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

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

edited by

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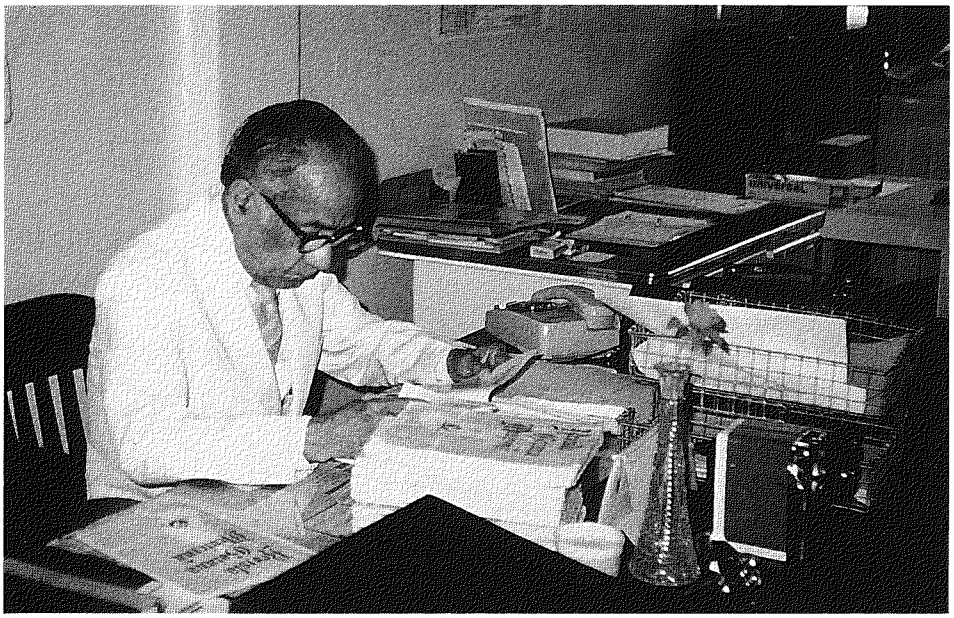
The present text reports on the data and materials
mainly collected during the period from 1992
to 1993 in Ecuador, South America

The leishmaniases have recently been shown to be far more prevalent and of greater public health importance than was previously recognized. Rough estimates suggest that some 350 million people are at risk of acquiring the infection and that approximately 12 million are currently infected. This represents a significant burden for the health services of the countries affected, which need to establish effective control programmes as part of the primary health care system. (World Health Organization, 1990, Control of the leishmaniases)



(Photo by Y. Hashiguchi)

Dedicated to late Dr. Jose Daniel Rodriguez M. on the 10th anniversary of his decease

Dr. Rodriguez's distinguished and long research career had been devoted to the study of many parasitic diseases caused by protozoons and helminths in Ecuador. Despite his medical background and dedication to tropical medicine, his special interest had been and remained wide-ranging, including the vector entomology of leishmaniasis, Chagas' diesease and malaria.

(Eduardo A. Gomez L. and Yoshihisa Hashiguchi, January 1994, Guayaquil, Ecuador)

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Plate 1. Landscape of an area endemic for Andean leishmaniasis, Huigra, Department of Chimborazo, Ecuador. **Above**, our field laboratory and its surrounding landscape in the endemic area of the Andes. **Below**, the center of town where Huigra station is located; the train is a main transportation system in the area.

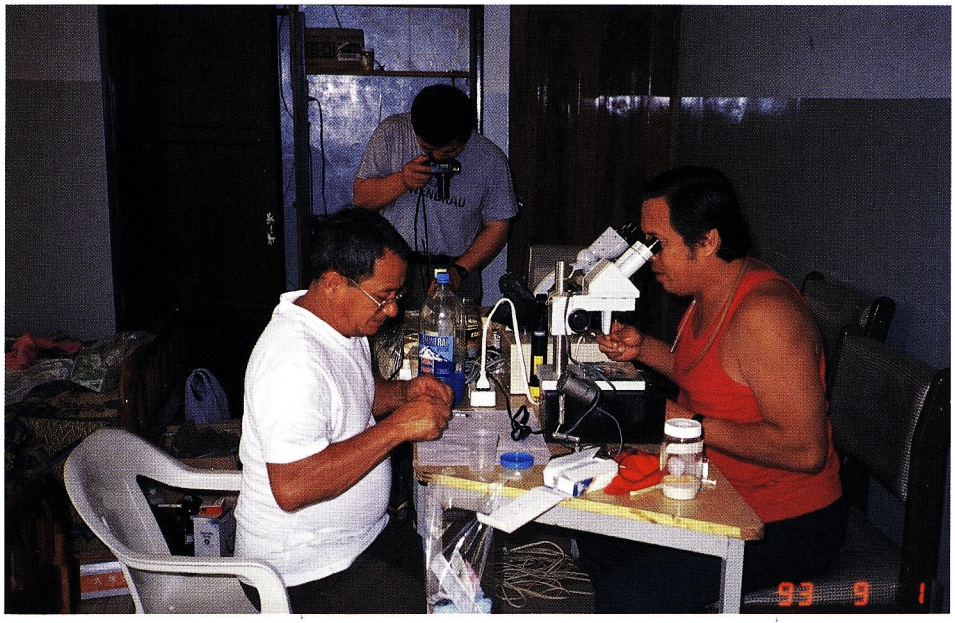

Plate 2. Showing, a part of our field activities in areas endemic for American cutaneous leishmaniasis. **Above**, dissections of vector sandflies of the disease. **Below**, a vistit to a primary school situated in the endemic area, Naranja Pata, Department of Chimborazo, Ecuador.

Foreword

Al revisar el material preparado para la publicacion de nuestro cuarto libro sobre la leishmaniasis en el Ecuador, me detengo a reflexionar sobre cuanto hemos trabajado, y me pregunto a la vez si lo que hemos logrado llena nuestras aspiraciones, o falta mucho por hacer todavía para alcanzar nuestro objetivo.

Buscando la respuesta pienso primero en cuánto hemos viajado a lo largo y ancho del Ecuador en la búsqueda activa de casos humanos, reservorios y vectores de la enfermedad. No es posible establecer exactamente cuántos miles de kilometros hemos recorrido yá, en nuestro ir y venir por las carreteras ecuatorianas, buenas y malas, seguras y peligrosas, desde 1982 hasta hoy; cuántas agotadoras horas a lomo de mulas y caballos subiendo lodosas y resbaladizas montañas, o simplemente caminando por remotos y rudimentarios caminos, o a lo largo de la vía férrea, en nuestra infatigable labor de estudiar la transmisión de la leishmaniasis en sus habitats naturales de la Costa, Sierra y Oriente.

Entonces pienso tambien en las decenas de miles de flebotomos (lutzomyias) capturados en tantas noches de riesgoso y minucioso trabajo realizado en tantos y tantos lugares de las montañas de nuestro país. Y luego la disección cuidadosa para su identificación y determinación de infeccion natural y aislamiento del parásito. Cuantos centenares de mamíferos estudiados para detérminar su condicion o no de reservorios. Animales silvestres, domésticos y peridomésticos, todos han pasado por nuestras manos y las lentes de nuestros microscopios.

I luego pienso en la gente, los enfermos, la gran mayoría de humilde condición social y económica; cuantos hemos atendido, cuantos medicamentos distribuidos y administradós gratuitamente; es asombrosa la cantidad de gente qué nos conoce, nos aprecia y agradece por la atención recibida. Ellos seguramente no comprenden la importancia de nuestro trabajo, no alcanzan a entender qué nos impulsa a viajar tanto y a tan remotos lugares solo para curar sus "llagas"; no se imaginan siquiera que han dado mucho más de lo que han recibido, no saben que la información epidemiológica, clínica y parasitológica que de ellos hemos obtenido, es invalorable, no solo porque es parte del fabuloso tesoro de la ciencia, sino que también es importante contribución para estructurar el beneficio futuro de la salud de la gente del compo.

Nosotros estamos eternamente agradecidos con nuestros pacientes. Gracias a su buena voluntad y colaboración, hemos podido realizar nuestro trabajo. Estoy seguro de no equivocarme cuando digo que ellos han dado mucho más de lo que han recibido.

Sigo meditando y llego a la inevitable pregunta: ¿Hemos obtenido buennos resultados?; busco la respuesta en las decenas de artículos y cuatro libros publicados; es indudable que hemos obtenido buenos resultados; actualmente, el mundo entero conoce la situación epidemiológica de la leishmaniasis en el Ecuador. Nuestras publicaciones se han distribuido por todas partes del planeta. Hemos incriminado a varias especies de *Lutzomyia* como probables vectores de la enfermedad; numerosos mamiferos han sido señalados como reservorios; se han aislado e identificado numerosas cepas de *Leishmania,* de insectos, animales y seres humanos, usando sofisticados metodos bioquímicos e inmunológicos; hemos avanzado mucho en el estudio de la biología de los vectores y en la ecologia y epidemiologia de los focos endemicos de la enfermedad. Ademas de lo anterior hay todavía muchas otras cosas que sería largo enumerar. Pero tal vez los más importante, es que nuestro grupo de investigación es cada vez más grande y experimentado; 43 investigadores de Japon (15), Ecuador (15), Paraguay (3), Estados Unidos (3), Peru (2), Escocia (1), Francia (1), Canada (1), Brasil (1) y Pakistan (1) participan rotativamente en las investigaciones que desde 1982 se han venido realizando. Pienso casi sin temor a equivocarme, que considerando a todos los grupo de investigación que se dedican al

estudio de la leishmaniasis en el mundo, el nuestro es el más numeroso, con un solo Jefe, el Dr. Yoshihisa Hashiguchi, que ha sabido dirigir al grupo con verdadera democracia científica.

Pero ha pesar de la gran cantidad de información obtenida y publicada, estoy seguro de que el objetivo final, es decir el control futuro de la enfermedad, está lejano todavía, no solo para nuestro país, sino para todos aquellos donde existe la leishmaniasis; más sin embargo, estoy seguro qué nuestro grupo magistralmente dirigido por el Dr. Hashiguchi, seguirá trabajando sin descanso hasta cuando sea posible, y por que nó, hasta lograr, tarde o temprano, nuestro objetivo final.

Hasta que ese día llegue, los habitantes de las zonas rurales ecuatorianas, seguiran viendonos pasar en carro, a pié o a caballo, en invierno o en verano, en el dia o en la noche, en la Costa, la Sierra o el Oriente, buscando incansablemente en el laberinto de la naturaleza, la información que nos permitirá un día, al fin, descifrar los ultimos acertijos que ante la ciencia plantea la leishmaniasis.

Eduardo A. Gomez L., M.D.
16 de Febrero, 1994
"Hotel Cachari", Babahoyo,
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Preface

The first phase of studies of leishmaniasis and its transmission in Ecuador was commenced in 1982 and continued until 1984, with the support of the Ecuadorian Ministry of Public Health and the Japan International Cooperation Agency (JICA). During this preparatory phase, we strongly felt that more detailed investigation of the disease should be done, in order to accumulate epidemiological data in different endemic areas of that country. Fortunately, we were able to carry out a continued study of leishmaniasis in Ecuador after 1986, through the financial support of the Ministry of Education, Science and Culture, Japan. In our field work, to date, we have principally aimed at obtaining a better understanding of epidemiological characteristics, including the mode of transmission of the disease in each endemic area of Ecuador. Therefore, human cases, sandfly vectors and mammalian reservoirs have been thoroughly examined, as well as any other factors relating to the transmission of leishmaniasis.

The results of our investigations including information on the causative agent, *Leishmania* spp., vector sandflies, *Lutzomyia* spp. and clinical forms of the disease were summarized in the past publications, Research Report Series Nos. 1, 2 and 3, entitled as "Studies on leishmaniasis and its transmission, with particular reference to Ecuador", appeared in 1987, 1990 and 1992, respectively. The current report, "Series No. 4", mentions the results of our studies of leishmaniasis obtained from field survey in Ecuadorian leishmaniasis-endemic areas and/or laboratory works during 1992 and 1993. Much of the materials and data collected have yet to be examined and analyzed. The results will be published in detail elsewhere at a later date, under the authority of all research workers participated in the study. A further intensive study of leishmaniasis and its transmission will be continued from 1994 onwards, with the main intention of employing molecular biological techniques to elucidate epidemiological and clinical features of leishmaniasis and its transmission in the New World.

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

Introduction

Protozoon parasites of the genus *Leishmania**, an obligate unicellular organism, produce a wide spectrum of clinical infections in both humans and vertebrate animals. In humans, clinical leishmaniasis ranges from a simple, often self healing cutaneous form to those producing destructive mucocutaneous ulcers of nasopharynges, uncurative diffuse cutaneous lesions, and a visceral form known as kala-azar, the sever chronic infection of the reticuloendothelial system, which is often fatal if left untreated. The disease is endemic in many tropical and subtropical regions in the Old and New World, and is classified as one of the six diseases selected by World Health Organization (WHO) for its special programme for Research and Training in Tropical Diseases, because of its importance (WHO, 1984). It is estimated that there are more than 12 million cases world-wide with about 400 thousand new cases recorded each year (Marinkelle, 1980); this number is however considered to be greatly underestimated. Leishmaniasis is transmitted by a tiny blood sucking female insect, the phlebotomine sandfly, of the genus *Lutzomyia* in the New World and by the genus *Phlebotomus* in the Old World. The protozoan parasite, *Leishmania*, has two morphological forms in the life cycle; amastigotes in the reticuloendotherial cells of vertebrates and promastigotes in the gut of vector sandflies. In nature, the life cycle is usually maintained among wild mammals and sandflies, and man contracts the disease (zoonosis) when he enters endemic areas for purposes such as colonization, road construction, plantation, agriculture, and etc.

Ecuador is one of the small countries in South America through which runs the equator; Colombia borders to the north and Peru borders to the southeast. The Andes mountain range crosses the country from north to south. It rises to altitudes of more than 5,000 meters above sea level and divides the country into the following three natural regions: a) the Pacific coastal regions, b) the Andes, and c) the Amazonian region extending eastward. The country has about 8,000,000 inhabitans, 4,160,000 of which correspond to the Pacific coastal regions, 3,160,000 live in the Andean highland, and the remaining 230,000 live in the Amazonian lowland (Teran, 1984). Each region of the country has specific features pertaining to the terrain, environment, and form of living of the inhabitants.

In Ecuador, leishmaniasis was first reported in 1920 by Valenzuela (Rodriguez, 1974) more than 70 years ago; however it has remained one of the least studied of Ecuadorian tropical diseases until recently. For many years the principal activity of investigation on the disease has been involved with clinical diagnosis, and this produced some eventually confirmed case reports. During the eleven years from 1982 to 1992, we have visited and studied almost all of the suspectable endemic foci of leishmaniasis in Ecuador, and also reviewed clinical cases reported in the literature. It was found that the disease is widespread in most provinces and is a considerable health problem in the country (Hashiguchi, 1987, 1990, 1992; Hashiguchi and Gomez, 1991, 1992). When the known endemic zones were separated into departments, nine are located in the Pacific coastal region: Esmeraldas, Los Rios, Manabi, Guayas, Cotopaxi, Pichincha, Bolivar, Cañar and El Oro, two are situated in the Andes: Azuay and Chimborazo, and four in the Amazonian region: Napo, Pastaza, Molona Santiago, and Zamora Chinchipe.

Regarding the causative agent of leishmaniasis in Ecuador, until 1987 when our research group

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* Scientific names of parasites in this paper 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, 2-6 July 1984 (Rioux, 1986).

began to characterize Ecuadorian isolates, it was only called *Leishmania* sp. or *L. (Viannia) braziliensis* mainly based on the following factors: clinical features of patients, geographic localization, behaviors in *in vitro* and *in vivo* cultures and serologic examinations. Recently, however, many *Leishmania* strains were isolated from patients, sandflies and mammalian reservoirs at different endemic areas of the country, and they were specifically identified: seven species of the genus *Leishmania* in the Pacific coastal region, *viz., L. (V.) braziliensis, L. (V.) panamensis, L. (V.) guyanensis, L. (Leishmania) amazonensis, L. (L.) mexicana, L. (L.) major*-like and *L. (V.) equatorensis*, two in the Andes, *L. (L.) mexicana* and *L. (L.) major*-like, and one in the Amazonian region, *L. (V.) braziliensis* (Mimori *et al.*, 1989; Hashiguchi, 1990, 1992; Armijos *et al.*, 1990; Grimaldi *et al.*, 1992). The parasites have been identified by molecular techniques such as zymodeme, schizodeme and serodeme analysis (Hashiguchi *et al.*, 1992). Recently, moreover, molecular karyotypes of some of the Ecuadorian *Leishmania* isolates were analyzed by pulsed field gel electrophoresis and Southern blot hybridization (Katakura *et al.*, 1993).

In the search for clinical forms of leishmaniasis in different endemic areas of all over the country, a great number of single or multiple cutaneous ulcers and a very small number of mucocutaneous cases were observed, but no visceral form was found in spite of our careful and intensive examinations. Bibliographically, only one case each has however been reported of visceral and diffuse cutaneous leishmaniasis without demonstration of the causative agents, *Leishmania* (Zerega, 1961;Rodriguez, 1974). In this issue, we are going to mention two parasitologically proven, interesting clinical cases; a diffuse cutaneous form and a generalized one with more than 300 ulcers on the body surface. Furthermore, the present report deals with the results obtained from surveys carried out at different areas endemic for leishmaniasis in Ecuador, including the Andean plateau. The study was done from various standpoints, such as epidemiology, parasitology, vector entomology, dermatology, immunology and molecular biology.

Yoshihisa Hashiguchi
Eduardo A. Gomez L.

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Chapter 1

Molecular Parasitology

1. Karyotype Similarity of *Leishmania* Isolates from Patients, Sandflies, and a Domestic Dog, Identifying the Major *L.* (*L.*) *mexicana* Strain as an Agent of Cutaneous Leishmaniasis in the Ecuadorian Andes

ABSTRACT. DNA karyotype of *Leishmania* isolates in three different towns in the Ecuadorian Andes was further examined by pulsed field agarose gel electrophoresis. Karyotype similarity was detected in twelve isolates; four human isolates and one canine isolate in Paute, four human isolates and one sandfly isolate in Huigra, and two sandfly isolates in Alausi. Horizontal distances between Paute and Alausi, and between Alausi and Huigra, are about 80 km and 20 km, respectively. Chromosomal DNA banding pattern of these isolates was characterized by an ordered chromosomal ladder, especially by the presence of four low molecular weight chromosomes of 220, 250, 280 and 325 kilobases. Since some of the isolates have been already identified as *L.* (*Leishmania*) *mexicana* by zymodeme and schizodeme analysis, these results suggest that the *L.* (*L.*) *mexicana* strain with a defined karyotype is widely distributed and a major agent of cutaneous leishmaniasis in the Andes of Ecuador.

Introduction

In our consecutive survey of leishmaniasis in Ecuador, we have discovered patients with cutaneous leishmaniasis in the Ecuadorian Andes. The causative *Leishmania* species was determined as *L.* (*Leishmania*) *mexicana* and *L.* (*L.*) *major*-like parasites by analyzing kinetoplast DNAs and isoenzymes of the isolates (Hashiguchi *et al.*, 1991). DNA karyotypes of these species were recently determined by pulsed field agarose gel electrophoresis (PFGE) and DNA banding pattern of *L.* (*L.*) *mexicana* could be easily distinguishable from that of *L.* (*L.*) *major*-like parasites (Katakura *et al.*, 1992, 1993). Thus, we take advantage of karyotype analysis by PFGE for identification of new isolates in epidemiological studies in Ecuador, especially in the Andean regions. In the present study, we examined DNA karyotypes of additional *Leishmania* isolates from patients and sandflies in different regions in the Andes. Taken together with previous observations, we specified the *L.* (*L.*) *mexicana* strain with a defined karyotype as a major agent of cutaneous leishmaniasis in the Ecuadorian Andes.

Materials and Methods

Parasites

Details of twelve *Leishmania* isolates used in the present study are summarized in Table 1.1.1. Some of these isolates have been previously identified as *L.* (*L.*) *mexicana* by isoenzyme electrophoresis, kinetoplast DNA fingerprints (Hashiguchi *et al.*, 1991). Parasite isolation was performed as described before (Gomez *et al.*, 1987). In short, biopsy materials were obtained by aspiration of the margins of active skin lesions from patients or by the liver

Table 1.1.1. *Leishmania* isolates used in the present study

Species	Designation*	Geographic origin
Ecuadorian isolates		
L. (L.) mexicana		
	MHOM/EC/88/Paute1	Paute, Azuay
	MHOM/EC/88/Paute2	Paute, Azuay
	MHOM/EC/88/Paute29	Paute, Azuay
	MHOM/EC/88/Paute103	Paute, Azuay
	MCAN/EC/88/PauteInu2	Paute, Azuay
	IAYA/EC/92/ALI3	Alausi, Chimborazo
	IAYA/EC/92/ALI4	Alausi, Chimborazo
	MHOM/EC/92/HU1	Huigra, Chimborazo
	MHOM/EC/92/HU3	Huigra, Chimborazo
	MHOM/EC/92/HU4	Huigra, Chimborazo
	MHOM/EC/92/HU6	Huigra, Chimborazo
	IAYA/EC/92/HUI1	Huigra, Chimborazo
L. (L.) major-like		
	MHOM/EC/87/G-09	Quininde, Esmeraldas
WHO reference strains		
L. (L.) amazonensis		
	MHOM/BR/73/M2269	Para, Brazil
L. (L.) mexicana		
	MHOM/BZ/82/BEL21	Belize

*, Designation code: Host (M = Mammalia, CAN = *Canis familiaris*, I = Insecta, AYA = *Lutzomyia ayacuchensis*)

puncture from domestic dogs. The biopsy samples were inoculated into culture tubes with biphasic blood agar based USAMRU medium (Difco blood agar medium) and incubated at 25℃. When promastigotes were found in the gut of sandflies, the organisms were collected and inoculated into the nose or the footpads of hamsters. Several weeks later, biopsy materials were taken by aspiration from the animals and passed into the cultures.

Pulsed field agarose gel electrophoresis (PFGE)

PFGE was performed as described previously (Katakura *et al.*, 1992, 1993). In short, agarose blocks containing 1 x 10^9 promastigotes were treated with 1-2 mg/ml proteinase K (Sigma) and 1% sarcosyl (Sigma) in 0.5 M EDTA, pH 8.0 at 50℃ for 2 days. The resultant blocks were electrophoresed in 1.5 % agarose at 180 V in 0.5 x TBE (TBE = 90 mM Tris, 90 mM boric acid and 2 mM EDTA, pH 8.0) at 12℃ with a pulse interval of 60-70 for 36-37 hr using a turn-table type PFGE apparatus (Cross Field Gel Electrophoresis, ATTO Corp., Tokyo, Japan). After the electrophoresis, the gel was stained with 0.5 mg/ml ethidium bromide in 0.5 x

TBE for 20 min, destained with 0.5 x TBE for 30 min and photographed. Chromosomal DNAs of *Saccharomyces cerevisiae* (FMC) were used as the molecular standard.

Results and Discussion

In our previous paper, we reported a similarity of schizodeme profile of *L.* (*L.*) *mexicana* isolates among eight from humans (MHOM/EC/88/Paute1, 6, 7, 23, 25, 27, 29 and 103), one from a dog (MCAN/EC/88/PauteInu2) and one from a sandfly (IAYA/EC/89/ PAI1). These parasites were all isolated in Paute (elevation 2,300m-2,500m), a small village in the Andes (Hashiguchi *et al.*, 1991). Of these isolates, four human isolates (MHOM/EC/88/ Paute1, 27, 29 and 103), and the canine isolate were performed for karyotype analysis and revealed to be an identical chromosome banding pattern (Fig. 1.1.1 A, lanes 4-8) (Katakura *et al.*, 1993). Similarities in both kinetoplast DNA fingerprints and genomic DNA karyotypes of those isolates strongly suggest that the *L.* (*L.*) *mexicana* strains in Paute belong to a homologous population. The DNA karyotype of the Ecuadorian *L.* (*L.*) *mexicana* is featured by the presence of four low molecular weight chromosomes of 220, 250, 280 and 325 kb, in which chromosomes 1 and 4 showed strong ethidium bromide staining intensities. This chromosomal banding pattern was not seen in WHO reference strains of *Leishmania* including *L.* (*L.*) *mexicana* (MHOM/BZ/82/BEL21) and *L.* (*L.*) *amazonensis* (MHOM/BR/73/M2269) (Fig. 1.1.1 A, lanes 2 and 3). A resemble chromosomal ladder was seen in a rodent isolate in Belize (MNYC/BZ/62/WR592) (Grögl *et al.*, 1991), but not in other strains of *L.* (*L.*) *mexicana* complex reported (Scholler *et al.*, 1986; Grögl *et al.*, 1991; Lighthall and Giannini, 1992). DNA karyotype of *L.* (*L.*) *major*-like, the other agent of cutaneous leishmaniasis in the Andes of Ecuador (Hashiguchi *et al.*, 1991), was totally different from that of *L.* (*L.*) *mexicana* isolates (Fig. 1.1.1 B, lane 9).

In addition to Paute, we found patients with cutaneous leishmaniasis in Alausi (elevation 2,300 m) in the Department of Chimborazo. This town is located about 80 km north of Paute. One of human isolates in Alausi (MHOM/EC/89/AL1) was identical to the *L.* (*L.*) *mexicana* strains in Paute in terms of schizodeme and zymodeme (Hashiguchi *et al.*, 1991). With respect to sandflies, three of 49 *Lutzomyia ayacuchensis* captured in Alausi in 1991

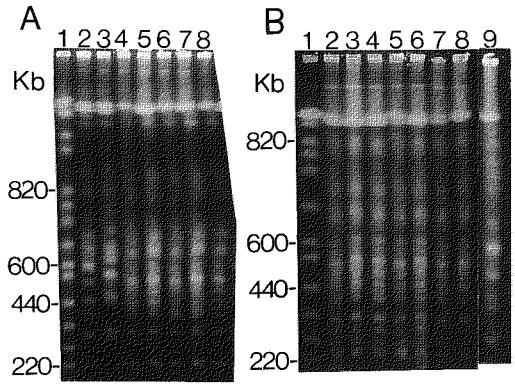

Figure 1.1.1. Separation of chromosomal DNAs of *Leishmania* isolates in the Andean Ecuador by pulsed field gel electrophoresis. This technique was performed using 1.5% agarose run at 180 V with a 70-sec pulse time for 37 hr (A) and a 60-sec for 36 hr (B) in 0.5 x TBE buffer. Panel A: lane 1, *Saccharomyces cerevisiae*; lane 2, *L.* (*L.*) *amazonensis* (MHOM/BR/73/M2269); lane 3, *L.* (*L.*) *mexicana* (MHOM/BZ/82/BEL21); lane 4, MHOM /EC/88/Paute27; lane 5, MHOM/EC/88/Paute29; lane 6, MCAN/EC/88/PauteInu 2; lane 7, MHOM /EC/88/Paute103; lane 8, MHOM/EC/88/Paute1. Panel B: lane 1, *Saccharomyces cerevisiae*; lane 2, IAYA/EC/92/ALI3; lane 3, IAYA/EC/92/ALI4; lane 4, IAYA/EC/92/HUI1; lane 5, MHOM/EC/92/HU1; lane 6, MHOM/EC/92/HU3; lane 7, MHOM/EC/92/ HU4; lane 8, MHOM/EC/92/HU6; lane 9, *L.* (*L.*) *major*-like (MHOM/EC/87/G-09).

and 1992 were positive for leishmanial promastigotes (Gomez *et al.*, 1992). DNA karyotypes of two of the sandfly isolates (IAYA/EC/92/ALI3 and 4) were analyzed and we found that they were almost identical to that of *L.* (*L.*) *mexicana* isolates in Paute (Fig. 1.1.1 B, lanes 2 and 3). Furthermore, in 1992, we confirmed cutaneous leishmaniasis patients in Huigra (elevation 1,300m), also in Chimborozo, located about 20 km southwest of Alausi. Four human isolates (MHOM/EC/92/HU1, 3, 4 and 6) and one sandfly (*Lu. ayacuchensis*) isolate (IAYA/EC/92/HUI1) showed DNA karyotypes similar to the *L.* (*L.*) *mexicana* strains in Paute (Fig. 1.1.1 B, lanes 4-8). Thus, our DNA karyotype analysis of *Leishmania* isolates strongly suggests that this defined strain of *L.* (*L.*) *mexicana* is widely distributed at least in Department of Azuay and Chimborazo in the Andes of Ecuador and that the parasite is transmitted by *Lu. ayacuchensis,* and domestic dogs may play a role as the reservoir host

Isolation of *L.* (*L.*) *mexicana* was also reported by other investigators from patients in Alausi (Armijos *et al.*, 1990), and in the western Andean cordillera of Colombia at an elevation of 1,500m (Corredor *et al.*, 1990). Although no karyotype analysis of these isolates have been described, it is possible that our specified strain of *L.* (*L.*) *mexicana* in this study is distributed in Colombia as well as in Ecuador. Clinical aspects of leishmaniasis in Paute, Alausi and Huigra closely resemble "Uta" caused by *L.* (*Viannia*) *peruviana* infections in the Peruvian Andes (Lumbreras and Guerra, 1985; Lainson and Shaw, 1987). However, molecular karyotype analysis revealed that *L.* (*V.*) *peruviana* belonged to *L.* (*V.*) *braziliensis* complex (Dujardin, *et al.*, 1993a) and that chromosome size polymorphism was seen among *L.* (*V.*) *peruviana* isolates from different regions in the Andean Peru (Dujardin, *et al.*, 1993b).

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2. Species Specific Monoclonal Antibodies Raised against *Leishmania* (*Viannia*) *equatorensis*

ABSTRACT. Monoclonal antibodies were raised against promastigote of *Leishmania* (*Viannia*) *equatorensis*, which was recently described as a new species (Grimaldi *et al.*, 1992). BALB/c mice were immunized with homogenate of *L.* (*V.*) *equatorensis* promastigotes derived from ten days old *in vitro* culture. Fusions of immunized spleen cells with P3-X63-Ag8,6.5.3. myeloma cells resulted in the production of six monoclonal antibodies (MAbs) against *L.* (*V.*) *equatorensis*. Among those, five MAbs 9F4, 7H6, 3A7, 8C1 and 1G5 were revealed to be species-specific for *L.* (*V.*) *equatorensis* by ELISA against a cross-panel of *Leishmania* strains. These MAbs will therefore be useful additions to the panel of antibodies available for the identification of *Leishmania* species. On the other hand, another MAb 7A6 cross-reacted to species of both *L.* (*V.*) *braziliensis* complex and *L.* (*Leishmania*) *mexicana* complex. By indirect immunofluorescent antibody (IFA) test, MAbs 9F4, 7H6 and 7A6 appeared to bind the surface and cytoplasm of *L.* (*V.*) *equatorensis* promastigote. MAbs 3A7 and 1G5 bound only to flagellum. On Western blot analysis, MAb 3A7 recognized set of bands ranging from 110 to 170 kDa. On the other hand, MAb 1G5 recognized a different set of molecules ranging 200-250 kDa.

Introduction

Leishmania (*Viannia*) *equatorensis* was originally isolated from a sloth and from a squirrel captured in Naranjal, Department of Guayas in Ecuador (Hashiguchi *et al.*, 1985; Gomez *et al.*, 1987). This parasite was characterized by isoenzyme electrophoresis, reactive patterns with monoclonal antibodies, and restriction-endonuclease fragment patterns of kinetoplast DNA and described as a new species (Grimaldi *et al.*, 1992). These results suggest its close relation to the parasites of *L.* (*V.*) *braziliensis* complex. Other biological characteristics such as the host range or the geographical distribution have little been known, however. Specific probes for the identification of this species are necessary for further investigation. In this report, we describe the development of MAbs against *L.* (*V.*) *equatorensis* and show their specificities.

Materials and Methods

Parasites

Leishmania promastigotes shown in Table 1.2.1 were cultured at 21°C in Schneider's Drosophila medium supplemented with 15% heat-inactivated fetal bovine serum and 50 μg/ml of gentamycin.

Mice

Four weeks old female BALB/c mice were purchased from Nihon Clea Co., Ltd., Tokyo. These

Table 1.2.1. *Leishmania* used in this study

Species	Designation
L. (V.) equatorensis	MCHO/EC/82/Lsp1
L. (V.) braziliensis complex	
L. (V.) braziliensis	MHOM/BR/75/M2904
L. (V.) guyanensis	MHOM/BR/75/M4147
L. (V.) panamensis	MHOM/PA/71/LS94
L. (L.) mexicana complex	
L. (L.) mexicana	MNYC/BZ/62/M379
L. (L.) amazonensis	MPRO/BR/72/M1845

BALB/c mice and C.B-17-*scid* mice were housed in Specific Pathogen-free Animal Care Facility of the Central Institute for Experimental Animals, Kawasaki, Japan.

Immunization of mice

At the tenth day of promastigotes culture of *L.* (*V.*) *equatorensis*, when parasite growth reaches to the stationary phase, organisms were harvested by centrifugation at 1600 x g using Kubota 5010 (Kubota Co., Ltd., Tokyo). The organisms were washed with Dulbecco's phosphate-buffered saline (PBS) three times. For immunization, after freezing and thawing, the promastigotes were suspended in PBS and sonicated on ice for 5 min, by ultra-sonicator UD-201 (TOMY SEIKO Co., Ltd., Tokyo) at output level 4. Female BALB/c mice were immunized intraperitoneally with homogenate of 1 x 10^7 promastigotes of *L.* (*V.*) *equatorensis* mixed with complete Freund's adjuvant. After 4 weeks, these mice were inoculated intraperitoneally with the same dose of promastigote homogenate. Eight weeks after the first immunization, these mice were finally boosted intravenously with homogenate of 1 x 10^6 promastigote.

Production of MAbs

Three days after the final boost, spleen cells from the immunized BALB/c mouse were fused with P3-X63-Ag8,6.5.3. myeloma cells using 50% polyethylene glycol solution, according to the method reported by Koher and Milsten (1975). Ten to the seventh fusion cells were plated to each well of 96-well multiplate and were cultured in RPMI-1640 containing 15% fetal bovine serum (FBS), 5% (v/v) supernatant of IL-6 producing cell culture (BriClone; Dainihon Pharm. Co., Ltd., Tokyo), hypoxantin (1 x 10^4 M), aminopterin (4 x 10^{-7} M) and thymidin (1.6 x 10^{-5} M) (HAT medium). After two weeks of incubation, the medium was replaced to RPMI-1640 containing FBS, growth factors (BriClone), hypoxantin and thymidin at the same concentration as HAT medium (HT medium). Supernatants from all wells showing cell growth were screened for the presence of antibody against *L.* (*V.*) *equatorensis* promastigotes by ELISA.

Hybridomas demonstrating antibody production were cloned by limiting dilution procedure at the concentration of 0.3 cells/well. The wells which presented only one visible colony were regarded as those which contain a clone. Immunoglobulin class and subclass of monoclonal antibodies were determined by using the MonoAb ID kit (Zymed Laboratories, Inc., San Francisco, CA). To get much MAb, sera or ascites were obtained from *scid* mice inoculated with each hybridomas intraperitoneally. The MAbs were purified with ammonium sulfate precipitation method from both sera and ascites.

Preparation of antigens for ELISA

Washed parasites from ten days old culture were suspended in a protease inhibitor solution (1 mM phenyl-methyl-sulfonyl-fluoride, 50 mM N-p-tosyl-L-lysin chloromethyl keton, 15 mM leupeptin, 2 mM EDTA 2Na in 10 mM Tris-HCl, pH 7.8) and sonicated on ice for 5 min, using an ultrasonicator UD-201 at the output level 4. Then 1% Triton X-100 was added to the homogenate. After incubation on ice for 30 min, samples were centrifuged at 10,000 x g using Kubota 1700 for 30 min at 4°C, then supernatants were removed to the fresh tube. Protein concentration was determined by DC Protein Assay (BIO-RAD Co., Ltd., Tokyo).

ELISA

Prior to the experiments, optimum concentration of antigens and second antibodies were determined by testing at serial dilutions of each reagents. The optimum conditions were as follows. The wells of 96-well polystyrene microplate (Inter. Med. Co., Ltd., Tokyo) were coated at 4°C overnight with 10 μg/ml of each antigens (100 μl/well) diluted with 0.1 M carbonate buffer (pH 9.5). After washing with 0.05% Tween 20-PBS (PBS-T), each well was incubated for 1 hr at room temperature with a blocking agent containing bovine milk proteins (Block Ace ; Dainihon Pharm. Co., Osaka). The test solution was applied (100 μl/well) and incubated at 37°C for 1 hr.

The wells were then washed and 100 μl of horseradish peroxidase-labeled anti-mouse Ig (Amersham. Co., Ltd., Tokyo) diluted to 1:6000 was applied to each well and incubated at 37℃ for 1 hr. After washing, mixture of 0.04% *o*-phenylenediamine and 0.003% H_2O_2 in phosphate-citrate buffer (pH 5.0) was added to each well, and incubated for 30 min. The reaction was stopped by adding 20 ml of 6 N sulfuric acid. The absorbance at 490 nm was measured with a microplate reader (Model 450; BIO-RAD Co., Ltd., Tokyo). For negative control, supernatant of myeloma cells or sera from intact *scid* mice was used.

IFA test

Promastigotes were harvested at stationary stage of growth, washed, and resuspended in PBS. Washed parasites were air-dried onto multispot slides and fixed in cold methanol for 10 min, dried, and stored at -80℃ until use. Slides were washed in PBS for 5 min, 3 times, and incubated with MAb for 1 hr at room temperature. The slides were washed with PBS (10 min, 3 times) and incubated with FITC-conjugated anti-mouse immunoglobulin (1: 20 dilution)(DAKO, Japan Co., Ltd., Tokyo) for 1 hr at room temperature. The slides were washed with PBS (10 min, 5 times) and mounted with buffered glycerol (glycerol: PBS = 9:1). The fluorescence was observed under a BH-2 Olympus (Tokyo) fluorescence microscope.

Western blot analysis

Protein samples from *L. (V.) equatorensis* promastigote were separated on 8%-18% gradient gel by SDS-PAGE under reduced condition. Sample buffer contained 0.2 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 2% 2-mercaptoethanol and 0.001% bromophenol blue. Separated proteins were electrolytically transferred to a nitrocellulose membrane using transfer buffer (39 mM glycin, 48 mM Tris base, 0.0375% (w/v) SDS and 20% methanol). The nitrocellulose membrane was treated with a blocking solution containing 0.5% Tween 20, 8 mM Na_2HPO_4, 2 mM NaH_2PO_4, and 0.85% NaCl (pH 7.4) three times. The nitrocellulose membrane was incubated (1 hr, room temperature) with Block Ace solution, cut into strips, and then incubated with MAbs (1 hr, room temperature). The membrane was washed with PBS containing 0.5% Tween 20 (15 min and 5 min, 4 times), and incubated with rabbit anti-mouse immunoglobulin conjugated with horseradish peroxidase (1: 5000 dilution) (Amersham Co., Ltd., Arlington, IL) for 1 hr at room temperature. The membrane was washed with PBS (15 min and 5 min, 4 times), incubated with chemiluminescence-enhanced reagents (ECL; Amersham Co., Ltd., Arlington, IL), and then the membrane was exposed to the hyper film (Amersham Co., Ltd., Arlington, IL) for 3 min to 5 min.

Results

The fusion was carried out using spleen cells from BALB/c mice immunized with homogenate of *L. (V.) equatorensis* (MCHO/EC/82/Lsp1) promastigote. The average hybridoma growth efficiency was 51%, of which 14.1% of hybridomas secreted antibodies against *L. (V.) equatorensis*.

Prior to making hybridomas, sera of mice immunized with *L. (V.) equatorensis* homogenates were examined for cross reactivity to other *Leishmania* species. The sera reacted only with *L. (V.) equatorensis* antigens, although sera from mice infected with *L. (Leishmania) amazonensis* cross-reacted with all of the strains used.

Six hybridoma lines (9F4, 7H6, 3A7, 7A6, 8C1, and 1G5) were selected based upon their reactivity on ELISA using *L. (V.) equatorensis* promastigote soluble antigens and were used for further examination on cross reactivity against cross-panel of *Leishmania* species by ELISA (Table 1.2.2). End-point titers (Venkatesan and Wakelin, 1993) of MAbs 9F4 and 7H6 were less than 10 ng/ml, of MAbs 3A7 and 1G5 were 100 ng/ml and of MAbs 8C1 and 7A6 were 1000 ng/ml. MAb 9F4, 7H6, 3A7, 8C1 and 1G5 did not cross-react with other *Leishmania* species, *L. (V.) braziliensis*, *L. (V.)*

Table 1.2.2. Characterization of monoclonal antibodies

Hybridoma lines	Subclass of Ig	End-point titre*
9F4	IgM, k	< 10
7H6	IgG2a, k	< 10
3A7	IgM, k	10^2
7A6	IgM, k	10^3
8C1	IgG1, k	10^3
1G5	IgM, k	10^2

* End-point titre was defined as the antibody concentrarion (ng/ml) at which the positive sample dilution equilibrates to a negative sample (Venkatesan and Wakelin, 1993).

panamensis, L. (V.) guyanensis, L. (L.) mexicana and *L. (L.) amazonensis* (Table 1.2.3). MAb 7H6 and 9F4 reacted more than twenty times stronger against *L. (V.) equatorensis* than other species of leishmanial parasites used in this study. On the other hand, MAb 7A6 showed some cross-reactivity with *L. (V.) panamensis* and *L. (L.) amazonensis* (Table 1.2.3).

To localize the antigen molecules, IFA test was carried out on methanol-fixed *L. (V.) equatorensis* promastigotes. The results were summarized in Table 1.2.4. The two different fluorescence patterns were distinguished. The fluorescence with MAbs 9F4, 7H6 and 7A6 appeared on surface and cytoplasm of the parasite. The strong fluorescence was observed near kinetoplast. MAbs 3A7 and 1G5 reacted only with flagellum of the promastigotes. No fluorescence was observed with 8C1.

Western blot analysis of soluble proteins of *L. (V.) equatorensis* promastigotes revealed that 3A7 and 1G5 recognized different bands. MAb 3A7 appears to recognize at least 5 components ranging from 110 to 179 kDa. MAb 1G5 recognized a different set of components ranging from 200 to 250 kDa (Fig. 1.2.1).

Table 1.2.3. Specificity of monoclonal antibodies by ELISA

Concentration	MAbs (ng/ml)	9F4 1	7H6 1	3A7 10	7A6 100	8CI 100	IG5 100	Immunized serum -	Mouse Ig -
Antigens									
L. (V.) equatorensis		1.476	1.166	0.284	0.767	0.570	0.472	2.425	0.095
L. (V.) braziliensis complex									
L. (V.) braziliensis		0.147	0.094	0.105	0.213	0.257	0.296	0.249	0.148
L. (V.) panamensis		0.102	0.103	0.117	0.115	0.321	0.251	0.178	0.217
L. (V.) guyanensis		0.092	0.084	0.120	0.402	0.288	0.374	0.204	0.132
L. (L.) mexicana complex									
L. (L.) mexicana		0.100	0.073	0.117	0.225	0.179	0.286	0.286	0.278
L. (L.) amazonensis		0.160	0.081	0.137	0.368	0.209	0.302	0.302	0.156
BSA		0.099	0.085	0.097	0.230	0.297	0.272	0.224	0.217

Table 1.2.4. Reactivity of MAbs by IFA test

Hybridoma lines	IFA reactivity	Antigen location
9F4	+	surface, cytoplasm*
7H6	+	surface, cytoplasm
3A7	+	flagellum
7A6	+	surface, cytoplasm
8C1	-	-
1G5	+	flagellum

*, Not all promastigotes were reactive with 9F4

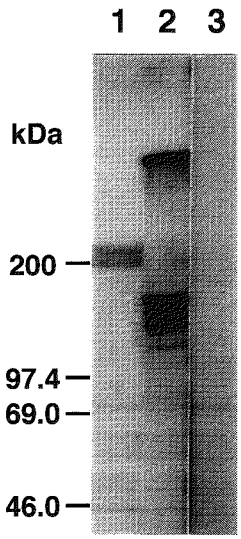

Figure 1.2.1. Western blot analysis of *Leishmania (Viannia) equatorensis*. *L. (V.) equatorensis* soluble protein reacted with monoclonal antibodies raised against *L. (V.) equatorensis*. Lanes 1-3 represent 1G5, 3A7 and mouse Ig, respectively.

Discussion

To date, many *Leishmania* parasites have been isolated from wild and domestic animals in Central and South America (Mimori *et al.*, 1989; Hashiguchi *et al.*, 1990; Grimaldi *et al.*, 1991). Most of *Leishmania* parasites which were isolated from systematically lower animals has not been well characterized. For epidemiological study of *Leishmania* in animals, identification of parasites with specific probes is an essential need.

L. (V.) equatorensis was isolated from sloth (*Choloepus hoffmanni*) and squirrel (*Sciurus granatensis*) during a survey of potential reservoir hosts of leishmanial parasite. Recent reports suggested this parasite is a member of *L. (V.) braziliensis* complex based upon the serodeme analysis carried by the use of MAbs specific for other species (Grimaldi *et al.*, 1992). However, to understand the taxonomical and genetical distance of *L. (V.) equatorensis* from other species of *Leishmania*, serodeme analysis using MAbs raised against this new species might provide more information.

Therefore, in this study, we produced MAbs against this new species. Among 6 MAbs examined for characterization, 5 MAbs in total, 9F6, 7H6, 3A7, 8C1 and 1G5 were specifically reacted with *L. (V.) equatorensis*. The serum from mouse immunized with *L. (V.) equatorensis* antigens reacted only with *L. (V.) equatorensis* antigen. Moreover, *L. (V.) equatorensis* is different from other New World *Leishmania* in its morphology and proliferating manner (Hashiguchi *et al.*, 1990). These facts suggested that this species is quite different from other *Leishmania*. Specific reaction of MAbs 9F6, 7H6, 3A7, 8C1 and 1G5 with *L. (V.) equatorensis* suggested the existence of unique epitopes of *L. (V.) equatorensis*. These MAbs might be very useful for identification of this species, characterization of species specific antigen molecules and epidemiological survey for animal leishmaniasis.

MAbs 3A7 and 1G5 reacted only with flagellum by IFA test. However, these recognized different size of antigen molecules; 3A7 recognized at least 5 bands that were ranged from 110 to 170 kDa, while 1G5 recognized a different set of molecules ranging from 200 to 250 kDa. These suggested that they recognized different components of flagellum of the

parasite, and might be very useful to analyze the structure and function of it.

Although MAbs 9F4, 7H6 and 7A6 reacted with *L. (V.) equatorensis* on ELISA and IFA test, these gave no signs on Western blot analysis. This kind of problem sometimes occur on MAb (Jaffe and Rachamim, 1989). One possible explanation could be that the presentation of specific antigenic determinants is influenced by the different antigen fixation methods used in the three assay systems. Further work is required to confirm this.

One MAb, 7A6, is cross-reacted with members of both *L. (V.) braziliensis* complex and *L. (L.) mexicana* complex. The present study showed that this parasite has antigens common with species of *L. (V.) braziliensis* complex and *L. (L.) mexicana* complex, suggesting that the antigen recognized with 7A6 commonly expressed on parasites belonging to the genus *Leishmania*. Grimaldi *et al.* (1992) examined cross reactivity of *L. (V.) equatorensis* using monoclonal antibodies specific for *L. (V.) braziliensis, L. (L.) mexicana, L. (L.) tropica,* and *L. (L.) donovani,* complex. And only MAbs specific for members of the *L. (V.) braziliensis* group were reacted with *L. (V.) equatorensis*. Interestingly, MAb 7A6 reacted to *L. (L.) amazonensis* a bit stronger than *L. (V.) braziliensis* and *L. (V.) guyanensis*.

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Chapter 2

Experimental Leishmaniasis

1. Histopathological and Ultrastructural Comparison of Experimental Animal Leishmaniasis caused by Different Strains of *Leishmania* (*Leishmania*) *mexicana* Isolated from Patients with Diffuse and Localized Cutaneous Lesions

ABSTRACT. In order to make a search for some factors relating to different disease forms caused by *Leishmania* strains or species, histopathological and ultrastructural comparisons were made. For this purpose, hamsters were infected experimentally with promastigotes of *Leishmania* (*Leishmania*) *mexicana* strains isolated from patients with two types of clinical forms, diffuse cutaneous (DCL) and localized cutaneous leishmaniasis (LCL). No histopathological and ultrastructural findings providing clear differentiation between DCL and LCL strains were recognized. The experimental animals used were divided into the following two groups. Hamsters in group A were infected with *L.* (*L.*) *mexicana* isolates from a patient with DCL, and the remaining animals in group B were infected with the parasite, *L.* (*L.*) *mexicana*, isolated from patients with LCL. Macroscopically, no remarkable difference on the inoculated sites was noticed between both groups after the lst month of promastigote inoculation. After the 2nd and 4th month of inoculation, small or large nodules were observed on the inoculation site of animals in both groups. The large nodules were found relatively more in numbers in the animals of group A than those of group B. No cutaneous dissemination and/or metastasis was recognized in any animals of both groups. Histopathologically, granulomatous changes were observed in all the microscopical sections of nose and footpad of hamsters infected experimentally. In the nose and footpad sections, a large number of neutrophils were observed in the animals of group A, while histiocytes and lymphocytes were dominant in those of group B. In ultrathin sections amastigotes were located in the dermis extracellularly and intracellularly. Degeneration of parasites was observed inside the macrophages in group B sections only. Morphologically, any clear differentiation was not found in light- and ultra-microscopical observations between the amastigotes of *L.* (*L.*) *mexicana* from experimental animals of the two groups.

Introduction

In Ecuador, cutaneous leishmaniasis is widely endemic and the disease is distributed in areas from lowland of the Pacific coast and Amazonian areas to highland of the Andes (Hashiguchi and Gomez, 1991; Hashiguchi *et al.*, 1992). In the country localized cutaneous leishmaniases (LCL) are found frequently, but diffuse cutaneous forms (DCL) are very rare; only one parasitologically confirmed case has been reported to date (Bhutto *et al.*, 1992; our unpublished data). The former type of the disease (LCL) is a clinical form in which the lesion is limited to the originally infected site of human skin,

and it afterwards may or may not changes from nodular lesion to ulcerative one and/or multiple lesions. On the other hand, the latter one (DCL) is a form in which nodular and plaque lesions spread all over the body, and these disseminated lesions persist for a long time. These DCL lesions are extremely difficult to treat with available drugs, such as meglumine antimoniates and others.

DCL is reported from various countries of the New World (Convit and Kerdel-Vegas, 1965; Convit *et al.*, 1971) and Old World (Bryceson, 1969, 1970). The present isolates of the parasites from patients with DCL and LCL in Ecuador were thoroughly characterized as *L.* (*L.*) *mexicana* by molecular biological techniques. In Ecuador, *L.* (*L.*) *mexicana* is considered to be responsible for a variety of disease forms ranging from localized simple and mild lesions to generarized diffuse type of lesions, with a wide range of geographical distributions from lowland to Andean highland (Hashiguchi *et al.*, 1987, 1990, 1992; Hashiguchi and Gomez, 1991; Armijos *et al.*, 1990; Katakura *et al.*, 1992). It, however, still remains obscure that why the same species of *Leishmania*, *L.* (*L.*) *mexicana*, cause such different disease forms. To date, various studies have been performed to evaluate the differential features between the two clinical forms. In Ethiopia Schurr *et al.* (1987) examined human skin biopsy specimens, and found parasite and host cell differences between DCL and LCL patients infected with the Old World *Leishmania*, *L.* (*L.*) *aetiopica*. On the other hand, Petersen *et al.* (1982) examined DCL patients infected with the New World *Leishmania*, *L.* (*L.*) *pifanoi*, from the Dominican Republic, and considered the DCL as an immunosuppressive disease due to the defect of cell mediated immune mechanisms, especially monocyte suppression of antigen specific lymphocyte responses. The current study was designed aiming at a search for some responsible factor(s) causing the two different disease forms, DCL and LCL, from clinical, histopathological and ultrastructural points of view, using biopsy materials from hamsters infected experimentally.

Materials and Methods

Hamsters

Male Syrian hamsters (SLC. Co., Japan) with 150-200g of weight and 11 weeks of age, were used in this study.

Parasites and mode of infection

The animals were divided into two groups, A and B, with 9 animals each. The first group (A) was infected with *L.* (*L.*) *mexicana* (MHOM/EC/90/INH 690) isolated from a patient with DCL from Esmeraldas, Ecuador. The second group (B) was infected with *L.* (*L.*) *mexicana* (MHOM/EC/88/PT7) isolated from a patient with LCL from Paute, Ecuador. Based on molecular biological data, such as zymodeme, serodeme and schizodeme analyses, the parasites isolated from each patient were identified as *L.* (*L.*) *mexicana*. The *L.* (*L.*) *mexicana* promastigotes of log-phase *in vitro* were inoculated into the nose and hind footpads of hamsters. Each animal received $1x10^7$ promastigotes per inoculation site. The evolution of lesions was observed and recorded periodically and autopsies were taken at 1 month, 2 months and 4 months of duration after the inoculation.

Histopathology

The animals were sacrificed at different time intervals after promastigote inoculation, and skin biopsy specimens were taken from the nodular sites of inoculated sites (lesions). The specimens taken were divided into two parts. One part was fixed in formalin for hematoxylin-eosin sections, and the other part was used for the fixation using 2% paraformaldehyde and 2% glutaraldehyde for ultrathin sections. All the specimens were processed as described in previous paper (Bhutto *et al.*, 1992).

Results

Clinical observations of hamsters

One month after inoculation: No remarkable dif-

ference was noticed on any inoculated site of both A and B group animals except for few hemorrhagic crusts on the nose that may be caused by the injection during *L.* (*L.*) *mexicana* promastigote inoculation.

Two months after inoculation: All the nine animals of group A showed small nodules on the nose (Fig. 2.1.1a). Four animals of this group (A) developed large nodules over the one or both footpads (Fig. 2.1.1b). In group B all the nine animals also developed small nodules over the nose and footpads (Fig. 2.1.2), but no large nodule was observed in this group.

Four months after inoculation: The animals infected experimentally with *L.* (*L.*) *mexicana* strain isolated from the patient with DCL (group A) showed large nodules on the nose in five (62.5%) out of eight hamsters and footpads in six (75.0%) out of eight. On the other hand, the animals infected with the strain of same species, *L.* (*L.*) *mexicana*, isolated from LCL patient (group B) developed large nodules on the nose in two (25.0%) of the eight hamsters, and footpads in 2 (25.0%) of the eight.

Light microscopic observations

In the current study, histopathological observations were conducted only on the skin biopsy specimens taken from hamsters at two months after inoculation of DCL and LCL strains of *L.* (*L.*) *mexicana*. Histologically, all the specimens of group A and B animals were consistent with granulomatous reactions. In group A sections tissue reaction was extended from upper dermis to subcutaneous layer (Fig. 2.1.3). The group A sections were diffusely infiltrated with neutrophils, histiocytes, lymphocytes and mast cells. In these sections neutrophils were predominant (Fig. 2.1.4), and the perivascular infiltration of lymphocytes was also noticed (Fig. 2.1.5); the center of lesion was occupied with parasitized macrophages. In group B sections, tissue reactions were almost similar to those in group A (Figs. 2.1.6 and 2.1.7), showing infiltration consisted of neutrophils, histiocytes, lymphocytes and mast cells; plasma cells and eosinophils were also present. In these sections, histiocytes and parasitized macrophages were dominant (Fig. 2.1.8).

Electron microscopic observations

In both group sections parasites were found intracellularly and extracellularly. Most of the parasites located inside the parasitophorous vacuoles of macrophages (Fig. 2.1.9a-c). In group A sections no degeneration of parasites was seen at this stage. However, in group B sections, the degeneration was visible in both the nose and footpad specimens (Fig. 2.1.10a-c). Morphologically, any difference was not found either in the size or light- and ultra-microscopical structure of amastigotes in both group animals (Fig. 2.1.11a-c).

Discussion

In the current study, histopathological and ultrastructural comparisons were made in hamsters infected experimentally with two strains of *L.* (*L.*) *mexicana* isolated from patients with DCL and LCL, in order to investigate some factor (s) uderlying the formation of different clinical types of leishmaniasis. However, no clear differentiation was found between the histopathological and ultrastructural features of experimental animals infected with the two strains, DCL and LCL. Clinically, our observation revealed that the animals infected with DCL *L.* (*L.*) *mexicana* strain developed large nodules, and they were more in numbers than those of the animals infected with LCL strain. This finding may show more active growth of nodular lesions in the animals infected with DCL strain as compared with those infected with LCL strain. However, the precise reason that why the difference was observed between the two groups, is unknown. In leishmaniasis, many species and/or many strains of the same species of the genus *Leishmania* cause a variety of clinical forms in each endemic area of the New World and Old World. For the variation of disease forms, various theories and explanations have been given to date. Scott and Sher (1986) showed a spectrum of susceptibility of *Leishmania* strains of intra-

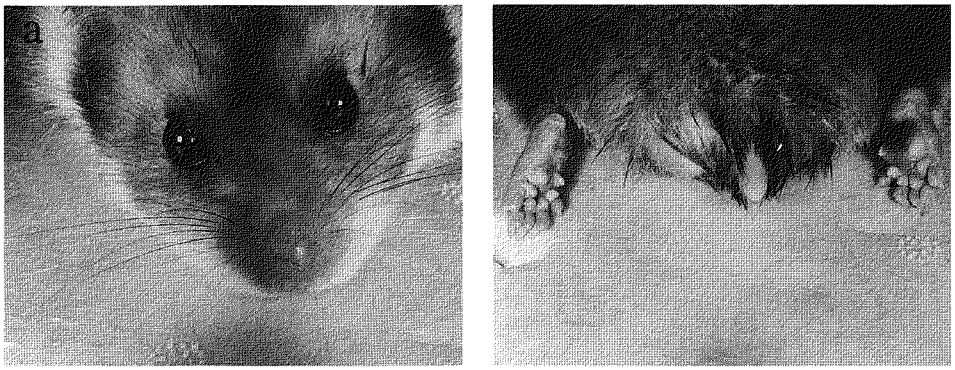


Figure 2.1.1 a and **b**. Appearance of nodules on the nose and footpads of animals infected with *L.* (*L.*) *mexicana* isolated from the patient with diffuse cutaneous leishmaniasis (DCL) at 2 months of duration after promastigote inoculation.

cellular killing by murine macrophages, and they considered the macrophages as a main factor that play the different role in killing the parasites in different strain of parasites. With regard to DCL and LCL, Schurr *et al.* (1987) worked with cutaneous leishmaniasis (CL) in Ethiopia using electron microscopy, proposed the different function of macrophages that may or may not control the intra-

Figure 2.1.2. Appearance of nodule on the nose of animals infected with *L.* (*L.*) *mexicana* isolated from the patient with localized cutaneous leishmaniasis (LCL) at 2 months.

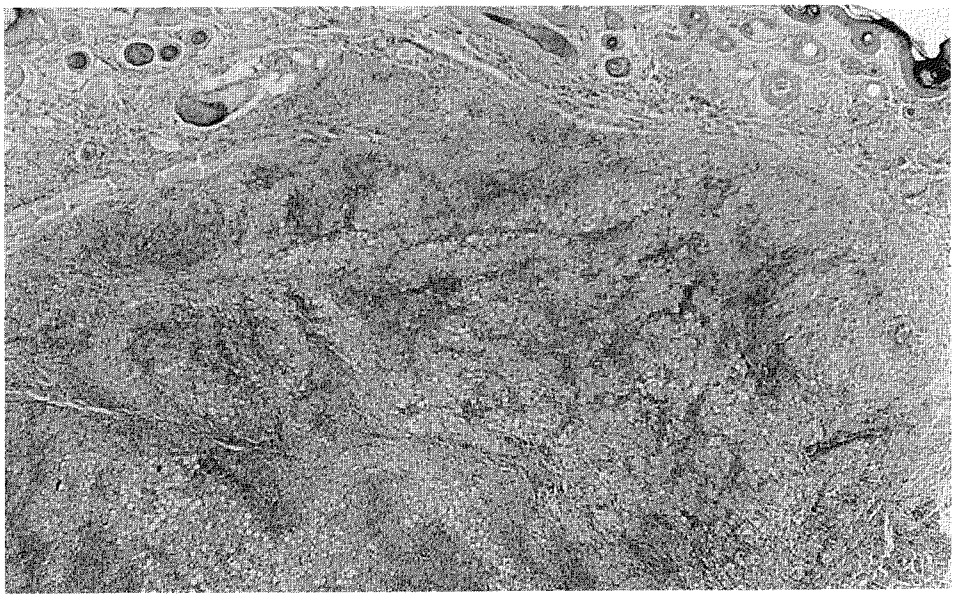


Figure 2.1.3. Low magnification view of a section from the nose of animals infected with *L.* (*L.*) *mexicana* isolated from DCL patient. Nodular changes are visible from middle dermis to lower dermis (H & E stain x20).

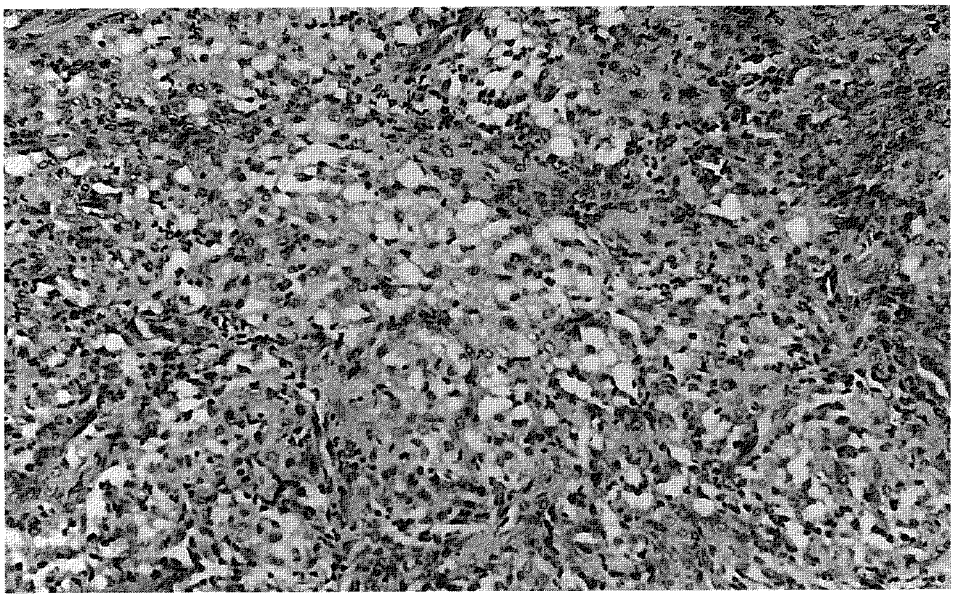


Figure 2.1.4. High magnification of Fig. 2.1.3. Polymorphonuclear cells are dominant in the highly vacuolated dermal area (H & E stain x50).

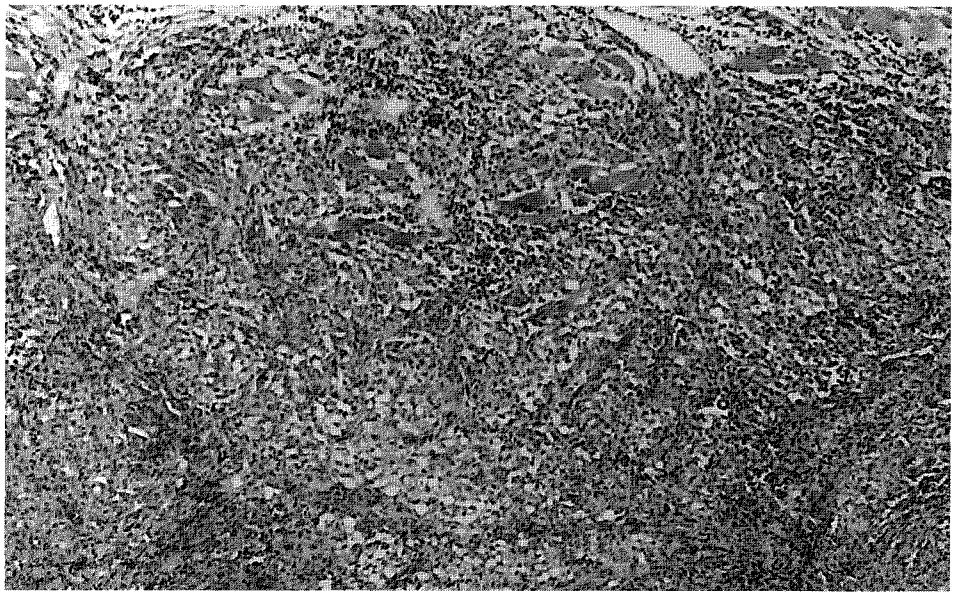


Figure 2.1.5. Section from the footpad of the animal infected with *L.* (*L.*) *mexicana* isolated from the patient with DCL; perivascular infiltration of lymphocytes and formation of the vacuoles (H & E stain x50).

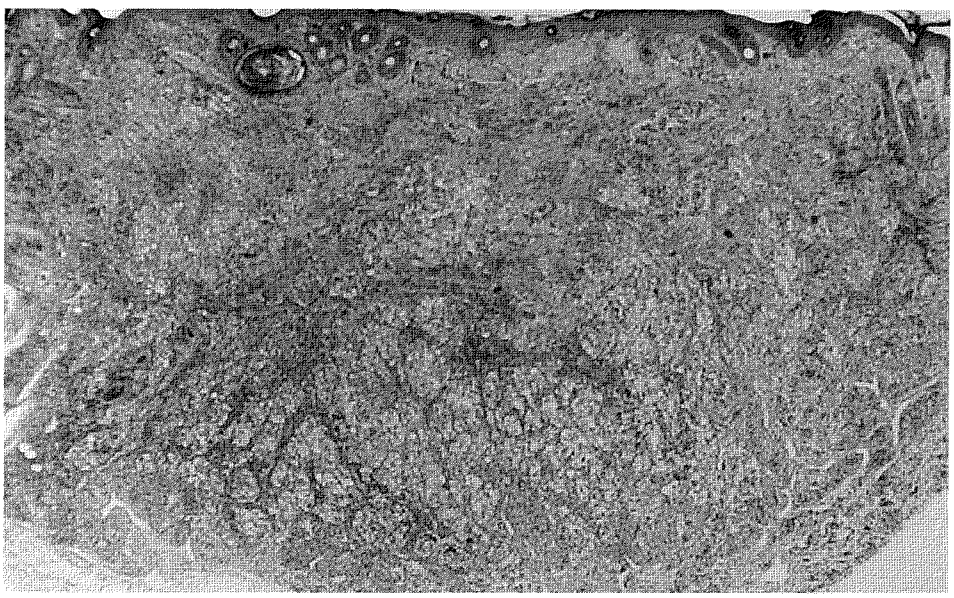


Figure 2.1.6. Section from the nose of the animal infected with *L.* (*L.*) *mexicana* isolated from the patient with LCL. Granulomatous changes are visible in the lower most dermis (H & E stain x20).

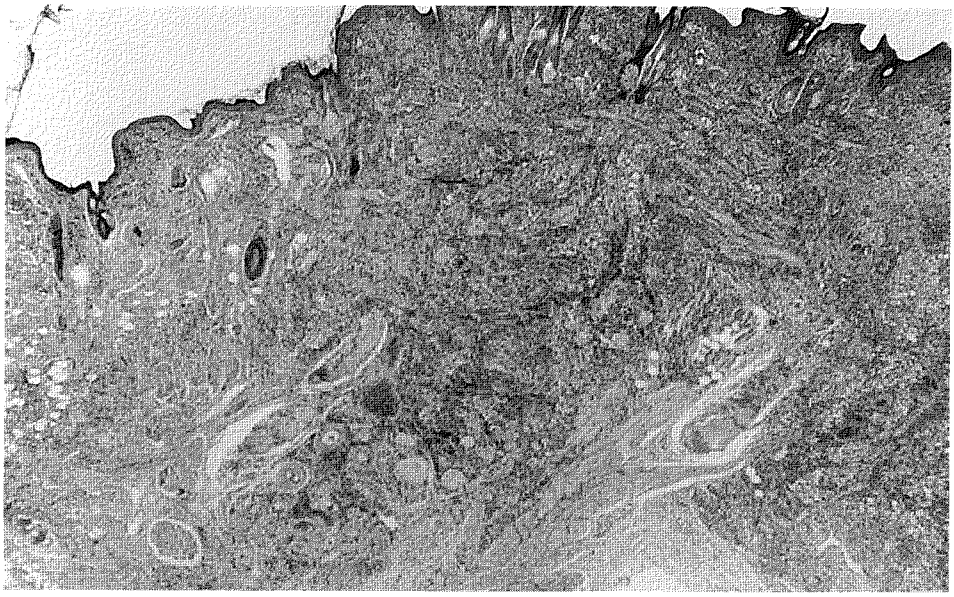


Figure 2.1.7. Section from the footpad of the animal infected with *L.* (*L.*) *mexicana* isolated from the patient with LCL (H & E stain x20).

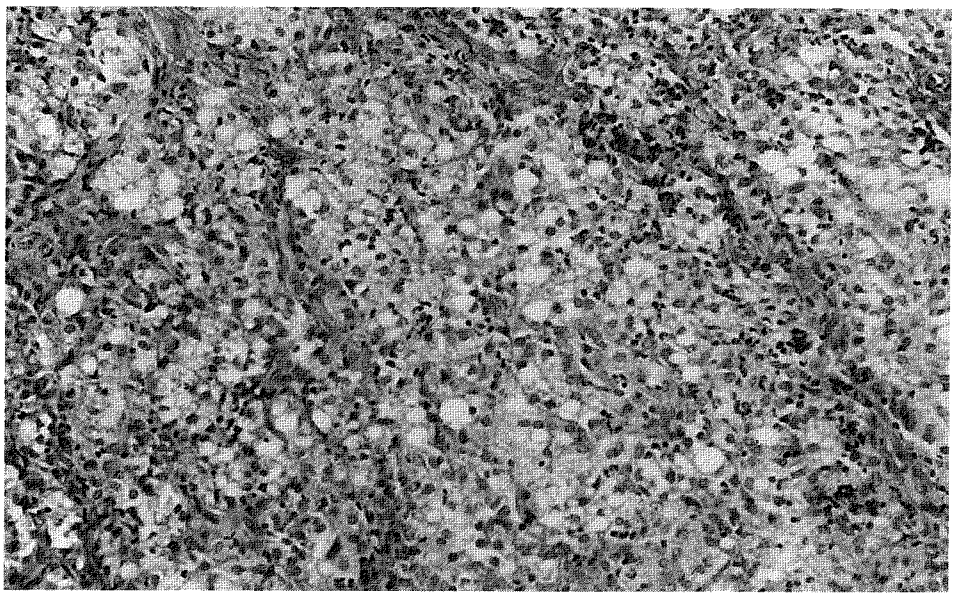


Figure 2.1.8. High magnification of Fig. 2.1.6. Many amastigotes are infiltrated in the vacuolated section (H & E stain x50).

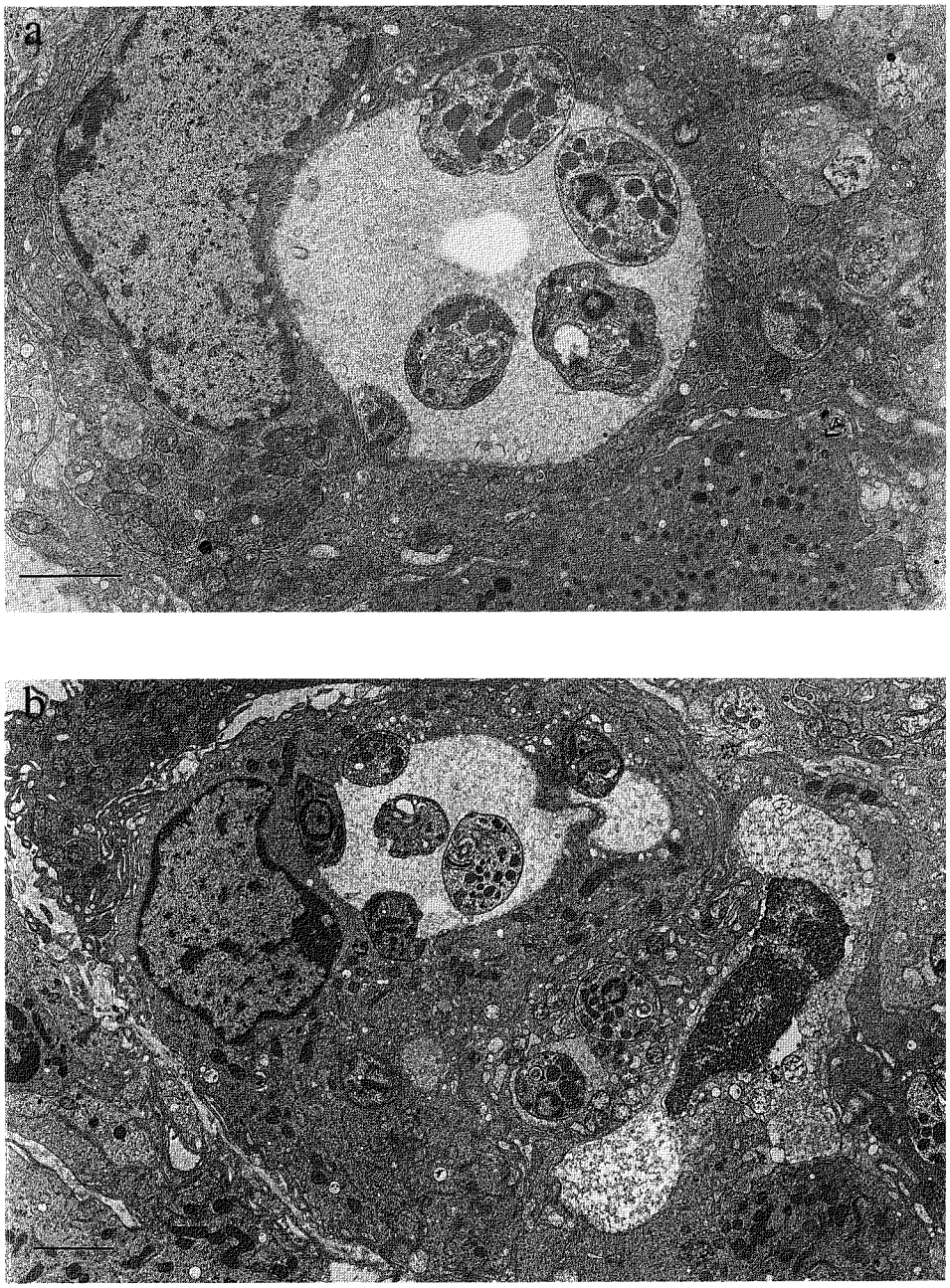


Figure 2.1.9. Electron micrograph from the animal infected with *L.* (*L.*) *mexicana* isolated from the patient with DCL. Phagocytized amastigotes are seen inside the vacuoles of macrophages in the sections of nose (**a**), right footpad (**b**), and left footpad (**c**). Note all the phagocytized parasites are still intact (Bars, a-c=2μm).

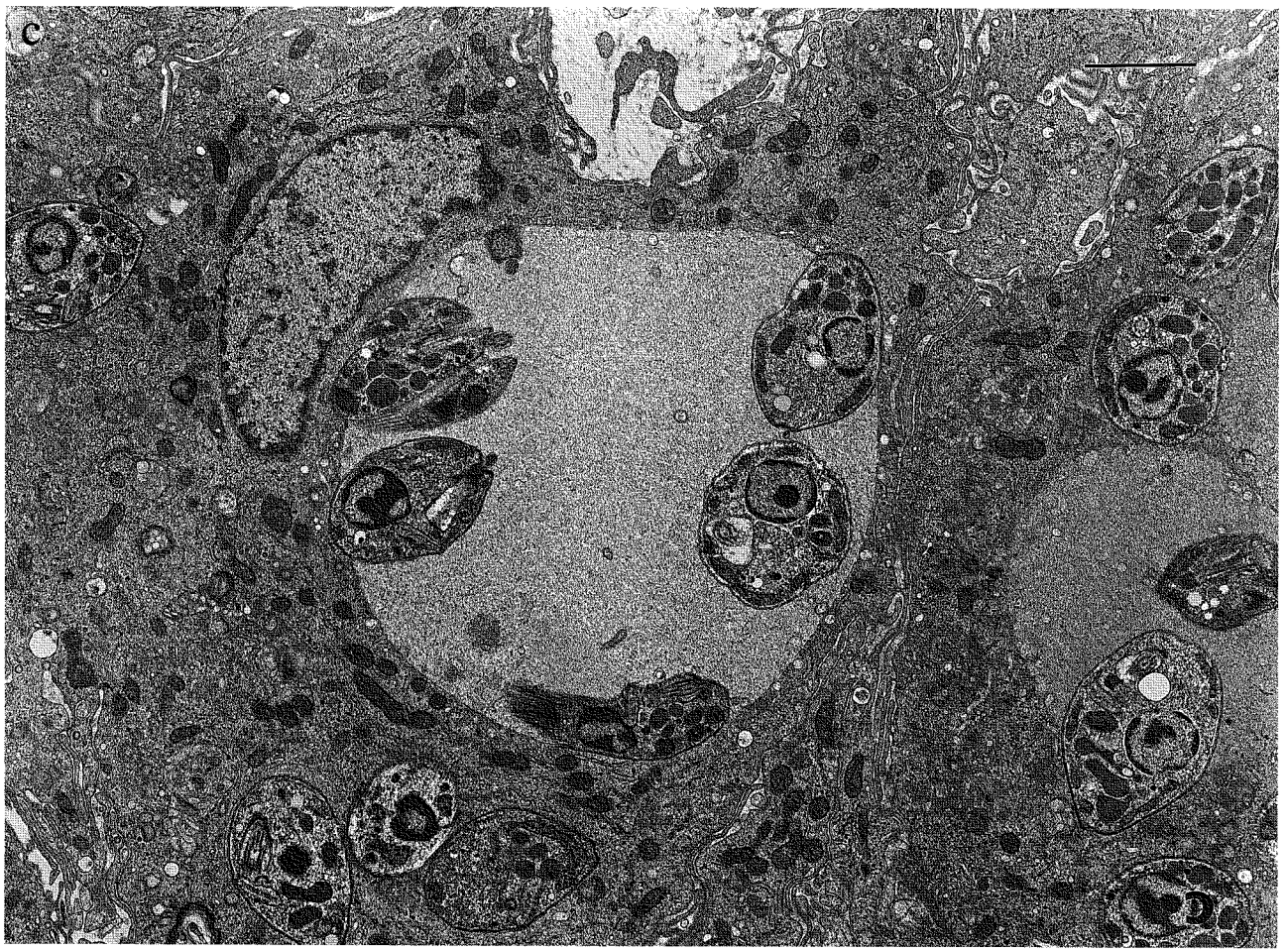


Figure 2.1.9 c (continued).

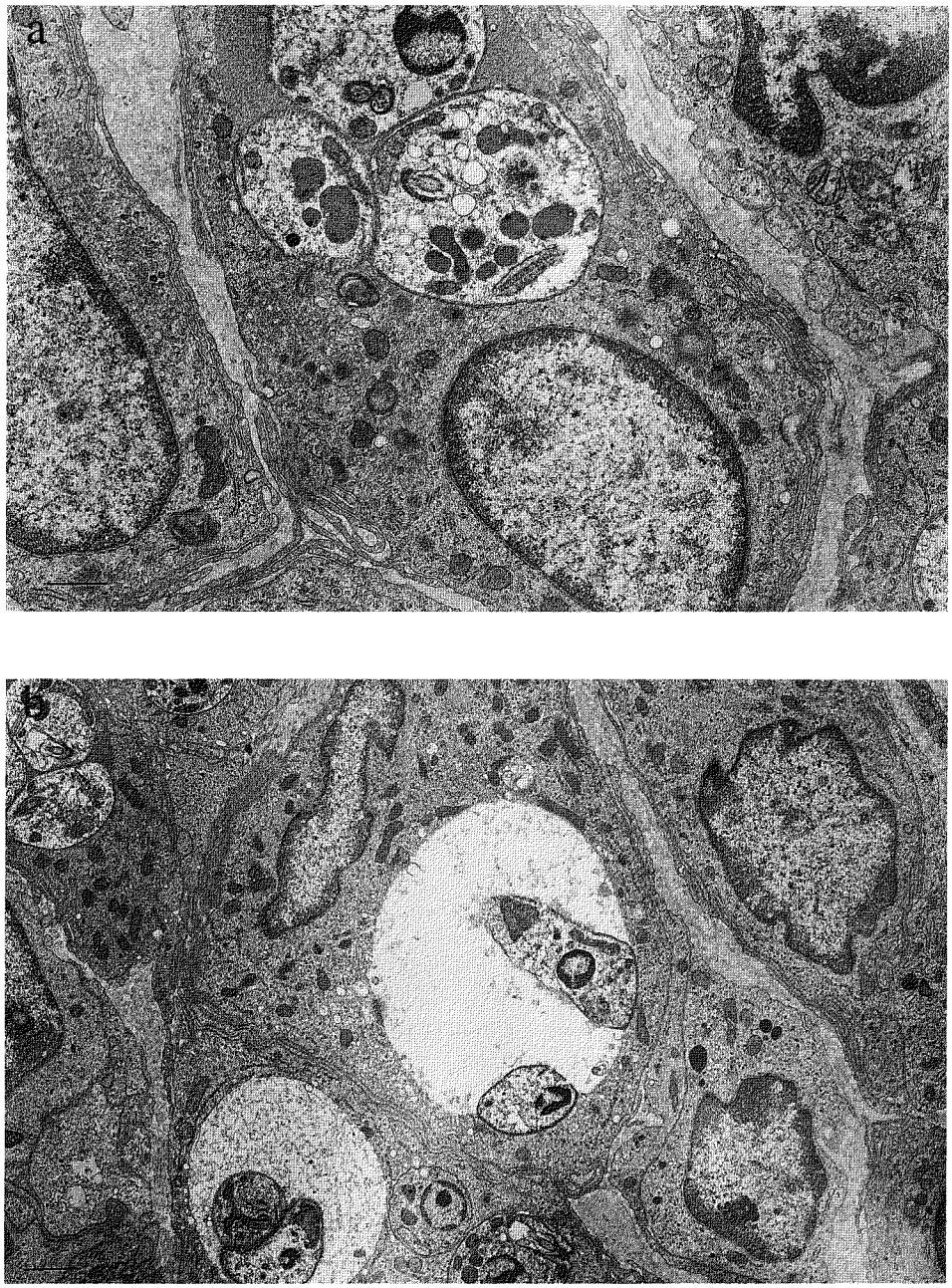


Figure 2.1.10 **a**, **b** and **c**. Electron micrograph from the animal infected with *L.* (*L.*) *mexicana* isolated from the patient with LCL. Note that almost all the phagocytized and non-phagocytized amastigotes are under the degeneration process (Bars, a,=1μm; b=2μm; c=500nm).

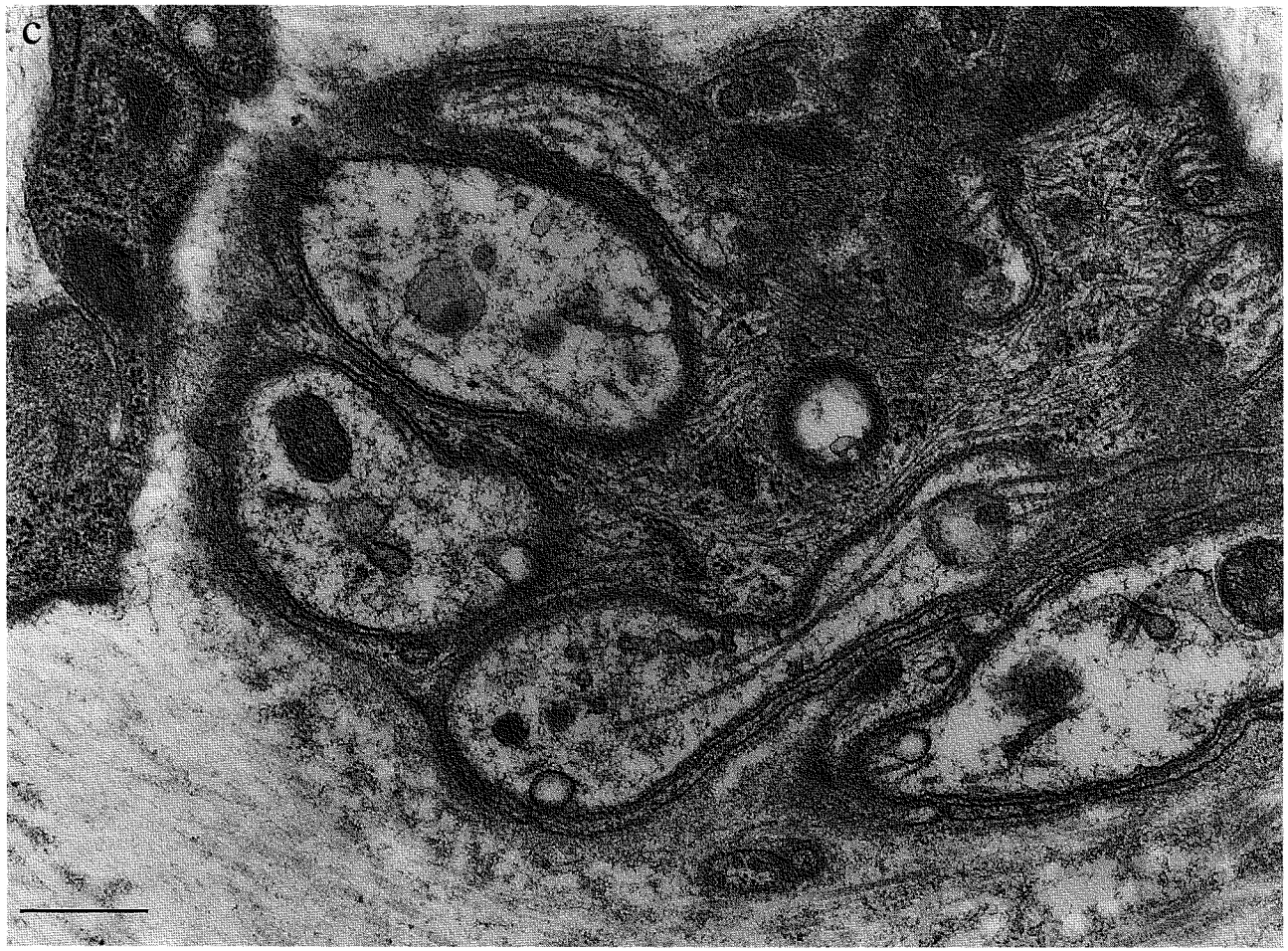
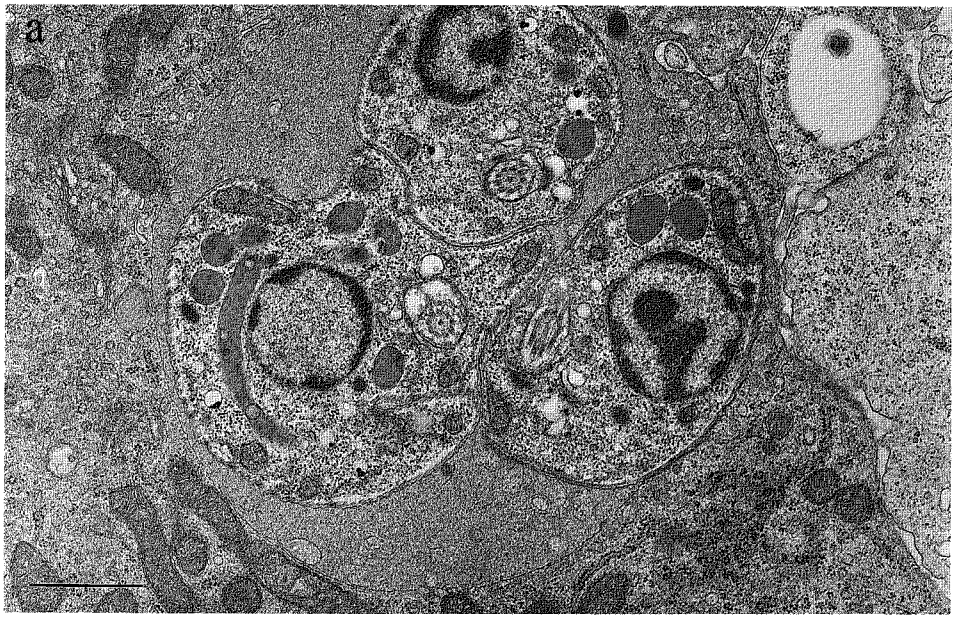


Figure 2.1.10 c (continued).



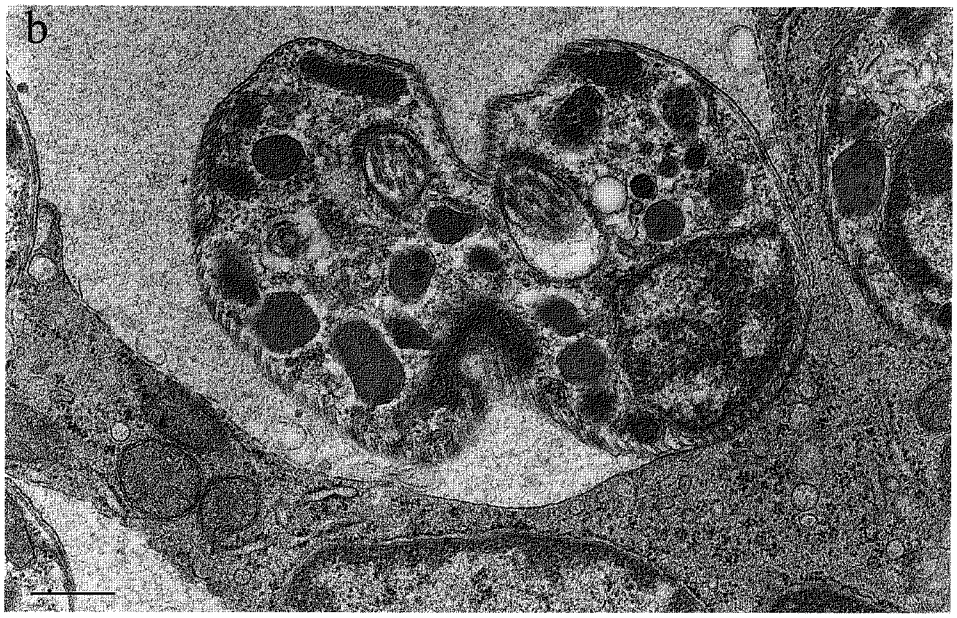


Figure 2.1.11 a and **b**. Ultrastructure of the *L.* (*L.*) *mexicana* parasites isolated from the patient with DCL (Bars, a=1μm; b=500nm). **c**. Ultrastructure of the *L.* (*L.*) *mexicana* parasites isolated from the patient with LCL (Bar=1nm).

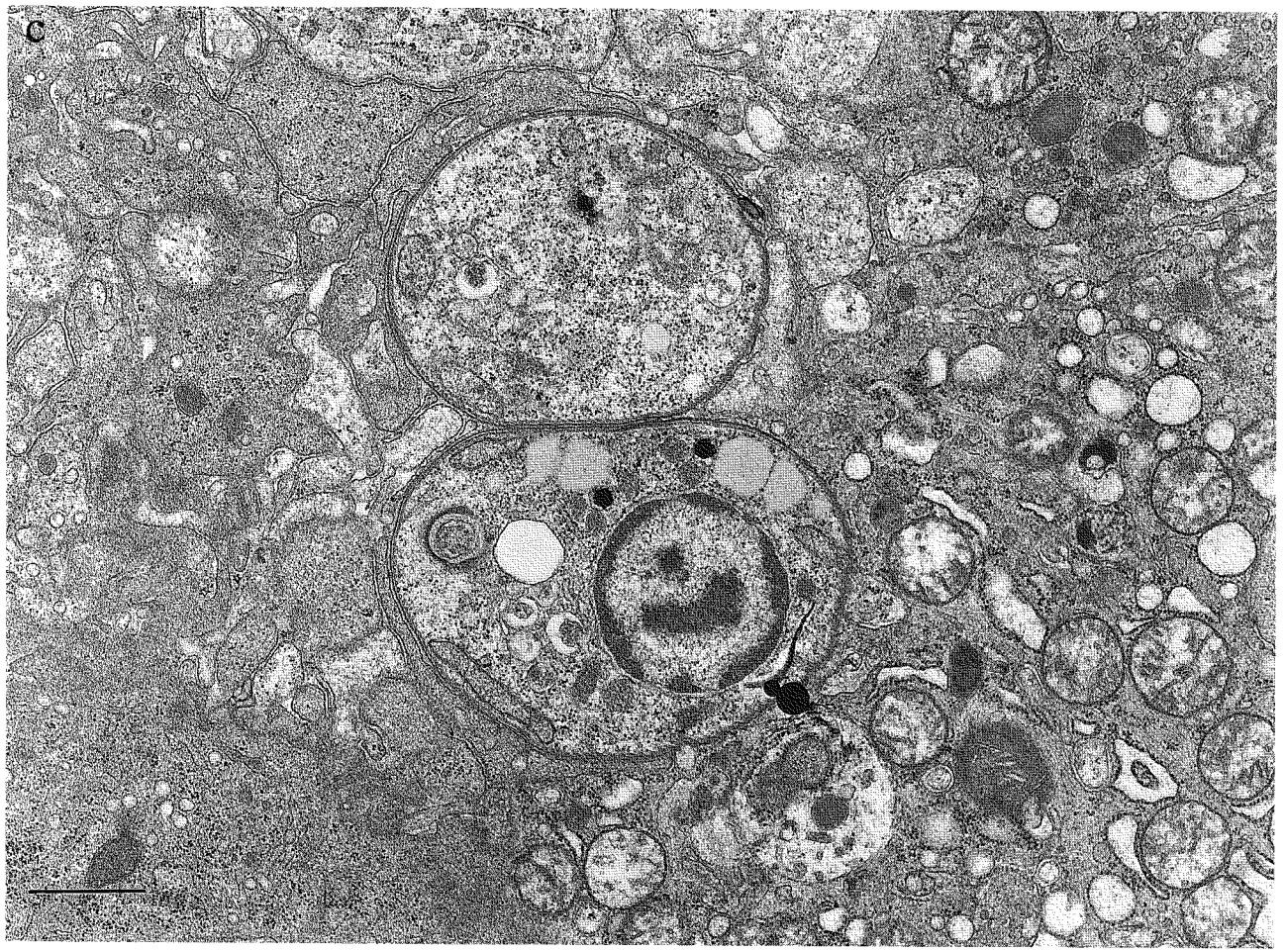


Figure 2.1.11 c (continued).

cellular growth of parasites between the two types of leishmaniasis. Recently, on the other hand, Kahl *et al.* (1991) made histopathological examinations of primary forepaw and metastatic lymph node, spleen, and liver lesions produced in golden hamsters infected with CL strains and mucocutaneous leishmaniasis (MCL) strains of *L.* (*Viannia*) *braziliensis* isolated in Brazil, but they could not find any pathological features providing clear differentiation of the CL and MCL strains.

In our specimens, we found the degeneration of parasites inside the macrophages only in the specimens of animals infected with LCL *L.* (*L.*) *mexicana* strain but not in the animals infected with DCL strain. From these findings it looks likely that the macrophages were unable to destroy the parasites of DCL stain, or, the present DCL *L.* (*L.*) *mexicana* strain might be resistant and not phagocytized by the macrophage. This histopathological difference is consistent with our clinical findings such as the development of different size and number of nodules in both groups (A and B). The finding indicates the possibility of involvement of some different mechanisms between the two groups. There was no difference in the number of phagocytic parasites inside the macrophages in both groups.

It is well known that CL patients show a delayed type hypersensitivity reaction against *Leishmania* antigen (Furuya *et al.*, 1989). On the other hand, when the *Leishmania* antigen was tested intradermally to DCL patients they showed no response to the antigen (Petersen *et al.*, 1984), suggesting the defect in cell mediated immune mechanism(s); our DCL patient also demonstrated no reaction against leishmanin skin test but showed positive reaction against other antigens, such as PPD, *Candida* and BCG (unpublished data). Petersen *et al.* (1982), furthermore, noted the involvement of suppressor cells in DCL patients and demonstrated an important role of suppressor cells in the pathogenesis of DCL. In our specimens the number of lymphocytes was lower in animals infected with DCL strains than those with LCL ones, but unfortunately the specificity of these cells was not checked in this study. Thus, as pointed out by the early work of Convit and Kerdel-Vegas (1965) and Convit *et al.* (1971), there must be some underlying host immune factors that play a major role in the transformation of one local nodule to multiple nodules spread all over the body.

In the present investigation, a few clinical and histopathological differences were observed between the animals infected with DCL and LCL strains of *L.* (*L.*) *mexicana* in the current study. These differences, however, were not recognized as major differential causative factors between DCL and LCL. In our experimental animals infected with DCL *L.* (*L.*) *mexicana* strain, no diffuse type of lesions was found. For a further investigation of differential factors between DCL and LCL, it will be necessary to search for a suitable animal model which produce similar cutaneous lesions as in human cases.

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2. Evaluation of Glucantime® (Meglumine Antimonate) Lots in anti-*Leishmania* Promastigote Activity *in vitro*

ABSTRACT. Growth inhibitory activity against leishmanial promastigotes was assessed with three manufacturing lots of meglumine antimonate (Glucantime®), which has been used for chemotherapy of leishmaniasis in Ecuador. A minimum twice difference in anti-promastigote activity was detected among Glucantime® lots. Effective concentration of the drug which inhibited promastigote proliferation by 50% (EC_{50}) varied with different *Leishmania* species, and EC_{50} values of the most effective lot were in a range of 20-38 mg/ml Glucantime® or 5.7-10.8 mg/ml antimony.

Introduction

Pentavalent antimonial drugs such as Pentostam® (sodium stibogluconate) and Glucantime® (meglumine antimonate) have long been used for treatment of leishmaniasis all over the world ever since these antimonial compounds were introduced in the 1940s (reviewed by Bryceson, 1987). However, unresponsiveness to pentavalent antimony in patients with leishmaniasis has been also a clinical problem (reviewed by Berman, 1988; Bryceson, 1987). Mechanism for the unresponsiveness is poorly understood. Host's genetic background or inherent resistance of the parasite to antimonials may be involved in inefficacy of the antimony therapy. Furthermore, lot variation of antimonial compounds in anti-leishmanial activity has been demonstrated, and this may be an additional reason for the clinical unresponsiveness (Jackson *et al.*, 1990). In Ecuador, Glucantime® has been the first choice chemotherapeutic agent against leishmaniasis (Hashiguchi and Gomez, 1990). However, little information is available concerning *in vitro* effect of meglumine antimonate against leishmanial promastigotes, and Glucantime® used in Ecuador has never been investigated in terms of lot variation. In the present study, thus, we examined *in vitro* anti-promastigote activity of three Glucantime® lots.

Materials and Methods

Parasites

In this study, we used three WHO reference strains, *i.e., Leishmania* (*Leishmania*) *amazonensis* MHOM/BR/73/M2269 (La), *L.* (*L.*) *garnhami* MHOM/VE/76/JAP78 (Lg), and *L.* (*L.*) *mexicana* MHOM/BZ/82/BEL21 (Lm), and also an isolate from *Choloepus hoffmanni* in Ecuador, *L.* (*V.*) *equatorensis* MCHO/EC/82/Lsp1 (Le) (Grimaldi *et al.*, 1992). Promastigotes were cultured at 24°C in Schneider's Drosophila medium (Gibco) containing 20% heat-inactivated fetal calf serum (HIFCS) and 100 mg/ml gentamycin.

In vitro drug susceptibility test

Drug susceptibility test of leishmanial promastigotes was performed as described before (Katakura *et al.*, 1993). Promastigotes were harvested from the late-log phase and resuspended in fresh Schneider's Drosophila medium containing 20% HIFCS at a concentration of $2x10^5$ per ml. The organisms ($4x10^4$) in a volume of 200 ml were added into each well of 96-well flat-bottomed microplates (Falcon). Three manufacturing lots of Glucantime® (Specia, Paris, France) were obtained in Ecuador. According to the subscription, each lot contains 300 mg/ml meglumine antimonate, and meglumine antimonate contains 28.35% (85 mg/ml)

antimony. Ten to forty microlitters of two-fold dilutions of each lot of Glucantime® were added into duplicate rows of the plate at final concentrations up to 60 mg/ml. The plate was sealed with a tape and incubated at 24°C for 4 days until control cultures containing no drug reach to the maximum growth. Ten microlitters of 20% glutaraldehyde were added into each well to fix the cells, and the number of parasites in each well 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 drug concentration which inhibited parasite growth by 50% (EC_{50}) was then determined.

Results and Discussion

A minimum twice difference in inhibitory effect on *in vitro* growth of leishmanial promastigotes was detected among three Glucantime® lots (Fig. 2.2.1). EC_{50} values of the most effective lot (referred to as lot A) were 20 to 38 mg/ml Glucantime® or 5.7-10.8 mg/ml antimony to promastigotes from four different *Leishmania* species, *i.e.*, 20, 24, 27 and 38 mg/ml Glucantime® for *L.* (*V.*) *equatorensis* (Le), *L.* (*L.*) *garnhami* (Lg), *L.* (*L.*) *mexicana* (Lm), and *L.* (*L.*) *amazonensis* (La), respectively. On the contrary, the other lots (referred to as lot B and lot C) appear to be twice to three times less effective than the lot A. EC_{50} values of the lot B and C against Le, Lg and La were all >60 mg/ml Glucantime®. Against Lm promastigotes, EC_{50} values of the lot B and C were 50 and 48 mg/ml Glucantime®, respectively.

A lot variation in cytotoxic activity against leishmanial promastigotes *in vitro* has been reported with both Pentostam® and Glucantime® (Jackson *et al.*, 1990). The authors developed a very sensitive radiorespirometric microtechnique to evaluate drug potency. Leishmanial promastigotes were preincubated with various ^{14}C-substrates (mainly ^{14}C-labeled L-amino-acids), and drug sensitivity was assessed by measurement of $^{14}CO_2$ release from drug-treated promastigotes in comparison to untreated controls. In this method a maximum 100 times difference in anti-promastigote activity was detected between Glucantime® production lots. The lot variation was not based on differences in antimony content, but may be due to problems inherent in the control of the carbohydrate moiety polymerization chemical reactions that occur during synthesis of the antimonial compounds (Jackson *et al.*, 1990).

The mode of action of pentavalent antimonials is poorly understood. Although sodium stibogluconate inhibited glycolytic enzymes and fatty acid beta-oxidation of amastigotes (Berman *et al.*, 1987), little information is available concerning meglumine antimonate. Interestingly, pentavalent antimonials are more effective against amastigotes in host cells *in vitro* than against promastigotes in culture (Berman *et al.*, 1982; reviewed by Neal, 1987). Glucantime® was active only at higher concentrations over 50 mg/ml against *L.* (*L.*) *enrietti*, *L.* (*V.*) *braziliensis*, and *L.* (*L.*) *pifanoi* promastigotes (Jiminez and Ercoli, 1965). With respect to *L.* (*L.*) *donovani*, ED_{50} value against promastigotes was over 20 mg/ml Glucantime® (Ullman *et al.*, 1989), whereas ED_{50} values against amastigotes in mouse macrophages were 1.39-2.68 μg/ml antimony (estimated to be 4.9-9.4 μg/ml Glucantime® (Neal and Matthews, 1982). Susceptibility of *L.* (*V.*) *panamensis* promastigotes to Glucantime® was biphasic; two EC_{50} values (0.5 and 35.0 mg/ml) were observed, suggesting mixed populations of parasites reflecting different response to the drug (Grögl *et al.*, 1989). In our study, EC_{50} values of one Glucantime® lot against promastigotes of different *Leishmania* species were from 20 to 38 mg/ml, which values are comparable to those reported by others above.

In Ecuador, patients with leishmaniasis are usually treated with intramuscular administration of Glucantime®, although a majority of patients were left untreated because of shortage of the drug (Hashiguchi and Gomez, 1990). Patients with simple cutaneous leishmaniasis were sometimes treated with perilesional administration of Glucantime® as reported in Saudi Arabia where intralesional injection of Pentostam® was made in treatment of cuta-

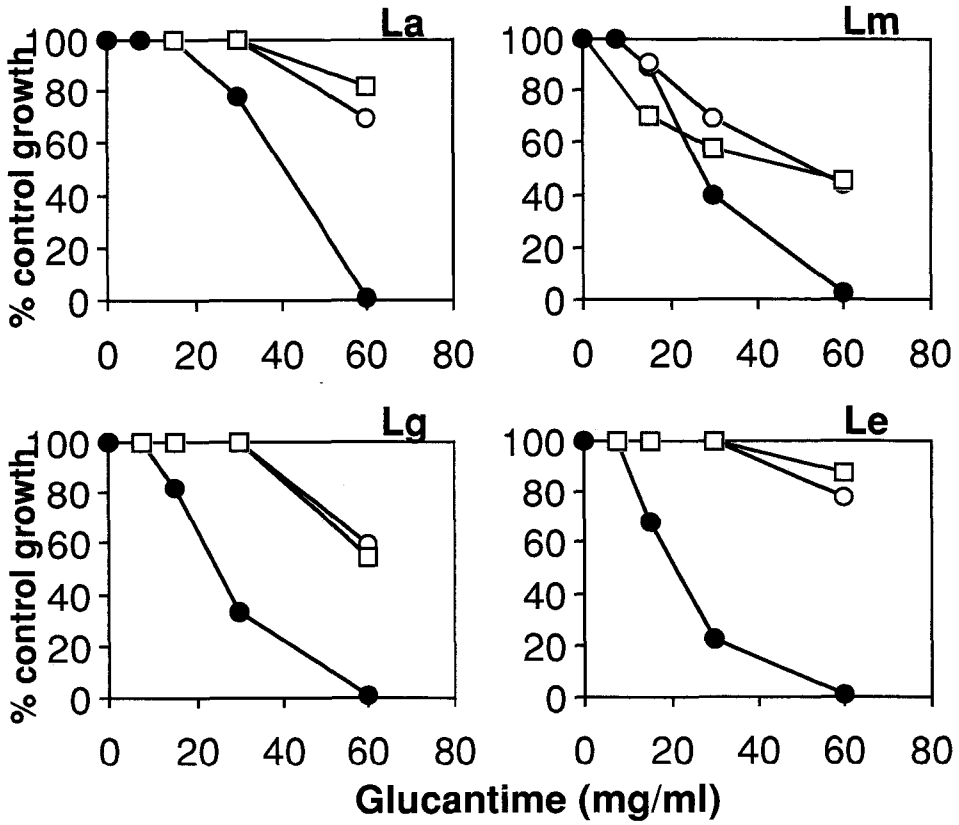

Figure 2.2.1. Anti-leishmanial activity of Glucantime® among manufacturing lots *in vitro*. Promastigotes of *L.* (*L.*) *amazonensis* (La), *L.* (*L.*) *mexicana* (Lm), *L.* (*L.*) *garnhami* (Lg) and *L.* (*V.*) *equatorensis* (Le) were cultured at 24°C for 4 days in the presence of various concentrations of Glucantime®, lot A (●), lot B (○) or lot C (□). Inhibitory effect of each lot on promastigote growth was determined as described in the text.

neous leishmaniasis (Kellum, 1986). We detected a minimum twice difference in anti-leishmanial activity among Glucantime® lots. Effect of Glucantime® against promastigotes *in vitro* may not reflect the effect against amastigotes *in vivo* (Neal, 1987), and lot variation observed in our study may not reflect the drug potency to eliminate amastigotes from host cells. However, drug potency must be carefully considered, because treatment of patients with insufficient dosage of pentavalent antimonials may cause serious relapse problems (Bryceson, 1987).

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Chapter 3

Vector Entomological Aspects

1. Studies on Species Composition of Sandflies and their Man-biting Activity in Leishmaniasis-endemic Areas of Ecauador

ABSTRACT. Biological features of several man-biting sandfly species were examined in two areas endemic for leishmaniasis. In the study site I (350m-600m above sea level) located at Andean slope, the population density, biting activity and natural infection with *Leishmania* promastigotes were thoroughly examined, and the results obtained were compared between 1983 and 1991/1993. The data revealed that there was a great difference between the two study points (1983 and 1991/1993). In the study site II (400m, above sea level) located at the cordillera of the Pacific coastal regions, species compositions and biting activities were mainly examined at the primary and secondary forest. Similar studies were also carried out inside the house sorrounded by secondary forest. In the area six man-biting species of the genus *Lutzomyia, viz., gomezi, serrana, trapidoi, shannoni, hartmanni* and *panamensis* were found. Among these, the first two species seemed to be most important from the point of view of the transmission of leishmaniasis.

Introduction

Lutzomyia trapidoi and *Lu. hartmanni* are the first species of anthropophilic sandflies incriminated as probable vectors of leishmaniasis in Ecuador (Hashiguchi *et al.*, 1985a). The biological features such as natural infections with *Leishmania* and their biting activities were previously examined in an Andean slope endemic for leishmaniasis in the country, during the period from 1983 to 1984 (Hashiguchi *et al.*, 1985a,b). Today, almost ten years later, similar studies have been done in the same place, in order to know if there might be any change in the species composition and/or the natural infection rates with the parasite. The results obtained are quite interesting, demonstrating different population densities and biting activities of sandfly species.

In the Pacific coastal regions of Ecuador, many sandfly species have been registered (Young and Rogers, 1984; Alexander *et al.*, 1992). Among them, *Lu. gomezi* has been suspected as a vector of leishmaniasis in the area. *Lu. serrana* is another important anthropophilic species collected and registered in the coastal regions of Ecuador (Rodriguez, 1956; Hashiguchi *et al.*, 1985a). Although *Lu. serrana* has not been found naturally infected yet, recent observations on the behavior suggest that this species could be role as a principal or one of the vectors of leishmaniasis. In order to know the behaviors of sandfly species, an investigation was also accomplished in Department of Manabi, Ecuador.

Materials and Methods

The present studies were conducted at the following two different areas endemic for leishmaniasis in Ecuador.

The study area I: Lu. trapidoi and *Lu. hartmanni* were studied in the same place where most biological evaluations of these two species were done during 1983 and 1984; the place named "El Chorro",

Ocaña, Department of Cañar, Ecuador, situated from 350m to 600m above sea level. Sandfly collections were made using protected human baits. The insects collected were dissected and identified, and then they were examined for natural infections with *Leishmania*. Hourly population densities and biting activity patterns were also analyzed based on the samplings in each collection site. The current data obtained were compared to the previous ones, in order to know the ecological changes of sandflies in the area during about ten years.

The study area II: Hourly biting patterns of six sandfly species were examined in an area endemic for leishmaniasis in the Department of Manabi, near the Pacific coast in Ecuador. Fly collections were performed in the primary and secondary forest; a part of collection was also conducted within the human dwellings surrounded by a dense forest.

Results and Discussion

As shown in Table 3.1.1, in 1983, *Lu. trapidoi* was the dominant sandfly species at 600m above sea level of Ocaña, a known leishmaniasis-endemic area with a natural infection rate of 7.7%. In the area *Lu. hartmanni* had a secondary role, with a lower population density and 3.3% of the natural infection rate with *Leishmania*. A similar situation was also found at 350m above sea level (data not shown). In 1991 another observation was also made at the same place, in order to check ecological situations. Surprisingly, *Lu. hartmanni* was the dominant species with a higher natural infection rate (5.5%), while the other species, *Lu. trapidoi* showed a much lower population density, and the natural infection was not found in any sandflies. In 1993 the situation was nearly same (Table 3.1.1).

Biting activities of *Lu. trapidoi* and *Lu. hartmanni* at the study site I was examined again, as shown in Table 3.1.2. This time, *Lu. hartmanni* was highly dominant throughout the night, just like *Lu. trapidoi* showed 10 years ago at the site (Table 3.1.2).

In the study site II, observation was done on the behavior of sandflies in two types of vegitations, *viz.*, the primary and secondary forest in the Department of Manabi, Ecuador. In the primary forest, the most important man-biting species was *Lu. gomezi* and then followed by *Lu. hartmanni* and *Lu. serrana* (Table 3.1.3). On the other hand, in the secondary forest, *Lu. gomezi* was most dominant species, then the numbers collected reduced gradually in the order of *Lu. serrana, Lu. trapidoi, Lu. hartmanni, Lu. panamensis* and *Lu. shannoni*. *Lu. hartmanni* was captured in a surprisingly low number at the secondary forest in this area (Table 3.1.4).

Domiciliary biting activity patterns of sandflies are quite different from those in forest of the area (II); *Lu. serrana* was highly dominant. Inside the house surrounded by a secondary forest, *Lu. serrana* was the most active man-biting species, followed by *Lu. trapidoi*, *Lu. gomezi* and *Lu. panamensis* (Table 3.1.5). A total of 103 males of these four species were also captured, but most of them (84; 81.6%) being *Lu. serrana*. It seemed to be very important to point out that although the four species were captured inside of the rural house at night, during the day (15:00 to 15:30) only *Lu. serrana* was captured in an important number (124) within 30 minutes of domiciliary captures. About one third of female *Lu. serrana* captured were engorged with blood; among the rest of flies, 20 were male specimens.

The changes of distribution, population density and natural infection with *Leishmania*, of two sandfly species, *Lu. trapidoi* and *Lu. hartmanni*, during the period of eight or 10 years at the same collection site, were not able to explain adequately. In order to answer this question, more detailed and long-term bioecological research works would be necessary, performing monthly observations throughout the year at the same place. Mammalian hosts should be identified in the area. Their population would influence density and dynamics of each *Lutzomyia* species, in relation to dietary preferences of sandflies. Some important change of mammalian host population might cause variation of the natural infection rates of these two sandfly species, *Lu.*

Table 3.1.1. Natural infections of *Lu. trapidoi* and *Lu. hartmanni* with *Leishmania* promastigotes in the study site I, Ocaña, Department of Cañar, Ecuador in 1983 and 1991/1993

Year	Altitude (m)	*Lu. trapidoi*		*Lu. hartmanni*	
		No. examined	(%)	No. examined	(%)
1983*	600	1061	7.7	275	3.3
1991	350	23	0	378	5.5
1993	600	19	0	437	5.9

*, Hashiguchi *et al.*, 1985a.

Table 3.1.2. Biting activity of two species of sandflies, *Lu. trapidoi* and *Lu. hartmanni*, expressed as hourly numbers and/or percentages, in study site I, Ocaña, Department of Cañar, Ecuador in 1983 and 1993

Lutzomyia species	Year	Hours						
		18-19	19-20	20-21	21-22	22-23	23-24	Total
trapidoi	1983*	25	178	275	142	175	141	936
		2.7%	19.0%	29.4%	15.2%	18.7%	15.1%	
hartmanni	1983*	2	34	27	21	17	34	135
		1.5%	25.2%	20.0%	15.6%	12.6%	25.2%	
trapidoi	1993	0	16	14	24	19	29	102
		0.0%	15.7%	13.7%	23.5%	18.6%	28.4%	
hartmanni	1993	69	119	117	202	311	287	1105
		6.2%	10.8%	10.6%	18.3%	28.1%	26.0%	

*, Hashiguchi *et al.*, 1985b.

trapidoi and *Lu. hartmanni*; animals might move from one to another habitat, and then the place in diet of sandflies might be taken by the other.

On the other hand, changes in distribution and/or population density of flies also strongly depend on the climatic or ecological conditions, directly relating to the breeding requirements of each sandfly species. In this case, many ecological factors, such as humidity, temperature, organic compositon of the soil, sugar sources, depredation, biological competition should be taken into consideration.

In leishmaniasis-endemic areas of the Department of Manabi, Ecuador, natural infections with *Leishmania* promastigotes have not been found yet in any of the six anthropophilic sandfly species mentioned above. By our intensive survey carried

Table 3.1.3. Hourly biting activities of six man-biting sandfly species during 19:00 and 06:00 at a primary forest of study site II, Km 100, Department of Manabi, Ecuador, in August 1991

	Species of *Lutzomyia*						
Hours	*gomezi*	*hartmanni*	*serrana*	*trapidoi*	*shannoni*	*panamensis*	Total
19-21	178	87	27	10	-	-	302
21-23	94	55	12	3	-	-	164
23-01	63	29	2	3	-	-	97
01-03	60	23	-	1	-	-	84
03-05	109	25	5	1	1	-	141
05-06	31	5	-	2	1	-	39
Total	535	224	46	20	2	-	827

Table 3.1.4. Hourly biting activities of six man-biting sandfly species during 19:00 and 06:00 at a secondary forest of study site II, Km 100, Manabi, Ecuador, in August 1991

	Species of *Lutzomyia*						
Hours	*gomezi*	*hartmanni*	*serrana*	*trapidoi*	*shannoni*	*panamensis*	Total
19-21	21	4	126	8	7	5	171
21-23	45	6	68	17	1	7	144
23-01	97	7	15	26	-	1	167
01-03	139	1	8	17	-	-	167
03-05	58	1	5	10	4	1	80
05-06	12	-	-	2	-	-	14
Total	372	19	242	80	12	14	743

out in the areas to date, however, some information is available to discuss a vectorial capacity at some extent. In the primary and secondary forest, *Lu. gomezi* seemed to be the most important man-biting species, followed by *Lu. serrana* playing a secondary role. Sandflies were collected inside the house surrounded by a dense forest during the night. The result suggested that the most important man-biting sandfly corresponded to *Lu. serrana*, though a few numbers of *Lu. trapidoi, Lu. gomezi* and *Lu.*

Table 3.1.5. Hourly biting activities of six man-biting sandfly species during 19:00 and 06:00 inside the house surrounded by a dense forest of study site II, Km 101, Department of Manabi, Ecuador, in August 1991

	Species of *Lutzomyia*						
Hours	*gomezi*	*hartmanni*	*serrana*	*trapidoi*	*shannoni*	*panamensis*	Total
19-21	-	-	85	6	-	2	93
21-23	8	-	112	11	-	5	136
23-01	3	-	127	3	-	1	134
01-03	-	-	98	-	-	-	98
03-05	-	-	105	-	-	-	105
05-06	-	-	42	-	-	-	42
Total	11	-	569	20	-	8	608
Males*	1	-	84	12	-	6	103

*, Accidentally caught.

panamensis were also captured during the night. In the day-time (resting site) collection, on the other hand, males and engorged females of *Lu. serrana* were found to be as the only species. The presence of males and engorged (gravid) females may strongly suggest that they are using the house as a breeding site. Moreover, based on the present observations, we speculated about the posibility of an adaptation of *Lu. serrana* to human dwellings. Namely, this species might find a suitable ecological condition for their resting and breeding. The leaves cover of the house not only provides shadow, humidity and fare temperature, but also a lot of organic materials existing among the leaves, because of the presence of a big number of rats, bats, cockroaches, spiders, beatles, mushrooms and other protagonists of life, there. To disclose behavioral factors of sandflies, *Lutzomyia* spp., in relation to leishmaniasis transmission, more detailed studies should be done in future.

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2. Examinations on Natural Infections of Sandflies, *Lutzomyia* spp., with *Leishmania* in an Endemic Area, Km 101, Department of Manabi, Ecuador

ABSTRACT. In a leishmaniasis-endemic area, Km 101, Department of Manabi, Ecuador, six man-biting sandfly species, *viz., Lu. gomezi, Lu. hartmanni, Lu. serrana, Lu. trapidoi, Lu. shannoni* and *Lu. panamensis,* were examined in order to search for natural infections with *Leishmania* promastigotes. A total of 2,530 flies were dissected, but no natural infection was found. In the text, transmission cycle of leishmaniasis in the area was briefly discussed.

Introduction

In the Pacific coastal regions of Ecuador, leishmaniasis had sporadically occured in some areas, especially in the north and north-eastern part of the Department of Manabi. In this area, after 1984 when an important climatic and ecological event, such as "El Niño" phenomenon and the construction of "Poza Honda" dam was found, human cutaneous leishmaniases were reported as an outbreak at different areas of the province (Alava *et al.*, 1994). Thus, the new focus was considered to be one of the most important endemic areas of leishmaniasis in Ecuador. The highest incidence was recognized during the period from 1989 to 1990 (Alava *et al.*, 1994). In the area, therefore, an entomological survey was necessary to complement epidemiological information on the disease.

The current paper presents the result of our preliminary entomological studies in the area. Sandfly population was diverse and abundant, especially during rainy season. A total of six man-biting species were found and examined so far. Although a total of 2,530 flies were dissected, natural infecions with *Leishmania* promastigotes were not found yet. The present result showed an intersting contrast when compared to other leishmaniasis-endemic areas of Ecuador.

Materials and Methods

Sandfly collections, using protected human baits, were performed in several sites of the leishmaniasis-endmic area of the Department of Manabi from 1990 to 1993. These collection sites were relatively close in distance each other. The dissection was carefully done by our routine procedure, and microscopic observations of phlebotomine gut and genitaria were also accurately accomplished (Hashiguchi *et al.*, 1985). Sandfly collections were performed in those places where several human cases of cutaneous leishmaniasis were reported (Alava *et al.*, 1994).

Results and Discussion

In the current study sites, six man-biting species of sandfies, *Lutzomyia* spp., were collected and dissected for identification and evaluation of natural infections with *Leishmania* promastigotes; the species identified were *Lu. gomezi, Lu. hartmanni, Lu. serrana, Lu. trapidoi, Lu. shanonni* and *Lu. panamensis.* The total numbers of each *Lutzomyia* species captured and dissected in different collections are shown in Table 3.2.1. No natural infection with *Leishmania* promastigotes was found in a total of 2,530 flies dissected. However, the present examination revealed some interesting information,

Table 3.2.1. Results of the dissection of man-biting sandfly species collected in a leishmaniasis-endemic area, Km 101, Department of Manabi, Ecuador; all negative for *Leishmania* promastigotes

Date	No. of *Lutzomyia* spp. collected and dissected						Total
	gomezi	*hartmanni*	*serrana*	*trapidoi*	*shannoni*	*panamensis*	
90-8-02	84	38	15	12	1	0	70
91-4-20	372	19	242	80	12	14	739
91-8-17	907	455	86	18	11	1	1478
92-8-08	47	77	5	0	0	13	142
93-1-26	0	37	1	0	0	2	40
93-1-29	5	4	49	0	3	0	61
Total	1335	630	398	110	27	30	2530

regarding to the transmission mode of leishmaniasis. First of all, as mentioned above, six anthropophilic sandfly species were found in the area. Among these species, only *Lu. serrana* was not yet incriminated as vector of leishmaniasis in Ecuador nor neighbouring countries (Young and Lawyer, 1987; Grimaldi *et al.*, 1989, 1993), though the species was found to be highly anthropophilic in the present collection site.

Lu. gomezi had been suspected as a vector of leishmaniasis in Panama (Christensen *et al.*, 1983). The species was also found naturally infected with *Leishmania* promastigotes in Mocache, Department of Los Rios, Ecuador (Gomez and Hashiguchi, 1987). In the present area, *Lu gomezi* seemed to be the most abundant man-biting species. *Lu. hartmanni* was a suspected vector of the disease in Ocaña, Department of Cañar, Ecuador, showing a high rate of natural infections with *Leishmania* (Hashiguchi *et al.*, 1985). Peripylarian promastigote infections of *Lu. hartmanni* were seen in two wild caught females in Santander, Colombia (Young and Lawyer, 1987). *Lu. trapidoi* was a presumptive vector of *L. (Viannia) panamensis* in Panama and Colombia (Young and Lawyer, 1987). In Ecuador the same species was found naturally infected with *Leishmania* promastigotes, showing a high rate, in Ocaña, Department of Cañar (Hashiguchi *et al.*, 1985). In this country *Lu. trapidoi* seemed to show a wide range of geographical distribution (Alexander *et al.*, 1992). *Lu. shannoni* was found naturally infected with *Leihmania* promastigotes in several countries of the New World, *viz.*, USA (Young and Perkins, 1984), Costa Rica (Zeledon and Alfaro, 1973), Panama (Johnson *et al.*, 1963), Colombia (Young and Lawyer, 1987), Brazil (Arias *et al.*, 1985; Ryan *et al.*, 1987) and French Guiana (Le Pont *et al.*, 1980). *Lu. panamensis* was regarded as a natural vector of *L. (V.) panamensis* in Panama (Christensen *et al.*, 1983).

As mentioned before, in Ecuador, a large numbers of parasitologically confirmed human cases of leishmaniasis were recently reported from the Department of Manabi (Alava *et al.*, 1994). The cases reported were registered at outpatient clinic of a national institute located in Portoviejo city; almost all of the patients recorded were from rural regions. Therefore, it was easily estimated that there might

be many self-cured or non-registered human leishmaniasis cases in the remote (rural) areas. It was also estimated that such an outbreak of the disease might be caused by the existence of one or more efficient sandfly vectors in this endemic area. Five of the present man-biting sandfly species except *Lu. serrana* were incriminated as probable vectors in other Central and South American countries (Young and Lawyer, 1987; Grimaldi *et al.*, 1989). Among these, three species, *Lu. trapidoi, Lu. hartmanni* and *Lu. gomezi,* were also suspected as vectors of the disease in other parts of Ecuador (Hashiguchi *et al.*, 1985; Gomez and Hashiguchi, 1987). Natural infections of sandflies with *Leishmania* in this endemic area might be actually very high, though no infection was recognized in the current study. This assumption might be supported by a high number of human cases reported in the area by Alava *et al.* (1994).

Most *Leishmania* isolates from the Department of Manabi have been identified as *L. (V.) panamensis* and *L. (V.) guyanensis* by isoenzyme analysis (Agatsuma and Furuya, unpublished data). With regard to their vectors *Lu. trapidoi* and *Lu. panamensis* might be probable vectors in the Department of Manabi, Ecuador, as reported in other countries, Colombia and Panama (Christensen *et al.*, 1983; Young and Lawyer, 1987). As factor(s) responsible for the present negative result of sandfly dissection in this study, the population size of mammalian (reservoir) hosts in the area should be taken into consideration. If the principal reservoir host might be a migratory species appearing only at certain period of the year, the natural infections of vector sandflies would not be found untill dissected just in that season. Clinical human cases in the area demonstrated that the majority of persons suffered from the disease during the end of rainy season, March, April and May (Alava *et al.*, 1994). Therefore, more detailed entomological survey should be conducted during these months in future, in order to find the natural infections. In conclusion, at the moment, all the six anthropophilic sandfly species found in the area should be considered as a suspected vector of leishmaniasis.

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3. Parity of Sandflies, *Lutzomyia* spp. Collected at Different Leishmaniasis-endemic Areas of Ecuador

ABSTRACT. Follicular developments of sandflies collected at both highland and lowland areas were thoroughly examined. The parous rate of *Lutzomyia ayacuchensis* was 9.0% (10/111) at night collection and 9.2% (9/98) at early morning collection in highland (Huigra, Department of Chimborazo). No difference between the rates of flies collected at two different times (night and early morning) was found. Parous rates of *Lu. gomezi* and *Lu. serrana* from lowland areas (Guayabales and San Sebastian, Department of Manabi) were 14.6% (7/48) and 0.0% (0/7), respectively. Some of *Lu. gomezi* collected showed the developmental stage II or III of follicles without any blood meals, suggesting an existence of autogeny individuals.

Introduction

Age determination of sandflies becomes an important factor to know the transmission mode of leishmaniasis, and also to evaluate the efficacy of insecticides. However, a few information on follicular developments of sandflies has been available to date, when compared with mosquitoes (Lewis, 1965; Magnarelli *et al.*, 1984; Yuval and Schlein, 1986; Wilkes *et al.*, 1980).

In the previous study, parous rates of *Lu. hartmanni* and *Lu. trapidoi* collected in early morning showed a higher rate than those caught during dusk in Ocaña, Department of Cañar, a lowland endemic area; 59.2% *vs* 33.3% in the former and 40.9% vs 20.0% in the latter (Eshita *et al.*, 1992). The current study was designed to compare follicular developments between highland and lowland sandflies, *Lutzomyia* spp., in relation to the transmission of leishmaniasis in endemic areas.

Materials and Methods

Sandflies were collected by protected human baits in leishmaniasis-endemic areas during the day and night time. Resting site collections were also made inside the houses in Huigra, Department of Chimborazo, located at 1,200m above sea level of the Andes and in Guayabales and San Sebastian, Department of Manabi, located at 400m-650m a.s.l. of the Pacific coast of Ecuador during July and September 1992. Sandflies collected were maintained at low temperature until dissected. Under stereo-microscope, fresh sandflies were dissected, and then their spermathecae were thoroughly examined for species identification. By observing dilatation of follicles, parous rate of each fly was estimated.

Results and Comments

Table 3.3.1 shows follicular development of *Lutzomyia* spp. from highland (Huigra) and lowland (Guayabales and San Sebastian) areas. In highland, parous rates of *Lu. ayacuchensis* were 9.1% (19/209); no marked difference was found between two collections made at early morning and night. On the other hand, in lowland parous rates of *Lu. gomezi* and *Lu. serrana* were 14.6% (7/48) and 0.0% (0/7), respectively.

As mentioned above, parous rates were estimated by observing dilatation of follicles. However, it was not easy to dissect the small sized ovary and also not easy to distinguish between the false dilatation by degenerated follicles and the dilatation just after ovulation. In our previous work with *Lu. hart-*

Table 3.3.1. Follicular development of highland and lowland sandflies, *Lutzomyia* spp. in Ecuador

Collection sites	No. of sandflies dissected	No. of female with developmental stages of the first follicles:					
		I	II	III	V	DF*	Suck-like dilatation
Huigra							
(resting site collection inside house)							
Lu. ayacuchensis	111	87	7** (6.3%)	1**	1**	5 (4.5%)	10 (9.0%)****
(human-bait collection in field)							
Lu. ayacuchensis	98	86	0	0	0	3 (3.1%)	9 (9.2%)****
						Parous rates: 19/209= 9.1%****	
Guayabales and San Sebastian							
(human-bait collection in field)							
Lu. gomezi	48	29***	5 (10.4%)	0	0	7 (14.6%)	7**** (14.6%)
Lu. serrana	7	6	1*** (14.3%)	0	0	0	0 (0.0%)
						Parous rates: 7/55= 12.7%****	

*, Degenerated follicles; **, females with red blood or brown blood meal; ***, females without blood meal; ****, parous rate (%) = No. females with dilatation / No. females dissected x 100.

manni and *Lu. trapidoi*, a clear difference of parous rates between sandflies collected in early morning and during dusk, obtaining a markedly high rate in the morning (Eshita *et al.*, 1992). In the present study, however, a low parous rate was found in each species of *Lutzomyia* from highland and lowland, indicating no significant difference between morning and night collections.

Some samples of *Lu. gomezi* caught in lowland showed the stage II and III of follicular development without any blood meals, suggesting a possibility of existence of autogeny. From the result obtained, it was considered that relationships between natural infections with *Leishmania* and seasonal autogeny

rates in endemic areas should be examined in future entomological surveys.

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4. A Preliminary Study on Susceptibility of Sandflies Against Fenitrothion (Sumithion)

ABSTRACT. Susceptibility of *Lutzomyia gomezi* adults against fenitrothion (Sumithion, Sumitomo Co., Ltd., Japan) was tested in Ecuador. Sandflies tested were collected by using protected human baits. Sumithion was diluted with acetone solution and treated onto synthetic boards and filter papers. The sandflies collected were confined onto the impregnated materials for different time intervals. Then, the exposed adults were transferred into insecticide-free small polystyrene containers. The numbers of knock-down sandflies were counted. A high rate of knock-dawn flies was observed within 60-80 minutes after 15 minutes exposure onto Sumithion-treated surface. Based on the result obtained, residual sprays of the insecticide used was briefly discussed from the view point of reducing biting chance of endophilic sandflies in leishmaniasis-endemic areas of Ecuador.

Introduction

It was frequently experienced that blood-fed and unfed endophilic sandflies took a rest on inside walls of houses even in the morning and during the daytime. From these field observatios, it was considered that such a long resting behavior of endophilic sandflies might strengthen residual effects of insecticides, not only reducing sandfly biting chance but also reducing *Leishmania* infection of persons. For this reason, we preliminary examined knock-down effects of an insecticide, Sumithion, against Ecuadorian sandflies, *Lutzomyia* spp.

Materials and Methods

All the sandflies, *Lu. gumezi*, were collected by protected human baits in Guayabales and San Sebastian, Department of Manabi, Ecuador. The flies collected were immediately used for susceptibility tests in the field under a careful maintenance using moisturized containers. Technical grade of fenitrothion (Premium-Sumithion = Sumithion; Sumitomo Chemical Co., Ltd., Japan) was diluted with acetone solution, and then was treated onto artificial boards at the ratio of 1 g active ingredient (AI) per square meter, and also treated onto filter papers (No. 2, Toyo Roshi Co., Ltd., Japan) at the ratio of 1% against the weight of filter paper. The boards and the filter papers were prepared on 22 August 1992 and wrapped with aluminum foil until used on 11 and 22 September 1992.

Sandflies, *Lu. gomezi*, were given a chance to contact onto the board or filter paper for 15, 30, 45 and 60 minutes, respectively, at approximately 25°C. The numbers of knock-down females were counted every several minutes, after they were transferred into insecticide-free tubes with wet paper. Ratios of the number of knock-down females per the number of females used were calculated in each test. Knock-down percentages of sandflies were corrected by Abbott's formula, if the control mortality existed between 5% and 20% as follows (WHO, 1970): % test knock-down − % control knock-down / 100 − % control knock-down x 100.

Results and Comments

Longevity of sandflies, *Lu. gomezi*, against insecticide-free (control) boards and filter papers was examined, and the results was shown in Table 3.4.1. All the flies exposed onto the insecticide-free

Table 3.4.1. The number of sandflies, *Lu. gomezi,* at different time intervals after transfer of the insects exposed to insecticide-free (control) boards and filter papers

Time intervals (minutes)*	Insecticide-free	
	Boards	Filter papers
0	0/8 (0 %)**	0/8 (0 %)**
60	0/8 (0)	0/8 (0)
70	0/8 (0)	0/8 (0)
80	0/8 (0)	0/8 (0)
90	0/8 (0)	0/8 (0)
100	0/8 (0)	0/8 (0)

*, Sandflies, *Lu. gomezi*, were transferred into new containers after exposure to insecticide-free materials during 60 minutes; each time shows the elapse after the transfer.

**, No. of knock-down females / No. of females tested (%).

Table 3.4.2. The number of knock-down sandflies, *Lu. gomezi*, at differnt time intervals after 60 minute exposure on Sumithion-impregnated board and filter papers

Time intervals (minutes)*	Insecticide-free	
	Boards	Filter papers
0	0/15 (0.0%)**	0/7 (0.0%)**
54	7/15 (46.7)	2/7 (28.6)
60	10/15 (66.7)	4/7 (57.1)
65	13/15 (86.7)	6/7 (85.7)
90	15/15 (100.0)	7/7 (100.0)

Remarks:

Lu. gomezi sandflies were exposed onto Sumithion-impregnated boards (1g/m²) or filter papers (1%, weight/volume) in polystyrene tubes for 60 minutes at 24°C. And then they were transferred into insecticide-free tubes with wet filter paper. Knock-down females were counted. The Sumithion-impregnated materials were prepared in 22 August 1992 and used for the experiments about 20 days later (11 September 1992).

*, Time intervals in incecticide-free containers after exposure of sandflies to Sumithion-impregnated materials.

**, No. of knock-down females / No. of females tested (%).

materials for 60 minutes were found to be alive in another (fresh) container at least 100 minutes after the transfer.

Susceptibility of *Lu. gomezi* against Sumithion-impregnated boards and filter papers in polystyrene tubes was examined preliminary (Table 3.4.2). Some knock-down sandflies were observed in fresh containers 54 minutes after they exposed onto boards or filter papers during 60 minutes. At 90 minutes of the transfer into fresh container all the flies tested were found knocked-down. There was no marked difference in numbers of knock-down females between those tested on boards and filter papers. The present preliminary test showed that Sumithion-impregnated materials remained effective against sandflies more than 20 days at least.

In the second experiment, field collected *Lu. gomezi* were exposed onto Sumithion-impregnated materials for different time intervals, 15, 30, 45 and 60 minutes, respectively (Table 3.4.3). The impregnated materials were used 21 days after the treatment. Knock-down females were observed 60 minutes later, even in a group of 15 minutes exposure onto the materials, and almost all the flies tested were knocked-down 80 minutes of the transfer. There was no marked difference in the numbers of knock-down females exposed on boards and filter papers at different time intervals against the insecti-

Table 3.4.3. The number of knock-down sandflies, *Lu. gomezi*, at different time intervals after exposure to Sumithion-impregnated boards and filter papers during 15, 30, 45 and 60 minutes

Time intervals (minutes)*	Sumithion-impregnated	
	Boards	Filter papers
15 minutes exposure to Sumithion		
0	0/6 (0.0%)**	0/6 (0.0%)**
15	0/6 (0.0)	0/6 (0.0)
60	4/6 (66.7)	0/6 (0.0)
70	6/6 (100.0)	5/6 (83.3)
80	6/6 (100.0)	6/6 (100.0)
30 minutes exposure to Sumithion		
0	0/6 (0.0%)	0/6 (0.0%)
30	0/6 (0.0)	0/6 (0.0)
60	2/6 (33.3)	4/6 (66.7)
70	5/6 (83.3)	5/6 (83.3)
80	5/6 (83.3)	5/6 (83.3)
90	6/6 (100.0)	6/6 (100.0)
45 minutes exposure to Sumithion		
0	0/8 (0.0%)	0/8 (0.0%)
35	3/8 (37.5)	3/8 (37.5)
45	3/8 (37.5)	3/8 (37.5)
60	7/8 (87.5)	6/8 (75.0)
70	8/8 (100.0)	8/8 (100.0)
60 minutes exposure to Sumithion		
0	0/8 (0.0%)	0/8 (0.0%)
35	2/8 (25.0)	4/8 (50.0)
50	5/8 (62.5)	6/8 (75.0)
60	5/8 (62.5)	6/8 (75.0)
70	8/8 (100.0)	6/8 (75.0)
80	8/8 (100.0)	7/8 (87.5)
90	8/8 (100.0)	7/8 (87.5)
100	8/8 (100.0)	8/8 (100.0)

Remarks:

Female sandflies, *Lu. gomezi*, were collected in Km 100 district, San Sebastian, Department of Manabi, on 12 September 1992. The sandflies were exposed onto Sumithion-impregnated boards or filter papers in polystyrene tubes for 15, 30, 45 and 60 minutes, respectively at 25°C. And then they were transferred into insecticide-free small containers with wet filter paper. The number of knock-down females in each trial were counted. Sumithion-impregnated materials were prepared on 22 August 1992 and used 21 days later.

*, Times (minutes) after transfer of sandflies exposed during 15, 30, 45, and 60 minutes, respectively, against Sumithion-impregnated materials.

**, No. of knock-down females / No. of females tested (%).

cide.

From the current data, applications of Sumithion on inside wall of houses may reduce man-biting chance, and also may lead to reduce domiciliary and/or peridomiciliary *Leishmania* infections. According to our field observations, man-biting sandflies invaded into houses and repeatedly landed on and off the inside walls of houses during night. Such a behavior of sandflies may strengthen the efficacy of Sumithion-treated walls of houses. In Ecuador there are some areas endemic for both malaria and leishmaniasis, especially in lowland regions. In these areas, therefore, residual spray for malaria control may be also effective for leishmaniasis control; sandflies, *Lutzomyia* spp., would be more sensitive for Sumithion than *Anopheles* mosquitoes (T. Ito, pers. commun.).

Residual efficacy of Sumithion seemed to be effective at least three months against endophilic *Anopheles* mosquitoes in the field (T. Ito, pers. commun.). Therefore, the spray on inside walls of houses in leishmaniasis-endemic areas in Ecuador may be fully applicable. Further trials using Sumithion should be done, in order to evaluate the efficacy at different durations of treatment.

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5. A Brief Note on Application of Insecticides for the Control of Endophilic Sandflies

ABSTRACT. In the text, sandfly control measures, especially chemical methods, were briefly discussed. At the moment, it was insisted that chemical control of sandflies is one of the most general and important methods for leishmaniasis prevention. Among many chemicals used as insecticides, the efficacy of DDT, malathion, fenitrothion, deltamethrin and permethrin was reviewed and evaluated. Moreover, recently developed juvenile hormone analogues, pyripfoxyfen and Sumilav was evaluated. The usage for sandfly control by using Sumilav-treated females was also discussed briefly.

Vector control is one of the main choice to prevent New World leishmaniasis, in the a lack of the protection and of suitable drugs against the infection. This concept is due to the successful control of sandflies in some countries during antimalarial campaigns (Pandya, 1983; Tesh and Papaevangelou, 1977; Vioukov, 1987). Control measures of sandfly can be grouped as chemical, biological, genetic and ecological methods. Chemical control of sandflies is one of the most general and important methods of leishmaniasis prevention, especially in cases of anthroponotic forms of the diseases (Vioukov, 1987).

In chemical control, long-term and permanent treatment of foci with residual insecticides formed in elements of the control program to eradicate anthroponotic cutaneous leishmaniasis in the USSR (Nadzharov and Gasan-Zade, 1980). Whole towns were treated and the 2g per square meter (active ingredient, AI) of DDT were used in the program. In public health importance, practical chemical methods for the control of sandfly vectors were summarized (WHO, 1984).

Various insecticides, DDT, malathion, fenitrothion, deltamethrin, permethrin and others have been used in sandfly control (Artem'ev *et al*, 1984; Pener and Wilamovsky, 1987; Le Pont *et al.*, 1989; Majori *et al.*, 1989; Falcao *et al.*, 1991; Maroli and Majori, 1991). In most of the cases, they were used successfully in houses. Residual spraying with these insecticides were needed a long duration of residual effect, because of the control of only adult sandflies.

DDT had 2 weeks residual effect when outside walls of buildings were treated (Saf'janova *et al.*, 1962). A pilot study to control *Lutzomyia umbrati lis*, the local sylvatic vector of *Leishmania* (*Viannia*) l*guyanensis* in Brazil, was investigated by spraying DDT on its favored diurnal resting sites, the bases of the larger forest trees. A marked reduction of the tree-base populations of the species was observed for 21 days following just one application of DDT emulsion in an area 200m square. The treated trunks were not occupied by the sandflies for at least eleven months (Ready *et al.*, 1985).

On the other hand, fenitrothion (Sumithion) may have a marked residual efficacy against *Lutzomyia* sandflies, because its residual efficacy against *Anopheles* mosquitoes continues about four months (Bruce-Chwatt, 1985). Sumithion may have more residual efficacy against sandflies than mosquitoes. It seems that the residual effect of insecticides is longer in a humid and cool climate than in a dry and hot zone. The duration of residual effect of insecticides may be varied in Ecuador, because of different environments in the lowland and highland leishmaniasis-endemic areas.

The effect of deltamethrin spraying on the sandfly populations in a focus of American cutaneous leishmaniasis in Viana, Espirito Santo State, Brazil was studied (Falcao *et al.*, 1991). The results show

a significant reduction in the number of sandflies inside houses in the treated area, compared with both the untreated area and the same area before spraying deltamethrin. Deltamethrin at 0.025 g/m^2 was sprayed inside and outside of houses in a sub-Andean village of Bolivia, located at 1,500m above sea level (Le Pont *et al.*, 1989). *Lu. longipalpis*, the local vector of visceral leishmaniasis, disappeared from houses for 9 months. As compared with pre-treatment data, the rate of engorged females in houses decreased by two, and their density was also reduced. However, *Lu. nuneztovari anglesi* that is the presumed vector of cutaneous leishmaniasis and a highly exophilic species in this area, did not correspond as expected. It may be limited to control exophilic sandfly species. Deltamethrin treatment showed a significant reduction in the number of sandflies inside houses in the treated area. Deltamethrin had a residual action outside houses for 12 months after spraying the insecticide for the control of phlebotomine sandflies in Brazil (Falcao *et al.*, 1991). Newly developed insecticide also may become an efficient insecticide for reducing the number sandflies inside houses.

The efficacy of permethrin-impregnated nets against *Phlebotomus perniciosus* and *Ph. papatasi* was evaluated in laboratory and field studies (Maroli and Majori, 1991). Permethrin showed low or any barrier effect against those sandflies. Mortality rate of the sandflies against the nets (1g/m^2) was greater than 90%, 24 hours after the contact with treated nets in laboratory test. Permethrin-impregnated curtains around windows reduced the number of sandflies entering the stables in cutaneous leishmaniasis foci of Italy (Moroli *et al.*, 1989; Maroli and Majori, 1991). And also, indoor use of wide-mesh cotton curtains impregnated with permethrin (1g/m^2) almost completely eliminated the occurrence of endophilic sandfly species. The use of permethrin-impregnated curtains could be an alternative to residual house-spraying for the control of endophilic vectors of leishmaniases.

Successful control of sandflies in some leishmaniasis-endemic countries was recognized during anti-malarial campaigns, however these results were achieved mainly with anthroponotic leishmaniasis, not with zoonotic leishmaniasis (Vioukov, 1987). Incidence of leishmaniasis may be reduced by insecticide spraying against malaria. These significant reductions occurred only in cutaneous leishmaniasis, but not in visceral leishmaniasis (Tesh and Papaevangelou, 1977). Visceral leishmaniasis disappeared from India during malaria program using residual insecticidal house-spraying. In this case, the sandflies were endophilic vector, *Ph. argentipes* (WHO, 1990). But the return of malaria in the mid 1970's was accompanied in parts of India by explosive outbreaks of leishmaniasis (Sen, 1975). The flying behavior of the sandflies may be changed. A mixture of anti-rabies campaigns, residual house-spraying for malaria control, and occasionally specific measures directed against sandflies, have reduced the incidence of leishmaniasis from several Mediterranean foci and Central Asia (Service, 1992). Control of malaria vectors, triatomines, flies, cockroaches and pests suggests mainly insecticide treatment of the inside walls in houses and is directed against endophilic vectors. This is why these controls would be effective epidemiologically with only endophilic sandfly species in the endemic areas.

It was reported from India that resistance had developed to DDT in *Ph. papatasi* during leishmaniasis control program, but not during anti-malarial campaigns in earlier years (Joshi *et al.*, 1979). Recently, an organophosphorus-resistant strain of *Culex quinquefasciatus* mosquitoes that were pressured with pyriproxyfen for 17 generations, showed no indication of increased tolerance to pyriproxyfen (Schaefer and Mulligan, 1991). We may need to consider restrictions on the use of insecticide with a long residual effect, and also to consider probability of the development of resistance to different insecticides by sandflies.

Insecticide treatment using DDT in burrows of animals for sandfly larvae, pupae and adults showed slight effect for short duration (Latyshev and Kryukova, 1941). Insecticides may not reach the

sandfly breeding sites, leaving live larvae and pupae, and also the duration of residual effect of insecticides may be short in a natural environment as compared to those in indoor houses. The recovery of sandfly densities to pre-control levels took 6 months after a single spraying of buildings with DDT in India (Vioukov, 1989).

Recently, juvenile hormone analogue, pyripfoxyfen, Sumilarv (Sumitomo Chemical Co., Ltd., Japan) has been developed for the control of mosquitoes, flies and cockroaches (Kawada *et al.*, 1989; Langley *et al.*, 1990; Syafruddin *et al.*, 1990). Sumilarv is several 100 times more effective against 4-day-old larvae of *Musca domestica* as well as 4th instar larvae of *Cu. pipiens pallens*, a vector of filaliasis. Sumilarv provided 100% inhibition of mosquito emergence for 12 weeks in the field (Technical brochure, Sumitomo Chemical Co., Ltd., Japan) and for more than two months and 20 days for *An. farauti* and *An. punctulatus*, respectively, in the Solomon (Suzuki *et al.*, 1989; Okazawa, 1991). Sumilarv may be also used for the sandfly control, if the drug is carried into animal burrows by Sumilarv-treated female sandflies. It may be able to make sandflies to carry Sumilarv into breeding sites, as reported by Schlein and Pener (1990).

Sandflies may be collected from sprayed and unsprayed indoor houses and in the open at night, either when they are resting on outside walls or when they are in the act of biting man or animals (WHO, 1970). In case of sandflies, vectors of leishmaniasis, the choice of control method can be made only after intensive epidemiological studies have identified the vectors and their behavior, the reservoir hosts, their biology and the circumstances in which man is at risk (WHO, 1970). The adults rest in soil cracks, rock crevices, limestone cave, tree hole, and animal burrows; larvae breed in soil (WHO, 1970).

The successful application for a chemical method of sandfly control demands integrated knowledge on distribution of the suspected vector species in endemic area, anthropophily, endophily, reservoir host, seasonal and daily rhythm of the behavior, long-term dynamics of the populations, longevity both of one generation and of individuals, as well as their susceptibility to insecticides. Combinations of the following two methods may be effective for sandfly control: 1) a chemical method with an auxiliary ecological procedure, 2) and ecological method with the auxiliary use of chemicals to control leishmaniasis (Vioukov, 1989). Development of auxiliary biological and genetic control, especially genetic engineering techniques, as well as combinations of chemical and ecological methods may strengthen approach to future sandfly control. They would become a part of integrated methods for control of these vectors.

The international workshop on leishmaniasis control strategies in Mexico in 1991 examined all possible control methods, community participation and health education, planting of eucalyptus and neem trees, rodent control to insecticidal control. One conclusion was that leishmaniasis control programs should ideally be incorporated into primary health care programs as part of integrated control measures for vector-borne diseases (Wijeyaratne, 1992). In these programs, there is still scope for searching integrated control methods based on either the systematic application of insecticides, the impregnation of nets or curtains, use of insecticides or chemosterilants in conjunction with an attractant (*e.g.*, sugar, pheromones, oil and others), because there is little evidence of insecticide resistance.

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Chapter 4

Seroepidemiological Aspects

1. Evaluation of ELISA in the Diagnosis of Leishmaniasis in Ecuador

ABSTRACT. The present study was designed to evaluate enzyme-linked immunosorbent assay (ELISA) as a diagnostic method in the diagnosis of cutaneous leishmaniasis in Ecuador. Ninty-five sera were obtained from inhabitants with any skin disorders who lived in three endemic areas, San Placido, Calceta and Junin, in the Department of Manabi. Based on clinical manifestations, these were divided into four groups; 11 had active leishmanial ulcer, 13 had leishmanial scar without record of treatment, 27 had leishmanial scar and had been treated after 1991 by us, and 44 were considered to be non-leishmaniasis. Serum from these individuals were subjected to ELISA. The antigens for ELISA were prepared from promastigotes of *Leishmania* (*Viannia*) *guyanensis* and *Leishmania* (*V.*) *panamensis*. Of these subjects 11 (100%), 5 (38.5%), 10 (37.0%), and 4 (9.1%) showed positive reactions by ELISA for *L.* (*V.*) *panamensis,* respectively. For *L.* (*V.*) *guyanensis* antigen, 11 (100%), 7 (46.2%), 11 (40.7%), and 8 (18.2%) of each group showed positive reaction. Among 12 subjects who received topical administration of parmomycin ointments, eight subjects showed reduced ELISA values either against *L.* (*V.*) *panamensis* or *L.* (*V.*) *guyanensis* antigen after the treatment. For 11 subjects, the results of ELISA, skin test, and clinical diagnosis were compared. Of these subjects, ten subjects showed compatible the results between ELISA and skin test, ten subjects were compatible using ELISA and clinical diagnosis. The present ELISA could be very useful for diagnosis and evaluation of the treatment in the endemic areas of Ecuador.

Introduction

American cutaneous leishmaniasis is known to occur in most provinces of Ecuador. Little information is, however, available on the epidemiological features of the disease in the country. This dearth of epidemiological information is partly due to the lack of a reliable diagnostic method in field studies. For diagnosis of leishmaniasis, many laboratory tests have already been reported (David *et al.*, 1993, Scott *et al.*, 1991, Reed *et al.*, 1990). Enzyme-linked immunosorbent assay (ELISA) is one of the simple and useful method to diagnose other parastic diseases (Goldin *et al.*, 1990, Dziegiel *et al.*, 1991, Bradly *et al.*, 1993). In this study, serum samples from the endemic area of Ecuador was tested by ELISA against antigens from two different species, *Leishmania* (*Viannia*) *guyanensis* and *L.* (*V.*) *panamensis*. Efficacy of ELISA for diagnosis of leishmaniasis was compared with clinical diagnosis and that of skin test.

Materials and Methods

Study area

Between 1991 and 1992, field studies were carried out in the communities of San Sebastian (Km

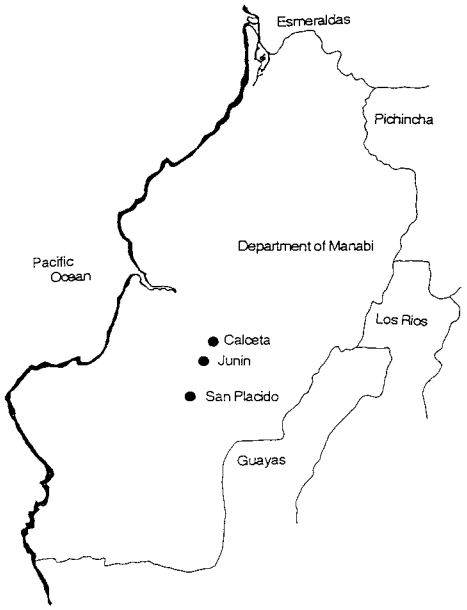

Figure 4.1.1. Location of leishmaniasis-endemic areas in the Department of Manabi, Ecuador, where the samples were collected.

103), Calceta (CLCT) and Junin (JNN), in the Department of Manabi, Ecuador (see Fig. 4.1.1). These study areas, Km103, CLCT and JNN, are located on the Pacific coastal dry areas of less than 300m in elevation.

Sera

Ecuadorian sera were obtained from patients living in endemic area of leishmaniasis who had any cutaneous lesion either by *Leishmania* or other agents. Patients were divided into four groups based on the clinical manifestations, first group had active cutaneous leishmaniasis lesion (Lsh-A, n=11), second group had leishmanial scar (Lsh-S, n=13), third group had leishmanial scar and had been treated after 1990 (Lsh-T, n=27) and the last group had non-leishmaniasis cutaneous lesion (N-Lsh, n=44). Sera were also obtained from students living in non-lendemic area in Ecuador (EST, n=33) and from Japanese in Japan who had no history of leishmaniasis (Jpn, n=44) (see Table 4.1.1). All subjects gave informed consent to participate in the study.

Leishmania *parasites*

Promastigotes of reference strains of *L. (V.) guyanensis* (MHOM/BR/75/M4147) and *L. (V.) panamensis* (MHOM/PA/71/LS94) were cultured at 21°C in Schneider's Drosophila medium supplemented with 15% heat-inactivated fetal bovine serum and 50 µg/ml of gentamycin. At stationary phase of parasite growth, organisms were harvested by centrifugation at 1,600 x g using Kubota 5010 (Kubota Co., Ltd., Japan). The organisms were washed with Dulbecco's phosphate-buffered saline (PBS) at least 3 times. The pellet was stored at -80°C until use.

Table 4.1.1. Classification of the patients in endemic area

Designation	Clinical diagnosis
Lsh-A (n=11)	Patients with active leishmaniasis lesions
Lsh-S (n=13)	Patients with leishmanial scar
Lsh-T (n=27)	Patients with leishmanial scar and have been treated after 1990
N-Lsh (n=44)	Patients with cutaneous lesions but not leishmaniasis

Antigen preparation

Washed parasites were suspended in a protease inhibitor solution (1 mM phenyl methyl sulfonyl fluoride, 50 mM *N-p*-tosyl-*L*-lysin chloromethyl keton, 15 mM leupeptin, 2 mM EDTA 2Na in 10 mM Tris-HCl, pH 7.8) and sonicated on ice for 5 min, using ultra sonicator UD-201(TOMY SEIKO Co., Ltd., Japan) of an output level 4. Then 1% Triton X-100 were added to the homogenate. After incubation on ice for 30 min, samples were centrifuged at 10,000 rpm using Kubota 1700 (Kubota Co., Ltd., Japan) for 30 min at 4°C, then supernatants were removed to the fresh tube and used as antigen. Protein concentration was determined by DC Protein Assay (BIO-RAD Co., Ltd., Japan).

ELISA

Preliminary, optimum concentration of antigens, serum, second antibodies and enzyme solution were determined as follows. The wells of 96-well polystyrene microplate (Inter. Med. Co., Ltd., Japan) were coated at 4°C overnight with 10 μg of each antigens (100 μl/well) diluted with 0.1 M carbonate buffer (pH 9.5). After washing with 0.05% Tween 20-PBS (PBS-T) three times, each well was incubated for 1 hr at room temperature with a blocking agent containing bovine milk proteins (Block Ace; Dainihon Pharm. Co., Ltd., Japan). The primary antibody (human serum) diluted at 1:100 was applied (100 μl/well) to each well and incubated at 21°C for 1 hr. The wells were then washed three times and 100 μl of biotinilated anti-human Ig (Amersham Co., Ltd., Japan) diluted at 1:20,000 was applied to each well and incubated for 1 hr at 21°C. After washing, streptoavidin-horse radish peroxidase (Amersham Co., Ltd., Japan) diluted at 1:5000 was applied (100 μl/well) and incubated for 30 min at 21°C. After washing mixture of 0.04% o-phenilen-diamine and 0.003% H_2O_2 in phosphate-citrate buffer (pH 5.0) was added to each well, and incubated for 30 min. The reaction was stopped by adding 20 ml of 6N sulfuric acid. The absorbance at 490 nm was measured using a microplate reader model 450 (BIO-RAD Co., Ltd., Japan).

Results

To determine the efficacy of antibody detection by ELISA in diagnosis of leishmaniasis, we examined sera obtained from endemic and non-endemic area of leishmaniasis using *Leishmania* promastigote soluble proteins as antigen. *L.* (*V.*) *guyanensis* and *L.* (*V.*) *panamensis* promastigotes were used as antigen source, because these two species are considered to be the major causative agents of human leishmaniasis in this area.

The ELISA values of samples from the two non-endemic areas are shown in Table 4.1.2. The OD values of the Japanese control group (Jpn, n=44) were 0.257 ± 0.086 against *L.* (*V.*) *panamensis*, and 0.197 ± 0.710 against *L.* (*V.*) *guyanensis*. EST (n=33) showed 0.315 ± 0.105 against *L.* (*V.*) *panamensis* and 0.249 ± 0.086 against *L.* (*V.*) *guyanensis*. Using two samples *t* test with Welchi's correction, the mean values of the two populations showed significant differences ($P < 0.05$).

The ELISA reactivity of Jpn and the Lsh-A (n=11) are shown in Fig. 4.1.2. All of the Japanese had OD values less than 0.550 against *L.* (*V.*) *panamensis* and 0.426 against *L.* (*V.*) *guyanensis*. All of the active leishmaniasis group had OD values more than 0.676 against *L.* (*V.*) *panamensis* and 0.534 against *L.* (*V.*) *guyanensis*. The mean plus 4 SD of the control group was 0.593 against *L.* (*V.*) *panamensis* and 0.481 against *L.* (*V.*) *guyanensis*. ELISA cut-off OD values were thus determined as the mean + 4SD of Jpn.

The ELISA results from Lsh-A, Lsh-S, Lsh-T, and N-Lsh are shown in Fig. 4.1.3 against *L.* (*V.*) *panamensis* and Fig. 4.1.4 against *L.* (*V.*) *guyanensis*. Five (38.5%) out of 13 were positive in Lsh-S, 10 (37.0%) out of 27 were positive in Lsh-T, and 4 (9.1%) out of 44 were positive in N-Lsh against *L.* (*V.*) *panamensis* (Fig. 4.1.3). Seven (64%) of Lsh-A showed OD values more than 1. In comparison, only one (7.7%) of Lsh-S, one (3.7%) of Lsh-T and one (2.3%) of N-Lsh were found >1 OD. The mean values in each group were as follows: Lsh-A, 0.941 ± 0.433; Lsh-S, 0.577 ± 0.369; Lsh-T, 0.524 ± 0.237;

Table 4.1.2. Result of sera from two non-endemic area by ELISA

Group	Antigen	
	L. (V.) panamensis	*L. (V.) guyanensis*
Japanese (n=44)	0.257 ± 0.086	0.197 ± 0.071
EST* (n=33)	0.315 ± 0.105	0.249 ± 0.086

*, Ecuadorian students living in non-endemic area.

and T-Lsh, 0.377 ± 0.195 (Table 4.1.3). ELISA against *L. (V.) guyanensis* antigen showed nearly the same results as to *L. (V.) panamensis* antigen. These results suggested that diagnosis by ELISA was in proportional to the clinical diagnosis

The 12 cases in Km 103 studied in 1992 had been diagnosed as active leishmaniasis and received therapy during the past one year. Lesion of those responded to the treatment resulting in transformation to the scar. Antibodies to both *L. (V.) pana-*

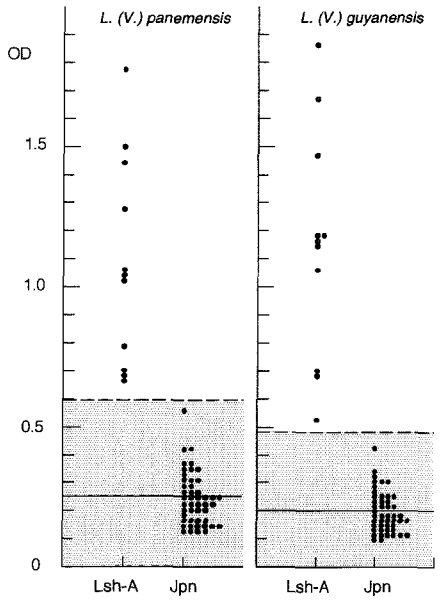

Figure 4.1.2. Comparison of the reactivity of sera from patients with active cutaneous leishmaniasis lesions (Lsh-A) and Japanese control (Jpn). Cutt-off values are in the shaded box.

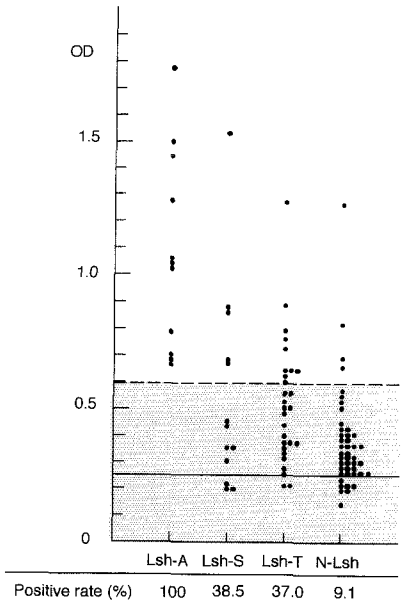

Figure 4.1.3. ELISA reactivity to *L. (V.) panamensis* antigen. Row Lsh-A: patients with active leishmanial lesions; Lsh-S: patients with leishmanial scar; Lsh-T: patients with leishmanial scar and have been treated after 1990; and N-Lsh: patients with cutaneous lesions but not leishmaniasis. Cutt-off values are in the shaded box.

Table 4.1.3. Members of each group and its mean value

Clinical diagnosis		Antigen	
		L. (V.) panamensis	*L. (V.) guyanensis*
Active leishmaniasis	(n=11)	0.941 ± 0.433	1.153 ± 0.409
Leishmaniasis	(n=13)	0.557 ± 0.369	0.579 ± 0.467
Leishmaniasis already treated	(n=27)	0.524 ± 0.237	0.534 ± 0.329
Non-leishmaniasis	(n=44)	0.377 ± 0.195	0.368 ± 0.283

mensis and *L. (V.) guyanensis* were determined in paired sera collected at the time of admission and after the treatment (Table 4.1.4). During 1 to 6-month interval between pre-treatment and post-treatment, antibody levels decreased in 8 cases against either *L. (V.) panamensis* and *L. (V.) guyanensis*. Eight out of eight cases in which antibody levels decreased had at least 5-month interval between sample collections. Only the case we could collect sera at different points after treatment, Km 103 92-1, showed an interesting kinetic of antibody level. Although one month after treatment antibody levels did not change greatly, after six months they rapidly decreased. Antibody levels increased in two cases, Km103 147 and 150.

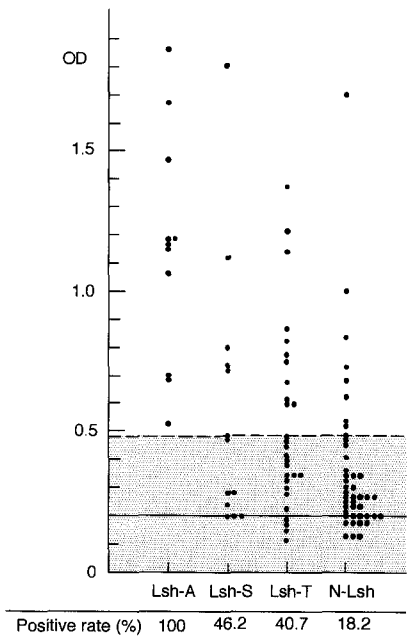

Figure 4.1.4. ELISA reactivity to *L. (V.) guyanensis* antigen. Row Lsh-A: patients with active leishmanial lesions; Lsh-S: patients with leishmanial scar; Lsh-T: patients with leishmanial scar and have been treated after 1990; and N-Lsh: patients with cutaneous lesions but not leishmaniasis. Cutt-off values are in the shaded box.

In JNN, skin test was performed on 11 cases (7 with active lesions, 1 with leishmanial scar, and 3 with non-leishmaniasis lesions). The results were compared with the result of ELISA and clinical diagnosis (Table 4.1.5). In 9 out of 11 cases, results of skin test, clinical diagnosis, and ELISA were compatible: 7 with active lesions, 1 with leishmanial scars, and 1 with non-leishmanial lesions. Although JNN 5 was positive for skin test, clinical diagnosis and ELISA were negative. On the other hand, JNN 13, was clinically diagnosed as non-leishmaniasis, but the results of skin test and ELISA were positive.

Discussion

A definitive diagnosis of cutaneous leishmaniasis requires demonstration of the etiological agent from lesion materials. However, the visual detection of protozoans in tissue samples and the isolation of

Table 4.1.4. The changes of ELISA reactivities before and after treatment

No.	ELISA values against						
	L. (V.) guyanensis			*L. (V.) panamensis*			Intervals
	before	after		before	after		
Km103 51	0.933 →	0.396*		0.950 →	0.521*		5M
Km103 54	0.598 →	0.609		0.400 →	0.475		21D
Km103 92	0.683 →	0.817		0.653 →	0.597*		6M
Km103 115	1.131 →	0.603*		1.219 →	0.603*		5M
Km103 120	0.149 →	0.144*		0.220 →	0.226		6M
Km103 147	0.226 →	1.209		0.269 →	0.787		20D
Km103 150	0.219 →	1.114		0.269 →	0.889		5M
Km103 163	0.773 →	0.803		0.794 →	0.792*		20D
Km103 174	1.250 →	1.375		1.016 →	1.259		5M
Km103 177	0.792 →	0.863		0.728 →	0.638*		5M
Km103 178	1.159 →	0.825*		1.172 →	0.720*		5M
Km103-92-1	0.915 →	1.004 →	0.734*	0.930 →	0.886 →	0.670*	1M, 6M

*, Samples reduced ELISA reactivity after treatment. D: day, M: month.

Table 4.1.5. Comparison of results of skin test, clinical diagnosis and ELISA

No.	Skin test	Clinical diagnosis	Results of ELISA against	
			L. (V.) guyanensis	*L. (V.) panamensis*
JNN 1	2+	Lsh-A	+	+
JNN 4	+	Lsh-A	+	+
JNN 5*	+	Lsh-N	-	-
JNN 6	-	N-Lsh	-	-
JNN 7	2+	Lsh-A	+	+
JNN 8	-	N-Lsh	-	-
JNN 9	3+	Lsh-A	+	+
JNN 10	3+	Lsh-A	+	+
JNN 11	2+	Lsh-A	+	+
JNN 12	2+	Lsh-S	+	+
JNN 13*	+	N-Lsh	+	+

*, Human cases in which results of ELISA were incompatible with those from skin test or clinical diagnosis.

parasites from active lesions by culture methods are often difficult (Weigle *et al.*, 1987). Two diagnostic procedures, *i.e.*, dermal scrapping smears for immediate diagnosis, and the taking of aspirates were recommended for a definitive parasitological diagnosis of cutaneous lesions. However, in our previous study in Ecuador, only 47.6% of subjects demonstrating active lesions were parasite positive by these methods (Furuya *et al.*, 1989). These methods are also very painful. Therefore, the difficulty was encountered in isolating parasites from the lesions. Another method usually used during epidemiological survey is clinical diagnosis by medical doctor. Although this method is carried out within very short time, to make certain diagnosis, medical doctors need sufficient experience. In the practical epidemiological survey in endemic areas, a diagnosis is usually made clinically or by immunoassay such as Montenegro skin test or ELISA, in addition to parasitological demonstration of the parasites from lesions (Mayrink *et al.*, 1979; Werner and Barreto, 1981). The present study was carried out to evaluate ELISA in the diagnosis of cutaneous leishmaniasis in the endemic areas of Ecuador.

The positive rate and the mean value of Lsh-A were clearly higher than those of other groups (Figs. 4.1.3 and 4.1.4 and Table 4.1.3). The mean values of individuals with leishmanial scars (Lsh-S and Lsh-T) were closer to the value of N-Lsh than Lsh-A (Table 4.1.3). This suggested the decrease of ELISA reactivity after the scar formation. Eight out of 12 cases decreased their values after treatment (Table 4.1.4) supported this suggestion. However, it will take nearly 5 months until resulting in reduction of the value after the treatment. Cases that showed increase of the values, Km103 147 and 150, might show the failure of the treatment.

Comparison of the mean values of two different non-endemic populations, Jpn control and EST, showed statistical difference between their values. The ELISA may allow us to identify individuals who had been exposed to leishmanial antigen but who have not developed clinical leishmaniasis. The fact that mean OD value of EST was higher than that of Jpn suggested that even if individuals who live in non-endemic area, the Ecuadorian has risks of being exposed to *Leishmania* higher than the Japanese. Therefore, this needs a consideration when individuals who live in endemic areas are subjected to the ELISA.

At a community JNN, 12 cases were tested by both ELISA and skin test, and compared with clinical diagnosis. The results of 9 (82%) cases were compatible on ELISA, skin test, and clinical diagnosis. In the cases such as JNN13 in which only a clinical diagnosis was negative, a possibility of leishmaniasis might be strongly suspected.

Diagnosis using ELISA can be used against New World cutaneous leishmaniasis. However, the evaluation of ELISA for diagnosis of cutaneous leishmaniasis has not been sufficiently exploited. In this paper, we evaluated the efficacy of ELISA as a method for diagnosis of cutaneous leishmaniasis. The present results showed that the ELISA might be highly specific for cutaneous leishmaniasis. For diagnosis and evaluation of treatment, the present ELISA could be very useful for the screening of leishmaniasis in the endemic areas in Ecuador.

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2. Serological Survey of the Domestic Dogs in Leishmaniasis-endemic Areas of Ecuador

ABSTRACT. Domestic dogs are considered to be a major reservoir of leishmanial parasite in northeast Brazil (Evans *et al.*, 1990) and southern Europe (Abranches *et al.*, 1991). In this study, to know endemy of leishmaniasis in domestic dogs as a reservoir host of human leishmaniasis in Ecuador, serological survey has been performed. Thirty-seven dog sera from two distinct endemic areas of leishmaniasis in Ecuador, Palm Junta (Km 103) in the Pacific coast region and Alausi in Andean region, were examined by ELISA. In Palm Junta, a high endemic area of human leishmaniasis caused by *Leishmania* (*Viannia*) *guyanensis* and *L.* (*V.*) *panamensis*, 11 and 9 dogs out of 20 showed positive against promastigote antigens of *L.* (*V.*) *guyanensis* and *L.* (*V.*) *panamensis*, respectively. On the other hand, in Alausi, where *L.* (*Leishmania*) *mexicana* and *L.* (*L.*) *major*-like parasites are considered as the main agents of human leishmaniasis, 10 out of 17 dogs also showed positive in ELISA against either antigen. Although positive rate of dogs in Alausi was higher than in Palm Junta, the average OD value of positives in Palm Junta was higher than that in Alausi against both leishmanial antigens. In *Leishmania* positive rates with age, old dogs showed higher positive rates than young ones.

Introduction

Leishmaniasis in man is widespread, from the countries around the Mediterranean, Africa, the Middle East, Asia and South America. In Ecuador, New World leishmaniasis caused by parasites of *Leishmania* (*Viannia*) *braziliensis* and *L.* (*Leishmania*) *mexicana* group widely distributes and has been an important public health problem. Although it is known that the domestic dog is a reservoir host of leishmanial parasites in northeast Brazil and southern Europe, the reservoir hosts have not been known well in Ecuador. To know endemy of leishmaniasis in domestic dogs and whether the dog is considered to be the reservoir host of the parasites in Ecuador, serological survey has been performed in two endemic areas of human leishmaniasis in the coastal and in Andean region using enzyme-linkedimmunosorbent assay (ELISA).

Materials and Methods

Study area

The investigation was carried out at Palm Junta (Km 103) in the Department of Manabi, located along Pacific coast and Alausi in the Department of Chimborazo, which is located in the mountainous region of the Andes. Palm Junta is situated at 150m-300m above sea level, where *L.* (*V.*) *guyanensis* and *L.* (*V.*) *panamensis* of *L.* (*V.*) *braziliensis* group are the causative agents of human leishmaniasis (Fig. 4.2.1). Alausi is situated at 2,300m-2,500m above sea level and it has been recently described as an endemic area of human leishmaniasis caused by *L.* (*L.*) *mexicana* and *L.* (*L.*) *major*-like parasites (Hashiguchi *et al.*, 1991) .

A total of 37 domestic dogs, 17 and 20 from Palm Junta and Alausi, respectively, were examined.

Sera

The blood was collected from the jugular vein,

Figure 4.2.1. Outline map of Ecuador, showing the study areas, Palm Junta (150m-300m above sea level), Department of Manabi, and Alausi (2,300m-2,500m a.s.l.), Department of Chimborazo, Ecuador.

allowed to coagulate and placed on ice. Serum was separated by centrifugation within the day and stored at -20℃ until use. Dog sera from non-endemic area of leishmaniasis were obtained from Dr. Ono of the Department of Veterinary Clinical Pathology, University of Tokyo. Domestic leishmaniasis either in human or dog has not been reported in Japan.

Promastigote antigen

Promastigotes of *L.* (*V.*) *panamensis* (MHOM/PA/71/LS94) and *L.* (*V.*) *guyanensis* (MHOM/BR/75/M4147) were cultured in Schneider's Drosophila medium supplemented with 10% heat inactivated fetal calf serum. Cultured promastigotes were washed three times with phosphate buffered saline (PBS) and sonicated in Tris-HCl (pH 7.8) containing 1 mM of phenyl methyl sulfonyl fluoride, 50 mM of *N-p*-tosyl-*L*-lysine chloromethyl ketone, 15 mM of leupeptin, 2 mM of EDTA-2Na and 1% of Triton X 100 on ice. Supernatant of the sonicated *Leishmania* promastigotes obtained by centrifugation was used as lysate antigen for ELISA.

ELISA

ELISA was performed as follows. One hundred μl of the antigen solution (10 μg/ml) was added into each well of 96-well microtitration plate and incubated for overnight at 4℃ . After washing three

times with 200 ml of 0.01 M PBS containing 0.05% Tween-20 (PBS-T), the blocking agent (25% Block Ace, Dainippon Seiyaku, in PBS-T) was added to the wells and incubated for 2 hours at room temperature. After washing, 100 µl of test serum diluted to 1:200 in the diluting agent (10% Block Ace in PBS-T) was added into each well and the plate was incubated for one hour at 37°C. After the wells were washed 3 times, 100 µl of rabbit anti-dog IgG (Amersham) diluted to 1:2000 was added into the each well and incubated for one hour at 37℃. One hundred µl of peroxidase-conjugated goat anti-rabbit IgG (Amersham) diluted to 1:9000 was added after washing. After incubation for one hour at 37℃, the wells were washed and then 100 µl of the reaction buffer that contained 0.4 mg per ml of orthophenylen-diamine and 0.03% H_2O_2 per ml in 0.05M phosphate citrate buffer was added to the each well. After incubation for 30 minutes in a dark box at room temperature, 20 µl of 6N sulfuric acid was added to each well to stop the reaction. Absorbance was read at 490 nm on an ELISA plate reader (Model 450 Micro-Plate Reader, BIO-RAD). Positive values were defined as those > 2 SD (standard deviation) above the mean of the 10 dog sera of the non- endemic area at the dilution of 1:200 according to the criteria by Evans *et al.* (1990).

Isolation of the parasites

For the isolation of leishmanial parasites from dogs, biopsy of nasal mucous membrane was performed and the specimens were cultured in blood agar slant overlaid with saline (Hashiguchi *et al.*, 1991).

Table 4.2.1. ELISA values of the control dog sera obtained from Tokyo, Japan, non-endemic area of leishmaniasis

	Antigen	
	L.(V.)guyanensis	*L.(V.)panamensis*
Mean ± SD	0.556 ±0.185	0.664 ± 0.264
Mean + 2SD	0.928	1.192

Results

The mean values plus 2 SD of the control sera were 0.928 against *L.* (*V.*) *guyanensis* and 1.192 against *L.* (*V.*) *panamensis* (Table 4.2.1). Therefore, ELISA values of the test sera over those values were defined as positive. Sera from 37 dogs were examined by ELISA and 23 (62.2 %) and 20 (54.1 %) dogs showed the positive values against the promastigote antigens from *L.* (*V.*) *guyanensis* and *L.* (*V.*) *panamensis*, respectively (Fig. 4.2.2).

In Palm Junta, twelve and nine out of 20 dogs showed positive against *L.* (*V.*) *guyanensis* and *L.* (*V.*) *panamensis*, respectively. All positives against *L.* (*V.*) *panamensis* were also positive against *L.* (*V.*) *guyanensis* (Fig. 4.2.3). In Alausi, 70.6% (12/17) of dogs showed positive either against *L.* (*V.*) *guyanensis* or *L.* (*V.*) *panamensis*. In Alausi ten dogs out of 11 *L.* (*V.*) *panamensis* positives also showed positive values against *L.* (*V.*) *guyanensis* (Fig. 4.2.3). Negative samples showed almost the same mean values among Palm Junta and Alausi. The average OD values of positive samples against both leishmanial antigens of Palm Junta were higher than those of Alausi (Fig. 4.2.4).

Sera from young dogs showed lower reactivity against both *Leishmania* antigens than older ones. Dogs that were below one year old showed 30.8% of positives against both parasites. Between two and three years old dogs showed 78.6% and 64.3% and in over four years old dogs, 88.9% and 66.6% of dogs showed positive against *L.* (*V.*) *guyanensis* and *L.* (*V.*) *panamensis*, respectively (Fig. 4.2.5).

Different positive rates between male and female dogs in each area were recognized. In Alausi, male dogs showed higher positive rate against both parasite antigens than female dogs, showing a rate of 78.6% (11/14) in male and 0% (0/3) in female dogs. In contrast, female dogs showed higher rates than

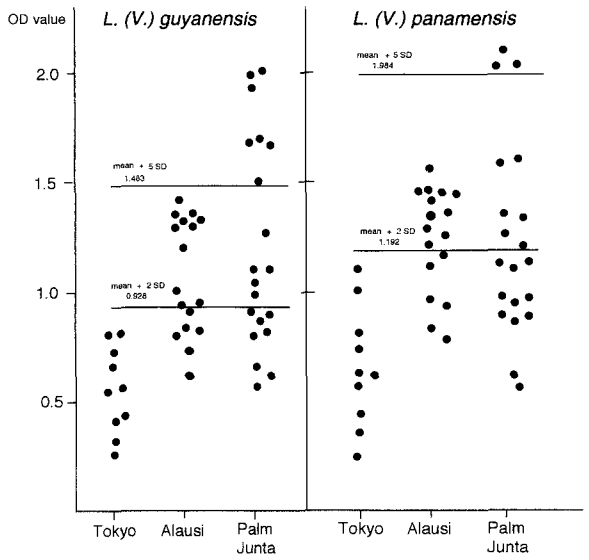

Figure 4.2.2. ELISA reactivity of dog sera obtained from two endemic areas in Ecuador (Alausi and Palm Junta) and non-endemic area (Tokyo, Japan) against *L. (V.) guyanensis* and *L. (V.) panamensis*. The reactivity was shown as the OD value.

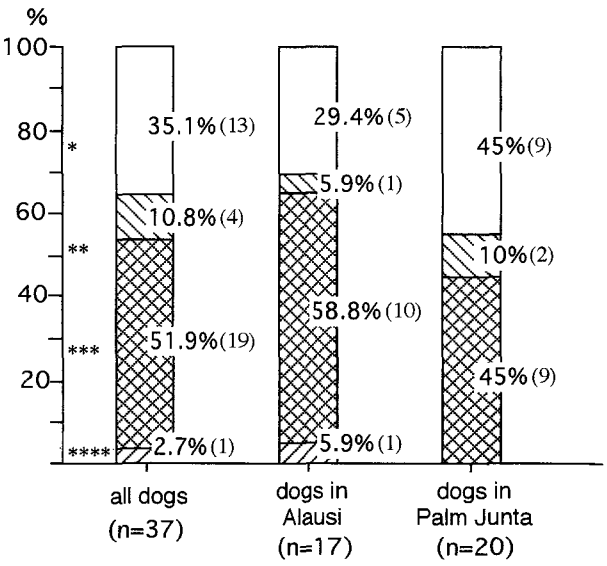

Figure 4.2.3. Positive and negative rates of anti-leishmanial antibodies of sera obtained from the domestic dogs in Palm Junta and Alausi, Ecuador. *, negative for both *L. (V.) guyanensis* and *L. (V.) panamensis* antigens; **, positive for *L. (V.) guyanensis* antigen; ***, positive for the both antigens; ****, positive for *L. (V.) panamensis* antigen.

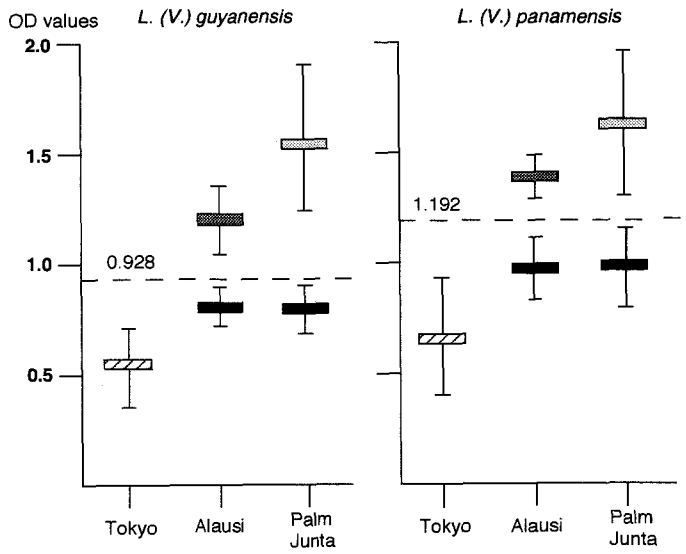

Figure 4.2.4. Average ELISA values of sera from dogs in Tokyo (non-endemic area of leishmaniasis), Alausi and Palm Junta (endemic areas of leishmaniasis in Ecuador). Leishmanial antigens obtained from *L. (V.) guyanensis* and *L. (V.) panamensis* were used for ELISA. The broken lines of 0.928 and 1.192 show the border lines between positive and negative ELISA values.

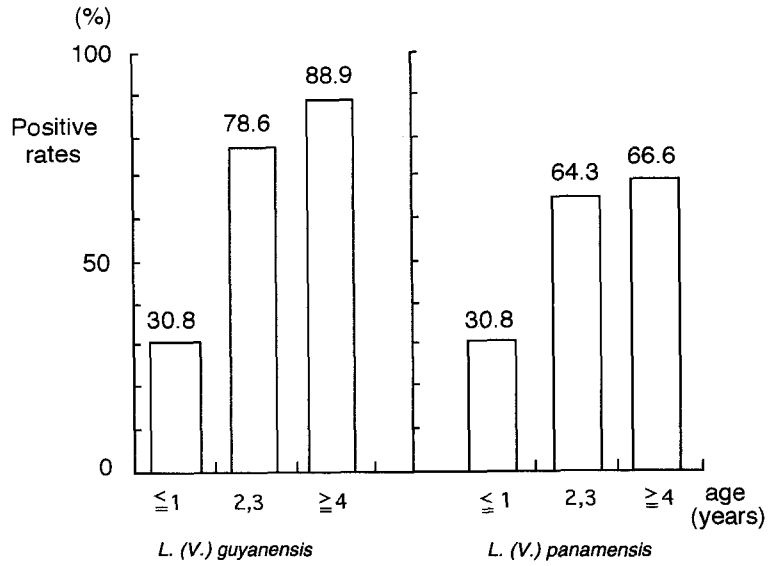

Figure 4.2.5. Difference in positive rates of ELISA reactivity with dog age. Dogs were divided into three age groups; less than 1 year old, 2 and 3 years old, and more than 4 years old.

Table 4.2.2. Positive rates of male and female dogs against *L. (V.) guyanensis* and *L. (V.) panamensis* at Alausi, Department of Chimborazo and Palm Junta, Department of Manabi, Ecuador

Antigen	Study area			
	Alausi		Palm Junta	
	Male	Female	Male	Female
L. (V.) guyanensis	78.6% (11/14)	0% (0/3)	42.9% (6/14)	83.3% (5/6)
L. (V.) panamensis	78.6% (11/14)	0% (0/3)	42.9% (6/14)	50.0% (3/6)

males in Palm Junta; the rates were 42.9% in males and 83.3% in females against *L. (V.) guyanensis*, and 42.9% in males and 50.0% in females against *L. (V.) panamensis* (Table 4.2.2).

In the cultivation of biopsy materials from nasal mucous membrane in the dogs, no growth of *Leishmania* was observed.

Discussion

The domestic dogs are considered to be a major reservoir of *L. (L.) chagasi* that causes visceral leishmaniasis in northeast Brazil (Evans *et al.*, 1990). *L. (L.) mexicana* parasites were isolated from dogs on Andean areas of Ecuador (Hashiguchi *et al.*, 1991). In this study, although the isolation of parasites from dogs was not successful, dog sera reacted strongly with the antigens from the causative agents of human leishmaniasis, *viz.*, *L. (V.) guyanensis* and *L. (V.) panamensis*. From the results obtained, the dogs are suspected to be the major reservoir hosts for *Leishmania* parasites in Ecuador.

In addition, two dogs out of three that have been taken care by the patients with leishmaniasis showed positive ELISA value against the both leishmanial antigens. Although seven dogs against *L. (V.) guyanensis* and three dogs against *L. (V.) panamensis* showed high OD values as above 5 SD in Palm Junta, there were no dogs that showed the OD values above 5 SD in Alausi. As the distribution of *L. (V.) guyanensis* and *L. (V.) panamensis* has not been reported in Andean region, the positive reaction of dogs in Alausi where *L. (L.) mexicana* and/or *L. (L.) major*-like parasites are endemic in human may be cross reaction. Although careful attention must be paid on the cross-reactivity with *Trypanosoma cruzi*, which is also endemic in Ecuador, an antigen that can react with some kinds of *Leishmania* parasite would be useful for diagnosis of leishmaniasis in any endemic area.

It was shown in the current study that the positive rate of the dogs in Alausi was higher than in Palm Junta against leishmanial antigens. In Andean region it may be considered that there are fewer species of mammals than in Pacific coast and Amazonian areas (Mimori *et al.*, 1992). The dogs might have more chances of infection by *Leishmania* in Andean region than in other regions.

The old dogs seem to have more chances of encountering *Leishmania* than young ones, and old ones may be infected sequentially and/or continuously by the parasite. That might be why old dogs showed higher positive rates against both *Leishmania* antigens than younger ones.

In human leishmaniasis, Hashiguchi *et al.* (1984)

reported that no marked sex difference on *Leishmania* infection rates was found. In the present study, there are different infection rates between male and female dogs. Although sex distribution of domestic dogs in the study areas is unknown, the following results were obtained. The infection rate of female dogs was higher than that of males in Palm Junta, and the females' were lower than the males' in Alausi. There might be different preference for male or female hosts in different *Leishmania* species. In order to clarify these points, however, more detailed investigation should be done in future.

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3. Further Epidemiological Studies of Andean Leishmaniasis, with Special Reference to Huigra, Chimborazo, Ecuador

ABSTRACT. Studies on autochthonous Andean leishmaniasis in Ecuador have been done in three important foci, *viz.*, Paute (2,300m-2,500m above sea level), Alausi (2,300m-2,500m) and Huigra (1,200m-1,500m). The disease forms in these foci were found to be similar to each other in the point of epidemiological, clinical and parasitological features. It was suggested, however, that in Huigra the ecological characteristics, including vector biology, were quite different from other Andean leishmaniasis-endemic areas.

Introduction

In 1986 when Andean leishmaniasis was reported for the first time from Ecuador, we commented that the vector seemed to be completely different, though the disease was clinically similar to Peruvian Uta (Hashiguchi *et al.*, 1987). According to our recent studies, it was confirmed that not only the vector but also the parasite were completely different from those of Peru (Hashiguchi *et al.*, 1992). In that country, the causative agent of the disease is *Leishmania* (*Viannia*) *peruviana* (*L.* (*V.*) *braziliensis* ?) and the suspected vectors are *Lutzomyia verrucarum* and *Lu. peruensis* (Lainson *et al.*, 1979; McMahon-Pratt *et al.*, 1982; Herrer, 1982; Kreutzer *et al.*, 1983; Romero *et al.*, 1987). In Ecuador, however, two species of the genus *Leishmania*, *L.* (*Leishmania*) *mexicana* and *L.* (*L.*) *major*-like, seemed to be involved in Andean leishmaniasis, and only one species of sandfly, *Lu. ayacuchensis*, was considered as a probable vector so far (Takaoka *et al.*, 1990; Gomez and Hashiguchi, 1991; Hashiguchi *et al.*, 1991). According to our recent research, differences might not exist only between Peru and Ecuador, but also exist among Ecuadorian foci. Of the foci, Huigra, an area endemic for the disease in the Andean slope, seemed to show its own epidemiological characteristics. Therefore, in this study, more detailed epidemiological surveys, such as human case, vector and reservoir examinations, were conducted in and around Huigra, Ecuador.

Materials and Methods

The study site

Huigra, was evaluated by geographical and ecological observations, in order to correlate these aspects with the transmission of Andean leishmaniasis. The endemic area, Huigra (2°20'S, 78°58'W), located at 1,200m-1,500m above sea level, and the population was about 2,000 persons. Altitude of leishmaniasis-endemic areas seemed to be a well-known determinant and influential factor for the ecology of the disease. In Huigra, for example, the vegetations still kept some aspects of typical alpine Andean flora, with sparsity of plants at the higher places. However, around the town which extended from one slope to another in narrow valley and in surrounding farms, flora was completely different from that found in other endemic areas of Ecuador, such as Paute (2,300m-2,500m above sea level) or Alausi (2,300m-2,500m). Very tall trees like guarumo, guabo, laurel, cedro, nogal, poma rosa, teca, aguacate, mango, orange and mandarina were generally found around the town of Huigra (Fig. 4.3.1). The people also cultivated cocoa, coffee, tomatoes, corn, potatoes, beens, jitomatoes, onion, carrots, and other products found in lowlands and higlands. The dense forests around Huigra were much more like those in the Andean slopes than the highland, especially in the density and height of plants, temperature, humidity and organic soils. Water was abundant in the area when compared with other endemic

areas, Paute and Alausi. People got piped water coming naturally from humid mountains and small river falls, and river branches were found in the field. Such conditions are absent in Paute or Alausi, where water supply was always a problem to be solved. In the present study site, Huigra, socio-economical parameters were also considered. For example, the railway has been, is, and will be still for a long time, a main medium of transportation of the people in Huigra. Bussines, trademarket, communications and touristic, domestic and entertaiment activities, occurred and developed along the railway (Fig. 4.3.2). When the train came once or twice a day, the town of Huigra was awake and noisy. However, if it did not, because of any land-slide covering the railroad or other inconvenience, the town would be sleepy and remain completely quiet.

Human cases

In order to find human leishmaniasis cases, suspective persons with cutaneous ulcers were registered, and examined by direct smears, tissue cultures and skin testing. In part, a serological screening of the inhabitants was also accomplished. All subjects gave informed consent to participate in this study.

Entomological survey

Entomological examinations, such as *Lutzomyia* species compositions, their natural infections and man-biting activities, were mainly done in and around Huigra. To know the vertical distribution of a principal vector, *Lu. ayacuchensis*, in the Andes, sandfly collections were also made at four lower sites (Figs. 4.3.3 and 4.3.4), *viz.*, Olympo (820m above sea level), Ochoa (650m), Naranja Pata (500m) and Ventura (300m) and two higher sites, Chanchan (1,500m-1,700m) and Alausi (2,300m-2,500m), in addition to the main study site, Huigra. Sandfly collections were made by using protected human baits. Flies were also collected directry from walls and ceiling inside of the houses during the day and night time. All the flies collected were dissected, and *Leishmania* promastigotes were isolated when the natural infections were ascertained microscopically. Parasites isolated from sandflies were firstly inoculated into the nose or footpads of golden hamsters. And then as the second step the isolation of parasites was done by performing syringe aspiration at the inoculation site of the animals. Fly collections were conducted in the field, peridomiciliary and domiciliary areas, from 18:00 to 20:00 on different days.

Reservoir host survey

Based on an information that in Paute a probable principal reservoir host seemed to be rodents (Gomez and Hashiguchi, 1990), we caught rats and mice in and around the houses where human cases were found and parasitologically confirmed. Furthermore, other animals, such as opossums and squirrels, were examined by performing cultures of tissue materials (liver, spleen and skin).

Identification of parasites isolated

The parasites isolated were identified by karyotype analysis using pulsed field gel electrophoresis.

Results

Human cases

The characteristic of clinical features of leishmaniasis in Huigra corresponded to that from Peru (Uta) and from other areas (Paute and Alausi) of Ecuador. Patients were mostly children and in Huigra they were between three months and two years old, being younger than those found in Paute and Alausi. Lesions were always small except secondary infections (Fig. 4.3.5), mostly located on the face, and very rich of amastigote forms of the parasite. The duration of evolution was estimated from four to 12 months in average. The incidence of Andean leishmaniasis in Huigra was very low like other endemic areas, Paute and Alausi, Ecuador, and only eight parasitologically confirmed cases were recorded in Huigra, during 1991 and 1993 as shown in Table 4.3.1.

Figure 4.3.1. Landscapes of Andean slopes in the study sites. **Above**, Rio Panama (1,100m above sea level): a humid dense forest with river branch at Andean slope from where sandflies were also collected. **Below**, Huigra (1,200m-1,500m a.s.l.): Andean flora and houses along railway at where epidemiological surveys including skin testing and sandfly collections were mainly done in and around the area.

Figure 4.3.2. The railway (**above**) along a river, "Rio Chanchan", down to the center of Huigra, and the train (**below**) with many agricultural products and turists, just passing our study site, Huigra Viejo (1,200m above sea level), at Andean slope.

Figure 4.3.3. Landscapes of lower forest areas of the two study sites at where sandfly collections were made. **Above**, Ventura (300m above sea level): a community established along railway in the area. **Below**, Naranja Pata (500m a.s.l.): houses surrounded by humid dense forest along railway.

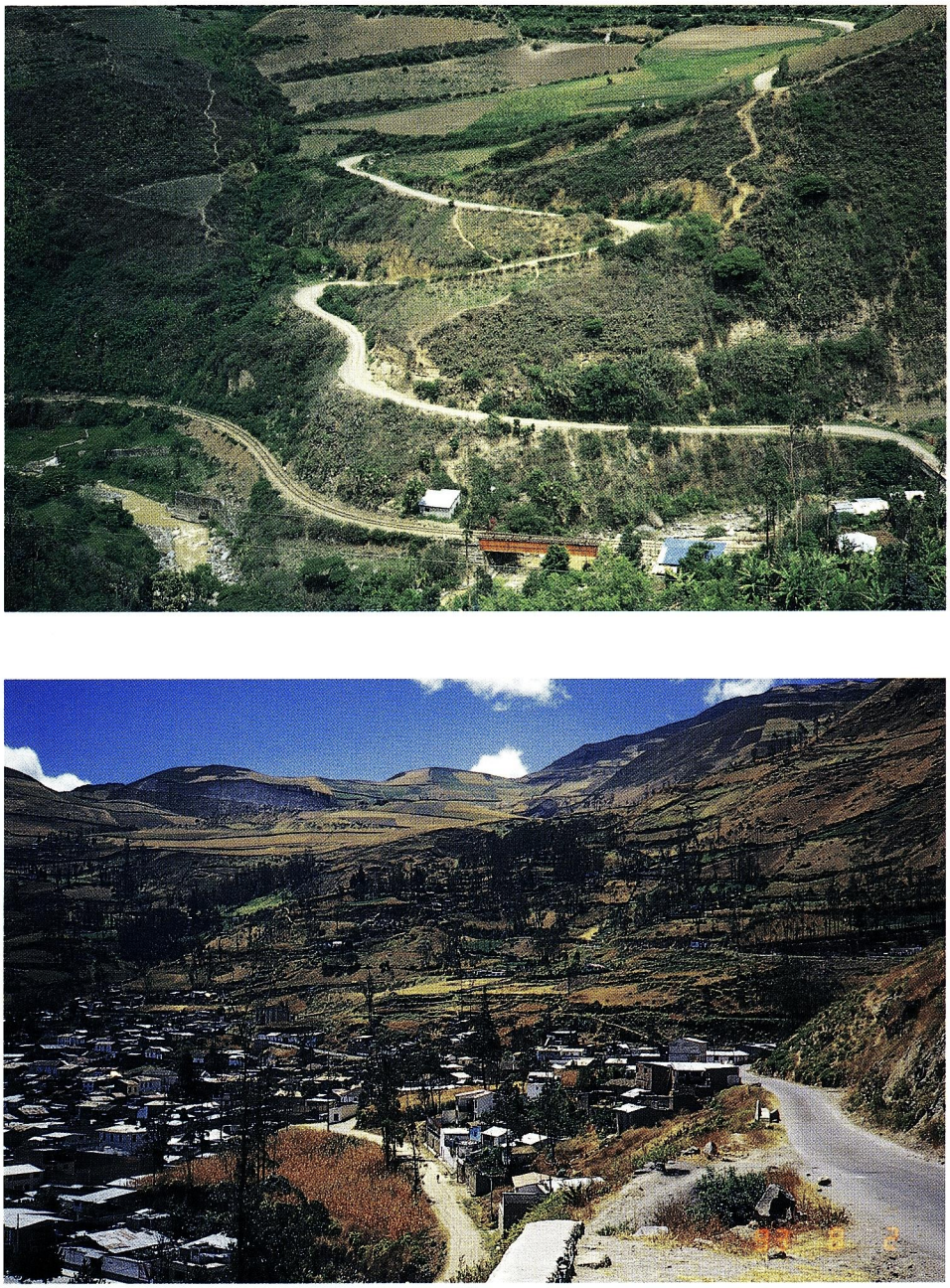

Figure 4.3.4. Landscapes of Andean highland in the study sites. **Above**, Chanchan (1,500m-1,700m above sea level): in this site sandflies were collected outside and/or inside of the houses. **Below**, Alausi (2,300m-2,500m a.s.l.): the town of Alausi where sandfly collections and epidemiological surveys were done.

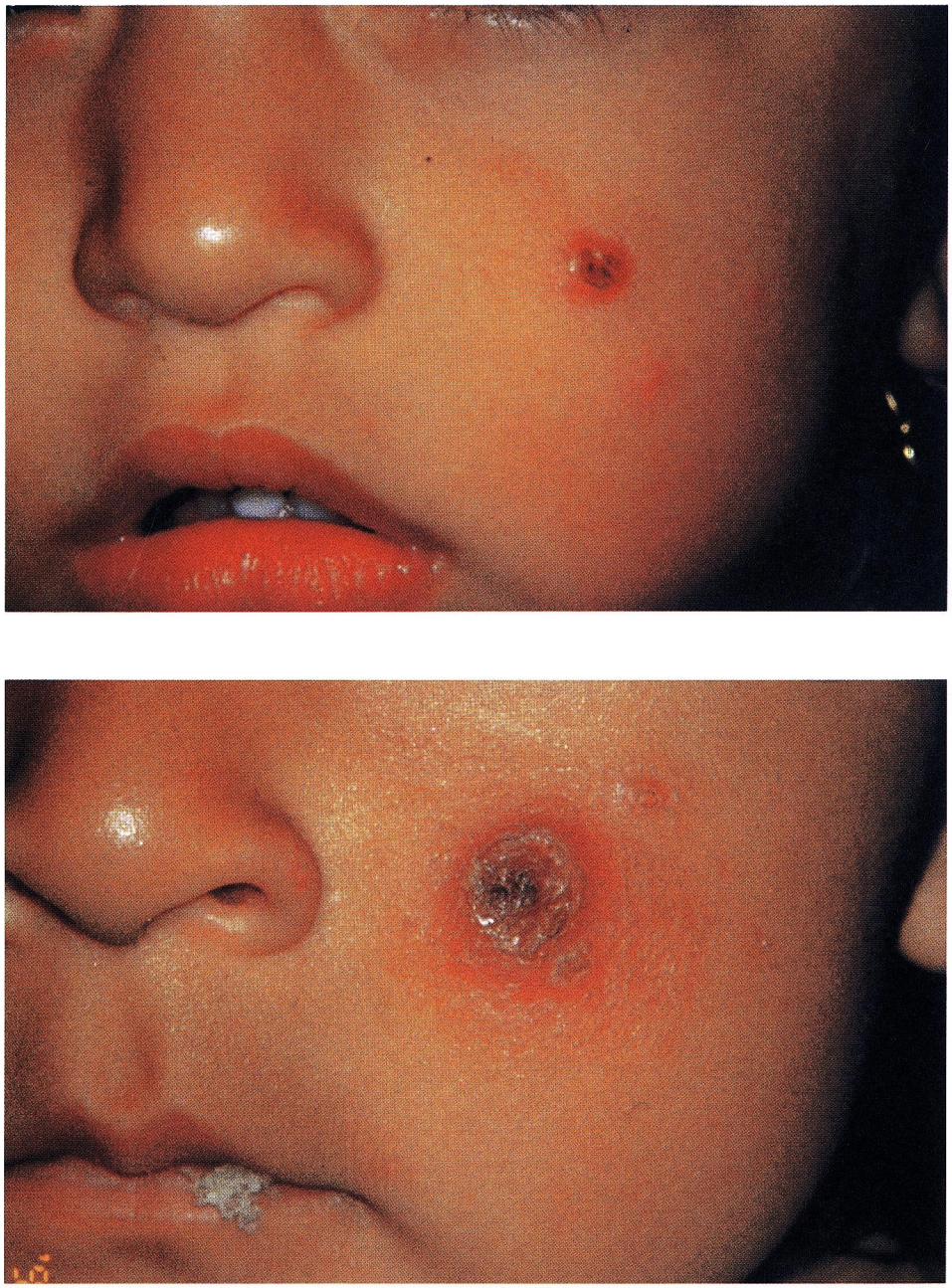

Figure 4.3.5. Patients with Andean leishmaniasis ulcers on the face. **Above**, a 1-year-old girl: one small cutaneous lesion on the face, Huigra. **Below**, a 2-year-old girl: multiple cutaneous lesions with secondary infections on the face, Alausi.

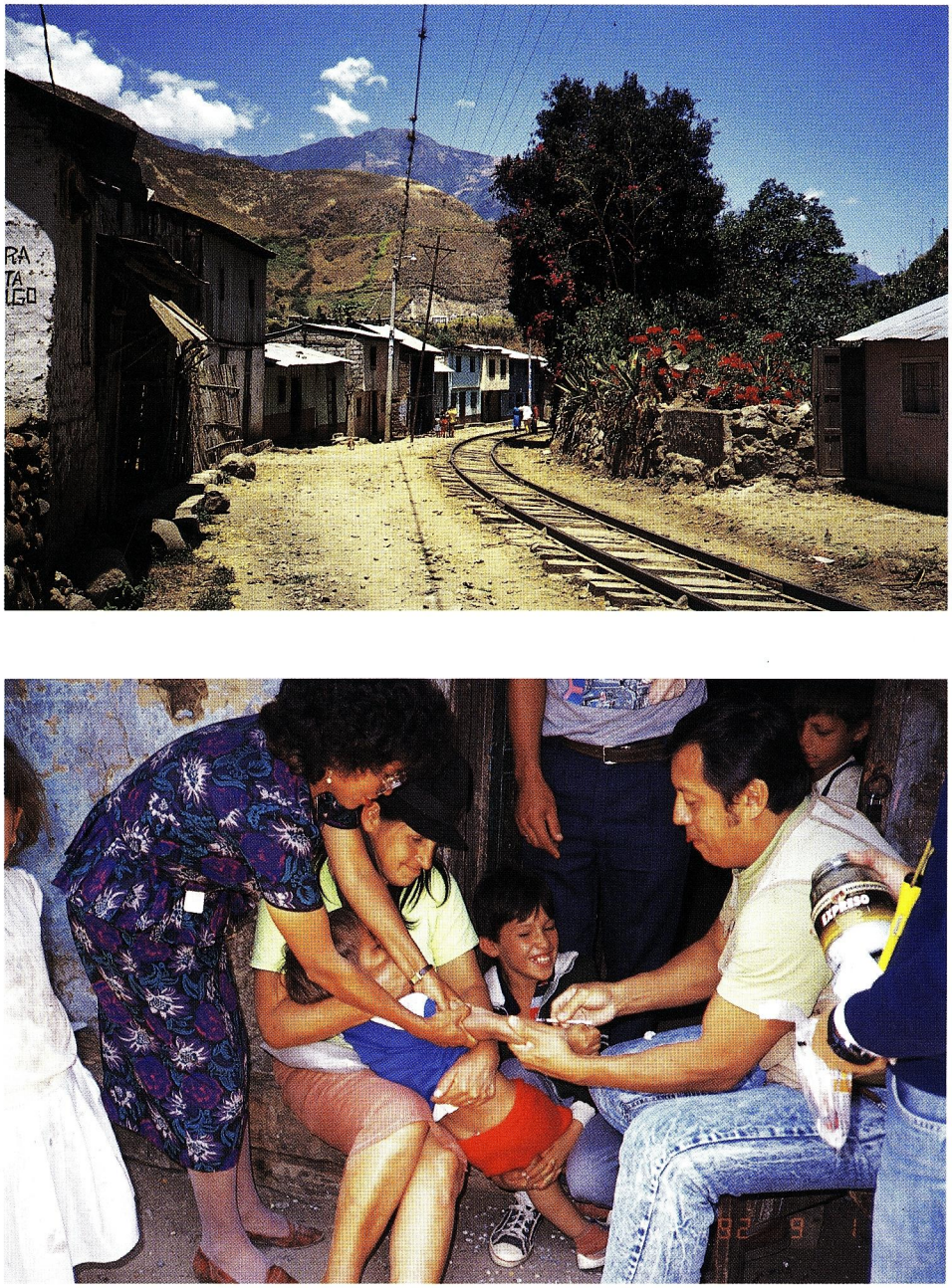

Figure 4.3.6. Landscape of Huigra along railway (**above**), and skin testing (**below**) made in the area, performing house to house visits, by Dr. Eduardo A. Gomez L. and a nurse, Fabiola Sulca in Huigra.

Table 4.3.1. Clinical and parasitological findings in human cases of cutaneous leishmaniasis seen in Paute, Alausi and Huigra, Ecuador

Age*	Sex	No. of lesions	Size of lesions (in mm)	Site of lesions	Duration (months)**	Smear#	*Leishmania* spp.##
Paute (Hashiguchi *et al.*, 1991)							
6Y	F	1	15 x10	face	14	+	*major*-like
5Y	F	1	3 x 3	face	7	+	*mexicana*
5M	M	1	5 x 5	arm	3	+	*major*-like
5M	M	2	5 x 4, 4 x 3	face	4	+	*mexicana*
9M	M	1	2 x 2	face	3	+	*mexicana*
Alausi (Hashiguchi *et al.*, 1992)							
3Y	M	3	5 x 5, 3 x 3, 5 x 5	face	24	+	*mexicana*
5Y	M	1	5 x 3	face	3	+	*mexicana*
7M	M	1	5 x 5	face	5	+	*mexicana*
8M	F	1	2 x 2	face	4	+	*mexicana*
Huigra							
1Y	F	2	1 x 1, 1 x 1	face	4	+	*mexicana*
2Y	F	3	3 x 5, 1 x 1, 1 x 1	face	18	+	*mexicana*
9M	M	4	5 x 5, 3 x 3, 3 x 3, 3 x 3	face	3	+	*mexicana*
2Y	M	1	3 x 2	arm	8	+	*mexicana*
3M	F	2	22 x 2, 3 x 3	face	1	+	*mexicana*
1Y	F	1	5 x 5	face	5	+	*mexicana*
1Y	F	1	5 x 3	face	9	+	*mexicana*
6M	M	1	2 x 3	face	4	+	*mexicana*

*, Y = years; M = month.

**, Duration of disease at the time when the patient was first seen.

#, + = positive smear and/or culture.

##, Identification based on molecular characterization of isolates.

Intradermal skin test was performed on 66 persons living along the railway (Fig. 4.3.6 and Table 4.3.2). Of these subjects, 44 (66.7%) showed a positive reaction. In patients positive for skin test, 20 (45.5%) were less than 10 years old. In this age group, five (25.0%) had active lesions, four (20.0%) had typical scars, and 11 (55.0%) had no lesions. In the remaining age group (10 years of age or over), none had active lesions, eight (33.3%) had typical scars, and 16 (66.6%) had no lesions. Among the

Table 4.3.2. Results of skin tests (ST) performed in inhabitants living along railway in Huigra, Ecuador

No.	Age*	Sex**	ST	Lesion***	No.	Age	Sex	ST	Lesion
1	14M	F	+	2(A)	34	2M	F	-	0
2	41Y	F	-	0	35	2M	M	-	1
3	30M	F	+	3(A)	36	3Y	F	-	0
4	9M	M	+	4(A)	37	23Y	F	+	0
5	2Y	M	+	1(A)	38	42Y	F	-	0
6	6M	M	+	1(A)	39	30Y	M	-	0
7	27Y	M	-	0	40	37Y	F	+	0
8	21Y	F	-	0	41	23Y	F	-	0
9	28Y	M	-	0	42	1Y	M	-	0
10	31M	F	+	0	43	3Y	M	-	0
11	34Y	F	+	0	44	5Y	F	-	0
12	7Y	F	+	1(C)	45	8Y	M	+	0
13	2Y	F	+	0	46	10Y	F	-	0
14	17Y	M	+	1(C)	47	8M	M	-	0
15	15Y	M	+	1(C)	48	12Y	F	+	0
16	11Y	M	-	0	49	19Y	F	+	0
17	26Y	M	-	1(C)	50	11Y	M	+	0
18	24Y	M	+	2(C)	51	40Y	F	+	0
19	36Y	M	+	3(C)	52	8Y	M	+	0
20	21Y	F	+	3(C)	53	6Y	F	+	0
21	5Y	F	+	0	54	3Y	M	+	0
22	4Y	M	+	1(C)	55	45Y	M	+	0
23	2Y	F	-	0	56	30Y	F	+	0
24	6Y	M	+	0	57	11Y	F	+	0
25	19Y	F	-	0	58	6Y	M	+	0
26	3Y	M	-	0	59	4Y	F	+	0
27	2M	F	-	0	60	32Y	M	+	0
28	36Y	M	+	0	61	31Y	F	+	0
29	36Y	F	+	0	62	12Y	M	+	3(C)
30	13Y	M	+	0	63	7Y	F	+	0
31	5Y	M	+	1(C)	64	11Y	M	+	1(C)
32	9Y	F	+	1(C)	65	27Y	F	-	0
33	61Y	F	+	0	66	16Y	F	+	1(C)

*, M: month, Y: year; **, M: male, F: female; ***, A: active; C: cured.

subjects negative for skin test, only one (4.5%) had a scar, but the remainder, 21 (95.5%), revealed no lesions.

Entomological study

The following five anthropophilic species of sandflies were collected from different study areas, as shown in Table 4.3.3. The species identified were *Lu. ayacuchensis*, *Lu. hartmanni*, *Lu. nevesi*, *Lu. gomezi* and *Lu. serrana*. The presence of a greater number of anthropophilic species in Huigra than in Paute (2 spp.) and Alausi (2 spp.) might show a completely different ecological feature, including the transmission mode of Andean leishmaniasis.

Although only *Lu. ayacuchensis* was found naturally infected with *Leishmania* promastigotes to date, showing a rate of 0.6% (6/910), further attention should be paid to other four anthropophilic species. So far, these negative *Lutzomyia* species were captured in a very low number during limited periods, excluding their probable participation in the transmission of leishmaniasis in the areas at the moment. However, in other seasons or times of the year, they might be higher in numbers, and also might be infected naturally. In this study, two of the six infected fleis were captured inside of a house where parasitologically confirmed human cases were found.

Parasite identification

Leishmania parasites isolated from humans and sandfies were characterized by karyotype analysis, and identified as *L.* (*L.*) *mexicana* (see Chapter 1.1)

Reservoir host survey

In total, the following 25 wild mammals were caught and examined for *Leishmania* infections in and around Huigra, by performing tissue cultures and/or direct smears: *Rattus rattus*, 20; *Sciurus granatensis*, 2; *Didelphis paraguayensis*, 2; and *Mus musculus*, 1. Of these, two *R. rattus* were positive for *Trypanosoma* sp. However, all the animals examined were negative for *Leishmania*. By the present survey, it was found that there were many mammals in Huigra and lower dense forest. They were "guatusa" (*Dasyprocta punctacta*), "guanta" (*Cuniculus paca*), "armadillo" (*Dasypus novencinctus*), "comadreja" (*Caluromys lanatus*), "zorro" (opossum) (*D. paraguayensis*), "cuzumbo" (*Potos flavus*), "perico ligero" (*Choloepus hoffmanni*) and "oso hormiguero" (*Tamandua tetradactyla*). The majority of these species was abundant especially in lower forest areas.

Discussion

From the results obtained, it was found that clinical features of Andean leishmaniasis in Huigra were not largely different from those of other two endemic areas, Paute and Alausi, Ecuador. The ecological factors of Huigra might not affect clinical features of the disease, including its incidence and prevalence. As to the causative agent, the parasite isolated from humans and sandflies was identified as *L.* (*L.*) *mexicana*, as found in other two endemic areas of Ecuador (Katakura *et al.*, 1992). Thus, Andean leishmaniasis in Ecuador showed different causative agent and vectors from Peru. In that country, the former was known as *L.* (*V.*) *peruviana* (*L.* (*V.*) *braziliensis* ?) and the latter, *Lu. verrucarum*, belonging to *verrucarum* group, and *Lu. peruensis*, belonging to *vexator* group and subgenus *Helcocytomyia* (Table 4.3.4). In Ecuador, *Lu. ayacuchensis* was found to be naturally infected in three endemic areas, Paute, Alausi and Huigra (Takaoka *et al.*, 1990; Gomez and Hashiguchi, 1991; Hashiguchi *et al.*, 1992). Other anthropophilic sandflies were also observed; *Lu. osornoi* in Paute and *Lu. hartmanni* in Alausi, but they were negative for *Leishmania*. As mentioned above, in Huigra five anthropophilic species of the genus *Lutzomyia* belonging to different species or sub-species groups were found (see Table 4.3.4). Peruvian vectors of *L.* (*V.*) *peruviana* belonged to *vexator* and *verrucarum* group, while Ecuadorian vector of *L.* (*L.*) *mexicana* belonged to *vexator* group. In our previous study,

Table 4.3.3. Sandflies collected and dissected at different altitudes of leishmaniasis-endemic areas of Department of Chimborazo, Ecuador

Locality (altitudes)	*Lutzomyia* spp.	No. flies dissected	No. flies infected (%)
1. Alausi (2,300m)	*ayacuchensis*	89	5 (5.6%)
2. Chanchan (1,500m)	*ayacuchensis*	110	2 (1.8%)
	serrana	1	0
3. Huigra (1,200m)	*ayacuchensis*	974	6 (0.6%)
	nevesi	75	0
	hartmanni	37	0
	gomezi	4	0
	serrana	1	0
4. Olympo (820m)	*serrana*	36	0
	ayacuchensis	30	2 (6.7%)
	gomezi	13	0
	hartmanni	4	0
5. Ochoa (650m)	*ayacuchensis*	36	0
	nevesi	24	0
	gomezi	21	0
	hartmanni	3	0
	serrana	3	0
6. Naranja Pata (500m)	*hartmanni*	62	0
	nevesi	24	0
	gomezi	4	0
	trapidoi	2	0
	shannoni	1	0
7. Ventura (300m)	*hartmanni*	39	0
	trapidoi	20	0
	gomezi	3	0
	nevesi	3	0
	panamensis	2	0

Table 4.3.4. Species-groups of sandflies found in Andean leishmaniasis-endemic areas of Peru and Ecuador

Locality	Peru	Ecuador		
		Paute	Alausi	Huigra
Altitude (m)	900-3,000m	2,500m	2,300m	1,200m
Species of *Lutzomyia*	*verrucarum* (verrucarum)	*ayacuchensis* (vexator)	*ayacuchensis* (vexator)	*ayacuchensis* (vexator)
	peruensis (vexator)	*osornoi* (vexator)	*hartmanni* (vexator)	*hartmanni* (vexator)
				nevesi (verrucarum)
				serrana (verrucarum)
				gomezi (lutzomyia)

infected *Lu. ayacuchensis* was already found at as low as 850m above sea level (Hashiguchi *et al.*, 1992). In this study, it was found that the vertical distribution of *Lu. ayacuchensis* was recognized down to 650m above sea level, and also ascertained that around Huigra the ecological features, such as flora, mammalian fauna and sandfly populations, were even more complex than other endemic areas, Paute and Alausi, Ecuador.

Regarding to reservoir hosts, there might be a difference between Huigra and other two foci of Andean leishmaniasis in Ecuador, because of different ecology with abundant mammalian fauna in Huigra. In the present study, two of the six infected flies, resting on the inside wall of a house were found in Huigra. In this house, two human cases confirmed parasitologically and all the five of the family revealed positive for skin test. However, rats and mice captured inside of the house did not show any infection. Similar results were also obtained in other places where natural infection of sandflies collected in and around the house was observed. Therefore, there might be a high possibility of the existence of other animals than rats and mice, acting as reservoir host(s) of Andean leishmaniasis in Huigra; rats and dogs were already confirmed as reservoir hosts in Paute, Ecuador (Hashiguchi and Gomez, 1990).

In conclusion, the clinical feature of Andean leishmaniasis showed no change between Peru and Ecuador. In the present new focus, Huigra, there might be a different transmission mode of the disease, conditioned by abundant mammalian fauna and sandfly populations. In further study of Andean leishmaniasis, more detailed investigation should be done, in order to get more information on the potential for environmental and biological adaptation of the parasite, *Leishmania* spp. in the Andes.

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Chapter 5

Clinical Aspects

1. Diffuse Cutaneous Leishmaniasis: the First Report of a Parasitologically Confirmed Case in Ecuador

ABSTRACT. In the text, a typical case of diffuse cutaneous leishmaniasis confirmed parasitologically was reported for the first time in Ecuador. The patient was followed-up for more than four years, and clinical and laboratory examinations were throughly done, in order to confirm the disease form. The anergy to leishmanin antigen and the refractory response to chemotherapy were also demonstrated. The parasite isolated was identified as *Leishmania* (*Leishmania*) *mexicana* by isozyme and karyotype analysis.

Introduction

On 28 February 1989, a 18 years old young man from a rural area, Muisne, Department of Esmeraldas, Ecuador came to our outpatient clinic. The patient had previously been clinically diagnosed and treated as leprosy without confirmed diagnosis. By parasitological examinations, however, the subject was found to have thousands of *Leishmania* amastigotes from his hansen-like nodular lesions. After confirming negative reactions to Montenegro skin test antigen, and obtaining the isolation and characterization of the parasites, the patient was finally diagnosed as a typical case of diffuse cutaneous leishmaniasis (DCL) caused by *L.* (*Leishmania*) *mexicana*. In Ecuador, hitherto, only one clinical case was reported without performing any parasitological and immunological confirmations (Zerega, 1961). In the present study, typical features of the disease, such as response to treatment, dermatological evolutions and nodular lesions with abundant amastigotes, revealed definitely the first case of a parasitologically confirmed diffuse type of cutaneous leishmaniasis in Ecuador. This text deals with the detailed clinical findings including parasitological observations.

Materials and Methods

Life history as of February 1993

The patient (A.C.G.) was a 22 years old young man, mestizo, born in Esmeraldas city, Department of Esmeraldas, Ecuador, on 8 September 1970; he was not married but lived on free-union with a woman. He worked and studied. He had spent most of his life in San Ignacio (Muisne), a rural, mountaneous and remote area from Esmeraldas, where his family actually lived.

Evolution of the disease

The patient was a healthy person; he did not suffered from any important disease besides malaria (*Plasmodium vivax*) when he was a child. The patient referred that when he was 16 years old, he first noticed discrete papules on his left knee and right cheek; these started growing up, and at the same time, other similar lesions appeared, and were

spreading over the entire body; papules slowly evolved into infiltrated plaques and nodules, without any ulceration. During about two years (1986-1987), he received different treatments by different physicians, with no improvement; therefore, he decided to visit a dermatological dispensary in Guayaquil city. At this time, he was 18 years old, to be 19 in September 1989. Routinary examinations and laboratory tests were done at the dermatological dispensary, but no biopsy was done. Mitsuda reaction using lepromin was done, but the result was not registered. When stained smears of limph node material were examined, "globus-like" material was observed, and he was registered with the diagnosis of lepromatous leprosy on 24 August 1988.

Physical evaluation as of 24 August 1988

As of 24 August 1988, erithematous nodules were observed on the face, trunk, arms and legs. Nasal obstruction symdrome, epistaxis and probable paresthesias were found in legs. Pulse, temperature and blood pressure were normal, and circulatory, respiratory and urinary systems were also normal. As of 2 February 1989, the patient revealed uncountable nodular and plaque lesions spread all over the body, without compromise of palms of the hands and soles of the feet. Lesions without ulcerated were red-bluish, scaly and rough; diffuse cutaneous leishmaniasis was highly suspected and smear and culture materials were taken from several nodular lesions.

Diagnosis and management of the patient

Physical examinations were thoroughly done on the patient, and all the data including life and clinical histories were recorded. Various indirect diagnoses were performed employing Montenegro, Tuberculin, candidine and trichophytine skin tests. As a direct diagnosis, smear and culture materials were taken from lesions; the former was stained by Giemsa or Wright solution and the latter was characterized by zymodeme and karyotype analyses. The patient was carefully managed and followed-up. After final dosis of each treatment with Glucantime® and/or every six months he was thoroughly examined.

Results

Clinical evaluation

The evolution of lesions revealed a typical clinical feature for diffuse cutaneous leishmaniasis. It started with papules, and then developed into infiltrated plaques and many nodules without ulceration. Smooth and firm nodular lesions slowly became into scaly and rough nodules, or cheloid plaques. There were some periods of apparent improvement during Glucantime® treatment (Fig. 5.1.1A-D), but usually followed by more aggresive relapses during untreated terms after each medication (Fig. 5.1.2A-D). As of February 1993, five years after the onset of the disease, infiltrative lesions affecting the eyebrowns, nose and ears are the first evidence of the production of leonine face typical for diffuse cutaneous leishmaniasis (Fig. 5.1.3A-D). In the rest of the body surfaces cheloid shaped nodular lesions are distributed on the trunk, arms and legs (Fig. 5.1.4A-D).

Direct and indirect diagnosis

Enormous amount of *Leishmania* amastigotes were observed in stained smears from lesions. The parasites were isolated in culture *in vitro* and used for characterization. Zymodeme and karyodeme analyses revealed they were *L.* (*L.*) *mexicana* (see Chapter 1.1). Vacuolated macrophages (foam cells) were completely filled with amastigotes. There was an evidence showing the lack of lymphoid infiltration. Montenegro skin test, performed on 28 February 1989 for first diagnosis, revealed a negative result; further controls during last four years were always negative. On the other hand, Tuberculin, candidine and trichophytine skin tests showed positive reactions, demonstrating the existence of specific anergy against *Leishmania* antigen. When the patient was treated with Glucantime®, 10 mg/kg/day, during 25-30 days, parasite density on smear speci-

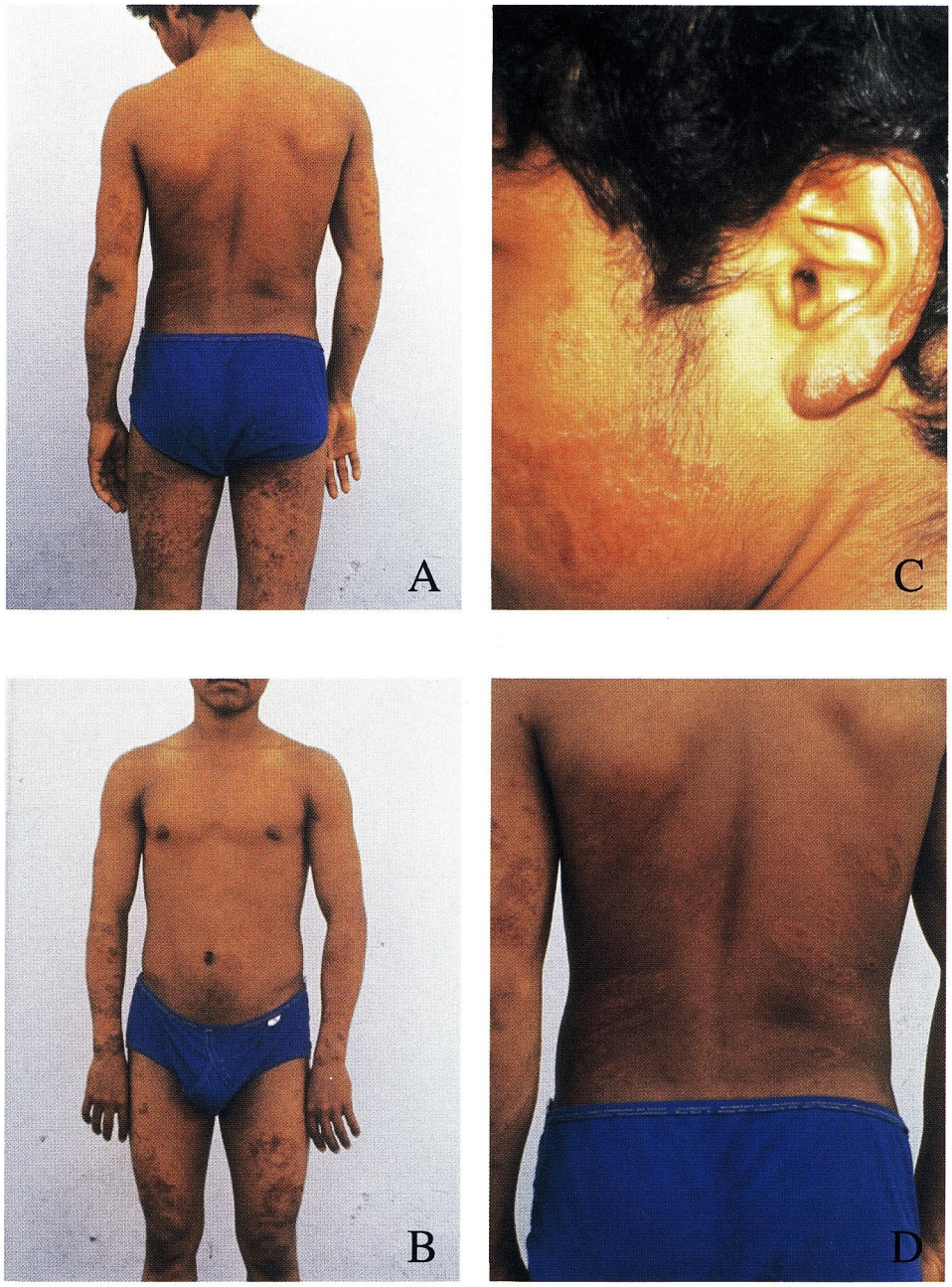

Figure 5.1.1A-D. A patient (A.C.G., 18 years old) sufferred from diffuse cutaneous leishmaniasis, confirmed parasitologically and treated with Glucantime® (meglumine antimonate). Nodular lesions showed flattened surfaces during the treatment. Photo, August 1986.

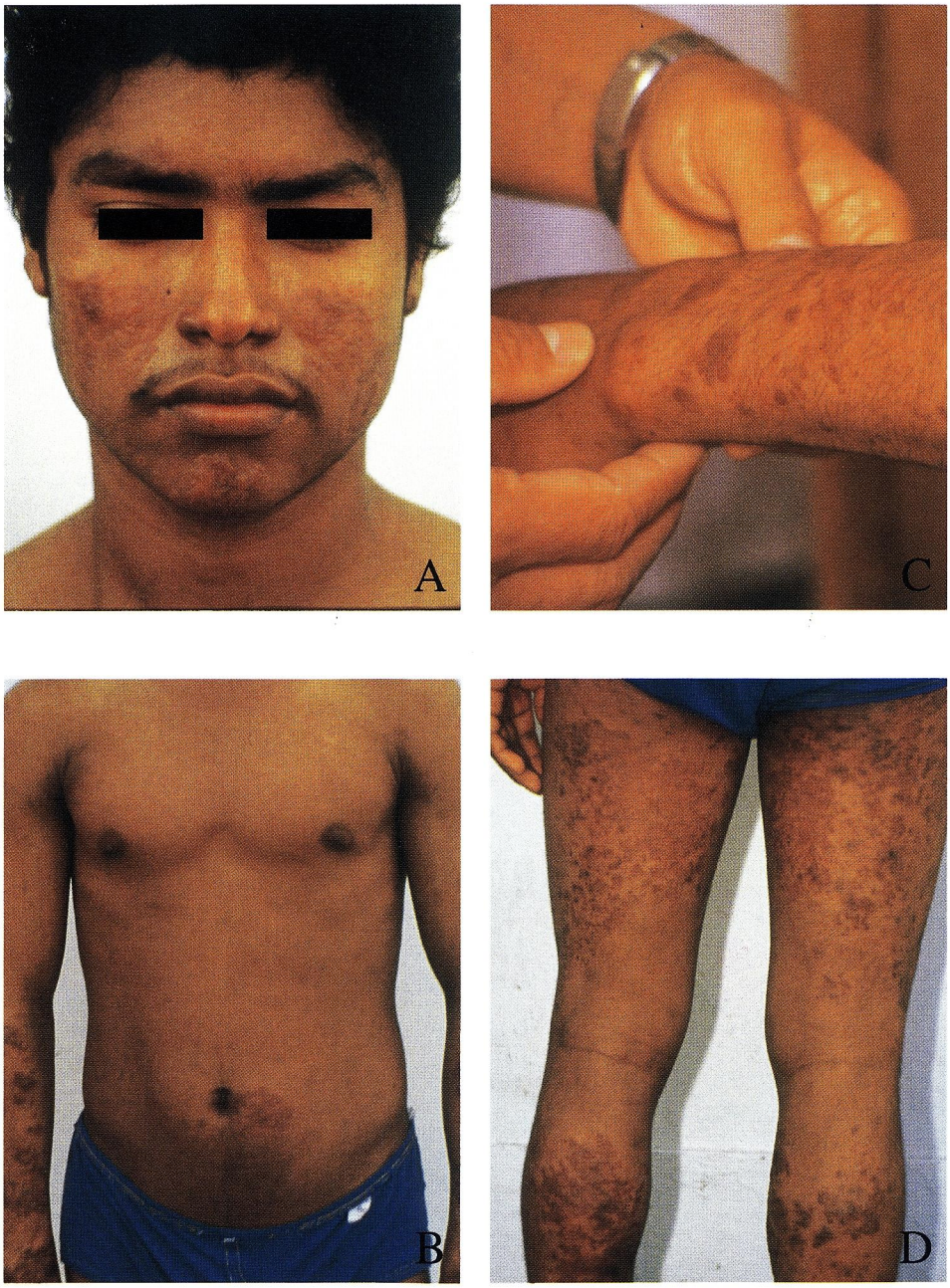

Figure 5.1.2A-D. Lesions of the patient (A.C.G.) treated with Glucantime® showed more aggresive relapes during untreated terms after each medication, demonstrating elevated surfaces. Photo, August 1988.

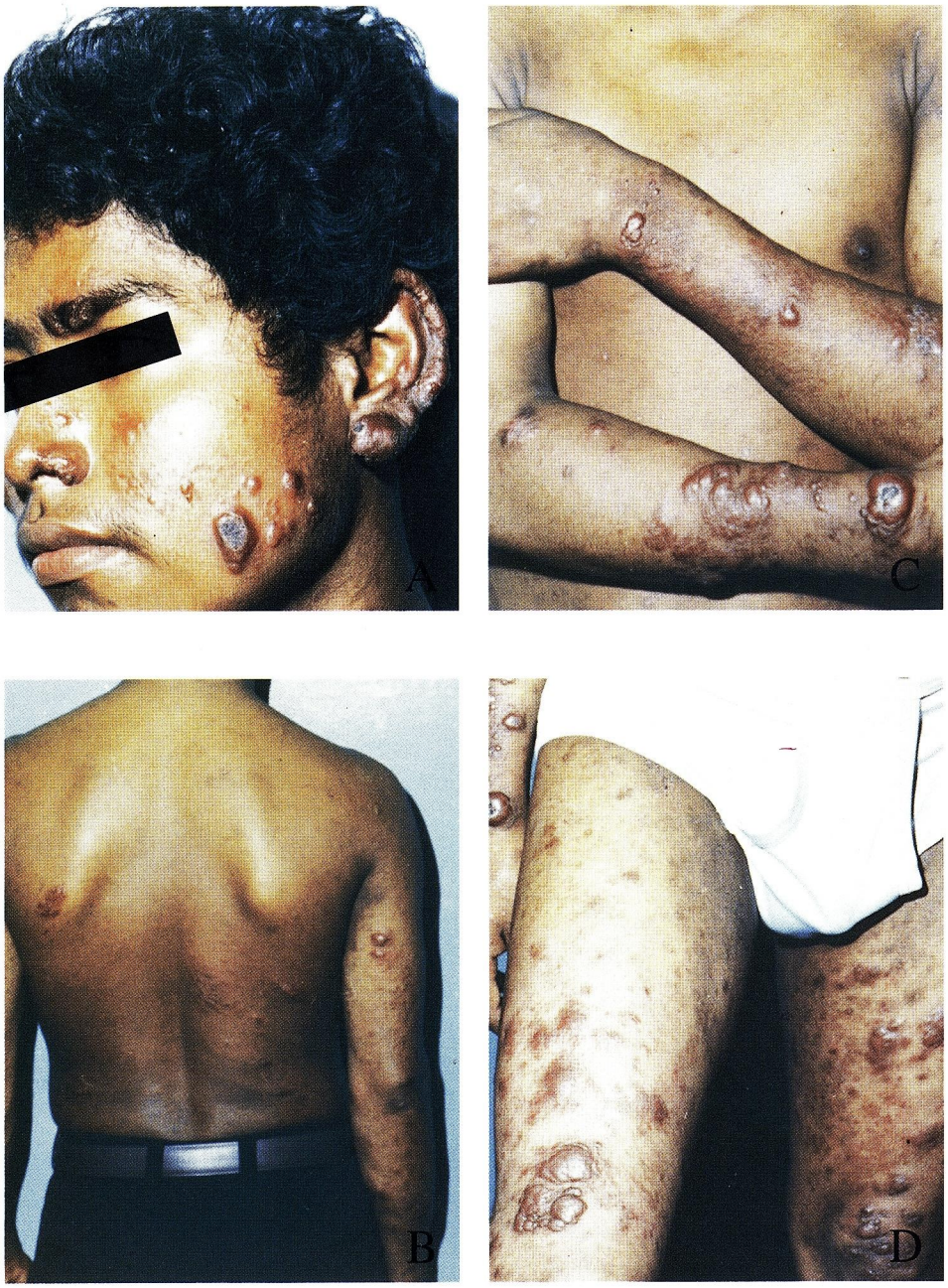

Figure 5.1.3A-D. More advanced features of the patient (A.C.G.) with diffuse cutaneous leishmaniasis, showing an early stage of lionine face. Photo, April 1992.

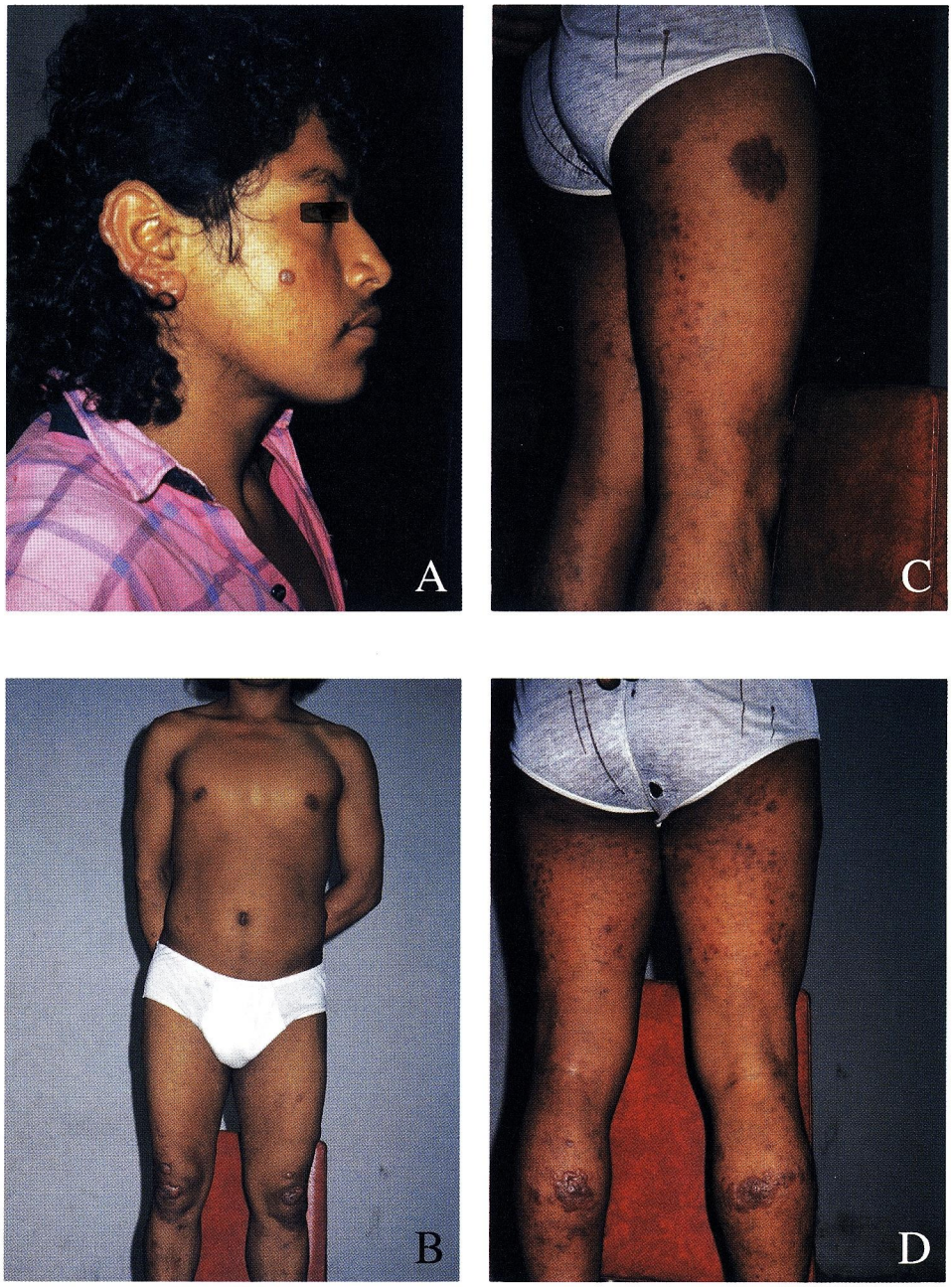

Figure 5.1.4A-D. Advanced features of the patient (A.C.G.) with diffuse cutaneous leishmaniasis, during medication with Glucantime®; by the treatment a part of lesions, again, showed slightly flattened surfaces. Photo, February 1993.

mens was significantly reduced immediately after the beginning of treatment, but never disappeared completely. Thus, relapses occurred followed five or six months intervals each of clinical improvements. Therefore, the patient was repeatedly treated with the same drug, Glucantime®, after each relapse. Regarding to the treatment of the present patient, more detailed results will be reported elsewhere.

Discussion

Diffuse cutaneous leishmaniasis which revealed a particular condition of classic cutaneous leishmaniasis, was actually not as rare as it was considered before. Many cases were recognized in the New World, especially in Brazil and Venezuela (Convit *et al.*, 1972; Barral *et al.*, 1990), and nowadays, it was a well characterized clinical entity as follows. The disease form originates as a single lesion which, with time, spreads metastatically giving rise to multiple non-ulcerated nodules (Leon *et al.*, 1990). The parasitism is typically intense with a heavy accumulation of parasitized and vacuolated macrophages in the lesions and there is a positive antibody response, but a negative cellular or delayed type hypersensitivity response to leishmanial antigen (Convit *et al.*, 1972; Leon *et al.*, 1990).

According to Simpson *et al.* (1968), Bryceson (1969) and Convit *et al.* (1972), the features of diffuse cutaneous leishmaniasis are defined as follows: 1) lesions are non-ulcerating nodules and plaques resembling lepromatous leprosy; 2) distinctive histopathology shows vacuolated macrophages (foam cells), packed with amastigotes, an abundance of parasites even in normal skin beyond the visible lesions and a lack of lymphoid infiltration; 3) no visceral involvement occurs; 4) the skin test reaction to leishmanin antigen is negative; 5) the condition is almost completely refractory to chemotherapy; 6) there is a slow but inexorable dissemination to the skin of all areas except the palms of hands and soles of the feet. Besides these six points, it is now recognized that the parasite causing diffuse cutaneous leishmaniasis has all the characteristics of *L.* (*L.*) *mexicana* complex (Grimaldi *et al.*, 1989).

With regard to Ecuadorian diffuse cutaneous leishmaniasis, Zerega (1961) described a case which clinically suggests the disease form, though he mentioned several ulcers and ulcerous lesions. The case, however, did not agree with the actually accepted concept of diffuse cutaneous leishmaniasis (point 1 mentioned above). Histopathology was also different, since it referred the presence of a predominantly plasmo-lymphoid inflamatory infiltration (disagreement with point 2). Montenegro skin test was not done; there was no reference to surveillance of evolution, neither information on resistance to treatment (lack of examinations at points 4, 5 and 6). Isolation (culture) and identification of the parasites from the patient were not performed; besides, no parasitological confirmation was done. Thus, this probable diffuse cutaneous leishmaniasis was not adequately examined and described. Recently, an uncommon but parasitologically confirmed case of generalized cutaneous leishmaniasis with 308 ulcers was reported (see Chapter 5.2); this case seemed to be caused by a metastatic dissemination of ulcerous lesions or multiple biting by infected sandflies. In the case, the patient had a satisfactory response to treatment with Glucantime®, in spite of its insufficient doses (12 amples); besides, the histopathology corresponded to simple cutaneous leishmaniasis. Unfortunately no Montenegro skin test was done, and parasite was not isolated neither identified.

In conclusion, the present patient was followed more than four years, and clinical and laboratory examinations were thoroughly accomplished, showing characteristic features to confirm the diagnosis of diffuse cutaneous leishmaniasis. The anergy to leishmanial antigen and the refractory condition to chemotherapy were also demonstrated as mentioned above. The parasite isolated was identified as *L.* (*L.*) *mexicana* by isozyme and karyotype analyses. This species, *L.* (*L.*) *mexicana*, was reported from several areas endemic for cutaneous leishmaniasis in Ecuador (Armijos *et al.*, 1990; Hashiguchi *et al.*, 1991), but no case of diffuse cutaneous one was

found, during our research work at different areas of the country (Hashiguchi and Gomez, 1991). Such a finding might confirm the concept that diffuse cutaneous leishmaniasis would be found only in persons with an abnormal or special immune response; *L.* (*L.*) *mexicana* would generally cause simple cutaneous lesions. In order to search for similar leishmaniasis cases, we visited San Ignacio, a community where the present patient came from, but no such a diffuse type form of the disease was found in his family and neighbouring people examined.

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2. Generalized Cutaneous Leishmaniasis: a Parasitologically Confirmed Case in Ecuador

ABSTRACT. A case of generalized cutaneous leishmaniasis with 308 crusty ulcers was reported. The patient, 40 years old mestiza female, came from Balao Chico, Department of Guayas, Ecuador. In the present case, no compromise of mucous membrane was found, in spite of dissemination of lesions all over the body surface. The patient was admitted to a hospital in Guayaquil city, Ecuador, with the diagnosis of small pox, paracoccidioid mycosis and staphylococcal infections. The clinical picture showed some controversies, and resulted in a difficult diagnosis. However, microscopical examinations of skin biopsies from lesions revealed *Leishmania* amastigotes. Thus, it was finally concluded that there might be coexistence of herpes zoster, which would partly support dissemination of ulcer lesions. Therapeutic response was very satisfactory with Glucantime® (meglumine antimonate), using a small dose (6.5 mg/kg/day) during 12 days. In this case, both metronidazole *per os* and its ointment were also used as a supplimentary treatment.

Introduction

Tropical dermatology enriches itself with knowledge of numerous clinical forms day to day. Leishmaniases in Ecuador show a wide range of clinical pictures that may confuse and complicate their diagnosis. Since cutaneous leishmaniasis ulcers constitutes predominant form in the country (Hashiguchi and Gomez, 1991), it is also true that such an entity varies according to some geographical, ecological and epidemiological factors. In addition, as an important factor, secondary infections with bacterial and fungal agents, must be taken into consideration. Existence of crusty ulcers and characteristic histopathology with abundant *Leishmania* revealed a typical cutaneous leishmaniasis. However, clinical evolution and abnormal distribution of ulcers showed a great difference from other cases of the disease.

The present case might be caused by metastatic dissemination of cutaneous ulcers under special conditions of the patient, and/or might be caused by multiple biting of infected sandflies with *Leishmania*. Therefore, the current paper reports an unusual case of cutaneous leishmaniasis, emphasizing on the clinical and laboratory findings.

Report of the Case

A 40 years old mestiza female patient, who lived with her husband in Balao Chico, Department of Guayas, Ecuador, a mountanous area located at 200m above sea level. She usually weared pants and blouses which were tucked up round the waist, while working. In the early phase, the lesion appeared on the lumbar region, where the skin was denuded, feeling localized burning. The patient's complaints appeared two months before consultation with fever, headaches, earaches, and pain at the joints that did not improve with analgesics and antipyretics.

Posteriorly, papulous lesions were seen, which became pustulous vesicles at the right lumber region and disseminated all over the body in 15 days, accompanied with generalized itching. On the physical examination the patient had a saburral tongue; lungs were clear; her abdomen was soft and there was no hepatomegaly nor splenomegaly. Marked right lumbar pain and lesions in this area resembling herpes zoster were recorded.

The patient was admitted for 16 days in another private hospital, and then was taken to a national hospital in Guayaquil city. She was admitted to the

hospital with the diagnosis of small pox, being registered No. 03738 in August 1979. As there was no improvement of her lesions on the 15th day of admittion, she was taken to receive differential diagnosis at a national institute in Guayaquil. Before obtaining detailed laboratory results, the presumptive clinical diagnoses were paracoccidioidomycosis, leishmaniasis or staphylococcal infections.

Characteristics of the ulcers

Most of the ulcers were crusty. When lifting the crusts they were excavated with a purulent fluid. The ulcers had different sizes ranging from 0.5mm to 20mm in diameter (Fig. 5.2.1A-D). Some had thick, prominent and adhered crusts that bleed easily when they were separated (Fig. 5.2.2A). Others were small crusty ulcers that leaved an excavation, and some were erythematous and papulous (Fig. 5.2.2B), and two confluent plagues were found on the lumbo-dorsal region (Fig. 5.2.2C).

Clinical and Etiological Diagnosis

Firstly, the present case was thought as paracoccidioidomycosis remembering a clinical form of generalized cutaneous paracoccidioidomycosis. Secondly, cutaneous leishmaniasis was considered, because of the presence of some characteristic ulcers with elevated borders on the face and the breast. Finally, as some lesions contained a purulent fluid, a disseminated staphylococcal infection was suspected. All the lesions did not improve or modify during two weeks, in spite of the antibacterial treatment, prior to our specific treatment for leishmaniasis.

Laboratory Diagnosis

No evolutive forms of *Paracoccidioides brasiliensis* were found in an examination of ulcer materials. In samples stained by Wright solution, numerous forms of amastigotes and some parasitized macrophages were observed, suggesting leishmaniasis. In order to determine if all ulcers were caused by the same agent, *Leishmania*, several samples from diverse regions of the body surface were taken at random (Fig. 5.2.2D). All the ulcers examined were positive for *Leishmania* amastigotes. Moreover, a biopsy of an ulcer located on the right leg was also made. Microscopic observation of the biopsy revealed *Leishmania* parasites, and also showed diffuse infiltration of the dermis with scarce intra- and extra-cellurar parasitic elements; the examination was made by Dr. F. Zerega.

In order to make a complete therapy of the patient, blood, urine and stool examinations were also performed. Cultures and sensitivity test to antibiotics were done with the purulent fluid of lesions. The results of blood examination were shown as follows. The first test: hematocrit, 34%; leukocytes, 10,850/μl; hemoglobin, 11.4g/dl; blood urea nitrogen (BUN), 30mg/dl; glucose, 105mg/dl; creatinine, 0.8mg/dl; alkaline phosphatase, 3.25 (King-Armstrong unit); and bilirubin, 0.5%. The second test: hematocrit, 35%; leukocytes, 5,500/μl; hemoglobin, 11.4g/dl; eosinophils, 11%; neutrophils, 51%; lymphocytes, 36%; basophil, 1%; and monocyte, 1%. Other laboratory examinations were made and the following results were obtained: urine sediment, pyuria 8-9 white blood cell per power field; purulent ulcers, positive for beta-hemolytic streptococcus; sensitivity test, sensitive for erythromysin, cephalexin, amikacin, ampicillin; and stool examination, positive for *Trichuris trichiura, Ascaris lumbricoides* and hook worms.

Treatment of the Case

Before the definitive diagnosis, the patient was treated with ampicillin, anti-histamine, vitamine C during 15 days at a private hospital in Guayaquil, but no modifications or improvements of complaints and cutaneous lesions were found. After the confirmed diagnosis as leishmaniasis for the patient, the treatment with Glucantime® (meglumine antimonate)

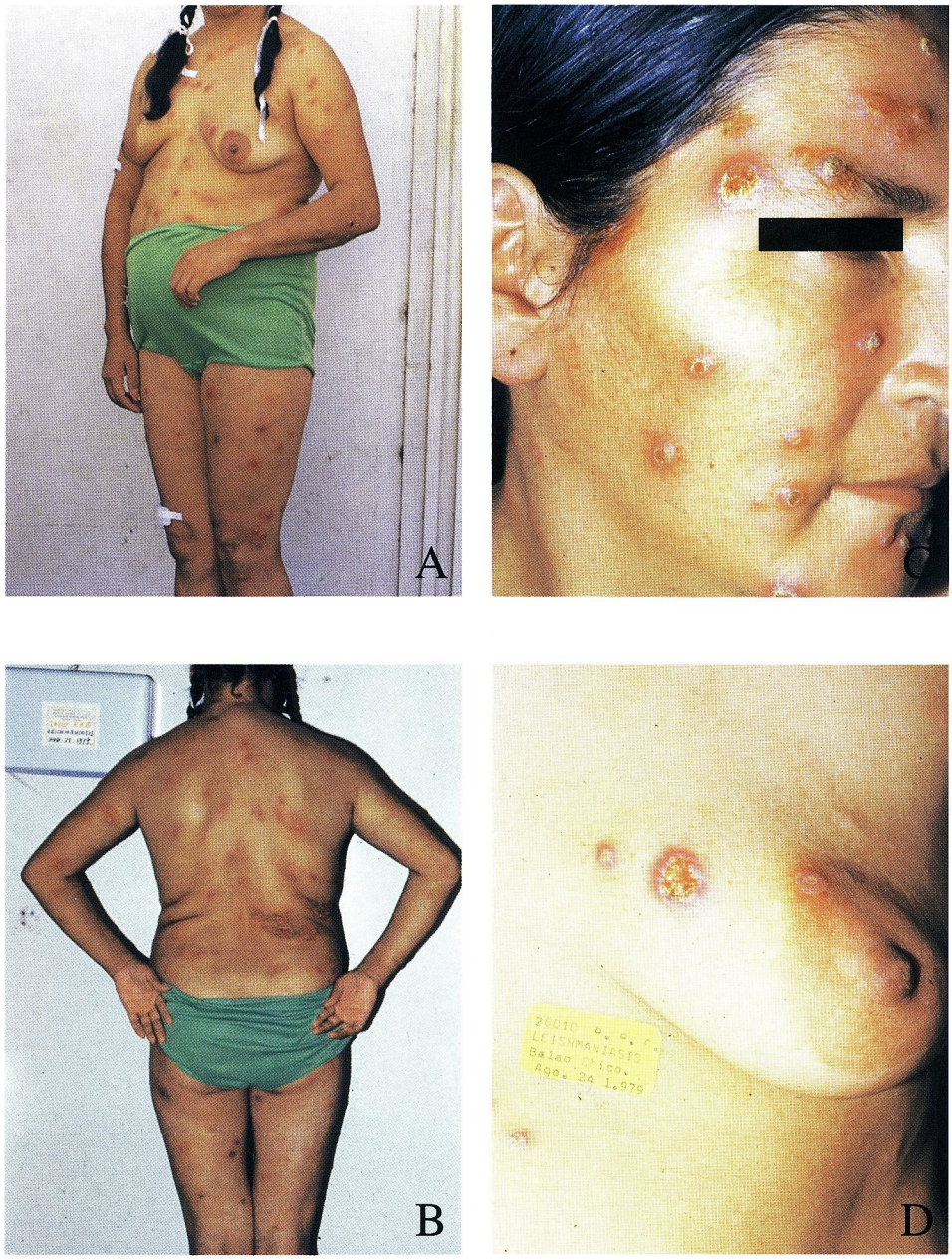

Figure 5.2.1. Showing detailed pictures of the present patient, 40 years old mestiza female, from Balao Chico, Department of Guayas, Ecuador. **A**, Disseminated ulcers all over the body surface. **B**, Ulcers on the back, more confluent on the right lumbar region. **C**, Crusty ulcers and some purulent lesions. At the fronto-temporal region there is a more characteristic ulcer. **D**, At the mammary region another characteristic ulcer is seen.

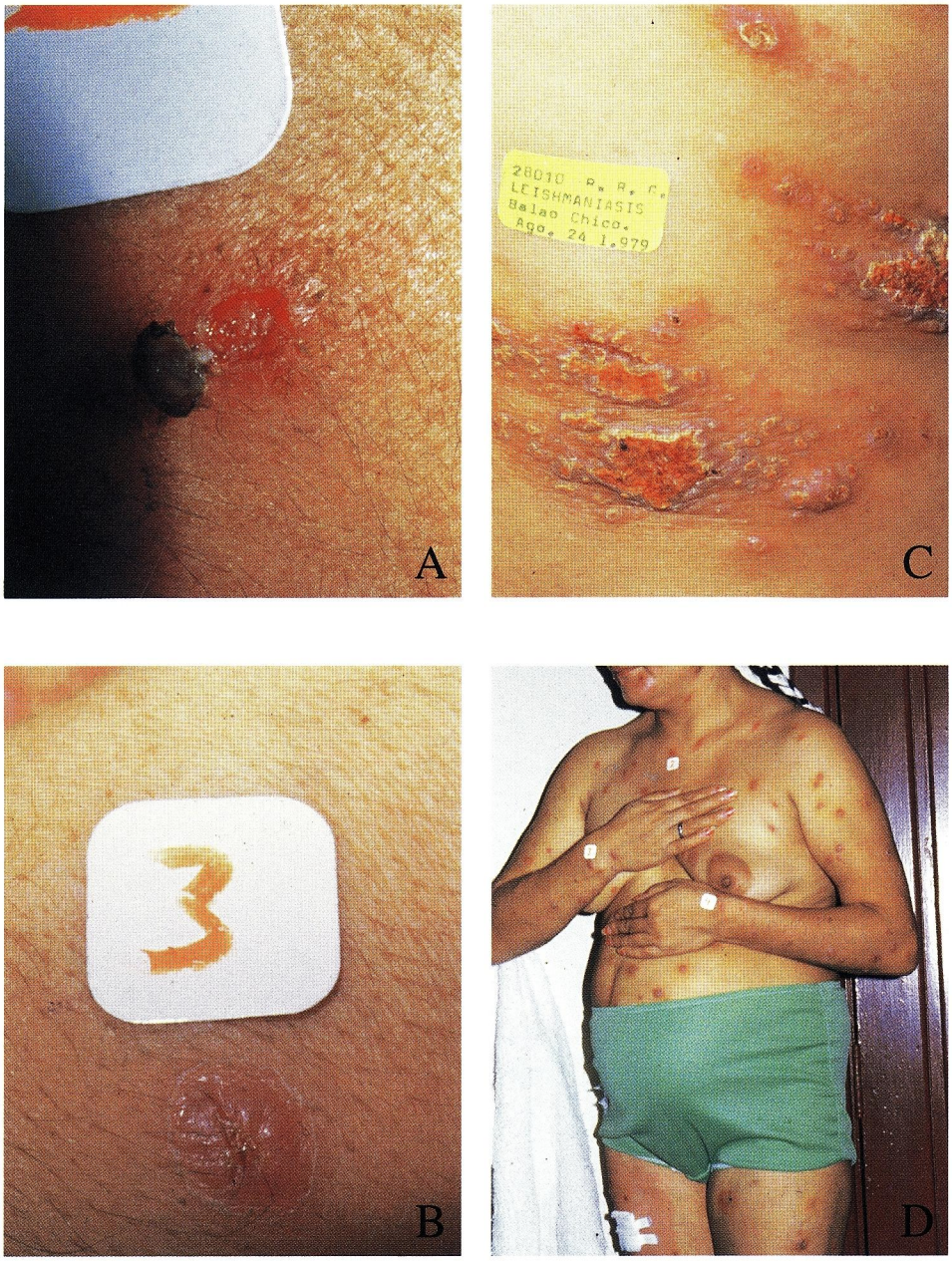

Figure 5.2.2. Showing different ulcers of the patient. **A**, A small excavated lesion on the cheek, lifted crust with an erythematous border. **B**, Papulous, umbilicated and erythematous lesion, 0.5cm in diameter, on the right wrist. **C**, Two confluent plagues on the lumbo-dorsal region. Characteristic lesion of herpes zoster with small satellite formations. Note a lesion to the left that does not belong to herpes zoster. **D**, Note the atypical ulcers that were positive for amastigotes of *Leishmania*. On the right leg there is a gauze covering the site where biopsy was taken.

was started by injecting intramuscularly 5ml daily. On the 7th day of therapy, marked improvement was seen. In this case, as supplimentary treatment, ampicillin, anti-histamine and metronidazole (1.5g daily *per os*) and metronidazole ointment were given for the patient during 15 days. When metronidazole ointment was applied, it caused intense itching about 15 minutes later. However, it was observed that the healing process of leishmanial ulcers was accelerated, with decrease of itching. In summary, the present patient received the following treatment at different times during 35 days: Glucantime® 12 ampules (5ml each), metronidazole *per os* 1.5g, for 13 days; metronidazole ointment 3g, for 13 days; ampicillin 2g intravenously, for 7 days; and mebendazole 1.5g, for 3 days. By performing the present treatment, a satisfactory result was found on the 28th day of treatment (Fig. 5.2.3A and B). The patient was discharged from the hospital, taking into account that she would have to come back two months later for evaluation.

Comments

It was evident that the clinical pictures of the present case of leishmaniasis constituted a special form that must be thoroughly analyzed, in order to define the factors which cause dissemination of ulcers reported. To date, such a leishmaniasis case with multiple ulcers (308 ulcers) had not been commonly described. From the interview to the patient, it was suggested that both the inoculation site by infected sandfly and the appearance of the first lesions occurred on the right lumber region.

Because of the appearance of a confluent plague of many ulcers and their distribution on the intercostal nervous regions, the coexistence of herpes zoster in the case was strongly suspected. Metastatic dissemination of leishmaniasis might be occurred simultaneously; thus, there might be a coincidence of process of two different diseases, *viz.*, leishmaniasis and herpes zoster. In the present case, there were some lesions distributing on the dorsal midline. Such clinical findings were not observed generally in the case of herpes zoster. For this reason, the cutaneous lesions observed were considered to be leishmanial ulcers.

In order to define those lesions which were not typical for leishmaniasis, biopsy samples were taken at random from several areas of body surfaces as shown in Fig. 5.2.2D. The small ulcers (millimetres) were excavated, and revealed many amastigotes of *Leishmania* in smear specimens. The purulent lesions described previously, did not improve with antibiotics used.

There was only marked improvement of the lesions when Glucantime® was given to the present patient. Due to the magnitude of dissemination of lesions and the coexistence of herpes zoster, we considered that 12 ampules of Glucamine® might not sufficient doses to reach the established therapeutic action; the appropriate dose was considered to be 10-20 mg/kg/day. For this reason, as her body weight was 60 kg, she should have received three ampules per day. However, an evident improvement was observed by extremely lower doses of Glucantime® as mentioned above. In the present case, metronidazole *per os* and its ointment were also used, respecting its complimentary therapeutic action as found in other leishmaniasis cases; it acts as a parasite-static and/or inflamation decreaser, so, helps on healing, but does not kill the parasite (Walton *et al.*, 1974). In the present case, metronidazole also seemed to be effective.

The reasons why the patient had a great number of cutaneous lesions on the body surface, demonstrating 308 ulcers. Some immunological and/or physiological conditions of the patient might caused metastatic dissemination of cutaneous leishmaniasis lesions. Furthermore, the coexistence of a viral infection as herpes zoster might favor the dissemination of the disease. In this case, an appropriate follow-up was necessary, but it was not possible because of unknown reasons. In future study, however, the following points should be ascertained by visiting the patient in Balao Chico, Department of Guayas, Ecuador: 1) if she was completely cured,

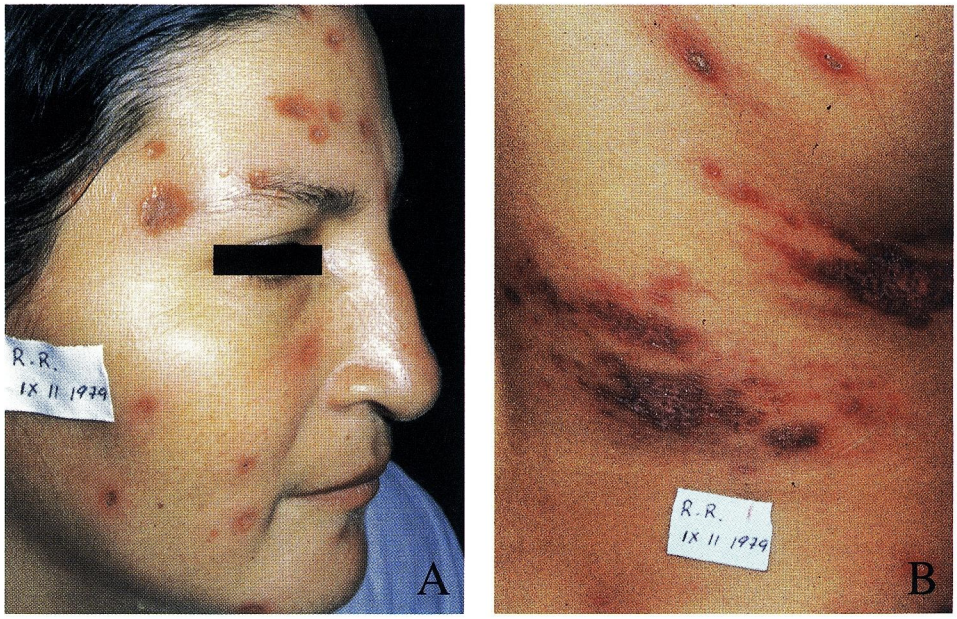

Figure 5.2.3. Pictures after 28 days of the treatment, on 11 September 1979. **A**, Note flattening of the lesions during healing process. The variety of size is quite visible. **B**, Note the improvement of the lesions considered as herpes zoster, as well as the surrounding lesions.

and 2) if the dissemination of lesions to the mucous membranes occurred or not.

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3. A Trial of Chemotherapy Using Anticancer Drug (Fluorouracil: 5FU) for Cutaneous Leishmaniasis in Ecuador

ABSTRACT. In this paper, a topical treatment of an anticancer drug ointment (fluorouracil: 5FU) against cutaneous leishmaniasis was evaluated. A total of 47 cutaneous leishmaniasis patients living in the village of Guayabales, Department of Manabi, Ecuador, were recruited for the study. 5FU ointment was prepared at the concentration of 2%. Among 7 patients treated with 2% 5FU ointment, one had a good improvement; two showed a slight improvement; and four showed no reaction. In addition, of these 7 cases, no complete cure was found within a month of treatment. It is reported that burning sensations were caused by the application of 10% paromomycin ointment. In this research, no patient with ulcerative lesions complained of burning sensation during the application of 2% 5FU ointment. It is concluded that 2% 5FU ointment may be usefull for the relatively small sized shallow ulcerative lesions, but not so effective against non-ulcerative lesions.

Introduction

Since topical treatment is not regarded as a first choice for patients with cutaneous leishmaniasis, it is needed today to search for more effective antiprotozoon drugs for the cutaneous leishmaniasis lesions, especially for dry, nodulous or plaque-type lesions. Fluorouracil (5FU) is known to be one of the anticancer drug and 5% 5FU ointment had been applied to the lesions of skin cancers, such as squamous cell carcinoma, as well as to precancer conditions, solar keratosis and Bowen's disease etc. (Kazuya, 1981). When 5% 5FU ointment is applied to the skin, the ointment makes erosions more easily than other anticancer drugs such as 0.5% bleomycin sulfate ointment. From this reason, we have decided to try to use low density 5FU ointment (2%) for topical treatment of cutaneous leishmaniasis.

Materials and Methods

Subjects

A total of 47 patients with cutaneous leishmaniasis living in the village of Guayabales, Department of Manabi, Ecuador, who consented were recruited for the present study. All the patients continued their daily activities during the treatment without hospitalization. Follow-up of the treatment was performed between July and September, 1992. Seven patients selected at random were treated with 2% 5FU ointment. The diagnosis was made based on clinical features of the disease, parasitological examination and leishmanin skin test. Clinical findings of some patients were demonstrated in Figs. 5.3.1 and 5.3.2. All the subjects gave informed consent to participate in the study.

Preparation of 5FU ointment

5FU ointment was prepared as follows: 10g of fluorouracil (Tokyo Kasei Co., Ltd., Tokyo, Japan) was dissolved in 10ml liquid vaseline. The dissolved fluorouracil was mixed with 500g of white vaseline. This mixture was then divided into 5g each in a small container.

Application procedure of topical ointment

The patients were given an oral instruction to apply the ointment two or three times a day. The applications were completely done by the patients themselves or their family members. Before the topical application, cleaning of the lesions using a soap and water was recommended to each patient.

Efficacy criteria for topical treatment

The effect of topical application was primarily judged by the clinical features. Photographs of the lesions were taken every two weeks, and the size and conditions of the lesions were carefully observed and recorded. Especially, ulcer sizes and indurations were measured at each clinical observation. The effects were graded into the following four criteria. 1) -, no change of the eruption during treatment; 2) +, slight improvement of the eruptions; and 3) ++, deffinate improvement of the eruptions. In this study, immediate evaluation was made at about one month after treatment with 2% 5FU ointment. The results of the early evaluation of each case were mainly reported in this text.

Results

The results were summarized in Tables 5.3.1 and 5.3.2. The number of the patients treated with 2% 5FU ointment was seven (2 males and 5 females). All the seven cases were followed up to the evaluation of topical treatment. Among the seven subjects, the mean age was 14.3-year-old; the mean time of obsevation was 29.4 days; mean number of lesions was 3.43 per patient. The treatment with 2% 5FU ointment produced positive improvement in one patient, a slight improvement in two patients and no change of the eruption in four patients (Figs. 5.3.1 and 5.3.2). When 2% 5FU ointment was applied, relatively small sized and shallow ulcer started to dry and the induration gradually tended to decrease, while these with plaque or nodulous type of lesion did not (Figs. 5.3.1. and 5.3.2) When 2% 5FU ointment was applied to the relatively large and deep ulcers, the lesions preferably began to get worse and the lesions of erosion became increased in size.

Profiles of several cases

Case 7 (file number G-116), a 9 years old female.

The lesion, which showed an ulcer, was located on the left thigh (Fig. 5.3.1A). The patient suffered from a painful ulcer for two months. For this patient, 2% 5FU ointment produced good reaction after 21 days of treatment. Positive improvement of the lesion was shown in Fig. 5.3.1B.

Case 5 (G-94), a 13 years old female.

The patient suffered from three ulcers sized from 10mm to 15mm in diameters on the right upper arm for a few months (Fig. 5.3.1C). 2% 5FU ointment and mercury chrome solution were given to the patient. One of the ulcers located at distal site of the right arm co-existent erosion became inceased in size. Other two lesions showed no changes. The condition of the patient after 34 days of treatment was shown in Fig.5.3.1D.

Case 4 (G-40), a 11 years old male.

The patient had three ulcers on the right cheek for a few months. An ulcer sized 10mm x 10mm was shown in Fig. 5.3.2A. For the patient 2% 5FU ointment and mercury chrome solution were given to the patient and the eruptions were gradually improved. A good result of treatment of the patient was shown in Fig. 5.3.2B, demonstrating much improved infiltration and induration of the eruptions after 34 days of the treatment.

Discussion

Many trials of topical applications to the lesions of cutaneous leishmaniasis had produced relatively good results (El-On *et al.*, 1988). However, problems about reactions of the lesions, metastasis to mucosae or viscera, possibility of transfer to mucocutaneous or visceral forms have not yet been solved. One of the most effective applications is paromomycin ointment (El-On *et al.*, 1988). However, the applications of paromomycin ointment have not been so effective, especially for the lesions, such as the dry nodulous and plaque-type lesions (Nonaka *et al.*, 1992). In this study the application of 5FU (anticancer drug) ointment to the lesions was tried. Usually, we, dermatologists, use the 5% 5FU ointment for the skin cancer such as squamous cell car-

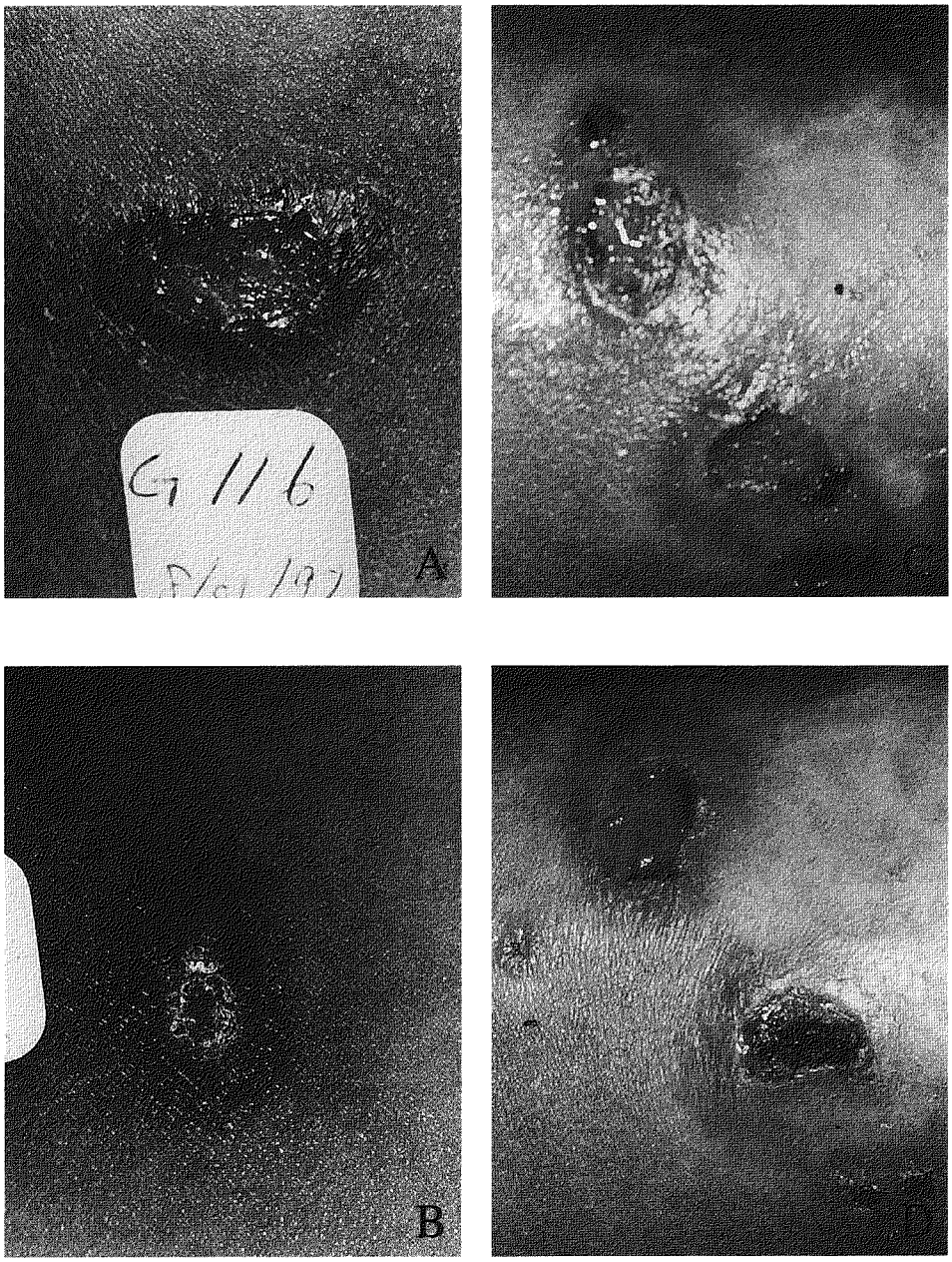

Figure 5.3.1. **A**, An ulcer of case 7. The size of the ulcer located on the extensor aspect of the left thigh was 20mm x 11mm. The figure shows the state of the lesion before treatment with 2% 5FU ointment. **B**, The same lesion (scar), showing positive improvement after 21 days of treatment. **C**, The ulcers of case 5 before treatment with 2% 5FU ointment. The size of ulcers located on the right upper arm was 15mm x 15mm before treatment with 2% 5FU ointment. **D**, The ulcer located on the most distal region of the right upper arm increased in size and erosion became apparent.

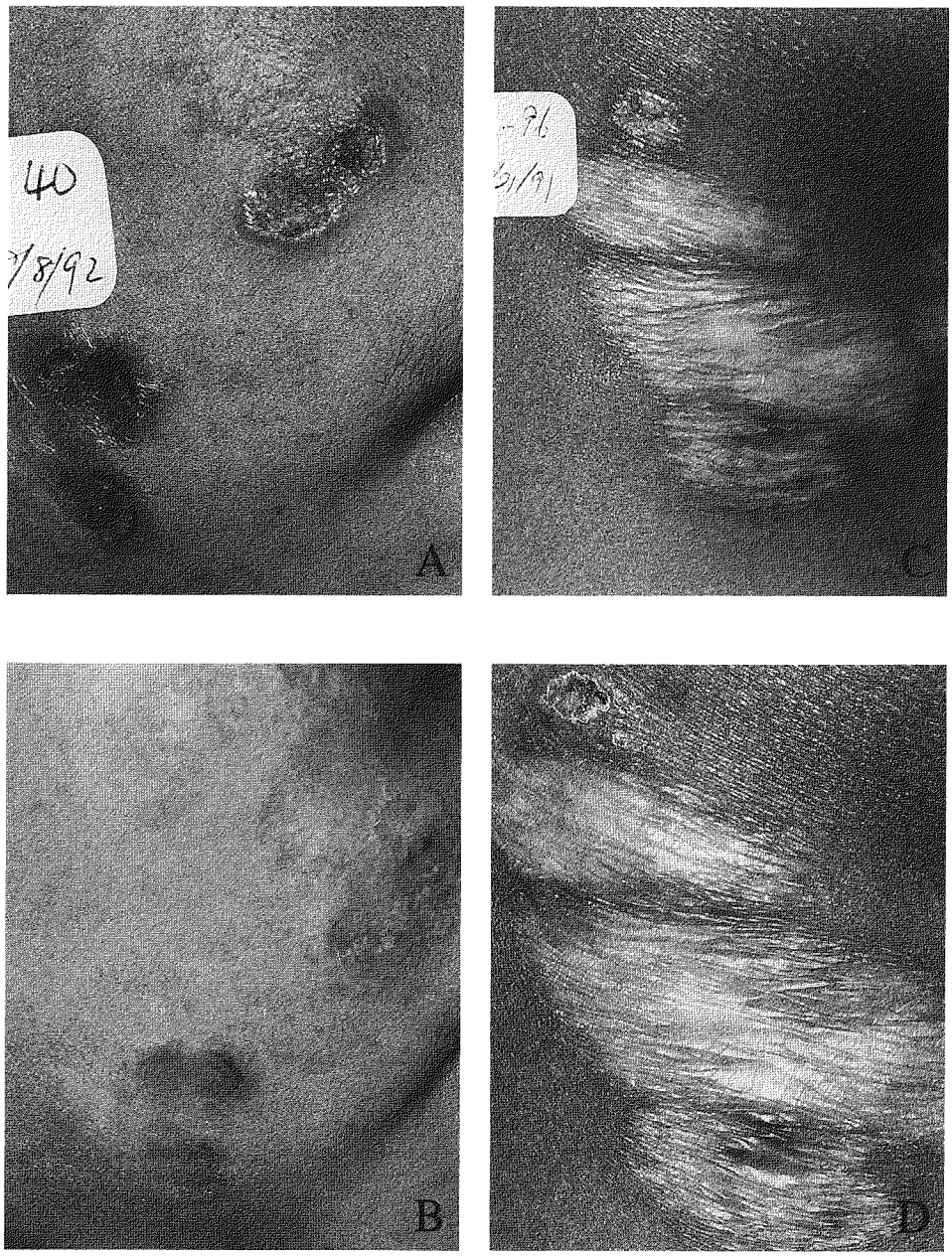

Figure 5.3.2. **A**, Ulcers of case 4 before treatment with 2% 5FU ointment. The size of the ulcer located on the right cheek was 10mm x 10mm. **B**, Induration and infiltration of the case 4 was much improved after 34 days of treatment. **C**, The eruption of case 6 (G-96) before treatment with 2% 5FU ointment. Ulcer, nodule, induration and scars are located at the region of the buttock flexor aspect of the left thigh. **D**, No marked improvement of the case 6 was found.

Table 5.3.1. Summary of the cutaneous leishmaniasis patients treated topically with 2% fluorouracil ointment in Guayabales, Department of Manabi, Ecuador

No.	File no.	Age	Sex	No. of lesions	Size of lesions in mm	Site of lesions	Symptom of lesions	Grade of effects*	Skin test
1	JNN-3	10	F	2	30x30, 30x30	l-leg	ulcer	(+)	+
2	JNN-23	43	F	1	7x6	r-arm	ulcer	(-)	+
3	G-32	6	M	4	5x5, 5x4,	r-knee,r-knee	nodule,scar		
					3x3, 5x10	l-knee,l-knee	nodule,scar	(-)	+
4	G-40	11	M	4	10x10, 10x10,	r-cheek,r-cheek	ulcer,ulcer		
					10x10, 13x10	r-cheek,l-elbow	ulcer,scar	(+)	+
5	G-94	13	F	5	15x15, 15x15,	l-arm,r-arm	ulcer,ulcer		
					15x15, 10x10,	r-arm,r-arm	ulcer,scar		
					10x10	r-arm	scar	(-)	+
6	G-96	8	F	7	30x20, 40x30,	l-thigh,l-thigh	scar,scar		
					20x10, 15x15,	l-thigh,l-thigh	scar,scar	(-)	+
					5x5, 5x5,	l-buttock,l-thigh	ulcer,nodule		
					5x5	l-thigh	induration		
7	G-116	9	F	1	20x11	l-thigh	ulcer	(++)	+

* -, No change of the eruption; +, slight improvent; ++, deffinate improvement.

Table 5.3.2. Effect of 2% Fluorouracil ointment after 21-34 days of the treatment of cutaneous leishmaniasis in Guayabales, Department of Manabi, Ecuador

Grade of effects*	Male	Female	Total
-	1	3	4
+	1	1	2
++	1	1	
Total	2	5	7
Mean age	8.5	16.6	14.3
Time of observation (days)	34	27.6	29.4
Mean number of lesions	4	3.2	3.4

* -, No change of the eruption; +, slight improvement; ++, deffinate improvement.

cinoma (Ikeda, 1981) or verruca vulgaris (Niimura, 1981); 5% 5FU ointment, however, makes erosions on the skin when it is applied. At first, therefore, application of low (2%) concentration of 5FU ointment was tried. In our preliminary study, there was no side effect such as burning sensation reported in the cases in which paromomycin ointment was applied (Nonaka *et al.*, 1992).

The present trial using 5FU ointment was not yet adequate to establish effective treatment of the cutaneous leishmaniasis, because of the short evaluation period and insufficient number of cases. However, 5FU ointment was found to be useful for small ulcerative lesions of the cutaneous leishmaniasis. Therefore, further investigations may require using various densities of 5FU ointment. For example, it may be a useful treatment that after making erosion on the nodulous and plaque-type lesions by application of 5% 5FU ointment, that 2% 5FU ointment should be applied to small ulcerative lesions. The inhabitants in the endemic areas usually use the mercury chrome solution for disinfection of wounds including the lesions of leishmaniasis. In the current survey, mercury chrome solution was prescribed to the patients after skin biopsy. It is reported that *Leishmania* was sensitive to mercury chrome *in vitro* and *in vivo* (Katakura *et al.*, 1993). It is necessary to observe the patients or lesions which are not treated with mercury chrome to evaluate the effectiveness of the topical treatment.

When any ointments are applied to such areas of the body as buttock flexor aspect of thigh and extensor aspect of upper arm, they may be easily removed by rubbing when sitting or wearing shirts. When oinment is applied to such regions, the treated lesions should be covered by gauge. For example, the topical treatment was not effective for the patients of Case 5 (Fig. 5.3.1C and D) and Case 6 (Fig. 5.3.2C and D). If their lesions were covered by bandages, it might have been possible to observe the good results. When we tried to use topical treatment for cutaneous leishmaniasis, it was the most important to perform parasitological investigation for each patient. We should select the patients infected by *Leishmania* species which never induce mucocutaneous or visceral type of leishmaniasis for the topical treatment.

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Chapter 6

Related Skin Diseases

1. Carrion's Disease: Histopathological Findings of the Cutaneous Verruga Nodules of an Ecuadorian Patient

ABSTRACT. Chronic verruga nodules taken from a patient with Carrion's disease were studied. Histologically, specimens of all the verruga nodules were consistent with that of granulomatous lesions that showed extensive infiltration of various types of cells along with the proliferation of capillaries. The sections were predominantly infiltrated with neutrophils and endothelial cells, while histiocytes, plasma cells, lymphocytes and mast cells were also visible to some extent. The blood vessels were dilated and many endothelial cells were located peripherally that were rounded and swollen, while a huge number of neutrophils invaded the vessels. Electron microscopically, large numbers of organisms were found and seen in different stages of the life cycle in the stroma. Furthermore, organisms were regularly seen either in close contact or existing inside the cytoplasm of neutrophils, suggesting the phagocytic role of these cells against the organisms. No organism was found inside the endothelial cells or histiocytes.

Introduction

Carrion's disease, also called as bartonellosis, is a bacterial disease caused by *Bartonella bacilliformis*. The disease is known to be transmitted by phlebotomine sandflies, *Lutzomyia* spp., blood sucking insects which also act as vectors of cutaneous and visceral leishmaniasis. Geographically, bartonellosis is reported only in the Central and South American countries bordering with the region of Andes mountain valleys, *i.e.*, Guatemala, Colombia, Ecuador, Peru, Chile and Bolivia (Carvajal *et al.*, 1978; Kreier and Ristic, 1981; Gray *et al.*, 1990; Schultz, 1968). Carrion, then a medical student at the Peruvian, Lima institute, in a self-inoculated experiment confirmed the disease as biphasic. This experiment proved fatal for him and he died later (Leonard, 1991). Verruga peruana, 2nd phase of the disease, is a cutaneous manifestation of the disease with characteristics of nodules that may occur without prevalence of the oroya fever, the first phase of the disease. Histologically, there has been considerable importance of verruga nodules. In early reports, Strong *et al.* (1914) mentioned that etiology of the verruga nodule is of viral in origin. Later it was suggested that the verruga nodules are due to the hyperplasia of the reticuloendothelial system (Weiss, 1967). After that, Recavarren and Lumbreras (1972) mentioned that verruga nodules are a tissue response against bacterias that later were phagocytized by verrucoma cells. Recently, there has been a report that suggested the histopathological similarities between the bartonellosis and Kaposi's sarcoma (Garcia, 1985). The aim of this study is to investigate and understand the nature and histopathology of cutaneous nodules of the Carrion's disease in an Ecuadorian patient.

Materials and Methods

A 10 years old Ecuadorian girl visited a nation-

al institute in Guayaquil, Ecuador, with the complain of the development of nodular lesions on the body. On the examination, the nodules ranged from 3mm to 5mm in diameter and were diffusely scattered on the lower portion of the body, particularly the hip, both knees and feet (Fig. 6.1.1A – D). They were symptom-less, erythematous and mostly dried. Some of them were hemorrhagic. Few of them resembled with molluscum contagiosum. There was no history of fever before onset of the nodules. On the basis of the clinical features and a typical history of residency in an endemic region, the diagnosis of verruga peruana stage of Carrion's disease (bartonellosis) was made. Six millimeters punch biopsies were taken from three different nodules of different sites before the treatment. The biopsied nodules were erythematous and slight scaly at the periphery. The specimens were divided into two parts. One part of biopsy material was fixed in 10% formalin, embedded with paraffin and stained with haematoxylin and eosin (H&E). Another part of the biopsy was cut into small pieces and fixed in cold 2% paraformaldehyde, 2% glutaraldehyde and processed with different concentrations of alcohol. Ultrathin sections were cut and stained with uranyl acetate and lead citrate, and then examined under JEM, 2000 EX; JEOL Japan, electron microscope.

Results

Light microscopic findings

Haematoxylin and eosin stained sections showed the hyperkeratosis, parakeratosis and mild acanthosis of the epidermis. There were dense infiltration of cells from the upper dermis to the lower dermis, and severe proliferations of small and large sized blood vessels were also visible (Fig. 6.1.2A). Mild edema was present just beneath the epidermis. Neutrophils and endothelial cells were predominant in the dermis in all the sections (Fig. 6.1.2B); while histiocytes, plasma cells, lymphocytes and mast cells were also visible. The blood vessels were surrounded by the hyperplasia of endothelial cells that were extraordinarily swollen (Fig. 6.1.2C). Almost all the blood vessels were occupied mainly with neutrophils and, to some extent, red blood cells. Overall histological features of the sections were similar to granulomatous reactions. Organisms were not detected in these sections.

Electron microscopic findings

Large number of *B. bacilliformis* organisms were found extracellularly in the stroma. Mostly they were in the form of colonies and seen under different stages of the life cycle (Fig. 6.1.3) where some of them were seen under the process of degeneration in these extracellular areas. Morphologically, organisms were situated in longitudinal and cross sections covered with the cell wall outside and one to two double-layered membranes inside (Fig. 6.1.4). The central zone of organisms was either electron-dense or electron-opaque. Organisms with extraordinary size were seen in the process of division by binary fission in the stroma (Fig. 6.1.5). Organisms were located only inside the cytoplasm of neutrophils where some of them showed degeneration (Fig. 6.1.6A and B). Beyond this, many organisms were trapped by the pseudopods (Fig. 6.1.7A and B) and cytoplasmic projections (Fig. 6.1. 8A and B) and found in the initial stage of phagocytosis. No organisms, alive or dead, were located inside the cytoplasm of endothelial cells and histiocytes.

Discussion

Carrion's disease is an immunological disease, and humoral antibodies against *B. bacilliformis* organisms have been determined by complement fixation test, haemagglutination test, ELISA and other methods (Howe, 1943; Knobloch *et al.*, 1985, García-Caceres and García, 1991). When we review the literature we find agreement with pathogenesis of the early phase of the disease in which organisms are shown to be phagocytized by reticuloendothelial system and red blood cells (Cuadra and Takano, 1969); however, there is controversal over the

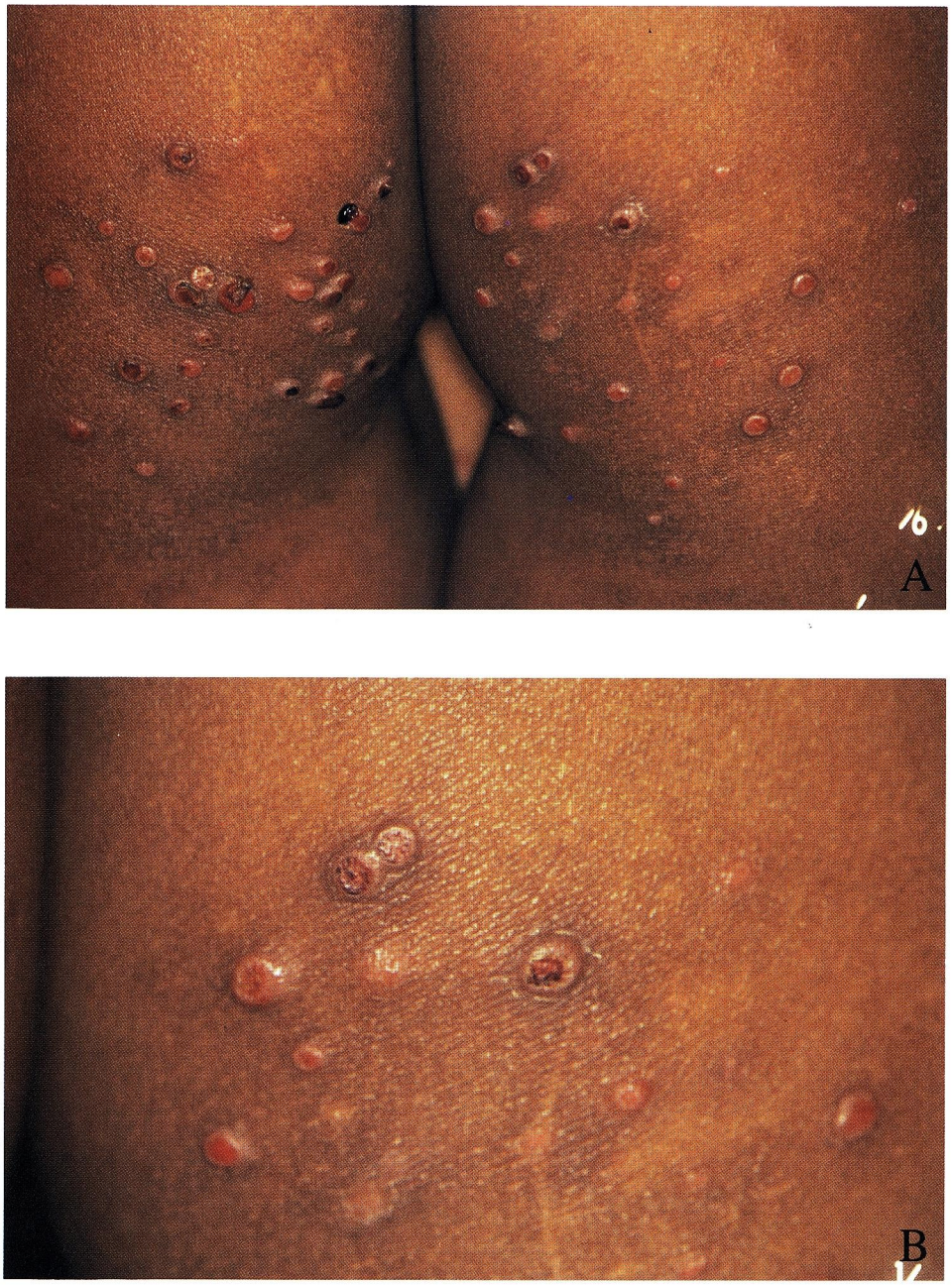
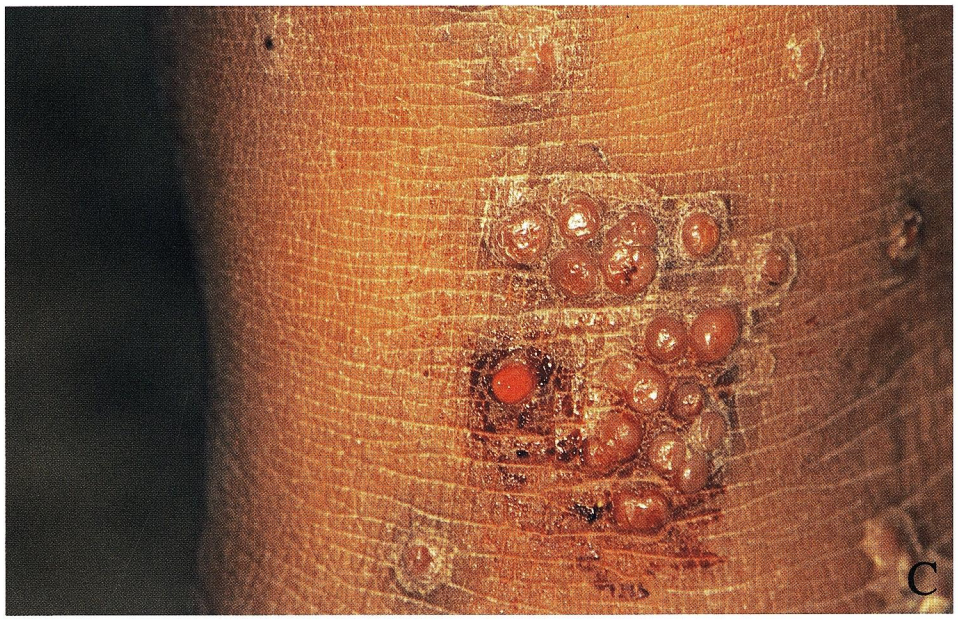


Figure 6.1.1. The clinical appearance of cutaneous lesions of Carrion's disease (bartonellosis). **A**, Bilateral involvement of gluteal region with the verruga nodules. **B**, High magnification of A., typical verruga nodules are visible in various forms and sizes, a few of them look like molluscum contagiosum. **C**, Papules and nodules are present on the knee, some of them are hemorrhagic. **D**, Hemorrhagic papules and nodules on the foot.



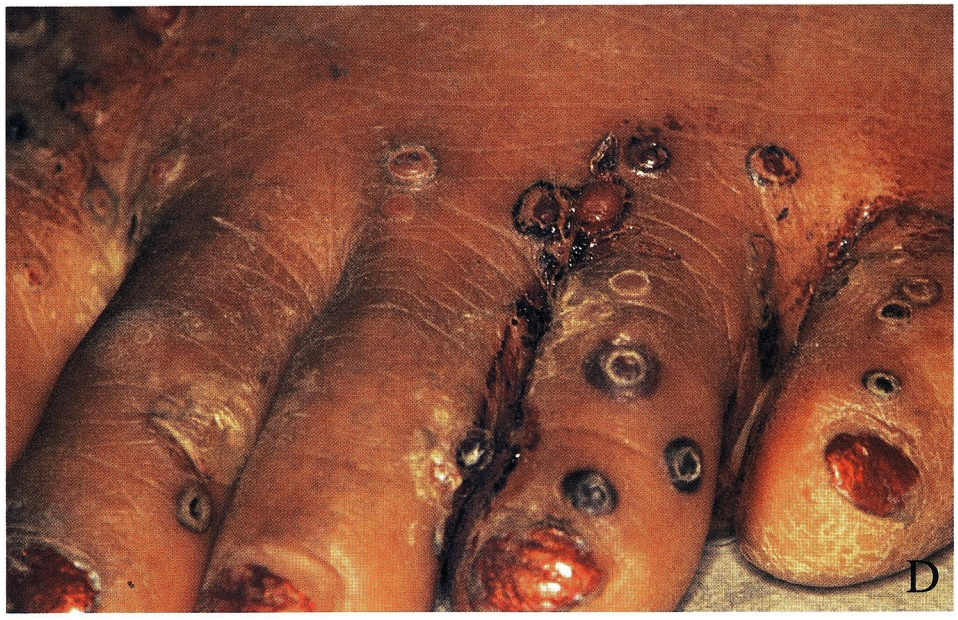


Figure 6.1.1C, D (continued).

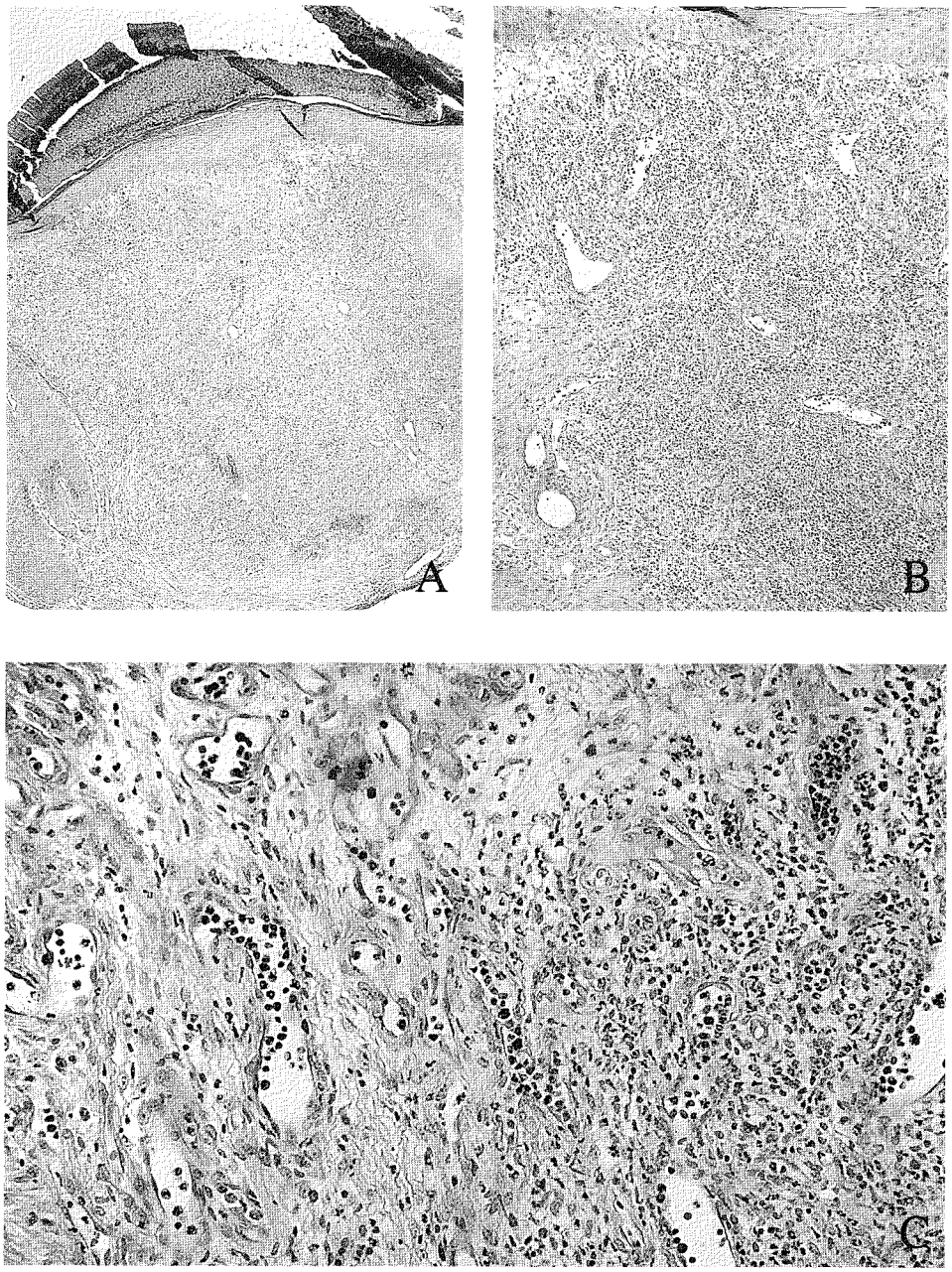


Figure 6.1.2. **A**, Light Photo micrograph of the nodule (low-magnification view). Note the deep dermal involvement of the lesion that covers the subcutaneous areas, and the proliferation of the blood vessels that is also visible. **B**, Severe infiltration of polymorphonuclear cells and edema of upper epidermis. **C**, High magnification of B, shows numerous blood vessels congested with the neutrophils. Dermal infiltration shows mainly neutrophils (H & E stain, A x20, B x50, C x100).

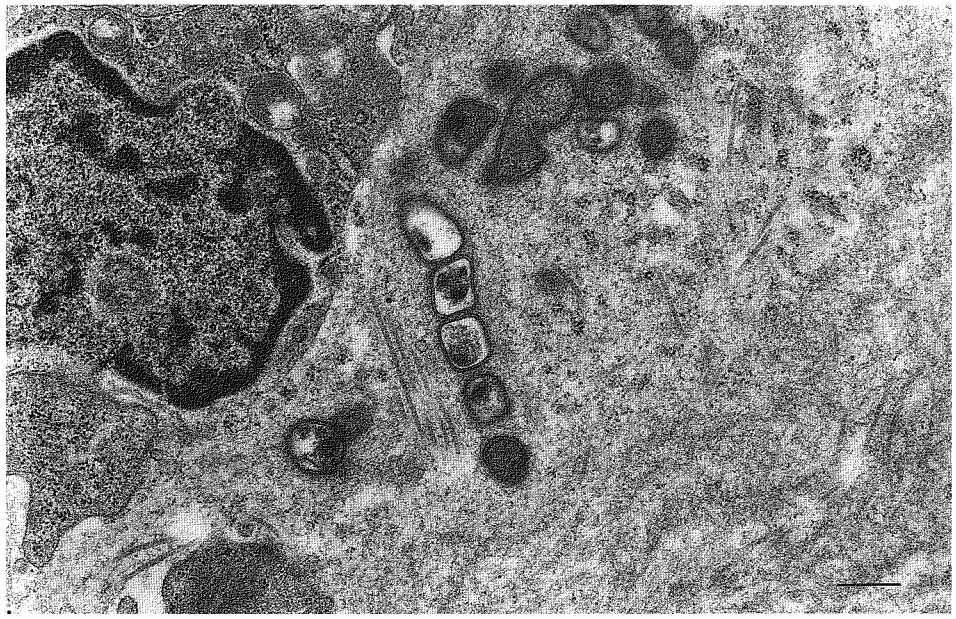


Figure 6.1.3. Electron micrograph. *Bartonella bacilliformis* organisms, in various life stages in the stroma. Some of them are in the degenerative stage (Bar=500nm).

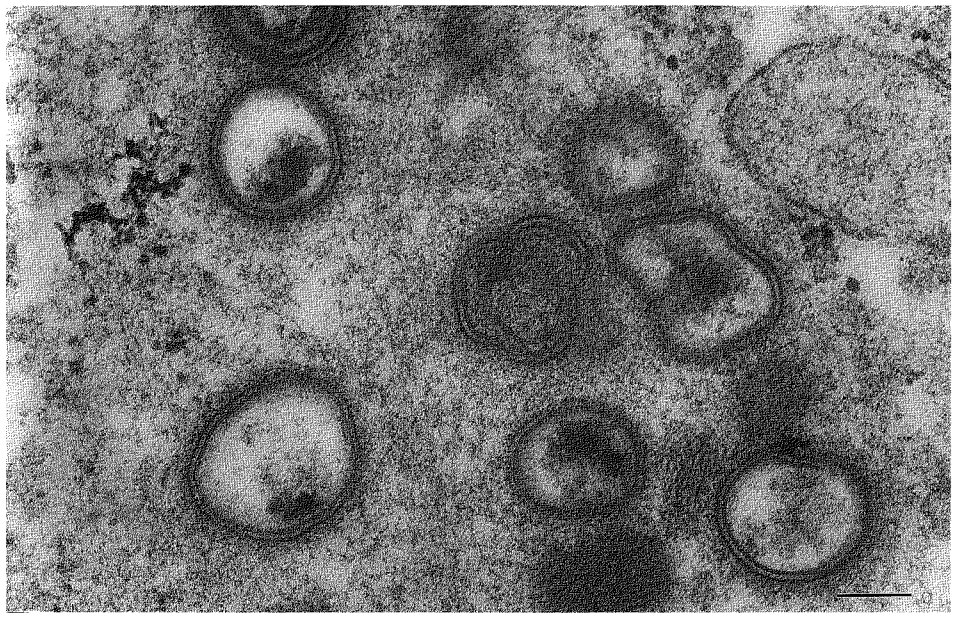


Figure 6.1.4. Ultrastructure of *Bartonella* organisms. All the organisms are shown in cross section covered with capsular and cell walls (Bar=200nm).

Figure 6.1.5. A longitudinal section of the *Bartonella* organism which is under the process of multiplication (Bar=200nm).

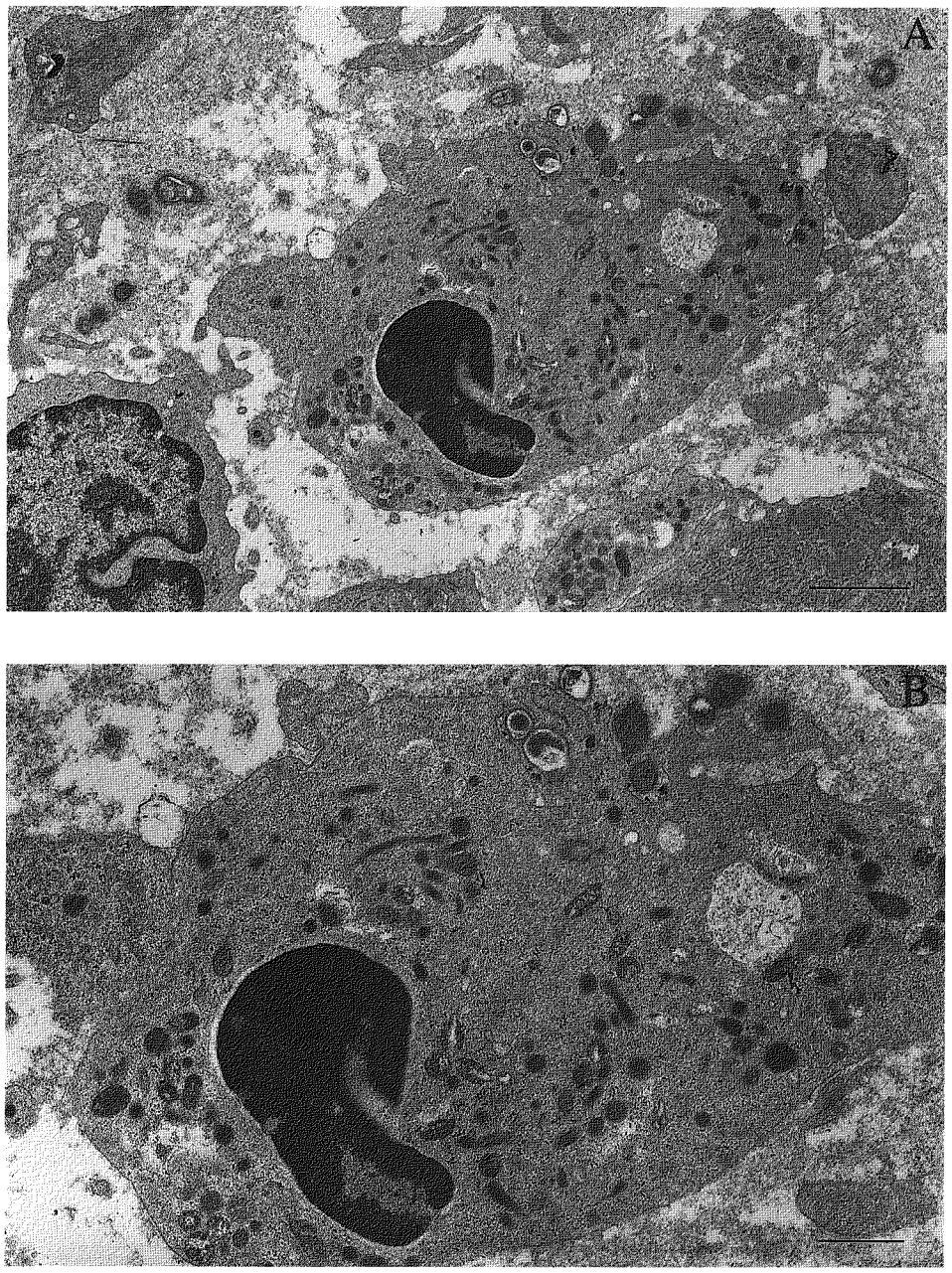


Figure 6.1.6. *B. bacilliformis* organisms phagocytized by the neutrophil. **A**, Certain organisms are trapped inside the cytoplasm while others are being trapped (Bar=2μm). **B**, High magnification of A, note the intracellular formation of phagolysosome representing the active stage of the cell (Bar=1μm).

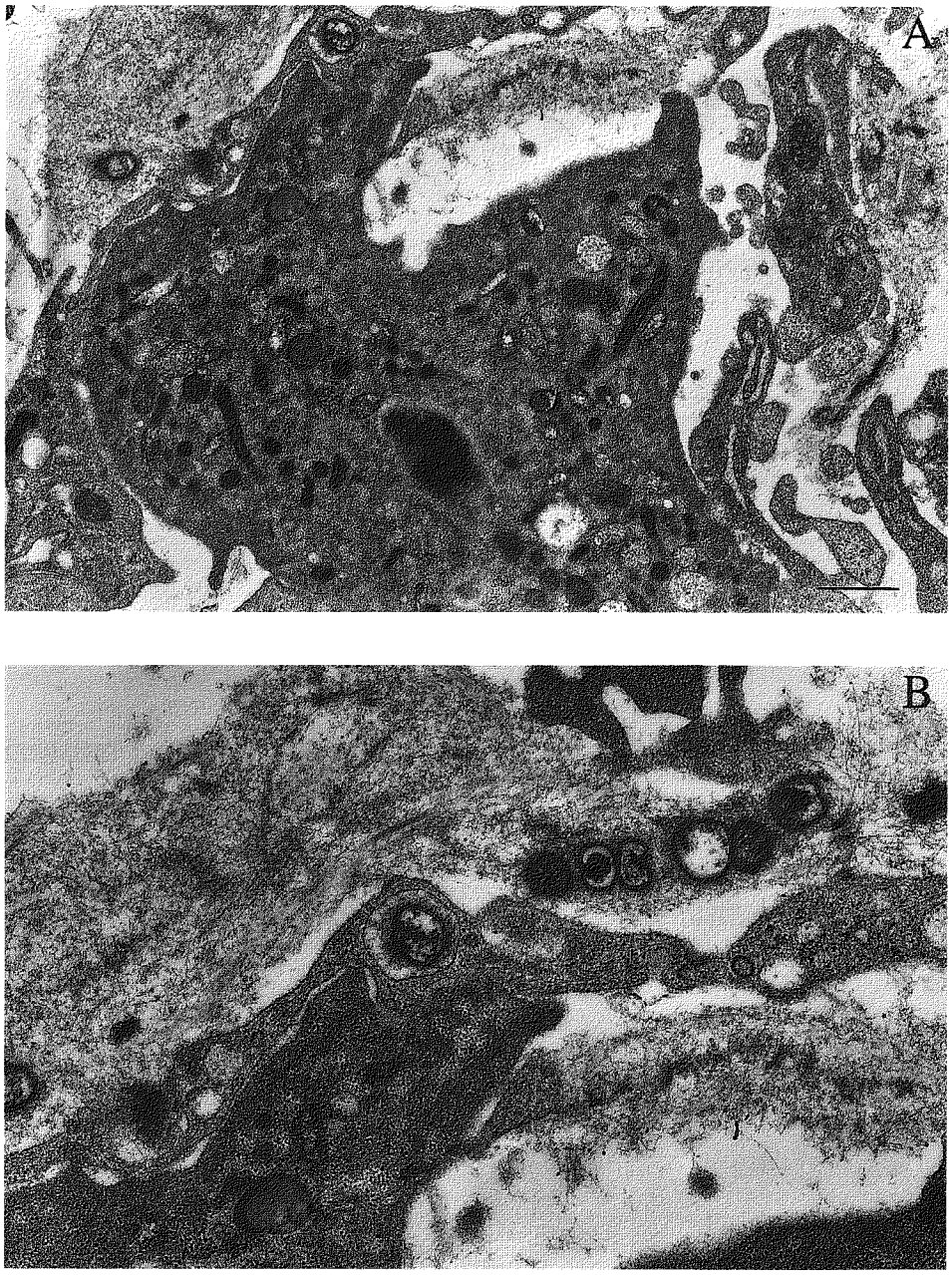


Figure 6.1.7. **A**, Organisms trapped by the pseudopods. Some of them are under the process of degeneration (Bar=1μm). **B**, High magnification of A (Bar=500nm).

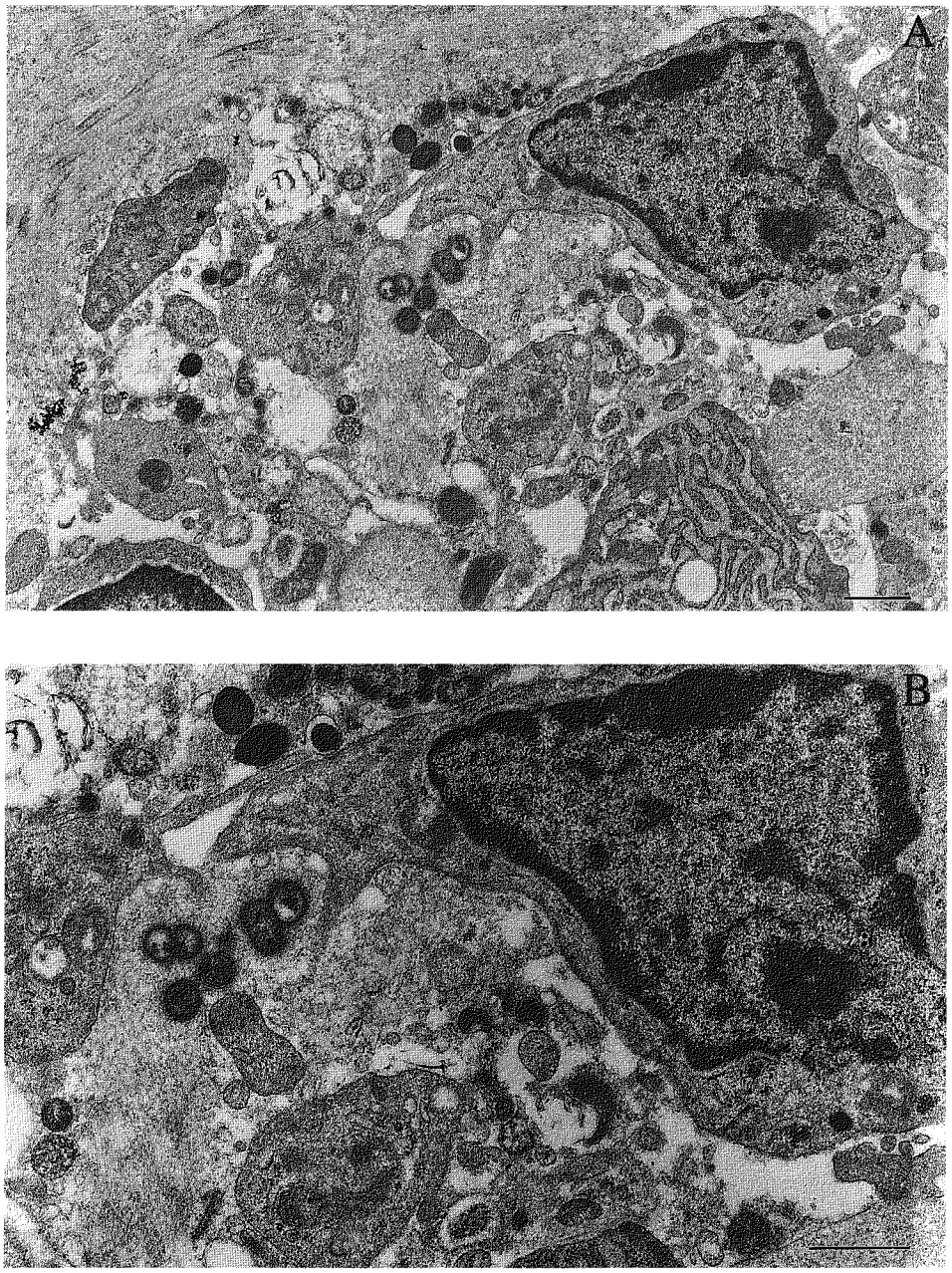


Figure 6.1.8. **A,** Elongated cytoplasmic projections of histiocyte covering the bacterias that may represent the initial step of phagocytosis (Bar=1μm). **B**, High magnification of A (Bar=1μm).

histopathological response in nodules. In various studies, it has been suggested that histiocytes and hemohistioblastic mesenchymal cells are fundamentally implicated and that the newly formed capillaries develop from them (Mackehenie and Battistini, 1922; Mackehenie and Weiss, 1926), while others considered that endothelial cells proliferates initially in the primary tissue reaction (Arias-Stella *et al.*, 1986; Rocha-Lima, 1913). However, there has been no report on a relationship between the neutrophils and cutaneous nodules of bartonellosis. We observed that neutrophils infiltrated and dominated the whole dermal areas as well as inside the blood vessels in the all sections. It seems likely that neutrophils were active under certain mechanism followed by the invasion of organisms in the tissues. Here it should be noted that neutrophils are capable of and responsive to following chemotactic factors through the vessel walls into the tissues. We propose that neutrophils may be considered as first line cells of the tissue reaction in bartonellosis.

With regard to the role of neutrophils and fate of organisms inside the nodules, we performed electron microscopic studies. Organisms were found only inside the cytoplasm of neutrophils that indicates the phagocytic role of these cells against the organisms. Urteaga and Calderon (1967) located these inside the cytoplasm of endothelial or histiocytic cells. Recavarren and Lumbreras (1972) described them inside the clear and dark cells and they called these cells as histiocytes or verrucoma cells. In our specimens we did not find organisms inside the endothelial cells or histiocytes despite an intensive search. In this study we did not investigate the mechanism of phagocytosis. However, Miller (1969) has shown the possibility of killing and lysis of gram-negative bacteria through the effect of hydrogen peroxide, ascorbic acid and lysozyme, the major microbicidal systems of the neutrophil. Arias-Stella (1987) noted either the epitheloid or pseudo-epitheloid pattern of infiltrated cells, or the appearance of predominantly spindle cells, suggested the neoplastic nature of the cutaneous nodules. Neither the spindle cells nor the carcinomatous changes were observed in our specimens. The histological changes were consistent with that of granulomatous reactions. Similar findings were observed by Goldman (1989). We suggest that the nature of the nodules is basically granulomatous but not neoplastic. On the basis of the described results, we conclude that histologically the nodules of Carrion's disease react like granulomas, and neutrophils play a role in the phagocytizing of *B. bacilliformis* organisms.

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2. Dermatological Survey in Rural Areas Endemic for Leishmaniasis and Urban Areas of Ecuador

ABSTRACT. Skin diseases found in an endemic area of cutaneous leishmaniasis in Ecuador were investigated whether there exists any skin disease and cutaneous changes that need to make differential diagnosis between the skin diseases and cutaneous leishmaniasis. Seven patients with leprosy were presented. Out of the various cutaneous changes found in these patients, leprosy should be considered as a possibility of misdiagnosis and therefore properly examined. High frequency of occurrence of skin diseases was found in pityriasis versicolor (17/257, 6.6%), paronychia blastomycetica (10/257, 3.9%), pityriasis rubra pilaris (2 cases), xerosis (6 cases) and pigmentary disorders (13/257, 5.1%). Number of atopic dermatitis was only two. There was no marked difference on incidence of cutaneous changes between rural and urban areas. Most of the cutaneous changes have a tendency to be influenced by the climate including sunlight exposure, life style and foods in the country. It seemed to be important to consider the role of these factors on the establishment and evolution of cutaneous leishmaniasis lesions.

Introduction

Clinically, patients with cutaneous leishmaniasis have various types of cutaneous changes such as insect bite-like papules, nodules, ulcers and erythematous plaques. It is therefore important to differentiate the diagnosis between cutaneous leishmaniasis and other skin diseases. It is reported that early nodular lesions of cutaneous leishmaniasis are similar to lepromatous leprosy (Jopling, 1984). We therefore investigated every cutaneous changes in endemic areas of cutaneous leishmaniasis, and also investigated those in some hospitals of Guayaquil city. In this study, the incidence of various cutaneous changes was compared between both rural and urban areas in Ecuador.

Materials and Methods

A total of 114 inhabitants living in the village of Guayabales, San Sebastian and Los Ranchos, Department of Manabi, Ecuador, located in endemic areas of cutaneous leishmaniasis, were dermatologically investigated. They visited our tentative clinic for the examination of cutaneous leishmaniasis that was carried out between July and September 1991 in those villages; at the same time, they were examined for other skin diseases. On the other hand, a total of 143 patients with skin diseases were examined at an outpatient clinic of skin diseases of contagious hospital in Guayaquil city, Ecuador.

Results

The result of examinations in rural areas was summarized in Table 6.2.1. The most frequent skin diseases found in rural area was dermatitis (61/257, 23.7%). Out of the dermatitis group, eczema chronicum (21 cases) was the most common, followed by contact dermatitis (13 cases). Only two children cases of atopic dermatitis were seen. Fifty one cases (51/257, 19.8%) of cutaneous leishmaniasis were recorded in these areas. The frequency of the fungal diseases was 17.1% (44/257). The diseases of appendages including acne vulgaris and xerosis were 30 (30/257, 11.7%). Two patients with pityriasis rubra pilaris were observed in children. The group of the bacterial diseases included seven cases of lep-

Table 6.2.1. Skin diseases observed in rural areas, San Sebastian (Km 103) and Guayabales (Junnin), Department of Manabi, Ecuador, in 1992

Skin disease	No. case	Skin disease	No. case
Diaper dermatitisa	1	Frunculus	1
Dermatitis linearis	1	Secondary infection	1
Eczema	4	Trauma	1
Eczema chronicum	6	Scabies	2
Contact dermatitis	1	Malignant skin tumor	2
Prurigo chronica	2	Basal cell carcinoma	1
Seborrheic dermatitis	2	Folliculitis	1
Paronychia blastomysetica	5	Syringoma	1
Tinea corporis	6	Pityriasis simplex faciei	3
Tinea pedis	1	Helides	1
Pityriasis versicolor	12	Lichen amyloidosis	1
Impetigo contagiosa	5	Acne conglobaata	1

rosy. The types of leprosy were as follows: borderline lepromatous leprosy, 1 case; borderline tuberculoid leprosy, 2 cases (Fig. 6.2.1); tuberculoid leprosy, 1 case; indeterminate leprosy, 1 case; and unknown types, 2 cases. All of the patients had sensory loss at the lesions and none of them, except one patient with borderline tuberculoid leprosy, had papule, nodule and ulcer with induration at the margin as seen in a patient with cutaneous leishmaniasis. The patient with borderline tuberculoid leprosy had pea-to-palm-sized infiltrated or non-infiltrated erythema or annular erythema on the face and trunk. Sensory loss was observed on his lesions. Skin tumors were relatively infrequent (11/257, 4.3%) and three patients with malignant skin diseases were seen (3/11, 27.3%), that is, basal cell carcinoma (BCC), 1 case; Bowen's disease, 1 case; and meibomian duct carcinoma, 1 case. The pigmentary disorder group such as chloasma (Fig. 6.2.2) was relatively frequent with the rate of 5.1% (13/257). These might be influenced by ultraviolet rays. Only one patient with scabies was found. The eruptions were miliary-sized-reddish-papules.

In the urban areas, as shown in Table 6.2.2, group of dermatitis (44/143, 30.8%) and diseases of appendages (24/143, 16.7%) were much more frequently observed than in the rural areas. Especially, 12 patients with contact dermatitis were observed in the urban areas, though only one case was found in rural areas. Two patients with collagen diseases (progressive systemic sclerosis, dermatomyositis) and two cases of bullous pemphigoid were seen in children at the outpatient clinic. One cutaneous leishmaniasis case from a rural area was diagnosed in the clinic. In the rural area, the frequency of dermatitis group was relatively low (17/114, 14.9%). Diseases caused by viruses, keratodermia, bullous disease and connective tissue diseases were not seen in the rural areas at all.

Comments

The number of the patients with atopic dermatitis was only two in an outpatient clinic in Guayaquil, Ecuador. Its frequency was lower than

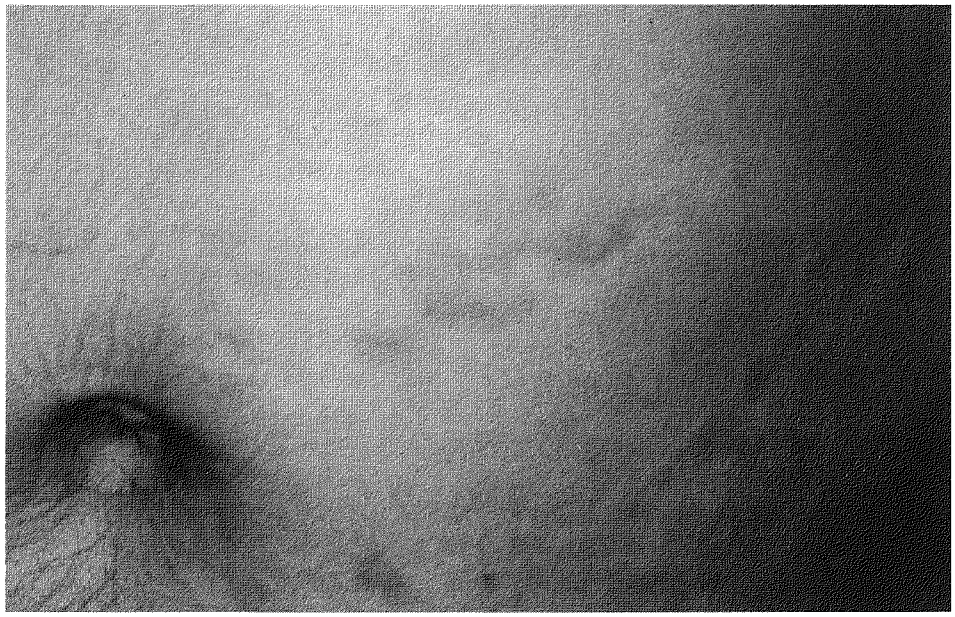

Figure 6.2.1. Patient with borderline group leprosy newly detected at an outpatient clinic of dermatological department of the contagious hospital in Guayaquil, Ecnador.

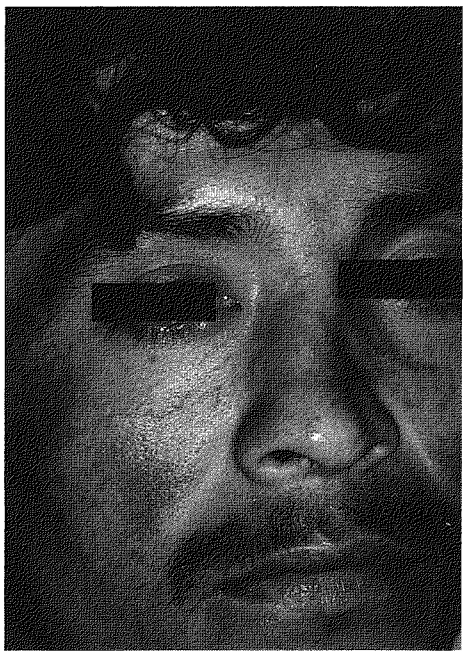

Figure 6.2.2 Patient with pigmentary disorders diagnosed as dyschromia.

that in the dermatological clinic in Japan, U.S.A. and European countries (Ueda, 1985; Uehara and Ofuji, 1987; Leung *et al.*, 1987). In these countries, the frequency of atopic dermatitis ranged from 2% to 11%. Recently, its frequency seemed to be more elevated in these countries. The number of seborrheic eczema was relatively large. The differences among those countries might be caused by the factors such as climate and food.

Five cases of leprosy were seen in rural areas and two cases of leprosy were found in urban areas (see Chapter 6.4). Three patients of them were newly detected. In 1991, the number of newly detected patients with leprosy in Ecuador was 110. In Okinawa, Japan, only five patients with leprosy were newly detected in 1992 (Hosokawa *et al.*, unpublished data). The prevalence rate of leprosy in Okinawa, Japan was 0.005 per 1,000 inhabitants in 1992, while in Ecuador it was 0.25 per 1,000 inhabitants in the same year, 1992. The latter country, Ecuador, therefore, seemed to be still an high endemic area for leprosy. The eruptions, such as

Table 6.2.2. *Skin diseases observed at an outpatient clinic in urban area, Guayaquil, Ecuador, in 1992*

Skin disease	No.case	Skin disease	No.case
Cutaneous leishmaniasis	1	Atopic dermatitis	2
Molluscum contagiosum	2	Xerosis(Vit.A)	6
Herpes genitalis simplex	1	Keratosis pilaris	1
Herpes zoster	1	Pityriasis rubra pilaris	2
Verruca vulgaris	2	Psoriasis vulgaris	4
Leprosy (BT,TT,BTH)	3	Folliculitis	3
Impetigo contagiosum	1	Acne vulgaris	8
Frunculus	1	Acne conglobata	2
Tinea corporis	3	Alopecia areata	2
Tinea pedis	3	Alopecia universalis	1
Tinea capitis	2	Penphigoid	2
Paronychia blastomycetica	4	Progressive systemic sclerosis	1
Candidiasis	1	Dermatomyositis	1
Pityriasis versicolor	3	Basal cell carcinoma	1
Hand eczema	1	Bowen's disease	1
Eczema nummulare	1	Meibonian duct carcinoma	1
Eczema chronicum	14	Naevus	1
Eczema sebborhoicum	6	Covernous angioma	1
Contact dermatitis	11	Strawberry mark	1
Stasis dermatitis	2	Oedema Quinckei	1
Erythrodermia	1	Schamberg's disease	1
Prurigo	2	Ephelides	1
Pruritus cutaneous senilis	1	Melanosis faciei feminina	1
Chloasma	2	Dyschromatosis	6
Insect bite	1	Ingrown nail	1
Trauma	1	Granuloma	1
Others	2		

papules, nodules, infiltrated erythemata, erosions and ulcers observed in a patient with leprosy, were clinically very similar to those of cutaneous leishmaniasis. For this reason, it was likely to be very important to make differential diagnosis between cutaneous leishmaniasis and leprosy. It was reported that the type of cutaneous leishmaniasis, especially disseminated anergic forms, most likely to be confused with lepromatous leprosy. According to Jopling (1984), the nodules were numerous and simulate those of lepromatous leprosy but were teeming with L-D bodies (*Leishmania* amastigotes). In the present study, the lesions of cutaneous leishmaniasis did not possess sensory loss. The ulcers of leprosy were relativery shallow and cured in a short time and no indurations were observed at the mar-

gins of the ulcers as seen in the patients with cutaneous leishmaniasis. Cutaneous leishmaniasis had various clinical features as mentioned above, such as insect bite-like papules, crusted-nodules as seen in fruncle, infiltrated erythema with or without shallow ulcers, erosions and deep ulcers with indurations at the margins. For this reason, the diagnosis was very difficult without the knowledge of cutaneous leishmaniasis. Therefore, it seemed to be important to investigate why varieties of the cutaneous changes occurred in patients.

Frequency of paronychia blastomycetica was 22.7% (10/44). This disease was observed on the fingers of women. It was considered that the blastomycetica was induced by kitchen work and scrubbing and washing cloths. Frequency of pityriasis versicolor was 38.6% (17/44). The high rate of occurrence might be caused by the tropical climate of Ecuador. It would be therefore important to consider the influence of the climate in the skin disorders and changes. It had been considered that pityriasis rubra pilaris might be caused by a deficiency in vitamin A and other vitamins in the past (Baden *et al.* 1987). Nutritional condition in some inhabitants, especially in a part of children living in urban areas, seemed to be poor. There were two patients with pityriasis rubra pilaris, but fortunately, no symptoms of the eyes existed. Nutritional condition also seemed to be one of the important factors which changed and modified the clinical symptoms of the skin disorder including the eruptions of cutaneous leishmaniasis. Six cases of xerosis that might also be caused by a deficiency of vitamin A in the past, were seen in the urban areas, but not in rural areas. We did not have a patient with pigmentary disorders diagnosed dyschromia in Okinawa, Japan (Hosokawa *et al.*, unpublished data). We felt that these symptoms seemed to be caused by the strong ultraviolet rays of the sunlight. Ultraviolet rays induce the local and mild immunodeficiency. Infrared rays causes high temperatures in the lesions with skin disorders. Climate, such as temperature and humidity, ultraviolet rays, food and life-style might have some influences on the skin diseases including cutaneous leishmaniasis. For this reason, it would be important to consider a role of these factors on the course of leishmaniasis. The study to know the role of fungi and bacteria on the evolution of cutaneous lesions are continued (Nishimoto *et al.*, 1992; De Coronel *et al.*, 1992).

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3. Seroepidemiological Surveys for Leprosy in Endemic Areas of Cutaneous Leishmaniasis in Ecuador

ABSTRACT. Serological examinations of leprosy in endemic areas of cutaneous leishmaniasis were carried out using sera collected during a survey for cutaneous leishmaniasis and several parasitic diseases in Ecuador. There was no correlation between prevalence rates for leprosy and seropositive rates of the antibodies (anti-PGL-I and LAM-B antibodies) in the subjects living in several provinces in Ecuador. Seropositive rates of anti-PGL-I antibodies of the leprosy patients and their families in Los Ranchos, Department of Manabi, were relatively high (84.6%, 11/13) in comparison with the average seropositive rates (42.2%, 154/365) of the subjects from other areas of Ecuador. It was suggested that seroepidemiological survey of families of leprosy patients might be useful for screening in a low endemic area, such as Department of Manabi, Ecuador.

Introduction

Leishmaniasis and leprosy are etiologically completely different diseases, but it has been known that the two diseases cause immunologically similar responses in their hosts (Bryceson, 1981). Therefore, it may be important to know seroepidemiological features of leprosy in endemic areas of cutaneous leishmaniasis. Two types of skin tests, leishmanin (Montenegro) test for leishmaniasis and lepromin test for leprosy, were made on several leprosy patients and their families. Using sera which were collected during surveys for cutaneous leishmaniasis and other parasitic diseases including leprosy, the value of anti-PGL-I (phenolic glicolipid-I) antibody and anti-LAM-B (lipo-arabino-mannan-B) antibodies were measured for immunological studies of leprosy. PGL-I is a major secretory product of *Mycobacterium leprae* (Hunter *et al.*, 1982). LAM-B is a complex glycolipid found in large amounts (15mg per g of bacilli) within the cell walls of *M. leprae* and *M. tuberculosis* (Gaylord and Brennan, 1987). The present paper reports the result of preliminary serological examinations of leprosy. Furthermore, based on the results obtained, a brief comment was also made on the screening system to detect leprosy patients in early stage of the disease.

Materials and Methods

Subjects examined

In this preliminary epidemiological survey of leprosy, an evaluation was made on serodiagnosis of the subjects from the following areas of Ecuador: Los Ranchos, Portoviejo and Guayabales, Department of Manabi; Echeandia, Department of Bolivar; Antepara, Machala, Piñas, Portovelo and Zaruma, Department of El Oro; Pedro Carbo, Guayaquil and Olon, Department of Guayas; Selva Alegre, Department of Esmeraldas; and other areas of Ecuador. A total of 365 subjects (153 males, 184 females and 28 unknown), with 3 to 73 years old, were examined for anti-PGL-I antibodies (IgG and IgM) and LAM-B antibodies (IgG and IgM). The mean age of subjects tested was 26.2-year-old in male, 25.4-year-old in female and 25.8-year-old in total (Table 6.3.1). In the present subjects from different areas, the following underlying diseases were reported: Department of Bolivar, cutaneous leishmaniasis, 15; El Oro, Chagas' disease, 15 and gnathostomiasis, 1; Guayas, cutaneous leishmaniasis, 13, Chagas' disease, 9, gnathostomiasis, 6, and toxoplasmosis, 4; Pichincha, cutaneous leishmaniasis, 17, and Chagas' disease, 1; Esmeraldas, cutaneous leishmaniasis, 25.

Table 6.3.1. Prevalence rates of leprosy in different areas of Ecuador

Departments	Prevalence rates (x1000)	No. patients and mean ages		
		male	female	total
1. Bolivar	0.63 - 0.91	8(16.4)*	7(28.9)	15(22.9)
2. El Oro	0.63 - 0.91	7(59.1)	7(40.6)	14(53.8)
3. Guayas	0.27 - 0.38	80(22.0)	98(18.0)	178(20.0)
4. Pichincha	0.10 - 0.16	9(23.8)	8(24.7)	17(24.2)
5. Manabi	0.10 - 0.16	9(24.1)	6(14.0)	15(23.2)
6. Esmeraldas	0.10 - 0.16	40(11.6)	58(12.6)	98(12.2)
7. Others	-	0	2(39.0)	2(39.9)
8. Unknown	-	?	?	26
Total		153(26.2)	184(25.4)	365(25.8)

*, Mean age (years old) in parentheses.

Sera

Serum samples from 365 subjects were examined. These sera were collected during the surveys for several infectious diseases, such as cutaneous leishmaniasis, gnathostomiasis, toxoplasmosis and Chagas' disease, including leprosy.

Skin test

The lepromin skin tests were performed in eight subjects suspected for leprosy. An amount (0.1 ml) of Mitsuda lepromin solution was injected intradermally on the flexor surface of the forearm using the small needle (a disposable needle; size 26G), and the skin test area was observed for erythema and induration (induration/erythema in mm) at 48 hours later. Erythema size of more than 11mm in diameter at the injection site was considered positive reaction, and reaction with 7mm x 10mm was considered an undetermined (+/-) Mitsuda early reaction. The diagnosis of leprosy was made on clinical and bacteriological grounds, according to the Ridley-Jopling classification (Ridley and Jopling, 1966).

Leishmanin skin tests (Furuya *et al.*, 1989) were made in 13 subjects suspected of leprosy (file number G-1-G-13). An amount (0.1 ml) of *Leishmania* promastigotes antigen solution was injected intradermally on the flexor surface of the forearm. The skin test area was observed for erythema and induration (induration/erythema in mm) at 48 hours later. Induration size of more than 5mm in diameter at the injection site was considered as a positive reaction.

Serological examination

Blood samples were collected by venipuncture. The sera were separated by using a centrifuge at several field laboratories in Ecuador. The sera were stocked in a freezer at the temperature of -20°C. The value of anti-PGL-I antibodies and anti-LAM-B antibodies were measured by enzyme-linked immunosorbent assey (ELISA) in a laboratory at the National Institute for Leprosy Research in Japan (Izumi *et al.*, 1993). Cut-off levels are as follows: PGL-I-IgG, 0.08 OD (optical density) units; PGL-I-IgM, 0.38 OD units; LAM-B-IgG, 0.25 OD units; LAM-B-IgM, 0.05 OD units. A criterion for considering the diagnosis was made as follows: PGL(+) and LAM(-), suspective leprosy; PGL(-) and LAM(+), suspective acid-fast bacteria infection

Table 6.3.2. Prevalence rates of leprosy and anti-PGL-I antibody positive subjects from different areas of Ecuador

Departments*	No. examined	Positive for PGL-I		
		Total (%)	Male	Female
1. Bolivar	15	3 (20.0%)	1	2
2. El Oro	16	3 (18.7%)	3	0
3. Guayas	198	61 (30.8%)	16 **	32**
4. Pichincha	18	10 (55.6%)	5	5
5. Manabi	15	12 (80.0%)	5	6
6. Esmeraldas	98	64 (61.2%)	23	41
7. Other areas	5	1 (20.0%)	0	1
Total	365	154 (42.2%)	53 (37.9%)	87(62.1%)

*, 1. Bolivar, Echeandia; 2. El Oro, five regions; 3. Guayas, Pedro Carbo and Guayaquil; 4. Pichincha, Puerto Quito and Quito; 5. Manabi, Los Ranchos; 6. Esmeraldas, Selva Alegre.
**, Sex of 13 subjects are unknown.

including leprosy; PGL-IgG(+) and IgM(-), suspective leprosy (old leprosy and spontaneously healing subjects); PGL-IgG(-) and IgM(+), suspective leprosy. Skin biopsies were made on two leprosy patients, G-1 and G-7, and skin slit smears were also taken from G-7.

Results

The results obtained were summarized as shown in Tables 6.3.2 to 6.3.8 and Figs. 6.3.1 and 6.3.2. Correlation between anti-PGL-I-IgG antibodies (PGL-IgG) and anti-PGL-I-IgM antibodies (PGL-IgM) of the subjects from different areas of Ecuador were summarized in Tables 6.3.2 and 6.3.3. Among 365 subjects, 154 (42.21%) were found to be seropositive in 53 males, 87 females and 14 unknown. A total of 22 sujects (22/154, 6.0%) were IgG- and IgM-positive. On the other hand, 25 (6.9%) of the 365 subjects were IgG-positive and IgM-negative and 107 (29.3%) were IgG-negative and IgM-positive. The remaining 211 (57.8%) of the 365 subjects examined were IgG- and IgM-negative. The subjects of file number EH57 and EH119 from Esmeraldas and G-7 from Manabi showed positive reaction to all the anti-PGL-I and anti-LAM-B antibodies examined.

The distribution of anti-LAM-B antibodies in the subjects with negative anti-PGL-I antibodies in the patients with cutaneous leishmaniasis, gnathostomiasis, Chagas' disease and others was summarized in Table 6.3.4. In 215 PGL-I seronegative persons, 132 (61.4%) were found to be LAM-B seropositive; 15 (7.0%), IgG-positive and IgM-positive; 16 (7.4%), IgG-positive and IgM-negative; 101 (47.0%), IgG-negative and IgM-positive; and 83 (38.6%), IgG- and IgM-negative.

The results of examinations of leprosy patients and their family in Los Ranchos, Department of Manabi were summarized in Tables 6.3.5 to 6.3.8. The number of subjects examined for serological tests of leprosy was 13 (7 males and 6 females). The results of skin tests were shown as follows. Only

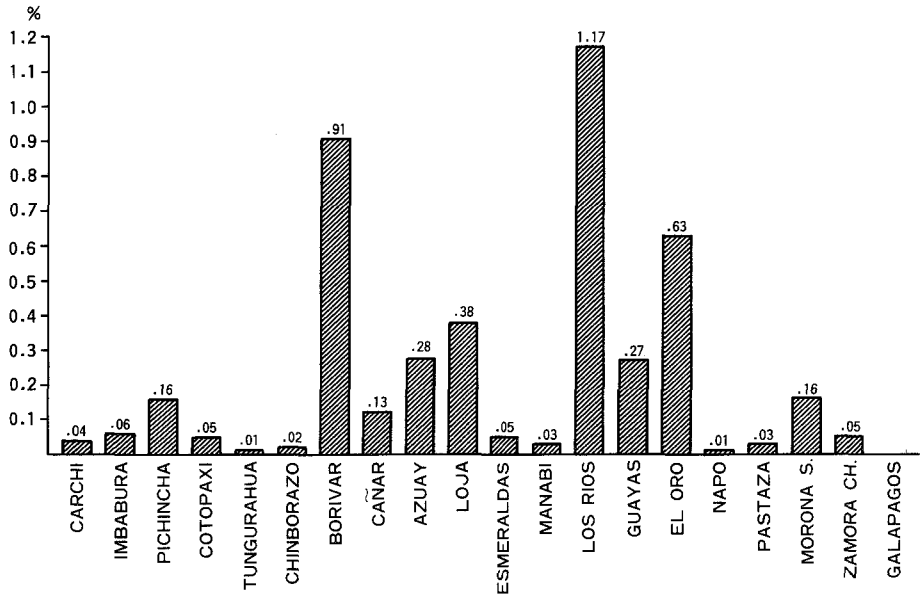

Figure 6.3.1. Prevalence rates of leprosy arranged by provinces (departments) of Ecuador; % x 1,000 habitants (modified from the results compiled by Jurado *et al.*, unpublished dada).

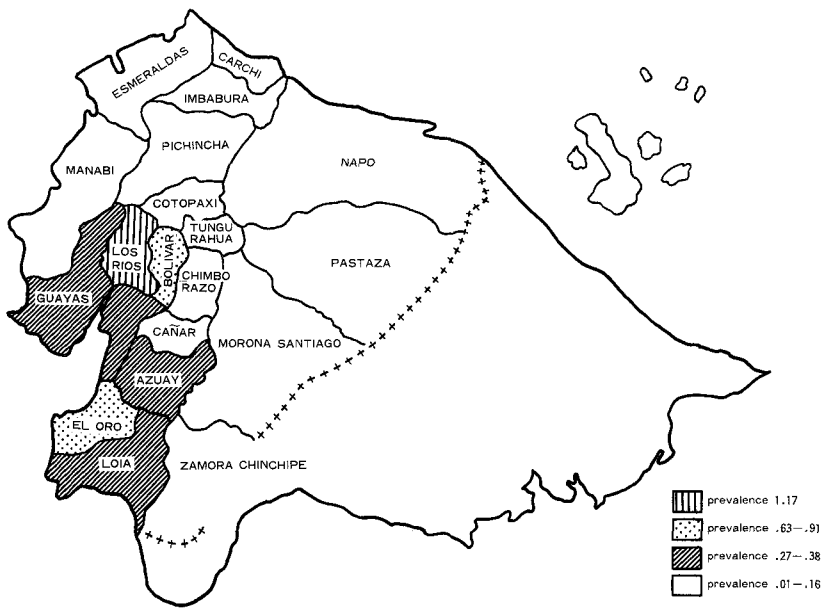

Figure 6.3.2. Geographical distribution of prevalence rates of leprosy in Ecuador; % x 1,000 habitants (modified from the results compiled by Jurado *et al.*, unpublished data).

Table 6.3.3. Correlation between anti-PGL-I (IgG) and anti-PGLI (IgM) antibodies of subjects from different areas of Ecuador

Departments and cities or villages	No. and (%) of subjects in each category				
	PGL(IgG)+ PGL(IgM)+	PGL(IgG)+ PGL(IgM)-	PGL(IgG)- PGL(IgM)+	PGL(IgG)- PGL(IgM)-	Total
1. Bolivar	0(0.0)	1(6.7)	2(13.3)	12(80.0)	15
Echeandia	0	1	1	7	9
San Francisco	0	0	1	5	6
2. El Oro	0(0.0)	2(12.5)	1(6.3)	13(81.2)	16
Antepara	0	1	0	0	1
Machala	0	0	0	2	2
Piñas	0	0	1	4	5
Portovelo	0	1	0	4	5
Zaruma	0	0	0	2	2
Others	0	0	0	1	1
3. Guayas	4(2.0)	8(4.1)	49(24.7)	137(69.2)	198
Pedro Carbo	3	6	41	115	165
Guayaquil	0	0	2	13	15
Olon	1	1	0	2	4
Others	0	1	6	6	13
4. Pichincha	2(11.1)	2(11.1)	6(33.3)	8(44.5)	18
Puerto Quito	2	1	6	8	17
Quito	0	1	0	0	1
5. Manabi	4(26.7)	1(6.6)	7(46.7)	3(20.0)	15
Los Ranchos	4	0	7	2	13
Portoviejo	0	0	0	1	1
Guayabales	0	1	0	0	1
6. Esmeraldas	12(12.3)	11(11.2)	41(41.8)	34(34.7)	98
Selva Alegre	12	11	41	34	98
7. Other areas	0	0	1	4	5
Total (%)	22(6.0)	25(6.9)	107(29.3)	211(57.8)	365

Table 6.3.4. Distribution of anti-LAM-B antibodies in the subjects, negative for anti-PGL-I antibodies, from different areas of Ecuador

Departments	No. and (%) of subjects in each category				
	LAM(IgG)+ LAM(IgM)+	LAM(IgG)+ LAM(IgM)-	LAM(IgG)- LAM(IgM)+	LAM(IgG)- LAM(IgM)-	Total
1. Bolivar	1(8.3)	1(8.3)	6(50.0)	4(33.4)	12
Echeandia	0	1	3	3	7
San Francisco	1	0	3	1	5
2. El Oro	1(7.7)	2(15.4)	4(30.8)	6(46.1)	13
Antepara	0	0	0	0	0
Machala	0	0	1	1	2
Piñas	1	1	0	3	5
Portovelo	0	1	3	0	4
Zaruma	0	0	0	1	1
Others	0	0	0	1	1
3. Guayas	8(5.9)	9(6.6)	65(47.8)	54(39.7)	136
Pedro Carbo	7	8	58	42	115
Guayaquil	0	1	4	8	13
Olon	0	0	2	0	2
Others	1	0	1	4	6
4. Pichincha	3(37.5)	2(25.0)	0(0.0)	3(37.5)	8
Puerto Quito	3	2	0	3	8
Quito	0	0	0	0	0
5. Manabi	0(0.0)	0(0.0)	1(33.3)	2(66.7)	3
Los Ranchos	0	0	1	1	2
Portoviejo	0	0	0	1	1
Guayabales	0	0	0	0	0
6. Esmeraldas	3(8.8)	2(5.9)	20(58.8)	9(26.5)	34
Selva Alegre	3	2	20	9	34
7. Others	0	0	0	2(100.0)	2
Total (%)	16(7.7)	16(7.7)	96(46.1)	80(38.5)	208

Table 6.3.5. Leishmanin skin test and Mitsuda early reaction in three leprosy families in an endemic area of cutaneous leishmaniasis, Los Ranchos, Department of Manabi, Ecuador, in 1992

No. (File no.)	Age	Sex	Symptoms	Type of leprosy
1 (G-1)*	12	F	hypopigmented freckle with anesthesia	indeterminate leprosy suspected
2 (G-2)	10	F	none	
3 (G-3)	9	F	none	
4 (G-4)	4	F	none	
5 (G-5)	11	F	none	
6 (G-6)	38	F	none	
7 (G-7)**	41	M	annular and/or infiltrated erythemata, nodules with anesthesia	borderline-lepromatous
8 (G-8)	8	M	none	
9 (G-9)	15	M	none	not leprosy
10(G-10)**	31	M	neuralgia, eruption with anesthesia, general fatigue	borderline-tuberculoid
11(G-11)**	6	M	none	not leprosy
12(G-12)	3	M	none	
13(G-13)	56	M	neuralgia, anesthesia, general fatigue	leprosy***

*, Mitsuda early reaction is undetermined with 5.7 x 7.5mm (+/-); **, leishmanin skin test positives; ***, leprosy type is unknown.

one patient (*G-1*) showed undetermined (+/-) Mitsuda early reaction. Seven other subjects proved to be Mitsuda negative. Leprosy patients (*G-7*, G-10 and *G-13*) except *G-1* showed positive reaction to leishmanin skin test, while 10 subjects showed negative reaction to the test (Table 6.3.5). As shown in Table 6.3.6, a subject, *G-7*, showed all positive reaction against PGL-IgG and -IgM, and LAM-IgG and -IgM. He was a borderline lepromatous leprosy patient who still had active symptoms, such as infiltrated erythema, ulcerative lesions of feet and neuralgia on the four extremities. The patient was treated with multi-drug therapy (MDT). Other two leprosy patients, *G-10* and *G-13*, were positive for PGL-IgM, and LAM-IgG and -IgM. The symptoms of these two leprosy patient were stable. Although *G-10* patient with borderline tuberculoid leprosy had relatively deep ulcers on both soles, active symptoms, such as erythema, hypertrophy of peripheral nerves and neuralgia, were not observed. The type of leprosy in *G-13* was unknown and the subject had no specific clinical findings of leprosy, and thought to be a spontaneously healed subject. As shown in Table 6.3.6, all the leprosy patients were LAM-IgM-positive, and the subjects, *G-1*, *G-7* and *G-10* except *G-13*, were strongly positive for LAM-IgM, show-

Table 6.3.6. Values (OD unit) of anti-PGL-I and anti-LAM-B antibodies of leprosy patients and their families in an area endemic for cutaneous leishmaniasis, Los Ranchos, Department of Manabi, Ecuador, in 1992

File no.	PGL-I		LAM-B	
	IgG	IgM	IgG	IgM
G-1	0.177 (+)	0.804 (+)	0.06 (-)	0.191 (+)
G-2	0.018 (-)	0.461 (+)	0.151 (-)	0.101 (+)
G-3	0.075 (-)	1.535 (+)	0.042 (-)	0.281 (+)
G-4	0.012 (-)	0.766 (+)	0.388 (+)	0.102 (+)
G-5	0.101 (+)	0.899 (+)	0.031 (-)	0.150 (+)
G-6	0.064 (-)	0.587 (+)	0.087 (-)	0.082 (+)
G-7	0.089 (+)	1.693 (+)	0.545 (+)	0.281 (+)
G-8	0.003 (-)	0.994 (+)	0.285 (+)	0.069 (+)
G-9	0.029 (-)	0.096 (-)	0.110 (-)	0.073 (+)
G-10	0.033 (-)	0.951 (+)	0.753 (+)	0.202 (+)
G-11	0.026 (-)	0.332 (-)	0.042 (-)	0.023 (-)
G-12	0.931 (+)	0.554 (+)	0.053 (-)	0.035 (-)
G-13	0.090 (-)	0.444 (+)	0.771 (+)	0.059 (+)

ing more than 0.190 OD units. Correlation between anti-PGL-I (IgG) and anti-PGL-I (IgM) antibodies in the patients with leprosy and their families was summarized in Table 6.3.7. The subjects, *G-1*, G-5, *G-7* and G-12, were positive for PGL-IgG and -IgM. On the other hand, G-9 and G-11 showed negative reaction for PGL-IgG and -IgM. There were no subjects with PGL-IgG-positive and PGL-IgM-negative reactions. The subjects, G-2, G-3, G-4, G-6, G-8, *G-10* and *G-13*, were PGL-IgG-negative and PGL-IgM-positive (Table 6.3.7). Correlation between anti-PGL-I (IgM) antibody and anti-LAM-B (IgG) antibodies in the patients and their families was summarized in Table 6.3.8. The subjects, G-4, *G-7*, G-8, *G-10* and *G-13*, were positive for PGL-IgM and LAM-IgG. Three patients with leprosy were included in this group. The subjects, *G-1*, G-2, G-3, G-5 and G-6, were PGL-IgM-positive and LAM-IgG-negative. There were no subjects who were PGL-IgM-negative and LAM-IgG-positive. The subjects, G-9 and G-11, were negative for PGL-IgM and LAM-IgG, while G-12 showed PGL-positive and LAM-negative (Table 6.3.6). The details of the clinical features of the two leprosy patients, G-7 and G-1, are described in Chapter 6.4.

Comments

Leprosy, as well as cutaneous leishmaniasis, has a wide range distribution in the world. Both diseases are included among the six most important infectious diseases which the World Health Organization (WHO) planned to stop from being an endemic. The most recent authoritative estimate of total number of leprosy cases in the world is between 10 to 12 million, with at least 3,737,375 cases registered by WHO in 1991 (Noordeen *et al.*, 1991). Also, estimated number of total patients with leishmaniasis in the world is almost the same with that of leprosy.

Table 6.3.7. Correlation between anti-PGL-I* (IgG) and anti-PGL-I (IgM) antibodies in the leprosy patients and their household contacts in Los Ranchos, Department of Manabi, Ecuador, in 1992

Category	PGL(IgG)+ PGL(IgM)+	PGL(IgG)+ PGL(IgM)-	PGL(IgG)- PGL(IgM)+	PGL(IgG)- PGL(IgM)-
File no.**	*G-1*,G-5 *G-7*,G-12	none	G-2,G-3,G-4 G-6,G-8,*G-10* *G-13*	G-9,G-11
No. of leprosy	2	0	2	0
Total	4	0	7	2

*, PGL-I: phenolic glycolipid-I; **, italics show leprosy patient.

Table 6.3.8. Correlation between anti-PGL-I* (IgM) and anti-LAM-B** (IgG) antibodies in leprosy patients and their household contacts in Los Ranchos, Department of Manabi, Ecuador, in 1992

Category	PGL(IgG)+ LAM(IgG)+	PGL(IgG)+ LAM(IgM)-	PGL(IgG)- LAM(IgM)+	PGL(IgG)- LAM(IgM)-
File no.***	G-4,*G-7*,G-8 *G-10,G-13*	*G-1*,G-2,G-3 G-5,G-6.G-12	none	G-9,G-11
No. of leprosy	3	1	0	1
Total	5	6	0	2

*, PGL-I: phenolic glycolipid-I; **, LAM-B: lipo-arabino-mannan-B; ***, italics show leprosy patients.

Most of the leprosy patients (76%) have been found in Asia where there are over populated districts. On the other hand, leprosy in the countries of Central and South America has not been paid much attention, when compared to Asia. This may be due to the reason that the total numbers of patients in American countries are relatively small.

In 1991, 110 subjects were newly diagnosed as leprosy in Ecuador (Jurado *et al.*, unpublished data). The prevalence rates of leprosy in different Ecuadorian provinces (departments) are shown in Tables 6.3.2. In some regions of Ecuador, the prevalence rates are very high. For example, the rate in Department of Los Rios showed 1.17 per 1,000 habitants. Persons living in the regions, where the prevalence rate is over 1.0 per 1,000, would be exposed to serious danger of infection whether they have or not contact with leprosy patients. The immediate counter plan for chronic infectious disease should be considerd as well as cutaneous leishmani-

asis.

The bacillus of leprosy (*M. leparae*) invades into the peripheral nerve at an early stage of infection. It is speculated that after the chemotherapy the organism continues to survive in the peripheral nerves and causes the relapse of leprosy and prolongs the peripheral nervous disorder. Leprosy can be cured if detected early enough. If the diagnosis is made in early stage, the prognosis is very good; the disease can also be prevented as well as those of cutaneous leishmaniasis. A few cases of leprosy experienced in the outpatient clinic in Okinawa, Japan, were cured in a few months (Hosokawa *et al.*, 1993). Therefore, early diagnosis is of great importance in leprosy as well as prevention of the disease.

Recently, serodiagnosis of leprosy was considered as one of the useful methods for early diagnosis (Buchanan *et al.*, 1983). Although we could not examine large numbers of leprosy patients during the current survey, anti-PGL-I and anti-LAM-B antibodies were examined by using accepted sera from different areas of Ecuador. The seropositive rates of anti-PGL-I antibodies in Ecuador was high, showing a rate of 42.2%. Anti-PGL-I and anti-LAM-B antibody positive subjects would need detailed medical examination to rule out the disease by acid-fast bacteria. A total of 154 subjects (42.2%) were serologicaly suspected of suffering from *M. leprae* and 128 subjects (35.1%) were suspected of diseases caused by acid-fast bacteria including *M. leprae*. In the examination, tuberculosis, tuberculosis cutis and its related diseases, such as lupus vulgaris, tuberculosis verrucosa cutis, scrofuloderma, erythema induratum Bazin and infectious diseases of atypical mycobacteria, should be taken into consideration.

Izumi *et al.* (1993a, b) reported the distribution of anti-LAM-B antibodies in non-leprous sera in Japan and South Sulwasi, Indonesia. According to their studies, the numbers of anti-LAM-B(IgG) antibody positive non-leprous subjects in Japan and Indonesia were 18 (4.9%) out of 367 and 20 (12.4%) out of 161, respectively. In comparison, the positive rate of anti-LAM-B antibodies in Ecuador (32/127, 15.6%) was relatively high. From these results, it was suspected that there might be large numbers of inhabitants infected subclinically by the bacillus, and some of them have the possibility of showing symptoms of the disease.

In the present study, no correlation between the positive rates of antibodies (anti-PGL-I and anti-LAM-B antibodies) and the prevalence rate of leprosy was observed in each area of Ecuador. In Manabi, Ecuador, the prevalence rate of leprosy was 0.10-0.16 per 1,000 inhabitants, showing a relatively low rate. But the anti-PGL-I-seropositive rates of the patients and their family in Los Ranchos, Manabi (11/13, 84.6%) were higher than the average positive rate (154/365, 42.2%) of all subject in several areas of Ecuador. From the data shown in Table 6.3.7, it was considered that PGL-IgG and -IgM positive cases were bacteriologically and immunologically active patients.

As shown in Table 6.3.8, three leprosy patients were positive for PGL-IgM and LAM-IgG. It was considered that the combination of positive PGL-IgM and LAM-IgG might be useful for the serological diagnosis of leprosy. The value of anti-LAM-B (IgM) antibody was thought to be unreliable, because of the low cut-off value (>0.19 OD unit). But a strong positivity of LAM-IgM might be an indicator for the diagnosis of leprosy, because leprosy patients (*G-1, G-7* and *G-10*), except *G-13*, were strongly positive for LAM-IgM. As the subjects (G-5 and G-12) were positive for PGL-IgG and -IgM, they should be watched for the development of the disease symptoms, though no clinical findings of leprosy were observed in the present examination.

In the current examination, two leprosy patients, *G-7* and *G-10*, were seemed to be necessary to have medical treatment to prevent the magnification and secondary bacterial infection of ulcers on both feet. Another patient, *G-1*, with indeterminate leprosy should be treated with DDS and Rifampicin®. Furthermore, the relatives living in the village who had not yet been examined, should have a medical attention. From the results of examinations of leprosy families in Los Ranchos, Manabi, the usefulness of serodiagnosis was evaluated in the current

study. A further wide range of screening of leprosy families should be urgently planned and carried out in Ecuador.

Izumi *et al.* (1993a, b) reported that 30% - 35% of inhabitants living in endemic areas of leprosy in Japan were infected by the bacteria, and the rate of infection was high in young people. They, therefore, suggested that dermatological and serological health screening in an endemic area for leprosy should be done mainly for young people, such as kindergarten and school age children. In Ecuador, such a screening should also be done in surveys of other tropical infectious diseases including cutaneous leishmaniasis. With regard to personnels to support these activities, well-experienced paramedical staffs and volunteers probably would find out leprosy patients. If these personnels would be arranged effectively in a public health screening program, it might prove to be useful system in Ecuador. As to diagnostic tools of leprosy, in near future polymerase chain reaction (PCR) method using skin slit smear materials might be added to one of the useful, simple and easy-handling screening tests of the disease (Sugita *et al.*, 1993). In such a case, smear materials would be easily taken by the personnels mentioned above, and be brought to a laboratory for PCR tests.

In the current study, one of the purposes was to make a differential diagnosis about the lesions found in the patients with cutaneous leishmaniasis and leprosy. Jopling (1984) reported that the disseminated anergic form of cutaneous leishmaniasis, of which nodules were numerous and simulate those of lepromatous leprosy, was most likely to be confused with lepromatous leprosy. In the survey, the differential diagnosis was done by examining sensory function. Most of the cutaneous manifestatons in leprosy, such as size, form, site of lesions and distribution of eruptions including ulcers, were different from those of cutaneous leishmaniasis. Correlation of skin tests between cutaneous leishmaniasis and leprosy was partly found; three out of four leprosy patients showed positive reaction to *Leishmania* promastigotes antigen. From this result, it would be speculated that the specific defect of cell-mediated immunity for *M. leprae* might be covered by other activated cellular immunity.

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4. Case Reports of Leprosy from an Area Endemic for Cutaneous Leishmaniasis in Ecuador

ABSTRACT. Four cases of patients with leprosy were seen in an area endemic for cutaneous leishmaniasis, Los Ranchos, Department of Manabi, Ecuador. Two cases of leprosy (borderline lepromatous leprosy and indeterminate one) in a single family were reported. A nine banded armadillo kept by the family was examined, but no acid-fast bacillus was observed in the liver materials. Screenings of family members of leprosy patients were also discussed briefly.

Introduction

Cutaneous manifestations in leprosy sometimes showed a similarity to those of cutaneous leishmaniasis, and histopathologically, both diseases had a chronic infectious granuloma (Jopling, 1984). Therefore, both diseases should be restrictively differentiated in the endemic areas. During a survey for cutaneous leishmaniasis in Ecuador, leprosy patients were also examined. The current paper mainly deals with these two cases in detail. The patients gave informed consent to participate to the present examination.

Case Reports

Case 1 (G-7): a 41 years old male.

Family history. His grand father, an uncle, an aunt, a nephew, a cousin, and a daughter were reported as leprosy by interview to the family (Fig. 6.4.1).

Present history. About 15 years ago, eruptions appeared on the abdomen, but after a while, the eruptions spontaneously disappeared. However, similar eruptions reappeared on the face, the trunk and the lower extremities. At the same time, there was a steady rise in temperature, and neuralgia appeared in both extremities. In 1982, a doctor from the Welfare Ministry visited the patient, and he was diagnosed as leprosy after several examinations.

Treatment and process. A 100mg per day of DDS was prescribed regularly for the patient. The medication was continued for two years and then stopped. About eight years later, a doctor from the Welfare Ministry came to the village again, and started the medication of multi-drug therapy (MDT) for 14 months. His prescription was as follows: DDS (100mg/day), every day; B-663 (50mg/day), every day; Rifampicin® (600mg/day), once a month; B-663 (300mg/day), once a month. This prescription was according to MDT for multi-bacillary leprosy recommended by World Health Organization (Noordeen, 1992). The patient was a farmer. He worked usually outside the house. About six years ago, deep ulcers on both soles and both palm appeared and the symptoms gradually increased.

Present illness. Infiltrated erythemata with sensory loss were observed on the face (Fig. 6.4.2A). Nasal septum was destroyed and deformity of the nose was observed (Fig. 6.4.2B). Pea sized nodule with sensory loss was recognized on the earlobes (Fig. 6.4.2C). Annular erythemata and hypopigmented spots with sensory loss (anesthesia) were scattered on the trunk (Fig. 6.4.2D). The body surface was dry except for the axillary region, the epigastric region, the lower abdomen, the inguinal region and the anal region. The ichthyosis-like eruptions were seen on the region of the waist, the back, the extensor aspect of the legs and the dorsal aspect of the feet. There were deep ulcers on the soles of the feet (Fig. 6.4.3A). The big toes on the right and left feet were mutilated and the remaining toes on both feet were shortened (Fig. 6.4.3B and C).

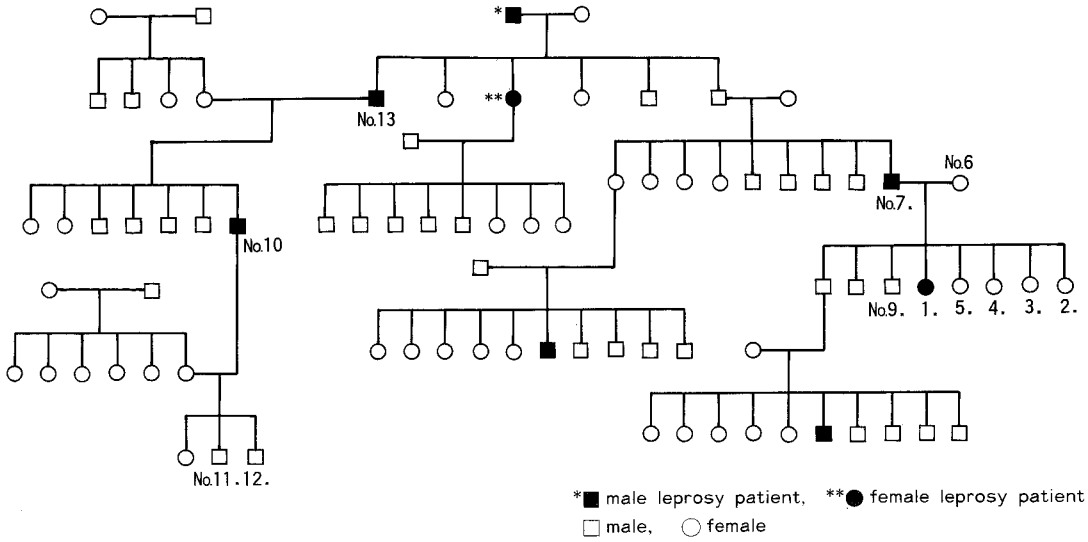

Figure 6.4.1. The pedigree of leprosy patients (Cases 1 and 2) reported in the text.

Bilateral ape-hands and claw-hands were seen (Fig. 6.4.3D). On the palm of the right hand, fissures caused by trauma were marked. There was no loss of hair at any region of the body. Hypertrophy of the ulnar nerves were palpated at the elbows.

Laboratory examination.

1) The lepromin test: negative.

2) Value of anti-phenolic glycolipid (PGL)-I and anti-lipo-arabino-mannan (LAM)-B antibody: PGL-I (IgG), 0.089 OD unit (positive); PGL-I (IgM), 1.693 OD unit (positive), LAM-B (IgG), 0.545 OD unit (positive), LAM-B (IgM), 0.281 OD unit (positive).

3) Examination of sensory function: Anesthesia was observed all over the body except for the scalp, the axillary region, the epigastric region, the inguinal and the anal region.

4) Histological findings of the specimen taken from the infiltrated erythema on the cheek: Rete ridge disappeared. Clear sub-epidermal zone was observed (Fig. 6.4.4A). Dermis was generally edematous. Although a relatively large number of epithelioid cells and lymphocytes infiltrated in the dermis, epithelioid cell granuloma was not observed. By acid-fast staining (Fite's staining), bacilli of *Mycrobacterium leprae* were observed (Fig. 6.4.4B and C). Biopsy index showed 3+; SFG index, 5; and SFG value, 1-2-1. By skin slit smear of the left earlobe, the bacilli of *M. leprae* stained by Ziehl-Neelsen's staining were found (Fig. 6.4.4D). Biopsy index showed 3+; SFG index, 4; and SFG value, 1-2-2. Nasal smear was negative for the bacilli. The patient was already diagnosed as borderline lepromatous leprosy.

Case 2 (G-1): a 12 years old female.

Family history. Father (Case 1) is borderline lepromatous leprosy as shown in Fig. 6.4.1, No. 7.

Present history. When we examined the family of the Case 1 (G-7) patient with leprosy, hypopigmented freckle on the extensor aspect of the left thigh of his daughter was noticed. The patient had

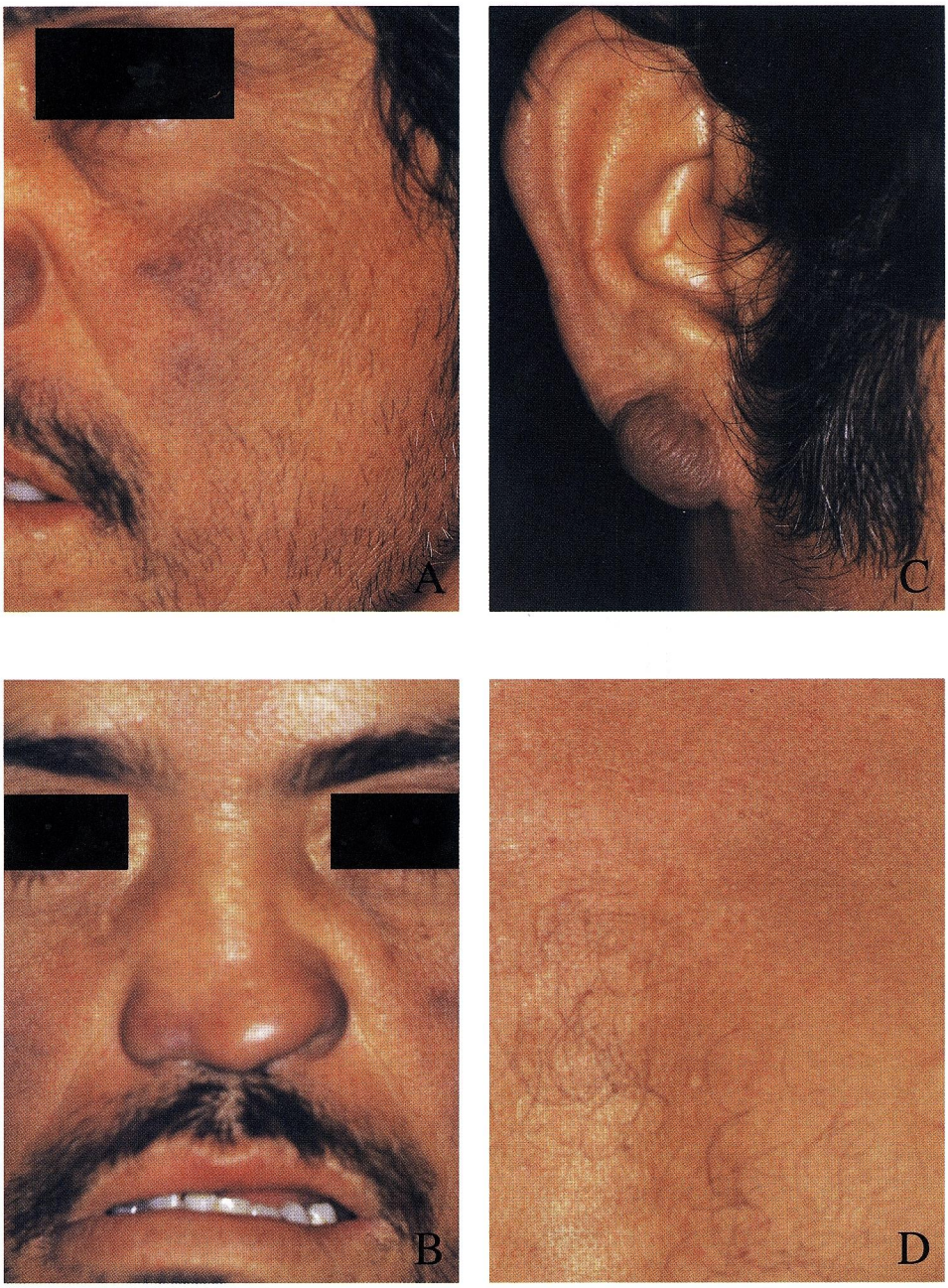

Figure 6.4.2. **A,** Infiltrated erythema with sensory loss on the face of Case 1 (G-7: 41 years old male); **B,** Deformity of the nose because of destruction of nasal septum; **C,** Pea sized nodule with sensory loss on the earlobe; **D,** Annular erythemata and hypopigmented spots on the trunk.

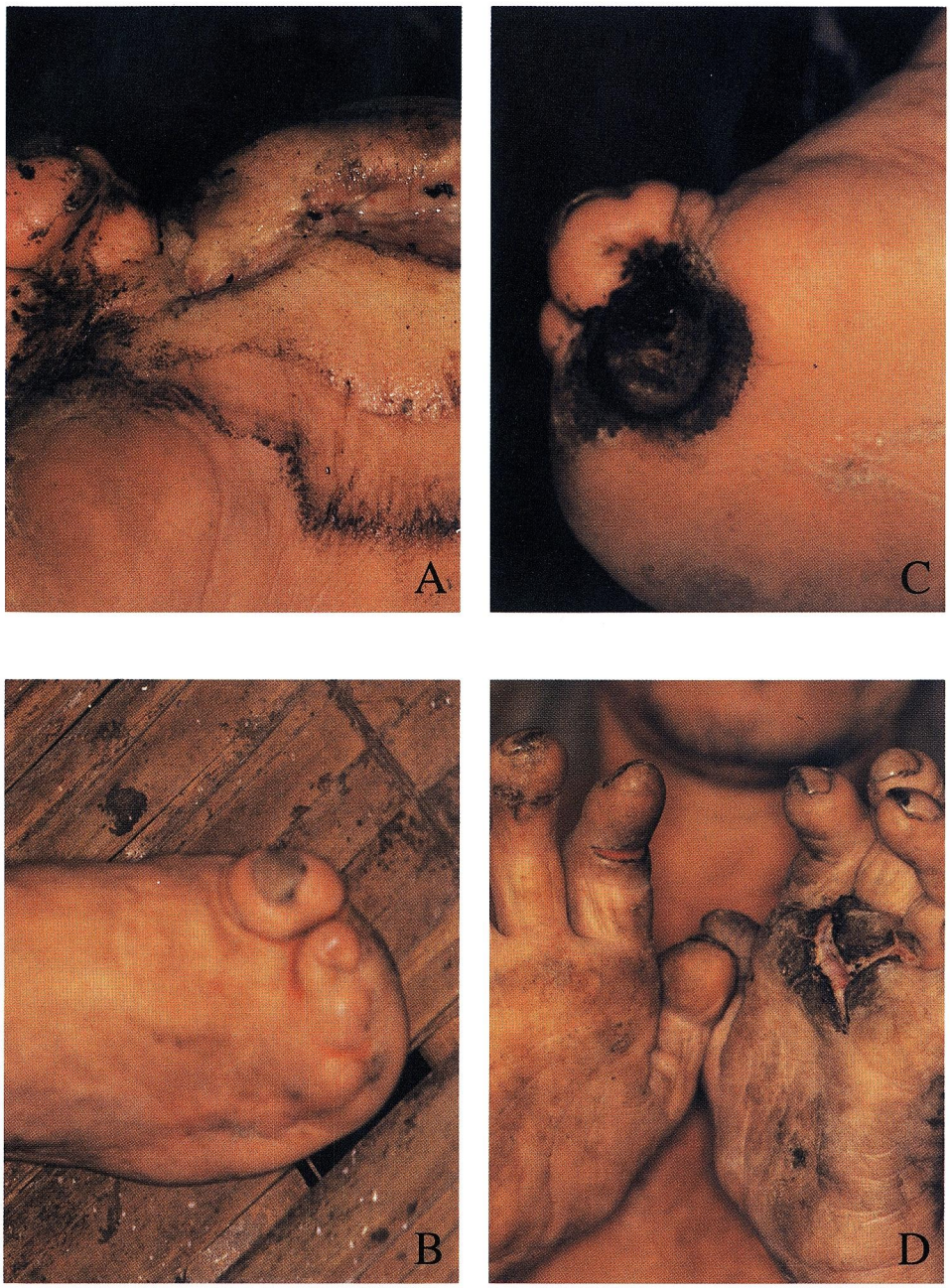

Figure 6.4.3. **A,** Deep ulcer on the sole of the patient (Case 1); **B,** His shortened toes; **C,** His mutilated toes on the foot; **D,** His ape-hand and claw-hand of both hands.

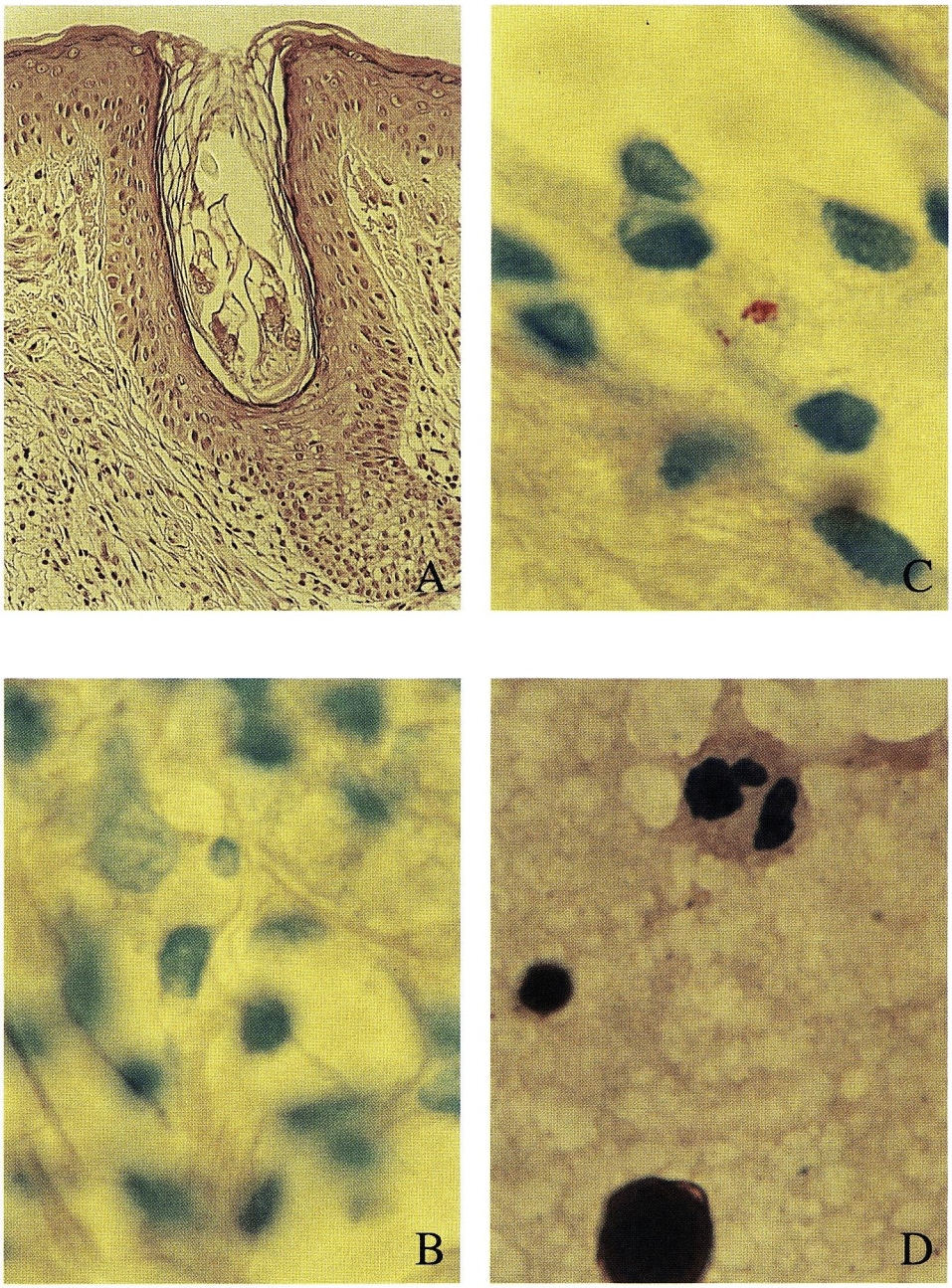

Figure 6.4.4. **A,** Clear subepidermal zones are observed, but no epithelioid cell granuloma is observed (HE, x100); **B** and **C,** The bacilli, *Mycobacterium leprae*, are observed (Fite's staining, x1,000); **D,** The skin smear was positive and the bacilli are observed (Ziehl-Neelsen's staining, x1,000).

been living in the house with her father in the house at the village of Los Ranchos, Depatment of Manabi since her birth.

Present illness. Palm sized hypopigmented freckle with anesthesia was observed on the extensor aspect of the left thigh (Fig. 6.4.5A,B). Hair loss was not observed at any region of the body surface. Hypertrophy of peripheral nerve was not palpated.

Laboratory examination.

1) Mitsuda early reaction (48 hrs.): 7.5mm x 7.5mm / 7.5mm x 7.5mm, undeterminable (+/-).

2) Value of anti-PGL-I antibody: IgG, 0.117 OD unit (positive); IgM, 0.804 OD unit (positive). Value of anti-LAM-B antibody: IgG, 0.060 OD unit (negative), IgM, 0.191 OD unit (positive).

3) Examination of sensory function: Sensory loss was observed only at the lesion site of the left thigh.

4) Histological findings of the specimen taken from the lesion of the left thigh: Small number of lymphocytes infiltrated around the capillaries and the appendages. There was no epithelioid cell granuloma in the dermis. No bacillus of *M. leprae* was observed in the skin tissue section stained by acid-fast staining. The patient was diagnosed as indeterminate leprosy according to the histological and clinical criteria of Redley and Jopling (1966).

Comments

In the present examination of leprosy in the village of Los Ranchos (Fig. 6.4.5C and D), no cutaneous manifestations similar to those of leishmaniasis were observed. Namely, the pea sized reddish nodule on the earlobes of the leprosy patient (Case 1), was soft and the induration of the lesions was not palpated. Although the deep ulcer on the sole of the patient had the sharply demarcated bank, it did not have the induration at the margin of the ulcer that was observed in cutaneous leishmaniasis. One of the most important factors for the differential diagnosis of leprosy was that there was anesthesia at the ulcer on the sole and the patient (farmer) could walk and work without pain. The hypopigmented freckle on the thigh of the indeterminate leprosy patient (Case 2), was not a cutaneous manifestation of cutaneous leishmaniasis. In the patient of Case 1, disorders of the peripheral nerves were not observed on the scalp, the axillary region, the epigastric region, the inguinal and anal region where the temperature of the body surface were relatively high. Similar symptoms were seen in patients with leprosy at an outpatient clinic in Okinawa, Japan (Hosokawa *et al.*, 1992). This phenomenon might clinically demonstrate the nature of *M. leprae*; the organisms multipied well in a relatively low temperature (Kosaka *et al.*, 1978).

In Ecuador, 110 patients with leprosy were newly diagnosed in 1991; the prevalence rate of leprosy in Ecuador was comparatively low (0.25 per 1,000 habitants in total), but the rate was high in some areas (Jurado *et al.*, unpublished data; see Chapter 6.3). For example, the prevalence rate was over 1.0 (1.17 per 1,000) in Department of Los Rios, while it was relatively low (0.10-0.16 per 1,000) in Department of Manabi, from where the present patients were reported.

Besides the two cases mentioned above, other two patients with leprosy (one with borderline tuberculoid and the other with unknown type) were examined in the current study. Furthermore, one indeterminated group of leprosy patient (child) was newly diagnosed in the area. In addition to these cases, the existence of other four patients with leprosy in this village was determined. Based on these examination and the interview, the figure of the pedigree was depicted, including relationships among leprosy patients in the endemic area of Los Ranchos, Department of Manabi, Ecuador (Fig. 6.4.1).

As to future group leprosy examinations, especially in a low endemic area, such as Department of Manabi, the screenings of leprosy family would be effective and useful in consideration of a program for epidemiological counterplan of the early diagnosis. In Okinawa, Japan, for example, group leprosy examinations have been done in almost all regions of the prefecture during about 30 years by special-

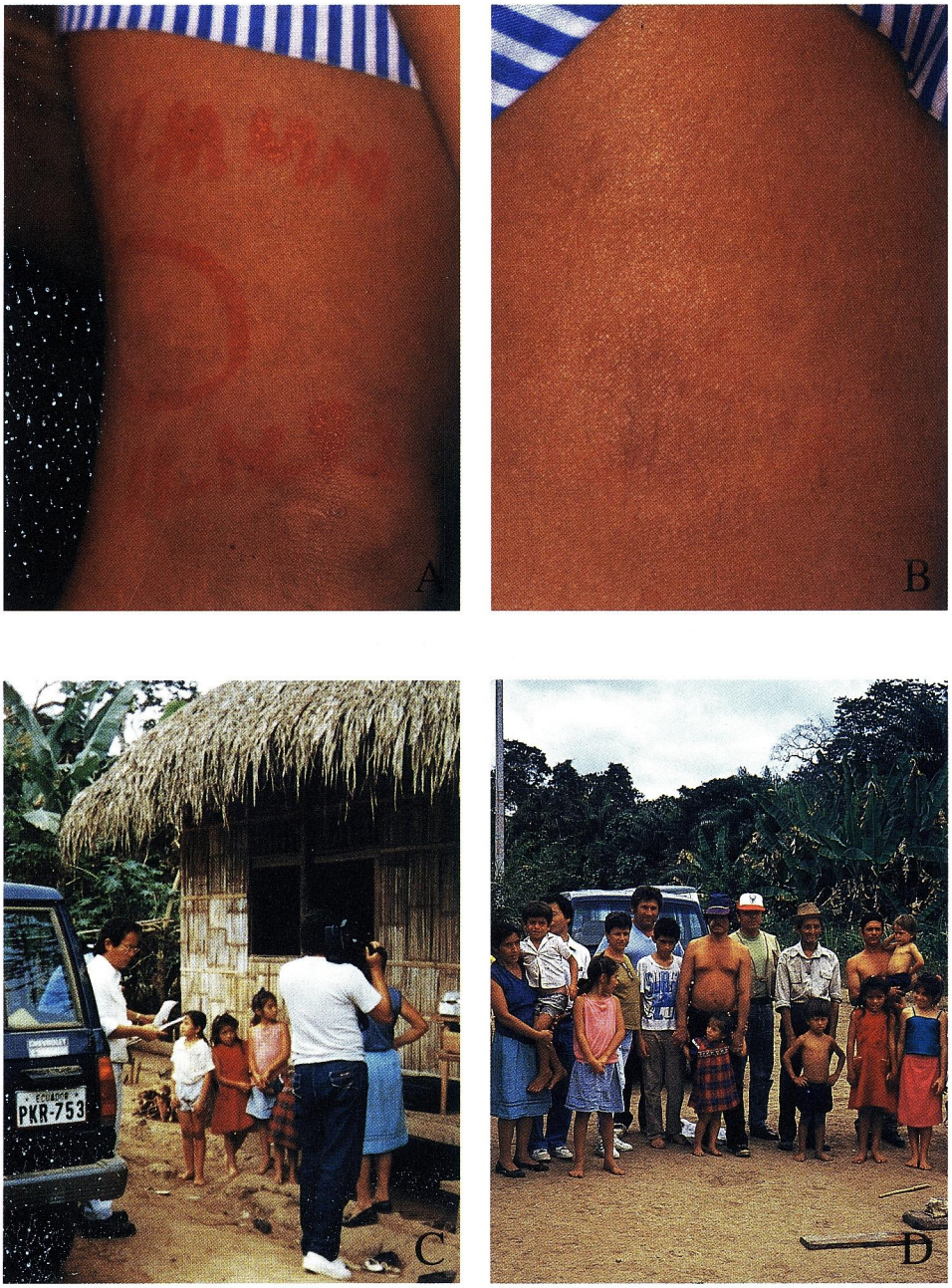

Figure 6.4.5. **A**, The lesion of indeterminate leprosy is marked with red line; **B**, Palm sized hypopigmented freckle with sensory loss; **C**, Showing, our research activities at the house of the present patients (Cases 1 and 2); **D**, Showing, the family members of Case 1 patient and the relatives; a part of them were depicted in Fig. 6.4.1.

ists who were appointed by the prefectural governor (Saikawa, 1989). In the case, members of the group examinations consisted of a leprologist, public health nurses from the public health center of each district, managers of leprosy and tuberculosis from the prefectural office, a dermatologist, an internist and a clinical technologist etc. The examination was called as a general medical check-up and not as a mass examination for leprosy, because in Okinawa, many of the inhabitants have been prejudiced against patients with leprosy. In the group examination, therefore, various examinations were done to find skin diseases, infectious diseases including tuberculosis, circulatory diseases such as hypertension, diabetes mellitus and other internal disorders. Dermatologist could prescribe medicine (ointments) for eczema, contact dematitis and dermatomycosis such as tinea pedis and pityriasis versicolor. The data from the medical check-up were proved to each subject by the public health center. If some pathological result was observed, detailed examination was done at the nearest hospital or public health center. Such a system of group leprosy examinations in Okinawa might be a good guide for a future program for the early diagnosis in Ecuaor. In condideration of economical and man-power resources, some modifications would be needed to implement this system of group leprosy examinations into Ecuador.

The patients (Cases 1 and 2) kept a nine banded armadillo for their food. In smear specimens of the liver, no acid-fast bacilli and *Leishmania* amastigotes were observed. It was reported that wild armadillos with leprosy were found in U.S. (Smith *et al.*, 1978; Marchiondo *et al.*, 1980). Although no such a report was described in Central or South America, the examination should be continued to ascertain whether leprosy was a zoonosis or not, and whether the nine banded armadillos were natural reservoirs of the pathogen in Ecuador.

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

ABSTRACT. In this study, fungous flora on the ulcer of the cutaneous leishmaniasis were examined, and the effect of fungi on the formation of the ulcers was investigated. In the current study from July to September 1992 in Guayabales and San Sebastian (Km 103; Ciento Tres), Department of Manabi, Ecuador, only 10 colonies of fungi were cultured from nine lesions of ulcerative cutaneous leishmaniasis. In the colonies, *Aspergillus* spp., *Penicillium* spp., *Mucor* spp., *Cladosporium* sp., and *Candida* sp., were identified by slide cultures. But it was difficult to determine whether these fungi would have a role causing the lesions, especially ulcerative lesions, because these fungi were frequently cultured as contaminants, and they would generally not produce such lesions as nodules, plaques or ulcers except in case of compromized host. In future study, histopathological approach and much more numbers of samples should be examined.

Introduction

According to Nonaka *et al.* (1990), clinical findings of the cutaneous leishmaniasis on the Pacific coast lowland areas were different from those observed in Andean highland areas of Ecuador. Namely, on the Pacific coast lowland, most of the lesions had a tendency to make large sized ulcer with sharp demarcated bank. On the other hand, in the Andean highland, the lesions were reddish nodule with small crust. Secondary fungous infections were suspected as to be one of the causes of the different clinical manifestations between lowland and highland leishmaniasis ulcers, in addition to the ecological factors, such as humidity, temperature, fungal and bacterial fauna, human behaviors etc. In order to obtain more information on the secondary infections of leishmaniasis patients, the current study was performed in hot and humid forest of the endemic areas on the Pacific coast lowland of Ecuador.

Materials and Methods

Samples were taken from the patients with cutaneous leishmaniasis patients on the Pacific coast lowland areas, San Sebastian and Guayabales, Department of Manabi, Ecuador. In this study, the samples were scratched from the cleaned lesions with cotton wool moistened with alcohol, using a flammed sterilized scalpel. And the samples were inoculated on the Sabouraud's dextrose medium with antibiotics at room temperature. The culture medium was putted into small sized glass tubes (20mm in diameter and 70mm in length). When a sufficient growth of fungi was observed, they were identified by slide cultures. The inoculated fungi on slide glass were stained by the solution of lactophenol cotton blue and then observed under the microscope.

Results and Comments

From nine lesions of cutaneous leishmaniasis, 10 colonies of fungus were isolated and identified; they were three strains of *Aspergillus* spp. (Fig. 6.5.1A and B), two strains of *Penicillium* spp., two strains of *Mucor* spp., one strain of *Cladosporium* sp., one strain of *Candida* sp., and one unknown strain of fungus.

In Ecuador, a similar study was carried out in

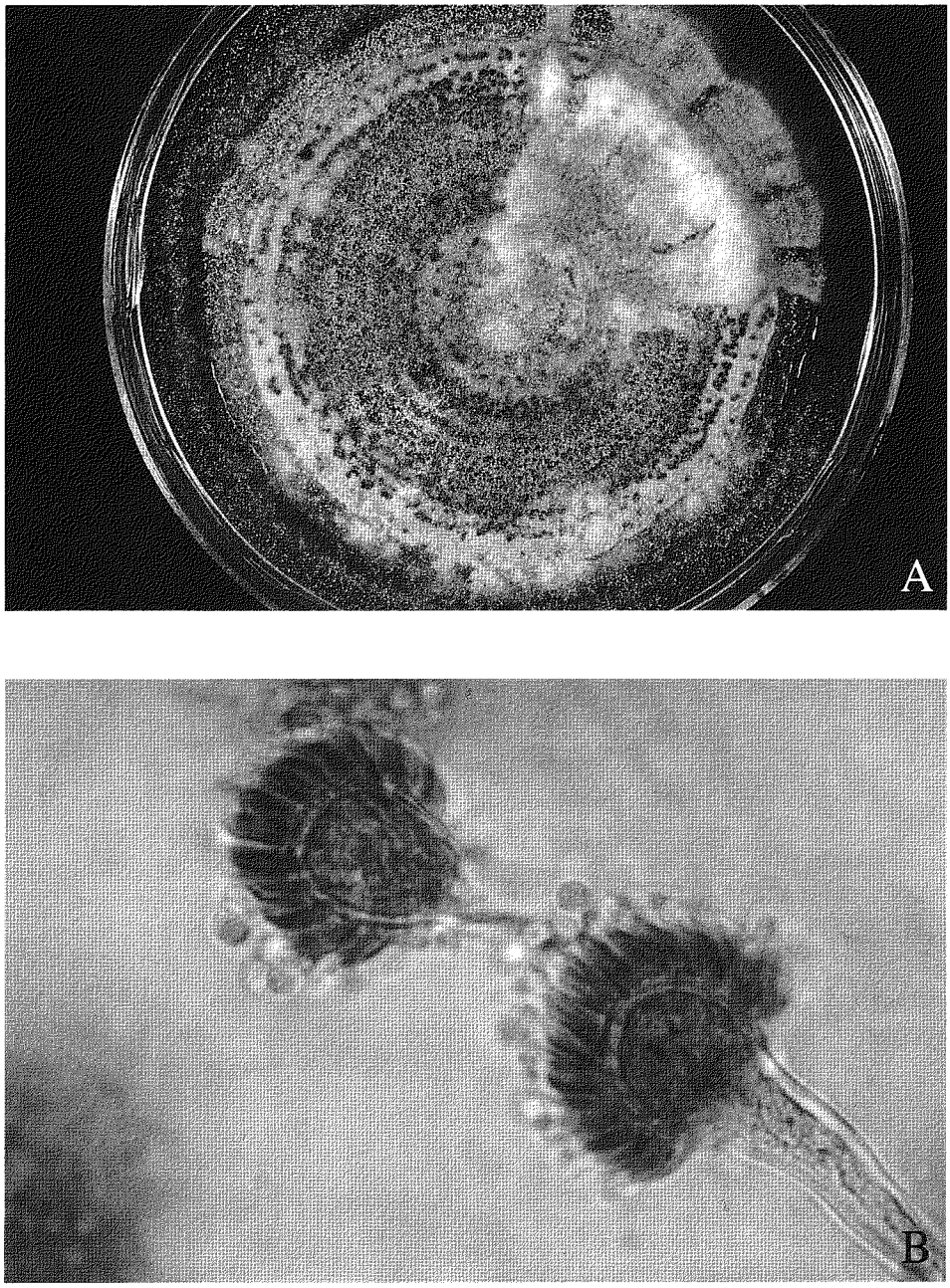

Figure 6.5.1. **A**, Colony of *Aspergillus* spp. cultured on Sabouraud's dextrose agar; **B**, The colony was identified as *Aspergillus* by slide cultures.

endemic areas of cutaneous leishmaniasis (Nishimoto *et al.*, 1992). In the current survey, almost the same result was obtained. It seemed to be rare for the isolated fungi to produce ulcers except in severe immunosuppresive hosts. *Sporothrix schenckii* and *Nocardia* spp. were known to make ulcers in human skin (Gavin *et al.*, 1964; Kasai *et al.*, 1982). *Aspergillus* spp. were rarely shown to produce plaque type lesions as cutaneous aspergillosis (Lever, 1990). In most of the Sabouraud's dextrose agar plates used, bacterial clonies were observed, demonstrating their common existence in most ulcerative lesions as well as fungi. In future study, an application of the antimycotic agent should be tried to find a clue for the influence of fungi to the ulcerative lesions. Histopathological mechanisms of the formation of ulcers by these organisms are still obscure. Therefore, more detailed histological examinations would be done. It was speculated that capillary embolism might be caused by large numbers of the micro-organisms including *Leishmania*. On the other hand, in the endemic area of leishmaniasis, the inhabitants did not have general nor basic information about a public health. And inadequate treatment of the lesions might be a cause of the formation of ulcerative lesions and the magnification of ulcers.

With regard to culture tubes, the glass bottle used in the current survey was very handy and easy to carry around the field. But the slanting surface of medium was too small. So, it would be very difficult to separate from each other, when two or more numbers of colonies of fungi appeared. And the size of the glass tube was too small to be sterilized enough by flame at the time of inoculations.

It might be difficult to diagnose cutaneous leishmaniasis only by the clinical findings without the experience of the disease, because the leishmanial eruption showed uncommon clinical findings. Other skin diseases, such as skin cancer, pyoderma gangrenosum, status dermatitis, erythema induratum bazin, polyarteritis nodosa and others, shoud be ruled out before the definitive diagnosis. The influence of secondary infections with fungi and bacterias on the formation of leishmanial lesions would be fully investigated; such a study would also be very important for the management of leishmaniasis treatment. Evolutions of ulcerative lesions by *Leishmania* parasites and other related factors should be investigated further.

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Summary

The present issue was mainly designed to compile the results of the field works carried out during the period from 1992 to 1993 at different areas endemic for leishmaniasis in Ecuador. Using materials collected in the field, furthermore, laboratory investigations were made in Ecuador and Japan, and the data were also mentioned in this text. The results obtained are summarized as follows.

Molecular biological and immunological findings

DNA karyotype of 12 *Leishmania* isolates, from three different areas of the Ecuadorian Andes, was examined by pulsed field agarose gel electrophoresis. A marked karyotype similarity was observed in all the isolates examined. Chromosomal DNA banding pattern of these isolates was characterized by an ordered chromosomal ladder, by the presence of four low molecular weight chromosomes of 220, 250, 280 and 325 kilobases. The results obtained suggested that *L.* (*Leishmania*) *mexicana* strain with a defined karyotype is widely distributed and a major agent of cutaneous leishmaniasis in the Ecuadorian Andes regions.

Monoclonal antibodies were raised against promastigotes of newly described *L.* (*Viannia*) *equatorensis*. Fusions of immunized spleen cells of BALB/c mice with P3-X63-Ag8,6.5.3. myeloma cells resulted in the production of six monoclonal antibodies (MAbs) against the parasite. Among these, five MAbs 9F4, 7H6, 3A7, 8C1 and 1G5 were found to be species-specific for *L.* (*V.*) *equatorensis*. By indirect immunofluorescent antibody test, MAbs 9F4, 7H6 and 7A6 appeared to bind the surface and cytoplasm of promastigotes of the parasite, while MAbs 3A7 and 1G5 bound only to flagellum. On Western blot analysis, MAb 3A7 recognized set of bands ranging from 110 to 170 kDa, MAb 1G5, however, recognized a different set of molecules ranging from 200 to 250 kDa.

Experimental findings using the Ecuadorian Leishmania *isolates*

In order to make a search for some factors relating to different disease forms caused by *Leishmania* strains or species, histopathological and ultrastructural comparisons were made. For this purpose, hamsters were infected with promastigotes of *L.* (*L.*) *mexicana* isolated from patients with two different types of clinical forms, *viz.*, diffuse cutaneous (DCL) and localized cutaneous (LCL) leishmaniasis. However, no clear difference was found between the two animal groups infected with DCL and LCL strains of the parasite, except the following points. In the nose and footpad sections of hamsters, a large number of neutrophils were observed in animals infected with DCL strains, while histiocytes and lymphocytes were dominant in those infected with LCL strains. In ultrathin sections amastigotes were located in the dermis extracellularly and intracellularly. Degeneration of parasites was observed inside the macrophages in animals infected with LCL strains only. No morphological difference was observed light- and ultra-microscopically in amastigotes of the parasites from animals infected with DCL and LCL strains.

In order to check lot variation of Glucantime® used in Ecuador, *in vitro* anti-promastigote activity was examined, by using three manufacturing lots of meglumine antimonate. A minimum twice difference in the activity was detected among the lots tested. Effective concentration of the drug which inhibited promastigote proliferation by 50% (EC_{50}) varied with different *Leishmania* species, and EC_{50} values of the most effective lot were in a range of 20-38 mg/ml Glucantime® or 5.7-10.8 mg/ml antimony.

Vector entomological findings

Biological features of several man-biting sandfly species were examined in two areas endemic for leishmaniasis, the Andean slope (site I) and the

Pacific coast (site II). In site I, the data obtained in 1991/1993 were compared with those in 1983; a marked difference was recognized in species composition of sandflies and natural infections with *Leishmania*, between the two study periods. In study site II, six man-biting species were collected in the primary and secondary forest. Among these, some were also captured inside the house, suggesting a possibility of the role of vectors of leishmaniasis in the area. In this study site, however, a total of 2,530 flies were dissected, no natural infections with the parasite was found to date. Parity of sandflies, *Lutzomyia* spp., was examined at different endemic areas of leishmaniasis. Some of *Lu. gomezi* showed the developmental stage II or III of follicles without any blood meals, suggesting an existence of autogeny individuals. To know a susceptibility of sandflies against fenitrothion (Sumithion), a preliminary study was conducted. Based on the results obtained, residual sprays of the insecticide were briefly discussed from the view point of reducing biting chance of endophilic sandflies, especially in Andean leishmaniasis-endemic areas of Ecuador. A bibliographical review was also made briefly on the application of insecticides for the control of endophilic sandflies.

Seroepidemiological findings

To evaluate enzyme-linked immunosorbent assay (ELISA) as a diagnostic method in leishmaniasis-endemic areas of Ecuador, 95 sera of the patients were examined. Based on clinical manifestations, these sera were divided four groups and subjected to ELISA; the antigens were prepared from promastigotes of *L. (V.) panamensis* and *L. (V.) guyanensis*. From the results obtained, it was found that the ELISA used could be very useful for both the diagnosis and the evaluation of treatment in endemic areas of the disease in Ecuador. In order to know endemy of leishmaniasis in domestic dogs as a reservoir host of human leishmaniasis in the country, a serological survey was performed. Thirty-seven sera from the Pacific lowland (Palm Junta) and the Andean highland (Alausi) were examined by ELISA, using two *Leishmania* antigens mentioned above. Although positive rate of dogs in Alausi was higher than in Palm Junta, the average OD value of positives was higher in the latter; older dogs showed higher positive rates.

A further epidemiological study of Andean leishmaniasis in Ecuador was carried out, especially in Huigra (1,200m-1,500m above sea level), Department of Chimborazo. The results obtained were compared to those in Alausi (2,300m-2,500m a.s.l.), Department of Chimborazo and Paute (2,300m-2,500m a.s.l.), Department of Azuay. The disease forms in these foci were found to be similar to each other. It was suggested, however, that in Huigra the ecological features, including vector and reservoir biology, were quite different from other endemic areas.

Clinical findings of leishmaniasis in Ecuador

In the text, a typical case of parasitologically confirmed diffuse cutaneous leishmaniasis was reported for the first time in the country. The patient was anergy to *Leishmania* antigen but not for other antigens, such as PPD and BCG, and was refractory against chemotherapy by Glucantime®. The parasite isolated was identified as *L. (L.) mexicana* by zymodeme and karyodme analyses. A rare case of generalized cutaneous leishmaniasis with 308 crusty ulcers was also reported. The clinical picture of this patient showed some controversies, showing herpes zoster, and resulted in a difficult diagnosis. However, microscopical examinations of lesions revealed abundant *Leishmania* amastigotes. Finally, it was concluded that coexistence of herpes zoster might have partly supported dissemination of lesions all over the body surface, though the infection by multiple biting of infected sandflies could not still be ruled out. A preliminary trial of chemotherapy using an anticancer drug, fluorouracil (5FU), was made against localized cutaneous leishmaniasis. From the trial, it was considered that 2% 5FU ointment would be useful for a relatively small sized, shallow ulcerative lesion, but not so effective against non-ulcerative lesions; no patients revealed burning sensation and other side effects by 2% 5FU

ointment applications.

Related skin diseases

Chronic verruga nodules taken from an Ecuadorian patient with bartonellosis was examined electron-microscopically; the disease was known to be transmitted by sandflies, *Lutzomyia* spp., vectors of leishmaniasis. Large numbers of organisms were found in different stages of the life cycle in the stroma. Furthermore, these organisms were regularly seen either in close contact or existing inside the cytoplasm of neutrophils, suggesting the phagocytic role of these cells against the organisms. No organism was found inside the endothelial cells or histiocytes. Skin diseases found in endemic areas of cutaneous leishmaniasis in Ecuador were investigated whether there would exist any skin disease and cutaneous changes that might need to make differential diagnosis for leishmaniasis. No marked difference on the incidence of cutaneous changes was found between rural and urban areas of the country. In an area endemic for cutaneous leishmaniasis, seven leprosy patients were found. The disease should be considered as a possibility of misdiagnosis and therefore properly examined. For leprosy, a serological survey was also performed using sera collected during surveys for leishmaniasis and other parasitic diseases. No correlation between prevalence rates and seropositive rates was observed in the current study. Two cases of leprosy, a borderline lepromatous leprosy and an indeterminate one, in a single family were reported in detail, with their pedigree. Fungi from patients were also examined, in relation to the evolution of leishmaniasis lesions.

Miscellanea

A Hymenolepid Parasite of Sandflies

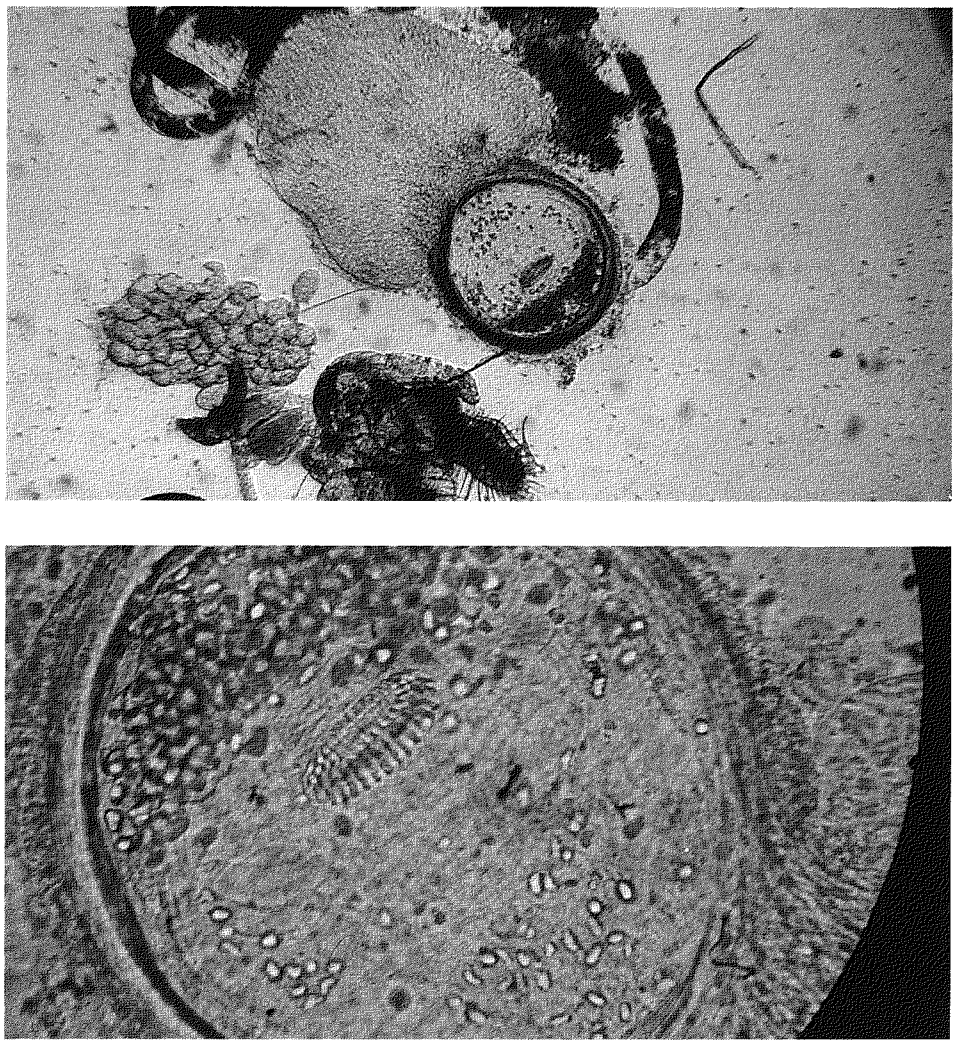

Plate 3. Cestode, a hymenolepid cysticercoid parasitic in *Lutzomyia gomezi* adult female. % incidence: 1 (0.13%) out of 768 sandflies; locality: Km 101 (Km Ciento Uno), Department of Manabi, Ecuador. **Above**, a cysticercoid was found with female genital organs and Malpighian tubules of the host. **Below**, large magnification of the cysticercoid, showing typical hooks at the center and calcareous bodies.

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. Infección 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 areas endemicas del Ecuador, las primeras fases de la investigación se canalizaron hacia las busqueda de las especies de flebotominós que estarian 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 manifestaciónes clinicas de la enfermedad en los pacientes, y sobre los aspectos taxonomicos y ecologicos de los insectos sospechosos de ser los vectores de la endemia. Sin embargo no se han hecho intentos para determinar definitivamente al vector o vectores principales de la enfermedad, mediante el hallazgo de la infeccion 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 mas importante o prioritario sera siempre conocer a los principales vectores en cada area endemica.

En el presente trabajo, usando cebos humanos, los flebotomus capturados fueron el nucleo de nuestra atención, desde Julio a Octubre de 1983, en siete diferentes sitios del area endemica de leishmaniasis escogida por nosotros, la zona de Ocaña, Provincia del Cañar. Solo encontramos dos especies antropofilicas del genero *Lutzomyia*, en esta area de estudio; ellas fueron identificadas como *Lu. trapidoi*, y *Lu. hartmanni*, basandonos en las caracteristicas morfologicas de su espermateca y armadura cibarial. Un total de 1,452 flebotominos de ambas especies capturadas, fueron sistematicamente disecados y examinados en busqueda 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 *Le. braziliensis*, de acuerdo a su aspecto morfologico y comportamiento en el vector, especialmente su ubicación en el tubo digestivo del huesped invertebrado.

Al examinar los ejemplares recolectados a diferentes alturas sobre el nivel del mar, 350m, 600m, 950m, 1,200m y 1,500m, *Lu. trapidoi* resulto ser la especie predominante en los sitios mas bajos, mientras que *Lu. hartmanni* lo fue en los lugares mas altos. De todos estos puntos, encontramos flebotomus naturalmente infectados con promastigotes de *Leishmania*, hasta los 1,200m de altura. La transmisión de la enfermedad, por tanto, se extiende hasta esta altitud, en el area de estudio. Ambas, *Lu. trapidoi* y *Lu. hartmanni*, visitaron al cebo humano durante toda la noche, para alimentarse. La mayoria 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 encontrandose ninguno positivo a partir de esa hora. Este hecho es atribuible al desarrollo del ciclo gonotropico, es decir flebotomus paridas y nuliparas, deduciendo que las paridas concurren a picar temprano. Por otra parte este fenomeno no pudo observarse en Lu. *hartmanni*, a los 600m, ya que a dicho nivel la captura del mismo fue escasa.

Asi, el resultado de este trabajo de investigación ha sido el descubrimiento de la infección natural con promastigotes del complejo, *Le. braziliensis*, en especies de *Lutzomyia* ecuatorianas, pro vez primera, lo que nos ha permitido automaticamente 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 pais, todo lo cual debera confirmarse minuciosamente antes del veredicto definitivo, como parte del largo camino que nuestro grupo debera aun recorrer revelando uno a uno los extraños secretos que la naturaleza guarda todavia sobre los complejos mecanismos de transmisión de las artropozoonosis, y entre ellas, la leishmaniasis tegmentaria 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.

Japanese 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 proyecto y desarrollo este trabajo, encaminado a la cria de phlebotomus en el laboratorio para trabajos de experimentación. Se capturo un buen numero de "progenitoras silvestres", y en frascos adecuadamente preparados con yeso humedo, se las traslado al laboratorio conjuntamente con machos de la misma especie escogida (*Lu. trapidoi*), para encerrarlos en una camara especial para la alimentación y copula. Las hembras gravidas fueron conservadas en frascos igualmente acondicionados hasta la oviposición, quedando luego los huevos depositados en los mismos recipientes, y guardados en camara humeda durante el tiempo de realización de la metamorfosis completa. A partir de 50 hembras gravidas obtuvimos 1,022 huevos, 706 larvas, 510 pupas y 498 adultos, quedando despues 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 Medicine 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 300m to 1,500m 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 500m, 1,000m, 1,300m and 1,500m 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, 500-1,000m and 1,300-1,500m ($0.01<p<0.05$, $x^2 = 5.314$), but not between 500m and 1,000m and between 1,300m and 1,500m. 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 reservoirs, 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 low-land 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. 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 eradicated 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 Serva 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 Investigaciónes 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, basandose en gran parte en la bibliografia publicada entre 1920 -el año en que se describio el primer caso humano- y 1989. La enfermedad es endemica en 14 de los 20 departamentos del pais. De 260 casos notificados, 239 (91.9%) eran de la forma cutanea, y 18 (6.9%), de la mucocutanea. Durante los 67 años transcurridos de 1920 a 1987, solo se registro un caso de la forma visceral y otro de la cutanea difusa. Tambien se analizan los conocimientos actuales sobre los vectores y los huespedes reservorios. En la acutualidad, se estan 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 (2%) of 97 *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, Psychodydae, 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 parasite. 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 were 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 10,000g 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 x 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 26kD, 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 Limoy

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 socio-economical and socio-medical 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 (2), 1992, 221-228

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 equatorensis* 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 (1), 1992, 123-130

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 nm (±0.17 S.D.) and mean width of 2.18 nm (±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 (2), 1992, 175-180

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.

Kochi, Japan: Kyowa Printing, Research Report Series,
No. 3, 1992, 1-182

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

Yoshihisa Hashiguchi (ed.)

ABSTRACT. The current text dealts 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 (1,300m above sea level) and Alausi (2,300 - 2,500m a.s.l.), Department of Chimborazo. In the areas school children, 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.

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.

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.

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 Sebastian (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.

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 was 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.

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 Sebastian (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

Findings on the Paraguayan leishmaniasis

A study was performed of the epidemiology of leishmaniasis in a newly established community in south-eastern Paraguay (Limoy, 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 sand flies, *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 indistiguishable 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.

Nishi Nihon Hihuka,
55 (4), 1993, 638-642

31. The Successful Treatment of 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 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 an insect-bite-like eruption on his left arm. The eruption had, gradually gotten worse despite of therapy. The patient visited our hospital on April 23rd, 1991. A huge amount of amastigote-like leishmaniae were 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 the upper dermis. We successfully cultivated *Leishmania* parasites isolated from the skin lesion which were identified as *Leishmania major* by a zymodeme analysis. Initially, an external remedy consisting of meglumine antimonate and povidone iodine was used, but was not effective. Therefore, an intralesional injection of meglumine antimonate was done. After 10 times injections, the ulcer and erythema eventually healed leaving only a pigmentation. The side-effects were limited to some localized pain following injection. Thus, intralesional injections with meglumine antimonate proved to be highly effective against the ulcerative lesion, while demonstrating no serious side effects.
(in Japanese with English summary)

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32. Molecular Karyotype Characterization of *Leishmania panamensis*, *Leishmania mexicana*, and *Leishmania major*-like Parasites: Agents of Cutaneous Leishmaniasis in Ecuador

Ken Katakura, Yoshitsugu Matsumoto, Eduardo A. Gomez L., Masato Furuya and Yoshihisa Hashiguchi

ABSTRACT. Molecular karyotypes of *Leishmania* isolates from patients with cutaneous leishmaniasis in Ecuador were analyzed by pulsed-field gel electrophoresis (PFGE) and Southern blot hybridization. The DNA karyotypes of *L. major*-like parasites were similar between two human isolates from a lowland coastal and a highland Andean region, but were apparently different from those of eleven World Health Organization reference strains including *L. major*. The smallest chromosome of 240 kilobases in *L. major*-like parasites was found to belong to the 715-class of small linear chromosomal DNAs, which have been shown to appear in some lines of *Leishmania*. Chromosome banding patterns of *L. mexicana* isolates exhibited a novel, ordered, chromosomal ladder, and were identical among four human isolates and one canine isolate from a restricted geographic region in the Andes. On the other hand, minor chromosome size polymorphisms were observed among three *L. panamensis* isolates from different endemic regions near the Pacific Coast. Chromosomal locations of dihydrofolate reductase-thymidylate synthetase and P-glycoprotein genes revealed further differences in chromosomal organizations among these *Leishmania* species in Ecuador. These results indicate that karyotype analysis by PFGE is useful for epidemiologic studies of leishmaniasis in Ecuador.

Nishi Nihon Hihuka,
1994, submitted

33. Dermatological and Parasitological Examinations of Leishmaniasis in Ecuador

Shigeo Nonak, Atsushi Hosokawa and Yoshihisa Hashiguchi

ABSTRACT. Dermatological and parasitological examinations were performed in four study sites of Ecuador, three leishmaniasis-endemic areas and an out-patient facility of the National Institute of Health. Cutaneous changes of each case due to leishmaniasis are described and compared with those described in the literature. A total of 18 leishmaniasis-positive cases were thoroughly examined in the current study. The most common manifestations in the cases examined were ulcers. These ulcers were clearly demarcated with an indurated periphery and a wet base. However, the line of demarcation was clearly recognized in the cutaneous manifestations of Ecuadorian leishmaniasis. It was noticed that the cutaneous manifestations in highland patients were mild compared to those in lowland patients. The small lesion with dry crust in the highland resembled the primary lesion (eschar) seen in tsutsugamushi disease. The longest duration of the eruption in our cases was 10 years seen in one case of lowland patients. However, almost all the cases healed within one year. Lymphnode swelling was frequently seen. The swelling was easy to palpate on the upper extremities, and it was asymptomatic. The histological findings in our cases coincided with the granulomatous phase.

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34. Leishmaniasis in an Endemic Focus on the Pacific Coast of Ecuador

Juan J. Alava P., Ana E. Mora de Coello, Eduardo A. Gomez L. and Yoshihisa Hashiguchi

ABSTRACT. In the current paper, we reviewed 1,296 leishmaniasis cases diagnosed at the outpatient facility of the national laboratory (INHMT-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.

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35. Histopathological and Electron Microscopical Features of Skin Lesions in a Patient with Baltonellosis in Ecuador

Abdul M. Bhutto, Shigeo Nonaka, Eduardo A. Gomez L. and Yoshihisa Hashiguchi

ABSTRACT. Chronic verruga nodules taken from a patient with verruga peruana were studied. Histopathologically, specimens of all the lesions that showed extensive infiltration of various types of cell along with the proliferation of capillaries. The sections were predominantly infiltrated with neotrophils and endothelial cells, while histiocytes, plasma cells, lymphocytes and mast cells were also visible in some extent. The blood vessels were dilated and many endothelial cells were located peripherally that were rounded and swollen, while the huge number of neutrophils was invaded inside the vessels. Electron microscopically, large number of organisms was found and seen under different stages of life cycle in stroma. Furthermore, organisms were regularly seen either close contact or being existed inside the cytoplasm of neutrophils, suggesting the phagocytic role of these cells against organisms. No organism was found inside the endothelial cells and histiocytes.

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3, 1994, 17-32

36. Comparative Observations of Golden Hamsters Infected with *Leishmania (Leishmania) mexicana* from Ecuadorian Patient with Diffuse and Localized Type of Cutaneous Leishmaniasis

Abdul M. Bhutto, Shigeo Nonaka, Masato Furuya and Yoshihisa Hashiguchi

ABSTRACT. In order to search for factors relating to different disease forms caused by *Leishmania* strains or species, histopathological and ultrastructural comparisons were made. For this purpose, hamsters were infected experimentally with promastigotes of *Leishmania (Leishmania) mexicana* (=*L. mexicana mexicana*) strains isolated from patients with two types of clinical forms, diffuse cutaneous (DCL) and localized cutaneous leishmaniasis (LCL). No histopathological and ultrastructural findings providing clear differentiation between DCL and LCL strains were recognized. The experimental animals used were divided into the following two groups. Hamsters in group A were infected with *L. (L.) mexicana*, isolated from a patient with DCL, and the remaining animals in group B were infected with the parasite, *L. (L.) mexicana*, isolated from patients with LCL. Macroscopically, no remarkable difference in the inoculated sites was noticed after the 1st month of promastigote inoculation. After the 2nd and 4th month of inoculation, small and large nodules were observed on the inoculation site of animals in both groups. The large nodules were found relatively more numerous in the animals of group A than those of group B. No cutaneous dissemination and/or metastasis was noted in the animals from both groups. Histopathologically, granulomatous changes were observed in all the microscopical sections of the nose and footpads of hamsters infected experimentally. In the nose and footpad sections, a large number of neutrophils were observed in the animals of group A, while, histiocytes and lymphocytes were dominant in those of group B. In ultrathin sections amastigotes were located in the dermis extracellularly and intracellularly. Degeneration of parasites was observed inside the macrophages in group B sections only. Morphologically, no clear differentiation was found in light- and ultra-microscopical observations between the amastigotes of *L. (L.) mexicana* from the two groups of experimental animals.