuador, a total of 132 cutaneous leishmaniasis patients were recruited for the topical treatment with two types of medications, *viz.*, paromomycin ointment and meglumine antimonate plus mercury chrome solution. The result indicated that paromomycin ointment may be quite useful for ulcerative lesions, but not so effective against non-ulcerative lesions. Meglumine antimonate plus mercury chrome solution seemed to be also effective for ulcerative lesions, showing more marked early dryness of the ulcers compared with the ointment.
(see Chapters 6.2 and 7.3)

Findings on the Paraguayan leishmaniasis

A study was performed of the epidemiology of leishmaniasis in a newly established community in southeastern Paraguay (Limóy, Department of Alto Parana). 59.1% of the 149 subjects examined revealed clinically positive for dermal and nasal (mucosal) lesions or dermal scars, while 49.7% showed positive for leishmanin skin test. Serious mucosal (nasal septum) lesions were observed in the following 41 subjects including two with loss of nasal septum; eight with ulceration; and 31 with erythema. In the community visited, the persons who had dermal and/or nasal problems had been treated with Glucantime®, without precise diagnosis. The socioeconomical and sociomedical aspects of *Leishmania* infection was also discussed in the text. In Paraguay,

nine species of sandflies, *Lutzomyia* spp. were caught by protected human bait and Shannon trap, in four areas hyper-endemic for leishmaniasis. By the dissection of 615 sandflies in total, a hind-gut infection with promastigotes indistinguishable from *Leishmania* was found in one (0.4%) out of 266 *Lu. whitmani*, suggesting a very low infection rate of vectors even in a hyper-endemic area.

(see Chapter 8.1 and 8.2)

Appendix

Abstract of Related Papers Published

Japanese Journal of Parasitology,
33 (5), 1984, 393-401

1. An Epidemiological Study of Leishmaniasis in a Plantation"Cooperativa 23 de Febrero" Newly Established in Ecuador

Yoshihisa Hashiguchi, Vicenta Vera De Coronel and Eduardo A. Gomez L.

ABSTRACT. An epidemiological study was performed on leishmaniasis in September 1982, in a plantation "Cooperativa 23 de Febrero" newly established in the region of Andean slope in Ecuador. The first immigration of inhabitants in this plantation started from August, 1977. Fifteen (15.8%) of the 95 inhabitants examined were diagnosed as positive for leishmaniasis with ulcers (active leishmanial lesions) on the skin. During the period between 1977 and 1982, a total of 57 (60.0%) of 95 examinees have suffered from the disease. Regardless to age and sex, leishmanial infections occurred almost evenly. The result indicated that the transmission of leishmaniasis had been occurring in a wide range of working and housing areas in the plantation. In most of the active patients, the onset occurred in July or August. The length of time between immigration and the onset of leishmaniasis ranged from 3 to 59 months, mostly 9 to 36 months in those with active leishmanial lesions. A large number of leishmanial lesions were located on the upper parts of the body exposed.

Revista Ecuatoriana de Higiene y Medicina Tropical, 34, 1984, 1-20

2. Infeccion Natural de Phlebotomus con Promastigotes de *Leishmania braziliensis* en una Area Endemica de Leishmaniasis en Ecuador

Vicenta Vera de Coronel, Yoshihisa Hashiguchi, Eduardo A. Gomez L., Tatsuyuki Mimori and Masato Kawabata

ABSTRACT. En el curso de nuestro estudio sobre el mecanismo de transmisión de la leishmaniasis en áreas endémicas del Ecuador, las primeras fases de la investigación se canalizaron hacia las búsqueda de las especies de flebotominos que estarían desempeñando el papel de vectores de la enfermedad (*Lutzomyia* spp.), por medio de la disección de especimenes capturados picando al hombre en la floresta. Hasta la fecha, en el Ecuador, se han realizado algunos trabajos de investigación sobre las manifestaciones clinicas de la enfermedad en los pacientes, y sobre los aspectos taxónomicos y ecológicos de los insectos sospechosos de ser los vectores de la endemia. Sinembargo no se han hecho intentos para determinar definitivamente al vector o vectores principales de la enfermedad, mediante el hallazgo de la infección natural en los insectos incriminados potencialmente.

Cuando la investigación se encamina a conocer el mecanismo de transmisión como paso previo a la adopción de probables medidas de control, lo más importante o prioritario será siempre conocer a los principales vectores en cada área endémica.

En el presente trabajo, usando cebos humanos, los flebotomus capturados fueron el núcleo de nuestra atención, desde Julio a Octubre de 1983, en siete diferentes sitios del área endémica de leishmaniasis escogida por nosotros, la zona de Ocaña, Provincia del Cañar. Sólo encontramos dos especies antropofilicas del género *Lutzomyia*, en ésta area de estudio; ellas fueron identificadas como *Lu. trapidoi*, y *Lu. hartmanni*, basándonos en las características morfológicas de su espermateca y armadura cibarial. Un total de 1,452 flebotominos de ambas especies capturadas, fueron sistemáticamente disecados y examinados en búsqueda de la infección natural, y el resultado fue que las dos resultaron positivas con promastigotes. Los flagelados observados fueron identificados al momento como pertenecientes al complejo *L. braziliensis*, de acuerdo a su aspecto morfológico y comportamiento en el vector, especialmente su ubicación en el tubo digestivo del huésped invertebrado.

Al examinar los ejemplares recolectados a diferentes alturas sobre el nivel del mar, 350 m, 600 m, 950 m, 1,200 m y 1,500 m, *Lu. trapidoi* resultó ser la especie predominante en los sitios más bajos, mientras que *Lu. hartmanni* lo fue en los lugares más altos. De todos estos puntos, encontramos flebotomus naturalmente infectados con promastigotes de *Leishmania*, hasta los 1,200 m de altura. La transmisión de la enfermedad, por tanto, se extiende hasta esta altitud, en el área de estudio. Ambas, *Lu. trapidoi* y *Lu. hartmanni*, visitaron al cebo humano durante toda la noche, para alimentarse. La mayoría de los picos de actividad de los vectores, se encontraron entre las 19:00 y 24:00 hs. Al disecar a *Lu. trapidoi* y *Lu. hartmanni*, encontramos que los naturalmente infectados, siempre fueron capturados entre las 18:00 y 24:00 hs, no encontrándose ninguno positivo a partir de esa hora. Este hecho es atribuible al desarrollo del ciclo gonotrópico, es decir flebotomus paridas y nuliparas, de-

duciendo que las paridas concurren a picar temprano. Por otra parte este fenómeno no pudo observarse en *Lu. hartmanni*, a los 600 m, ya que a dicho nivel la captura del mismo fue escasa.

Así, el resultado de este trabajo de investigación ha sido el descubrimiento de la infección natural con promastigotes del complejo, *L. braziliensis*, en especies de *Lutzomyia* ecuatorianas, pro vez primera, lo que nos ha permitido automáticamente incriminarlas fundamentalmente como los vectores principales de la leishmaniasis en una zona endemica ecuatoriana. Ademas una de estas especies, *Lu. hartmanni*, no ha sido antes señalada como vector en estudios previos realizados en Centro y Sudamerica, ni conocida con anterioridad en nuestro país, todo lo cual deberá confirmarse minuciosamente antes del veredicto definitivo, como parte del largo camino que nuestro grupo deberá aún recorrer revelando uno a uno los extraños secretos que la naturaleza guarda todavía sobre los complejos mecanismos de transmisión de las artropozoonosis, y entre ellas, la leishmaniasis tegumentaria americana.

American Journal of Tropical Medicine and Hygiene, 34 (3), 1985, 440-446

3. Natural Infections with Promastigotes in Man-biting Species of Sand Flies in Leishmaniasis-endemic Areas of Ecuador

Yoshihisa Hashiguchi, Eduardo A. Gomez L., Vicenta Vera De Coronel, Tatsuyuki Mimori and Masato Kawabata

ABSTRACT. In order to determine the vectors of leishmaniasis in Ecuador, 1,054 man-biting sand flies from the Department of Cañar were dissected and examined for promastigotes. There were 2 man-biting species, *Lu. trapidoi* and *Lu. hartmanni* in this endemic area of the disease. The infection rates were 7.7% in the former and 3.9% in the latter species, demonstrating the different rates in various localities and altitudes of the study areas. There was an association between infection rates and the time of day, suggesting some connection with biting activity of sand fly species. In collections using human bait at 7 study areas in 5 Departments, 6 man-biting species were recognized, indicating different dominant species in each area. It was assumed that the dominant species would play an important role as the principal vector of leishmaniasis in each endemic area. As to species determination of the present *Leishmania* promastigotes, suffice it to say that the parasites are *Leishmania* sp., presumably *L. braziliensis* s.l., until the isolates have been typed.

Annals of Tropical Medicine and Parasitology, 79 (5), 1985, 533-538

4. Biting Activity of Two Anthropophilic Species of Sandflies, *Lutzomyia*, in an Endemic Area of Leishmaniasis in Ecuador

Yoshihisa Hashiguchi, Eduardo A. Gomez L., Vicenta Vera De Coronel, Tatsuyuki Mimori and Masato Kawabata

ABSTRACT. The biting patterns of *Lutzomyia trapidoi* and *Lu. hartmanni*, vectors of leishmaniasis, were studied using a human bait in an endemic area on the Pacific slope of the Andes in Ecuador. The results suggest that *Lu. trapidoi* is primarily an early biter at dusk, with the first peak at 20:00-21:00 hours and the second at 03:00-04:00 hours; and that *Lu. hartmanni* bites more constantly throughout the night, with a pronounced peak between 23:00 and 24:00 hours. The biting activity, however, shows a marked variation at each site and between different collections at the same site. The activity and the biting places on man are discussed in relation to human infection with leishmaniasis in the area and the location of lesions on patients.

Transactions of the Royal Society of Tropical Medicine and Hygiene, 79 (1), 1985, 120-121

5. *Leishmania* Isolated from Wild Mammals Caught in Endemic Areas of Leishmaniasis in Ecuador

Yoshihisa Hashiguchi, Eduardo A. Gomez L., Vicenta Vera De Coronel, Tatsuyuki Mimori and Masato Kawabata

ABSTRACT. In total, the following 48 wild mammals were caught and examined for *Leishmania* infections in the two localities, Naranjal (N) and Ocaña (O): *Didelphis marsupialis*, nine in N and five in O; *Tamandua tetradactyla*, one and nil; *Choloepus hoffmani didactylus*, one and nil; *Sylvilagus braziliensis*, one and nil; *Dasypus novemcinctus*, one and one; *Sciurus granatensis*, four and one; *Rattus espinosus*, six and nil; *R. rattus*, one and nil; *Coendou bicolor*, two and nil; *Agouti paca*, two and nil; *Dasyprocta punctata*, two and nil; *Potos flavus*, eleven and nil. Of these animals, only three were positive for the parasite, namely, one *Choloepus hoffmani didactylus*, one of four *Sciurus granatensis* and one of 11 *Potos flavus* from Naranjal. Only cultures from the liver of these three animals were positive for *Leishmania*, those from the spleens being negative. In the light of future planning of control measures of the disease in Ecuador, it is thought to be important to make a search for the reservoir hosts in endemic areas. To determine the principal host in this country, however, more detailed such a work should be performed.

apanese Journal of Tropical Medicine and Hygiene, 13 (3), 1985, 205-2453

6. A Review of Leishmaniasis in the New World with Special Reference to its Transmission Mode and Epidemiology

Yoshihisa Hashiguchi

ABSTRACT. Leishmaniasis is a widespread protozoan disease in the New World from southern US at the north to northern Argentina at the south. The disease is principally divided into three forms, *i.e.*, cutaneous, mucocutaneous and visceral leishmaniasis, mainly based on the clinical manifestations in patients and on the species of the causative agents, *Leishmania*. The leishmaniases are well known as a considerable public health problem in endemic areas of the disease in the New World, except for Canada, Chile and Urguay where no such a disease occurs. In this review, an attempt was made to understand a global situation of the epidemiology of the New World leishmaniasis, laying an emphasis on the pick-up of known endemic areas, vectors and reservoir hosts of different species of the genus *Leishmania* in each country. From the information published hitherto, it was found that an intensive leishmaniasis research has been made in Central and South American countries, such as Belize, Panama, Venezuela and Brazil. The study, however, was poorly done in many other countries of the New World, without limiting endemic areas or deciding vectors and reservoir hosts of the disease. In the present text, the author emphasized on a future research importance of epidemiological characteristics including the transmission mode of New World leishmaniases, in order to search for suitable control measures in each endemic area of different countries. Most of the transmission of leishmaniasis in the New World have been found in dense tropical rain forests with various species of *Leishmania*, sand flies and mammals. In such circumstances of endemic areas of leishmaniasis in the New World, the difficulty of the prophylaxis and control has frequently been pointed out by several investigators. At the present situation of leishmaniasis research without a suitable vaccine and sufficient epidemiological data, ones have commented that the only control measure for New World leishmaniasis is to remove all the inhabitants of communities from regions at risk of the disease, or to perform thoroughly deforestrations around dwelling areas or working places. Past trials of several control measures, such as the spraying of insecticides, destruction of reservoir hosts, application of some vaccines and etc., were also briefly reviewed in the text. (In Japanese with English summary)

Revista Ecuatoriana de Higiene y Medicina Tropical, 36, 1986, 3-8

7. Primera Generación de Phlebotomus de Laboratorio en el Ecuador. El Metodo de Crianza, Mantenimiento y su Contribución al Futuro de la Investigación Cientifica en Epidemiologia Nacional

Eduardo A. Gomez L.

ABSTRACT. Dada la importancia que tiene el estudio de la transmisión de la leishmaniasis se proyectó y desarrolló este trabajo, encaminado a la cría de phlebotomus en el laboratorio para trabajos de experimentación. Se capturó un buen número de "progenitoras silvestres", y en frascos adecuadamente preparados con yeso húmedo, se las trasladó al laboratorio conjuntamente con machos de la misma especie escogida (*Lu. trapidoi*), para encerrarlos en una cámara especial para la alimentación y cópula. Las hembras grávidas fueron conservadas en frascos igualmente acondicionados hasta la oviposición, quedando luego los huevos depositados en los mismos recipientes, y guardados en cámara húmeda durante el tiempo de realización de la metamorfosis completa. A partir de 50 hembras grávidas obtuvimos 1,022 huevos, 706 larvas, 510 pupas y 498 adultos, quedando después de seis semanas completamente estudiado el ciclo evolutivo *in vitro* de *Lu. trapidoi*. A partir de la eclosión de los huevos las larvas fueron alimentadas con heces de conejo secas y pulverizadas.

Japanese Journal of Tropical Medicne and Hygiene, 15 (1), 1987, 7-15

8. Leishmaniasis in Different Altitudes on Andean Slope of Ecuador

Yoshihisa Hashiguchi, Eduardo A. Gomez L., Vicenta Vera De Coronel, Tatsuyuki Mimori and Masato Kawabata

ABSTRACT. An epidemiological survey was performed in a leishmaniasis-endemic area along highway which was established about 15 years ago on the Andean slope of Ecuador; the area ranged from 300 m to 1,500 m above sea level. In general survey, 64 (14.3%) of the 446 subjects examined were positive for leishmanial signs. In order to know leishmanial infections in relation to the altitudes of dwelling sites of subjects, analysis was made on 224 children with 5 to 15 years of age. At 4 different sites with 500 m, 1,000 m, 1,300 m and 1,500 m above sea level, the infection rates of the subjects from the individual sites were 17.4, 18.8, 5.6 and 8.8%, respectively. A statistically significant difference was recognized between the altitudes, 500-1,000 m and 1,300-1,500 m ($0.01<p<0.05$, $x^2 = 5.314$), but not between 500 m and 1,000 m and between 1,300 m and 1,500 m. Leishmanial infections of the children who came from forest and highway areas were compared in each altitude. But no significant difference was found between forest and highway dwellers at any study sites.

Annals of Tropical Medicine and Parasitology, 81 (6), 1987, 681-685

9. The Relationship between Severity of Ulcerated Lesions and Immune Responses in the Early Stage of Cutaneous Leishmaniasis in Ecuador

Tatsuyuki Mimori, Yoshihisa Hashiguchi, Masato Kawabata, Eduardo A. Gomez L. and Vicenta Vera De Coronel

ABSTRACT. The relationship was examined between the severity of ulcerated lesions and immune responses in 19 Ecuadorian patients in the early stages of New World cutaneous leishmaniasis. As an immunological assay, the humoral immune response was assessed by enzyme-linked immunosorbent assay (ELISA) and the cell-mediated response by delayed type skin test for leishmanial antigen (leishmanin test). There was a statistically significant correlation ($r = 0.61$, $p<0.01$) between the total area of ulcerated lesions and the reciprocal titre of ELISA in identical subjects. However, no significant difference was observed in the ELISA titre between patients with a single lesion and those with multiple lesions ($x^2 = 7.06$, $df = 5$, $p>0.01$). These results suggest that the severity of ulcerated lesions relates to the activation of both the humoral and cell-mediated immune systems in the early stage of New World cutaneous leishmaniasis.

Kochi, Japan: Kyowa Printing, Research Report Series No. 1, 1987, 1-174

10. Studies on New World Leishmaniasis and its Transmission, with Particular Reference to Ecuador

Yoshihisa Hashiguchi (ed.)

ABSTRACT. In the present text, results of field studies on several aspects of leishmaniasis epidemiology in Ecuador are presented. These aspects include parasite isolation and characterization, detection of natural infections of sand flies and mammalian hosts with *Leishmania*, and evaluation of immunological tools in the epidemiological survey. In addition, current knowledge of Ecuadorian leishmaniasis and its endemicity were reviewed. The following points were extracted from each chapter of this text.

Leishmaniasis investigations in Ecuador

Prior to 1982 the principal leishmaniasis research activity in Ecuador was limited to case reports and/or the treatment of patients in medical centers or hospitals, although some studies of vector entomology had been done by several investigators. Thereafter, transmission studies were initiated by the present workers, who detected natural infections of sand flies and wild mammals with leishmanial parasites in endemic areas. According to the articles published in Ecuador to date, there may be three or four clinical forms of the disease: cutaneous cases (CL), ca. 93% of the total; mucocutaneous (MCL), ca. 6 or 7%; and visceral (VL) and diffuse cutaneous ones (DCL). The last two forms have not yet been parasitologically proven in the country. Analysis of the data accumulated in medical institutions revealed that the disease had a country-wide distribution in Ecuador.

Ecology of areas endemic for leishmaniasis

The Andes divide the country into three natural regions: the Pacific coast including the Andean slope, the Andean and the Amazonian region. The majority of leishmaniasis cases reported was from the Pacific coast, followed by the Amazon. A few cases were also observed in the Andean highland or the mid-Andes. In the text, ecological features of each region relating to the mammalian and sand fly fauna, are taken into special consideration in discussion of disease transmission.

Parasite isolation and their characterization

We have isolated eight stocks, five from humans and three from wild mammals, in the present study. Identifications based on results of serodeme typing using monoclonal antibodies revealed that three of the five from humans are *Le. b. panamensis* (MHOM/EC/87/G05, MHOM/EC/87/G06 and MHOM/EC/87/G07) and all three from wild mammals are *Le. m. amazonensis* (MSCI/ EC/87/G02, MPOT/ EC/87/G03 and MTAM/EC/87/G04). The remaining stocks from humans require further investigation until they are fully characterized. Results of this will be reported elsewhere.

Natural infections of sand flies and wild mammals

One species of *Lutzomyia, Lu. gomezi*, was added to the list of Ecuadorian leishmaniasis vectors, in addition to the two known vector species, *trapidoi* and *hartmanni*. With regard to reservoir hosts, one species, *Tamandua tetradactyla*, was newly implicated. Of these other mammal species, *Potos flavus*, *Sciurus vulgaris* and *Choloepus h. didactylus*, which had already been listed as leishmaniasis reser-

voirs, the first two mammalian species were also positive for leishmanial parasites in the current study. A search for leishmaniasis reservoir hosts was also made by the immunological method using counter immunoelectrophoresis (CIE) in this study. The CIE technique revealed that the tissue extracts (antigen) of three arboreal species, *Didelphis marsupialis*, *Caluromys lanatus* and *Choloepus h. didactylus*, reacted immunologically with anti-leishmanial serum, producing precipitin lines. In the first two mammalian species, no natural infections with leishmanial parasites have parasitologically been observed. It was, however, suggested that these immunologically positive mammals play an important role as reservoirs of the disease in endemic areas of Ecuador.

Immunological diagnosis of the disease

The present immunological tools, skin test and ELISA, were highly sensitive and specific for cutaneous and mucocutaneous leishmaniasis in Ecuador. From the results obtained, it was concluded that these diagnostic method could be very useful in screening of the disease in epidemiological surveys.

Epidemiological findings

Andean leishmaniasis (uta) in Ecuador was first described from the mid-Andes (2,300 to 2,500 m above sea level). The suspected sand fly vector is *Lu. peruensis*, which was the only species collected during our field survey. No *Leishmania*-positive fly was found among 51 specimens dissected. In order to clarify epidemiological features such as human, reservoir and vector infections in this mid-Andes endemic area, a further investigation will be conducted by the present workers. Bacterial flora was isolated from highland and lowland leishmanial ulcers, in an attempt to determine the effect of bacterial concomitant infection on the development of the distinct skin manifestations. The prevalence rate of Gram-negative rods, but not Gram-positive cocci or anaerobic bacilli was apparently different between two types of ulcer, occurring in 18.2% of highland as opposed to 37.5% of lowland infections. Gram-negative rods were composed of such enterobacteria as *Escherichia*, *Serratia*, *Klebsiella* and *Enterobacter*. Histological examination showed inflammatory cell infiltrations mostly composed of small lymphocytes throughout the dermis in highland ulcers, while those from lowland cases restricted to the deep dermis. When the parasitologically-proven prospective leishmaniasis cases were reviewed, the most important period for transmission of the disease in Ecuador was considered to be during the rainy season, from October to April.

Most of the findings presented here can be considered as preliminary results of the investigation. Based on these basic data obtained, however, we hope to further elucidate the epidemiological features of leishmaniasis in the New World, with particular reference to Ecuador, in future studies.

Japanese Journal of Tropical Medicine and Hygiene, 15(2), 1987, 97-104

11. The Fate of *Leishmania braziliensis*, *L. donovani* and *Trypanosoma cruzi* in Diffusion Chambers Implanted into Hamsters and Mice -a Preliminary Study-

Yoshihisa Hashiguchi, Masato Furuya and Yoshisuke Okamura

ABSTRACT. *Leishmania braziliensis* and *L. donovani* were investigated for the transformation and survival in intraperitoneal (IP), subcutaneous (SC) and intrascrotal (IS) diffusion chambers implanted into hamsters and mice. For a comparison, *Trypanosoma cruzi* was also examined by using the same procedure. The 2 *Leishmania* species revealed an unexpectedly short survival time, and no transformation was observed in the parasites in chambers implanted into hamsters or mice. IS chambers seemed to provide a better condition for *L. donovani*, *L. braziliensis* and *T. cruzi*, as compared with IP and SC chambers in hamsters. In the study, no IS chambers were examined in mice because of too small size of the scrotum to insert the diffusion chamber. *T. cruzi* showed a considerably longer period of survival than *L. donovani* or *L. braziliensis* in mice, but not in hamsters. The trypanosome, *T. cruzi*, transformed from epimastigote to trypomastigote and amastigote in IP and SC chambers in mice. These results seemed to suggest that the factors responsible for the transformation and survival of the organisms might be greatly different between the 2 genera, *Leishmania* and *Trypanosoma*, and also between the 2 host animals, hamsters and mice.

American Journal of Tropical Medicine and Hygiene, 40(2), 1989, 154-158

12. Identification, using Isoenzyme Electrophoresis and Monoclonal Antibodies, of *Leishmania* Isolated from Humans and Wild Animals of Ecuador

Tatsuyuki Mimori, Gabriel Grimaldi, Jr., Richard D. Kreutzer, Eduardo A. Gomez L., Diane McMahon-Pratt, Robert B. Tesh, and Yoshihisa Hashiguchi

ABSTRACT. Six strains of *Leishmania* isolated from wild mammals and humans on the Pacific Coast of Ecuador were identified by isoenzyme electrophoresis and by their reactivity patterns to a cross-panel of specific monoclonal antibodies using a radioimmune binding assay. Single isolates from *Sciurus vulgaris*, *Potos flavus*, and *Tamandua tetradactyla* were identified as *Leishmania amazonensis*. Three other strains, isolated from cutaneous lesions of humans, were identified as *Leishmania panamensis*.

Japanese Journal of Tropical Medicine and Hygiene, 17(2), 1989, 149-155

13. Observations on the Validity of the Ovarian Accessory Glands of Seven Ecuadorian Sand Fly Species (Diptera: Psychodidae) in Determinating Their Parity

Hiroyuki Takaoka, Eduardo A. Gomez L., John B. Alexander and Yoshihisa Hashiguchi

ABSTRACT. Females of seven sand fly species caught on man in several leishmaniasis-endemic foci in Ecuador were examined to assess the value of the accessory gland secretions as an indicator of parity. It was found that parous females could be distinguished from nulliparous by the presence of granular secretions in the accessory glands in *Lutzomyia ayacuchensis*, probable vector of *Leishmania* in the Andean highlands of southern Ecuador. Examination of the female accessory glands was not a reliable method for determining parity in six other sand fly species caught in lowland areas, including *Lu. trapidoi, Lu. hartmanni*, and *Lu. gomezi*, three proven vectors of *Leishmania*, since glanular secretions were found in both parous and nulliparous females.

Nettai, 22, 1989, 68-82

14. A Brief Review of Central and South American Leishmaniasis, with Special Reference to Ecuador

Yoshihisa Hashiguchi

ABSTRACT. A brief review is given of recent developments in leishmaniasis research worldwide, including details of the transmission of the three clinical forms of the disease, *viz.*, cutaneous, mucocutaneous, and visceral one. Current knowledge of leishmaniasis in *Leishmania*-endemic regions of Ecuador is described, for each of the three geographical regions of the country, *i.e.*, Pacific coastal, Amazonian and Andean plateau. Particular emphasis is given to Andean leishmaniasis and its endemic area, a focus of the disease discovered by our field survey in 1986. Current leishmaniasis treatment methods such as perilesional administrations of antimonials and topical treatments such as thermotherapy and cream application are discussed, together with progress in the development of vaccines and new drugs. The continued importance of field studies in *Leishmania*-endemic areas is noted, these being necessary in understanding leishmaniasis epidemiology and in application of control measures. (In Japanese)

Nihon Iji Shinpo, No. 33397, 1989, 59-60

15. Leishmaniasis Research in Central and South America -Why Is It Necessary to Study Parasitic Diseases Which Are Not Prevalent in Japan ?-

Yoshihisa Hashiguchi

ABSTRACT. In the article an attempt is made to stimulate the interest of Japanese investigators in the field of parasitic and other infectious diseases. The author developed an understanding of the importance of leishmaniasis research in the Third world, through his own research experience on the disease in Ecuador and wanted to help promote a general understanding among medical workers on the necessity of international medical (research) collaboration in tropical regions of the world. In Japan, parasitic diseases have largely erradicated through the application of efficient control measures and sanitary improvements. This has resulted in a tendency for Japanese researchers to have little interest in parasitology and the control of parasitic diseases, at a time when research on these topics is urgently required in the Third world countries. (In Japanese)

Japanese Journal of Tropical Medicine and Hygiene, 17(4), 1989, 331-338

16. Epidemiological Survey of Leishmaniasis using Skin Test and ELISA in Ecuador

Masato Furuya, Tatsuyuki Mimori, Eduardo A. Gomez L., Vicenta Vera de Coronel, Masato Kawabata, and Yoshihisa Hashiguchi

ABSTRACT. The present study was designed to evaluate the intradermal skin test (ST) and the ELISA as diagnostic tools in the screening for Ecuadorian cutaneous and mucocutaneous leishmaniasis. The antigen for skin testing was prepared from ruptured promastigotes of *Leishmania braziliensis*. The ST and ELISA positive rates among 72 subjects with active dermal lesions were 81.1% (36/44) and 81.3% (52/64), respectively, while parasites were observed in 31 (44.9%) of 69 subjects presenting active lesions. In the parasites positive cases, all subjects proved to be positive for the two tests except for one in ST and two in ELISA. In 35 healed cases, the ST and ELISA positive rates were 86.2% (25/29) and 72.4% (21/29), respectively. On the other hand, the positive rate in subjects without clinical signs was only 3.8% in ST and 8.2% in ELISA. An epidemiological survey in Selva Alegre, Esmeraldas, revealed that among 115 inhabitants 38 were positive for the clinical signs, 10 active and 28 healed cases. Of these subjects 33 (86.8%) showed positive reactions against ST and/or ELISA. Based on the results obtained, therefore, we concluded that the present skin testing antigen and ELISA were very useful for the screening of leishmaniasis in the endemic areas of Ecuador.

Boletin de la Oficina Sanitaria Panamericana, 108(4), 1989, 296-307

17. Las Investigaciones sobre la Leishmaniasis en el Ecuador, 1920-1989

Yoshihisa Hashiguchi y Eduardo A. Gomez L.

ABSTRACT. Se examina brevemente el estado actual de los conocimientos sobre la leishmaniasis en el Ecuador, basándose en gran parte en la bibliografía publicada entre 1920 — el año en que se describió el primer caso humano — y 1989. La enfermedad es endémica en 14 de los 20 departamentos del país. De 260 casos notificados, 239 (91.9%) eran de la forma cutánea, y 18 (6.9%), de la mucocutánea. Durante los 67 años transcurridos de 1920 a 1987, solo se registró un caso de la forma visceral y otro de la cutánea difusa. También se analizan los conocimientos actuales sobre los vectores y los huéspedes reservorios. En la acutualidad, se están estudiando muchas cepas de *Leishmania* aisladas durante 1982 y 1988 por los autores. Hasta la fecha, mediante la electroforesis de isoenzimas y el empleo de anticuerpos monoclonales, una parte de ellas ha sido identificada como *Leishmania amazonensis*, procedente de animales salvajes, y *Leishmania panamensis*, originaria de seres humanos.

Journal of Medical Entomology, 27(4), 1990, 701-702

18. Natural Infections with *Leishmania* Promastigotes in *Lutzomyia ayacuchensis* (Diptera: Psychodidae) in an Andean Focus of Ecuador

Hiroyuki Takaoka, Eduardo A. Gomez L., John B. Alexander, and Yoshihisa Hashiguchi

ABSTRACT. In the Andean town of Paute, Ecuador, 2 of 97 (2%) *Lutzomyia ayacuchensis* Caceres and Bianchi were found to be naturally infected with *Leishmania* promastigotes. The parasites were confined to the midgut of the sand fly, indicating they did not belong to the subgenus *Leishmania* (*Viannia*).

Memorias del Instituto de Investigaciónes en Ciencias de la Salud, 14, 1990, 128-133

19. Phlebotomes of Paraguay: species identification in three endemic areas (Diptera, Psychodidae, Phlebotominae)

Alba Inchausti, Yoshihisa Hashiguchi and Antonieta de Arias

ABSTRACT. Sand fly catch was performed in four sites of three leishmaniasis-endemic areas of Paraguay, using Shannon trap and protected human bait collections. A total of 606 females of the genus *Lutzomyia* were dissected to examine the natural infections with *Leishmania* promastigotes; only one of *Lu. whitmani* was positive for the parsite. The following 8 sand fly species were identified (% shows species composition): *Lu. migonei* (11.0%), *Lu. shannoni* (13.6%), *Lu. intermedia* (20.3%), *Lu. walkeri* (0.2%), *Lu. whitmani* (51.4%), *Lu. fisheri* (2.6%), *Lu. longispinosa* (0.7%) and *Lu. cortelezzi* (0.2%).

Kochi, Japan: Kyowa Printing, Research Report Series No. 2, 1990, 1-238

20. Studies on New World Leishmaniasis and its Transmission, with Particular Reference to Ecuador

Yoshihisa Hashiguchi (ed.)

ABSTRACT. The present text dealt with the results obtained from surveys carried out in different leishmaniasis-endemic areas of Ecuador, from epidemiological, vector entomological, immunological and dermatological point of view. Particular emphasis was given to a recently discovered autochthonous Andean highland leishmaniasis, and comparison of this disease form with others in the Pacific coast and Amazonian lowland Ecuador. Moreover, currently available techniques in molecular biology was briefly reviewed and evaluated on their application to future studies of leishmaniasis epidemiology in Ecuador. Potential control measures against the disease in the country were also considered. The results obtained are summarized as follows.

Leishmaniasis and its endemic area of Ecuador

In the text the relationship between human activities and ecological factors in each of the endemic areas was discussed in terms of the disease transmission. American cutaneous leishmaniasis is highly prevalent in the Pacific coast and Amazonian lowland regions, although mucocutaneous forms are more frequent in the latter than the former. In the Andean highland of Ecuador, a recently discovered new type of the disease was found, and its ecology was compared with that of the both lowland disease forms.

Leishmania *isolates from humans and animals and their characterization*

In the present study 18 *Leishmania* strains from the Pacific coast and Amazonian lowland patients and 11 from Andean highland were isolated. The isolates were precisely characterized employing serodeme, zymodeme and schizodeme analysis. The Andean parasites were identified as *Le. pifanoi*, while in the Pacific coast region *Le. panamensis* was found and in the Amazon, *Le. braziliensis*. A part of the present strains isolated, however, still remained unknown. In distinct human leishmaniasis-endemic areas, 194 wild and domestic animals were examined, by performing liver punctures, of which 14 or 7.2% of the total were positive for protozoans. A strain from Andean domestic dogs was identified as *Le. pifanoi* but the majority still remained unidentifiable in spite of a precise characterization method. *Leishmania* isolates from humans and wild animals were examined by restriction enzyme analysis of kinetoplast DNA (kDNA). From the results of fragment patterns, three isolates from cutaneous lesions of patients from the Pacific coast lowland region were identified as *Le. panamensis*. On the other hand, the isolates from three wild mammals from the same region were identified as *Le. amazonensis*.

Sand fly fauna and human leishmaniasis vectors in Ecuador

In eight Departments of Ecuador where human leishmaniases are endemic, the phlebotomine sand fly was sampled. A total of 40 species was collected, of which at least 11 represented new records for Ecuador. This record increased the number of sand fly species of Ecuador to 56. In the country, three sand fly species of the genus *Lutzomyia*, *trapidoi*, *hartmanni* and *gomezi*, hitherto, had been recorded as *Leishmania*-vectors. In the present study, *Lu. ayacuchensis* from Andean plateau, Paute, Department of Azuay was found to be positive for *Leishmania* promastigotes. These Andean parasites were confined to the midgut of the fly, suggesting that they did not belong to a *Le. braziliensis* complex species. Monthly examination of the natural infection with *Leishmania* and the biting activity of the sand fly, *Lu. ayacuchensis* was performed in Andean leishmaniasis-endemic area, Paute. The results revealed that there is a marked monthly variation in both natural infections and biting activity, of the flies in the area suggesting a high transmission intensity during the rainy season. The validity of the ovarian accessory glands of seven sand fly species from both the lowland and

highland Ecuador was examined. It was found that in highland species parous females could be distinguished from nullipars by the presence of granular secretions in the gland but the feature is of no value in determining parity of lowland species.

Immunological findings

Partially purified skin test antigen prepared from *Le. panamensis* promastigotes was evaluated in 17 Ecuadorian patients with active cutaneous lesions caused by *Le. braziliensis* complex. Based on the results obtained, it was concluded that crude antigen and two fractions (FA-1 and FA-2) were useful for diagnosis of cutaneous leishmaniasis in Ecuador. Moreover, it was estimated that at least 5 antigens, approximately 66, 55, 45, 28, and 26 kilodalton polypeptides, were related to a specific delayed-type hypersensitivity in the New World disease. Skin test using the crude antigen was performed in two endemic areas of Ecuador, lowland and highland regions. The intradermal responses of the subjects from the two regions were compared each other. Recently discovered Andean leishmaniasis and its ecology

During studies made in 1986 and 1988, 25 patients less than 10 years of age were found to be positive for *Leishmania* parasites, demonstrating abundant amastigotes in smears taken from small cutaneous lesions. The disease symptoms were clinically similar to those exhibited by cases of uta caused by *Le. peruviana* reported from Peru. However, the causative agent and vectors of the Ecuadorian form were completely different; the former is *Le. pifanoi* and the latter, *Lu. ayacuchensis*, though the reservoir seems to be rats and domestic dogs in the endemic area. From examination of our preliminary data, it appears that the transmission cycle of Andean leishmaniasis involves variable overlapping of two sets of biological entities, with the degree of overlap governed by climatic conditions. Changes in the incidence and frequency of human cases of Andean leishmaniasis in this endemic area are considered to be the result of migrations of sand flies and rodents (principal reservoir host) among the three habitat categories.

Clinical findings of leishmaniasis in Ecuador

Cutaneous changes due to leishmaniasis were thoroughly examined dermatologically, histopathologically and parasitologically in different endemic areas of Ecuador. Special emphasis was given to the comparison between the lowland and highland disease in the country. The most common manifestation in lowland cases was a large wet-ulcer which was clearly demarcated, had an indurated periphery and a wet base. On the other hand, the highland patients had a small papule with dry crust resembled the primary lesion (eschar) seen in tsutsugamushi disease. Mean age of patients was 20.47 years in lowland, while it was 1.96 years in highland. In the lowland disease, the longest duration of the eruption in our cases was 15 years, but almost all the cases healed within one year. Lymphnode swelling was frequently seen; the swelling was easy to palpate on the upper extremities and asymptomatic. The histological findings in lowland cases coincided with the granulomatous phase. Thus, the present study revealed a marked difference in clinical findings of leishmaniasis patients between the lowland and highland of Ecuador.

Comment on combating leishmaniasis in Ecuador

Presently available perilesional administrations of antimonials and topical treatments are discussed, together with current progress in the research into vaccine and new antileishmanial drugs. In future application of control measures, moreover, it is important to better understand the epidemiological features of the disease in each endemic area, because the New World form of the disease manifest themselves in a variety of cycles in different endemic areas. In additon to individual protections such as use of mosquito net and repellents, sanitary education through community campains for people in endemic areas of Ecuador is also important for prophylaxis and/or partial protection.

Strategies for future molecular epidemiology in Ecuador

A series of procedures for the preparation of specific DNA probes which may be applied for future epidemiological survey on leishmaniasis in Ecuador have been briefly summarized in the text.

Bulletin of the Pan American Health Organization, 25(1), 1991, 64-76

21. A review of leishmaniasis in Ecuador

Yoshihisa Hashiguchi and Eduardo A. Gomez L.

ABSTRACT. The current state of knowledge on Ecuadorian leishmaniasis was briefly reviewed, largely from previous literature reported during the period from 1920 when the first human case was described in Ecuador, to the present. Of the 20 Departments of the Republic of Ecuador, 14 are endemic for the disease. Out of 260 cases reported, 239 (91.9%) were cutaneous (CL) forms, while 18 (6.9%) were mucocutaneous (MCL) ones. Only one case each of visceral (VL) and diffuse cutaneous (DCL) forms was reported during 67 years from 1920 to 1987. In the text current knowledges of the vectors and reservoir hosts reported are also reviewed. Many strains of *Leishmania* isolated during 1982 and 1988 by the authors are currently under study. Up to date only a part of them was identified as *Le. amazonensis* from wild animals and *Le. panamensis* from humans by using isoenzyme electrophoresis and monoclonal antibodies.

Japanese Journal of Tropical Medicine and Hygiene, 19(29), 1991, 209-217

22. Evaluation and characterization of partially purified skin test antigens prepared from *Leishmania panamensis* promastigotes

Masato Furuya, Shigeo Nonaka, Eduardo A. Gomez L. and Yoshihisa Hashiguchi

ABSTRACT. The present study was designed to evaluate skin test preparations prepared from *Leishmania panamensis* promastigotes in 30 active cutaneous leishmaniasis patients. The crude antigen preparation (CA) used was 10,000 g supernatant of the parasites-homogenate. The soluble extract was further resolved into 4 preparations (FA-1 to -4) with the aid of a Sephacryl S-200 gel filtration. There was no significant difference in the positive ratio and the average induration size between CA (10 μg protein/test) and Montenegro's antigen (MA; 5×10^6 parasites/test). The reactivity of the delayed-type hypersensitivity to 10 μg dose of CA was shown with much the same intensity in the 25 μg dose of CA. In FAs (10 μg protein dose, except for 7.5 μg in FA-4), the positive ratio was as follows: 90.0% in FA-1, 77.8% in FA-2, 75.0% in FA-3 and 37.5% in FA-4. The positive ratio and the intensity of skin test response in FA-4 were remarkably low in comparison with those in CA or MA. Significant difference was found in the intensity of response between FA-3 and CA or MA. Based on these results, therefore, we concluded that 10 μg protein dose of CA of *L. panamensis* and same dose of the fractionated preparations, FA-1 and -2, were very suitable for the diagnosis of cutaneous leishmaniasis in endemic areas of the New World. Furthermore, it was estimated that at least some or all of the 5 proteins, approximately 66, 55, 45, 28, and 26 kD, were related to a specific delayed-type hypersensitivity in cutaneous leishmaniasis of the New World.

American Journal of Tropical Medicine and Hygiene, 44(2), 1991, 205-217

23. Andean Leishmaniasis in Ecuador Caused by Infection with *Leishmania mexicana* and *L. major*-like parasites

Yoshihisa Hashiguchi, Eduardo A. Gomez L., Vicenta V. de Coronel, Tatsuyuki Mimori, Masato Kawabata, Masato Furuya, Shigeo Nonaka, Hiroyuki Takaoka, J. Bruce Alexander, Aida M. Quizhpe, Gabriel Grimaldi, Jr., Richard D. Kreutzer and Robert B. Tesh

ABSTRACT. Between 1986 and 1988, epidemilogic studies were carried out in a small rural community in an Andean region of Ecuador, where cutaneous leishmaniasis is highly endemic. A total of 25 human cases, positive for *Leishmania* parasites by culture and/or smear, were examined. Fourteen of the cases were in infants less than one year of age, suggesting intradomiciliary transmission of the disease. Clinically, many of these cases were similar to descriptions of "uta," a form of cutaneous leishmaniasis which occurs in Andean regions of Peru and is reportedly caused by *L. peruviana*. Of the 11 positive cultures obtained from human cases in the present study, eight were identified by molecular characterization as *L. mexicana* and three were identified as *L. major*-like. Two additional isolates of *L. mexicana* were also made from an infected dog and from a sand fly, *Lutzomyia ayacuchensis*, living in the region, thus implicating the latter species as possible reservoir and vector, respectively, of *L. mexicana* in this highland community. The significance and validity of recent isolates of *L. major*-like parasites from the New World are also discussed.

Transactions of the Royal Society of Tropical Medicine and Hygiene, 85(5), 1991, 592-594

24. Cutaneous Leishmaniasis in South-eastern Paraguay: a Study of an Endemic Area at Limóy

Yoshihisa Hashiguchi, Ofelia Arias, Domingo Maciel, Julio Mansur, Masato Furuya and Masato Kawabata

ABSTRACT. An epidemiological study was performed on leishmaniasis in a newly established community in south-eastern Paraguay. 149 persons, of 172 inhabitants, were thoroughly examined by clinical, parasitological and immunological (leishmanin skin test) examinations. 88 of those examined (59%) were clinically positive for dermal and nasal (mucosal) lesions or dermal scars, while 74 (50%) were positive by the leishmanin test. Of the 88 persons, 66(75%) were positive for both leishmanial (dermal and nasal) signs and skin test; these subjects were therefore considered to be leishmaniasis patients. Most of the patients (60%) had a single dermal lesion. Among the 66 leishmaniasis patients, serious mucosal (nasal septum) lesions were observed in the 41 subjects: 2 had destruction of the septum, 8 had ulceration and 31 had erythema. In this community the persons with dermal and/or nasal problems had been treated with meglumine antimonate (Glucantime®), without any precise diagnosis having been made by parasitological or immunological examination. The socioeconomical and sociomedical points of view aspects are discussed.

Annals of Tropical Medicine and Parasitology, 85(4), 1991, 407-411

25. Monthly Variation in Natural Infection of the Sandfly *Lutzomyia ayacuchensis* with *Leishmania mexicana* in an endemic focus in the Ecuadorian Andes

Eduardo A. Gomez L. and Yoshihisa Hashiguchi

ABSTRACT. In order to collect information on the role of *Lutzomyia ayacuchensis* in the transmission of leishmaniasis in a newly discovered Andean endemic focus in Ecuador, a longitudinal field study was carried out over 13 months. Monthly dissections were made of a minimum of 200 anthropophilic sandflies, collected at night during the month. A total of 2600 flies was separated from a small number of *Lu. osornoi*, another anthropophilic species in the area, and dissected; 95(3.65%) were naturally infected with *Leishmania mexicana* promastigotes. The parasites were always located in the sandfly midgut. The current study revealed a marked monthly variation both in natural infections with *Leishmania* and in biting activity of sandflies in the endemic area, demonstrating a high transmission rate during the period from the early rainy season to the early or mid dry season (February to July).

Memorias do Instituto Oswardo Cruz, 87, 1992, in press

26. Description of *Leishmania equatorensis* sp. n. (Kinetoplastida: Trypanosomatidae), a New Parasite Infecting Arboreal Mammals in Ecuador

Gabriel Grimaldi, Jr., Richard D. Kreutzer, Yoshihisa Hashiguchi, Eduardo A. Gomez L., Tatsuyuki Mimori and Robert B. Tesh

ABSTRACT. Characterization is given of a new parasite, *Leishmania eqatorensis* sp. n., which was isolated from the viscera of a sloth (*Choloepus hoffmanni*) and a squirrel (*Sciurus granatensis*), captured in humid tropical forest on the Pacific Coast of Ecuador. Data based on biological and molecular criteria, as well as numerical zymotaxonomical analysis, indicate that this parasite is a new species of the *L. braziliensis* complex. *L. equatorensis* is clearly distinguishable from all other known species within this complex, using the following molecular criteria: reactivity patterns with specific monoclonal antibodies, isoenzyme electrophoresis, and restriction endonuclease fragment patterns of kinetoplast DNA (k-DNA).

Memorias do Instituto Oswardo Cruz, 87, 1992, in press

27. New Records of Phlebotomine Sand Flies (Diptera: Psychodidae) from Ecuador

J. Bruce Alexander, Hiroyuki Takaoka, Yuki Eshita, Eduardo A. Gomez L. and Yoshihisa Hashiguchi

ABSTRACT. The phlebotomine sand fly fauna of Ecuador was surveyed in two 3-month collecting trips made in 1988 and 1990. A total of 12 provinces were visited, including three (Bolivar, Loja and Morona Santiago) from which no previous records of phlebotomines existed. Forty-six species were collected, 13 of which, together with 1 subspecies and 1 genus (*Warileya*) represented new records for the country. This survey increases the known number of species in Ecuador to 60. The distribution of Ecuadorian sand flies is discussed in the light of these new findings.

Japanese Journal of Tropical Medicine and Hygiene, 20 (1), 1992, 11-21

28. Ultrastructural Studies on Cutaneous Leishmaniasis in Ecuador

Abdul Manan Bhutto, Shigeru Okada, Shigeo Nonaka, Eduardo A. Gomez L. and Yoshihisa Hashiguchi

ABSTRACT. Ultrastructural observations were made of lesions of three Ecuadorian patients with cutaneous leishmaniasis. Parasites were located both within the macrophages, either inside the intracytoplasmic vacuoles (parasitophorous vacuoles) or free in cytoplasm and outside host cells. Amastigotes were rounded or oval with a mean length of 2.62 μm (±0.17 S.D.) and mean width of 2.18 μm (± 0.28 S.D.). Parasites showed degeneration intracellularly both within the vacuoles and in the cytoplasm of macrophages. Lymphocytes were seen in close contact with parasitized macrophages as well as directly attached to the parasites. Furthermore, spongiotic vesicle was observed in the epidermis where *Leishmania* parasites were found, surrounded by lymphocytes and other mononuclear cells. Amastigotes attached to mononuclear cells were also observed inside and between the keratinocytes. Mononuclear cells containing melanin granules showed amastigotes in their cytoplasm.

Annals of Tropical Medicine and Parasitology, 86, 1992, in press

29. Phlebotomine Sandfly Species and Examinations of Their Infection with *Leishmania* in Paraguay

Yoshihisa Hashiguchi, Tom Chiller, Alba Inchausti, Antonieta de Arias, Masato Kawabata and John Bruce Alexander

ABSTRACT. Nine species of sandflies, *Lutzomyia* (*Nyssomyia*) *whitmani* (Antunes and Countinho), *Lutzomyia* (*Nyssomyia*) *intermedia* (Lutz and Neiva), *Lutzomyia* (*Psathyromyia*) *shannoni* (Dyar), *Lutzomyia migonei* (Franca), *Lutzomyia* (*Pintomyia*) *fischeri* (Pinto), *Lutzomyia* (*Pintomyia*) *pessoai* (Countinho and Barretto), *Lutzomyia cortelezzii* (Brethes), *Lutzomyia walkeri* (Newstead) and *Lutzomyia* (*Trichopygomyia*) *longispinus* (Mangabeira), were caught, by human bait and Shannon trap, in four areas of Paraguay hyper-endemic for human leishmaniasis. *L. whitmani* and *L. intermedia* were the predominant species. All the species collected were found to be anthropophilic. Hindgut infections with leishmanial promastigotes were observed in only one (0.38%) of the 266 *L. whitmani* dissected. No *L. intermedia* were found infected, giving an overall infection rate of one (0.16%) of 615 flies dissected. The results indicate a very low rate of natural infection in endemic areas of Paraguay.

Nishi Nihon Hihuka, 1992, in press Successful treatment with intralesional injections of meglumine antimonate for cutaneon leismanilsis

30. Successful Treatment with Intralesional Injection of Meglumine Antimonate for Cutaneous Leishmaniasis

Motoi Takenaka, Taro Ohgami, Yoshihisa Hashiguchi, and Shigeo Nonaka

ABSTRACT. A 35 year-old male patient, had a walnut-size erythema with induration like a bank on his left upper arm. There was a nut-size ulcer at the center of the erythema. He had been interned at a desert in southern Iraq from August to November, 1990. In November, he noticed a insect-bite-like eruption on his left arm. The eruption had got worse against a therapy. The patient visited to our hospital on April 2nd, 1991. A huge amount of amastigote-like bodies of *Leishmania* was recognized in the smear specimen taken from the edge of the ulcer. A biopsy of the skin lesion revealed many histiocyte-like cells that had many granules in upper dermis. We successed cultivation of *Leishmania* parasites isolated from the skin lesion. They were identified as *Leishmania major* by zymodeme analysis. Initially, an external remedy consisted of meglumine antimonate and Povidone iodine was used, but it was not effective. Therefore, intralesional injections of meglumine antimonate were done. After 10 injections, the ulcer and erythema were healed. The side-effect was limited to some localized pain following injection. Thus, in this case intralesional injections with meglumine antimonate were highly effective against the ulcerative lesion, demonstrating no serious side effect. (In Japanese with English summary)