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

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Research Report Series No. 5

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edited by

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The present text reports on the data and materials
mainly collected during the period from 1994
to 1996 in Ecuador, South America

There could be some 12 million *Leishmania*-infected people in the world, and 350 million at risk, of whom some 1.5 to 2 million will be infected each year. Some 90% of the visceral leishmaniasis cases are reported from two regions - a wide zone in northeast India, Bangladesh and southern Nepal, and Sudan; and 90% of the cutaneous cases are found in Afganistan, Iran, Saudi Arabia and Syria in the Old World, and Brazil and Peru in the New World. Smouldering epidemics of visceral leishmaniasis in the India /Bangladesh /Nepal focus are thought to be killing 75000 people a year. War and in some areas military training in forests or deserts is associated with the disease. There is also a risk of the urbanization of these principally rural diseases, particularly where AIDS and leishmaniasis overlap. The diversity of the epidemiology of the different forms of the disease makes it impossible to control leishmaniasis with a single approach or tool, except perhaps a vaccine...

(Twelfth Programme Report, UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases -TDR-, 1995, Leishmaniasis, pp. 135-146)

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Plate 2. Examinations of patients with leishmaniasis at Hospital El Carmen, Ministry of Public Health, located at a town, El Carmen, Province of Manabi, Ecuador. **Above:** most of the patients here came to the examination from remote and mountainous areas. **Below:** examination of a patient with ulcer type of CL on his forearm by Drs. E.A.Gomez L., T. Mimori, M. Carvopiña and Mr. R. Sud A.

Plate 3. Field activities of our research team at endemic areas of cutaneous leishmaniasis (CL) in Ecuador. **Above:** An assembly at a primary school of CL-endemic area, La Esmeralda, Province of Los Rios, to give information on our research. **Below:** examination of inhabitants at a primary school, located at a rain forest CL-endemic area, La Mamey, Province of Esmeraldas.

Plate 4. Field activities of our research team in Ecuador. **Above:** dissections of vector sandflies by Drs. E.A.Gomez L., S. Suguri, Y. Hashiguchi and Mr. R. Sud A., at Hotel Majestad, located near CL-endemic area of Andean slope, La Troncal, Province of Cañar. **Below:** our special transportation system of laboratory equipments and others.

Preface

Our research activity on leishmaniasis in Ecuador was started in the year 1982 and continued until 1984, under the support of the Japan International Cooperation Agency (JICA) and the Ministry of Health, Ecuador. After 1986 to date, our project entitled "Studies on New World leishmaniasis and its transmission, with paritcular reference to Ecuador" was financially supported by the Ministry of Education, Science and Culture, Japan; and invaluable supports were obtained from Ecuadorian institutions, such as Catholic University, Guayaquil University and health centers at different endemic areas of the disease. During these long research terms, a considerable information was accumulated, and the details were reported in our Research Report Series Nos. 1-4 published in 1987-1994.

At the beginning of this project, the isolate and characterization of *Leishmania* organisms, the detection of natural infections of vector sandflies and reservoir host mammals, and the evaluation of immunological tools for diagnosis were made principally. In addition, the collection of available information on Ecuadorian leishmaniasis and the revision of leishmaniasis-endemic areas in that country were also carried out.

At the second phase of our research, the results obtained from surveys carried out in different leishmaniasis-endemic areas of Ecuador, were analyzed by multi-disciplinary investigators from parasitological, epidemiological, vector entomological, immunological and dermatological points of view. Particular emphasis was given to a recently discovered autochthonous Andean highland leishmaniasis, and a comparison of this disease form with others in the Pacific coast and Amazonian lowland of Ecuador was done. Moreover, a currently available technique in molecular biology was briefly reviewed and evaluated for the possibility of their application to future studies of leishmaniasis epidemiology in Ecuador.

At the third phase, Andean leishmaniasis was mainly investigated and compared to the lowland leishmaniasis, from parasitological, entomological and clinico-epidemiological points of view, employing light and electron microscopical findings. Local applicattion of ointment and lotion prepared in our project was tried to use for the treatment of cutaneous leishmaniasis patients.

At the fourth phase of the current project, DNA karyotype of 12 *Leishmania* isolates from different areas of the Ecuadorian Andes was examined. Furthermore, monoclonal antibodies were raised against newly described *Leishmania* species, in addition to various laboratory works using Ecuadorian isolates. Many dermatologic and therapeutic data obtained from field research were also accumulated in this research phase.

The present report (Research Report Series No. 5) mentions the results of field and laboratory works done during 1994 and 1996. Special emphasis was given to the oral treatment and its evaluation of cutaneous leishmaniasis patients with antimalarial drugs, mefloquine and artesunate. Much of the materials and data collected have yet to be examined and analyzed. The results will be published in detail elsewhere, and a further investigation will be continued from 1997 onwards, by the financial support of the Ministry of Education, Science and Culture, Japan.

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

Introduction

Leishmaniasis in Ecuador was first reported by Valenzuela in 1920, about 77 years ago; however it has remained one of the least studied of Ecuadorian tropical diseases until recently. For many years the principal research activity of leishmaniasis has been involved with clinical diagnosis and therapy, and this produced some eventually confirmed case reports. For example 260 clinical cases in total were reported in principal Ecuadorian medical journal during 67 years from 1920 to 1987. Only one each of diffuse cutaneous leishmaniasis and visceral one were reported, but no parasitological confirmation was made in both cases. Recently, a case of parasitologically confirmed diffuse cutaneous leishmaniasis was reported by our research group. Thus, in Ecuador the variety of cutaneous forms of leishmaniasis are prevalent at different endemic areas, such as Pacific and Amazonian regions and the Andes, showing about 95% of total prevalence. From 1982 to date, we have studied almost all of the susceptible endemic foci in that country. And it was found that leishmaniasis is widespread in most provinces and it is a considerable health problem there; out of the 21 provinces, 15 are reported as leishmaniasis-endemic areas. Thus, the disease occurs in many populations living in rural areas on both sides of the Andes, including those living in the Andean plateau. However, the actual numbers of leishmaniasis-patients in each endemic area are difficult to establish, because of insufficient medical and/or communicative systems in the area. During our epidemiological studies from 1982 to date in Ecuador, we made different types of field works in order to decide the causative agents, probable vectors and reservoir hosts of the disease in each endemic area, in addition to clinical and therapeutic studies. In sandfly collections, for example, we used several types of methods, such as Shannon trap, CDC light trap, Casteroil trap and protected human-bait collections, depend on the purpose of research. To date 70 species were recorded from different areas of Ecuador, of which only 4 were incriminated as probable vectors in Ecuador, isolating *Leishmania* parasites by fly dissection. At the lowland endemic areas of leishmaniasis, *Lutzomyia trapidoi* is the most important vector in that country, and then followed by *Lutzomyia hartmanni*, and *Lutzomyia gomezi*. At the highland endemic areas only one species, *Lutzomyia ayacuchensis* was proved to be positive for *Leishmania* organisms. As to reservoir hosts of leishmaniasis in Ecuador, 8 species of mammals were incriminated to date; they are 2 species each of sloths and squirrels, 1 species each of anteater, rat, kinkajous and canine, showing positive for *Leishmania* parasites in cultures of the viscera of these mammals. In Ecuador, 7 species of the genus *Leishmania* were isolated from patients, sandfly vectors or mammalian reservoirs in Ecuador. In the Pacific lowland areas including Andean slopes, *braziliensis*, *panamensis* and *guyanensis* of the subgenus *Viannia,* and *mexicana*, *amazonensis* and *major*-like belonging to the subgenus *Leishmania* were found. In the Andean plateau of 1200 m to 2700 m of the altitudes, 2 species, *mexicana* and *major*-like, were prevalent, and only 1 species, *braziliensis* was isolated from patients of different areas of the Ecuadorian Amazon. The remaining species, *equatorensis* was described as a new species of the subgenus *Viannia* group by our research group. This organism was isolated from the viscera of a sloth and a squirrel caught in humid tropical forest of Ecuador on the Pacific cordillera of about 1000 m of the altitudes.

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Chapter 1

A Brief Comment on the Changing Pattern of Leishmaniasis in the World

Leishmaniases are caused by a protozoan parasite of the genus *Leishmania*, and transmitted by a tiny blood feeding insect, sandfly, the genus *Phlebotomus* in the Old World and the genus *Lutzomyia* in the New World. About 20 species of *Leishmania* are responsible for a wide range of clinical manifestations in both humans and vertebrate animals as zoonosis. In humans clinical forms range from a simple, often self healing cutaneous type to those producing destructive mucocutaneous ulcers of nasopharynges, uncurative diffuse cutaneous lesions, and a visceral form known as kala-azar, the severe chronic infection of the reticuloendothelial system, which is often fatal if left untreated. Different types of the disease are endemic in many tropical and subtropical regions, and responsible for increasing health problems in large parts of the Old and New World, especially in developing countries. According to Ashford *et al*. (1992), the number of countries affected by visceral and cutaneous forms of leishmaniasis is 67 in total, of which 16 countries are distributed in Asia, 22 in Africa, 21 in Americas and 8 in Europe. In these affected countries, about 3 hundreds and 67 millions of people are estimated as a population at risk. In this estimation, it is noticeable that the number at risk with both visceral and cutaneous forms is extremely high in Asia, showing 2 hundreds and 71 millions of people. Futhermore, Ashford *et al*. (1992) estimated that in the world about 3 hundreds and 84 thouthands of people are annually sufferring from the disease; the highest number of annual cases of both visceral and cutaneous leishmaniasis is found in Asia, and then followed by Africa. Many serious problems, especially with visceral forms of leishmaniasis were reported in different countries of the world. For example, in India drug resistance is now a severe problem causing high death rates (Dhanda *et al*., 1996). In Sudan, the war zone is probably now the area with the highest incidence of the disease in the world (Ziljstra *et al*., 1991; Kemp, 1997); Lockwood (1991) noticed that visceral leishmaniasis (kala-azar) in the Sudan should be in the news.

In the New World, leishmaniases are also endemic in many areas of Central and South American countries, including southern parts of the US and northern to southern Mexico, and they constitute significant public health problems in each country. Control of leishmaniases in these New World regions is complicated by the variety of different *Leishmania* species and their diverse clinical forms, and also complicated by a unique epidemiological patterns of each *Leishmania* species. Furthermore, in many regions of the New World, two or more species are often sympatric. In the New World 18 species of the genus *Leishmania* are reported, and 15 of which are responsible for different types of human leishmaniasis cases (Grimaldi and Tesh, 1989). *Leishmania* species belonging to the subgenus *Viannia*, are shown as the causative agents of cutaneous and mucocutaneous leishmaniasis. Among the *Viannia* group, *Leishmania* (*Viannia*) *braziliensis* shows the most wide range of distribution, affecting many populations and causing cutaneous and mucocutaneous leishmaniasis. In the New World endemic areas of leishmaniasis, almost all forms of the disease are basically zoonoses; and man is usually an incidental host of the pathogenic *Leishmania* species. Cutaneous forms of leishmaniasis associated with the subgenus *Leishmania* group are also widely prevalent in the New World. *L.* (*L.*) *mexicana* is mainly reported from the Central American countries and from a part of the northern regions of the South America. The species, *mexicana*,

amazonensis and *pifanoi* are important as causative agents of diffuse type of cutaneous leishmaniasis; the clinical form is specifically anergy for *Leishmania* antigen, and is highly resistant for available antileishmanial drugs. In the New World *L.* (*L.*) *chagasi* is main species which causes the visceral leishmaniasis with a wide range of distribution from Mexico to Brazil. In the case of New World visceral forms, some records are done based on clinical or serological examinations without confirmation of the parasites on smears or cultures. For example, in our research field of Ecuador as mentioned before, 3 years old boy was reported as visceral leishmaniasis without parasitological confirmation; from our epidemiological survey in that country, however, we are sure that the visceral case reported would be misdiagnosed.

Recently, leishmaniases, especially its visceral forms are identified and noticed as one of the important opportunistic infections of acquired immunodeficiency symdrome (AIDS) in several areas of the world where both diseases distribute sympatrically. Since the mid-1980s there has been a dramatic increase in the number of *Leishmania* infections in human immunodeficiency virus (HIV) positive patients concurrent with the spread of the viral epidemic to areas traditionally endemic for leishmaniasis in the world (Gradoni and Gramiccia, 1994). In southern Europe, for example, especially in Spain, Italy and France, leishmaniasis is a growing problem with several hundreds of HIV co-infection cases (Olliaro and Bryceson, 1993). Similar problems are also reported from Asian countries, such as India, Bangladesh and Nepal where the severe visceral leishmaniasis is highly prevalent (WHO, 1995). In the patients with co-infections of *Leishmania* and HIV, clinical manifestations are unusual and diagnosis difficult, showing atypical secondary localizations in the gastric mucosa, rectum or skin, either in lesions or in normal tissue (Gradoni and Gramiccia, 1994); these patients need prolonged treatment and are liable to relapse (Altes *et al*., 1991). In Spain where the disease has been a compulsorily notifiable disease since 1982, almost 80% of the Spanish visceral leishmaniasis cases were immunodepressed patients, 60% of which were HIV-positives and the rest were basically divided between haematological disorders of the white blood cells, transplants, autoimmune disease and alcoholic haepatopathies (Alvar *et al*., 1989; Alvar, 1994). In the endemic area of leishmaniasis of southern Europe, more than 50% of visceral leishmaniasis in adults is now related to infection with HIV, suggesting that *Leishmania* and HIV co-infections are becomming more frequent in areas where both microorganisms are endemic (Gorgolas and Miles, 1994). In these areas of Europe, furthermore, intravenous drug abusers, who represent 60% of the HIV-positive cases, operate in periurban areas, where the drug traffic is uncontrolled. Therefore, the opportunity for both diseases, HIV and leishmaniasis, to overlap in these areas is optimized (Alvar, 1994). Tremblay *et al*. (1996) reviewed *Leishmania* and the pathogenesis of HIV infection based on the recently reported information on the putative cofactor role that the intracellular pathogen of the genus *Leishmania* may play in the pathogenesis of HIV infection. They commented that as both *Leishmania* and HIV can invade and replicate within macrophages, it is possible that interactions between both pathogens may exacerbate the process of *Leishmania* infection; thus, co-infection with *Leishmania* in HIV infected subjects may affect the pathogenesis of both AIDS and leishmaniasis diseases. Therefore, the medical community should be alerted to the inevitable rise in HIV and *Leishmania* co-infections in endemic areas and to potential interactions between these infectious agents (Tremblay *et al*., 1996).

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Chapter 2

Parasitology and Vector Entomology

1. Karyotype Analysis of *Leishmania* (*Viannia*) *panamensis* Isolated from Cutaneous Leishmaniasis Patients at the Pacific Coastal Regions in Ecuador

ABSTRACT. DNA karyotype of *Leishmania* isolates from cutaneous leishmaniasis patients at nine endemic areas in the Pacific coast of Ecuador was analyzed by pulsed field gel electrophoresis. All of 17 isolates, even isolates at the same endemic area exhibited different chromosomal DNA banding patterns. The size variation in the chromosomal DNAs was evident particularly in chromosomes between 400 and 800 kb. However, DNA bands of approximately 230, 350, 800 and 850 kb appeared to be conserved among these isolates. Since a similar DNA karyotype has been reported for the *Leishmania* (*Viannia*) *braziliensis* complex as well as three Ecuadorian *L.* (*V.*) *panamensis* isolates previously identified, these isolates in this study probably belong to *L.* (*V.*) *panamensis*. With regard to DNA karyotypes in *Leishmania* in Ecuador, it is worth to note that karyotype variation was evident among *L.* (*V.*) *panamensis* isolates in the Pacific coastal regions while karyotype homogeneity was detected in *L.* (*L.*) *mexicana* isolates in the Andes mountain regions (Katakura *et al*., 1993, 1994).

Introduction

DNA karyotype analysis is important for understanding chromosomal organization in eukaryote cells. For this purpose pulsed field agarose gel electrophoresis technique has been introduced for separation of chromosomal DNAs in yeast (Schwartz and Cantor, 1984) and parasitic protozoa including *Trypanosoma* (Van der Ploeg *et al*., 1984), *Leishmania* (Spithill and Samaras, 1995) and *Plasmodium* (Kemp *et al*., 1985).

In *Leishmania*, DNA karyotype is, in general, highly variable between species and strains (reviewed by Bastien *et al*., 1992; Lighthall and Giannini, 1992). Nevertheless, when molecular karyotype of certain strains showed extensive similarity, karyotype analysis is still useful for identification of *Leishmania* species and grouping of different isolates. In this respect we have reported on karyotype homogeneity among *L.* (*L.*) *mexicana* isolates in the Andean mountain regions in Ecuador (Katakura *et al*., 1993, 1994), strongly suggesting a common geographic origin of the parasites. On the contrary, DNA karyotypes of *L.* (*V.*) *panamensis* strains in the Pacific coastal regions in Ecuador were variable although we examined only three isolates from different areas of the northern part of Ecuador; Quininde, Zapallo Grande and Santo Domingo (Katakura *et al*., 1992, 1993). The results indicated the complexity and diversity of *L.* (*V.*) *panamensis* in the Pacific coast of Ecuador. In this study we analyzed additional 14 *Leishmania* isolates from patients at 6 different new endemic areas for further understanding of distribution and variation of *Leishmania* species in the Pacific coastal regions in Ecuador.

Materials and Methods

Parasites

Detail designations and geographic origins of *Leishmania* isolates in Ecuador and WHO reference strains used in the present study are shown in Table 1.1.1 and Figure. 1.1.1. Three Ecuadorian isolates, MHOM/EC/87/G05, G06 and G07 have been previously identified as *L.* (*V.*) *panamensis* by isoenzyme electrophoresis, monoclonal antibodies and kinetoplast DNA fingerprints (Mimori *et al.*, 1989, 1990). Parasite isolation was performed by aspiration of the margins of active skin lesions from patients followed by inoculation of the biopsy samples into culture tubes with biphasic human blood agar based USAMRU medium (Difco blood agar medium). Promastigotes grown in the medium at 25℃ were collected after several passages and cryopreserved in the liquid nitrogen before use.

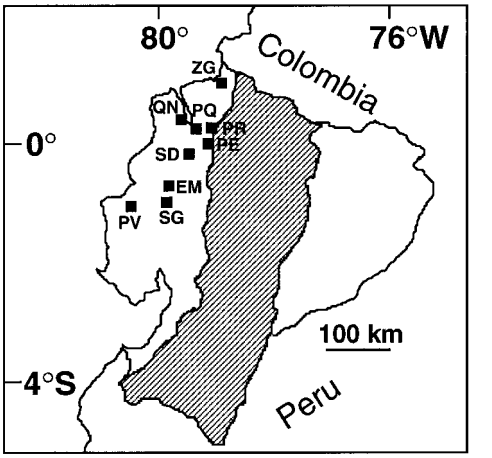

Figure 1.1.1. Geographic locations of *Leishmania* isolates in Ecuador used in this study. The shaded area on map indicates the Andean highland region more than 1000 meters above sea level. EM, El Moral; PE, Paraiso Escondido; PQ, Puerto Quito; PR, Paraiso; PV, Portoviejo; QN, Quininde; SD, Santo Domingo; SG, San Gabriel; ZG, Zapallo Grande

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

Species	Designation	Geographic origin	Abbreviation
Ecuadorian isolates			
L. (*V.*) *panamensis*			
	MHOM/EC/87/G05	Quininde (QN)	G5
	MHOM/EC/87/G06	Zapallo Grande (ZG)	G6
	MHOM/EC/87/G07	Santo Domingo (SD)	G7
L. (*V.*) *panamensis*?			
	MHOM/EC/90/EM50	El Moral (EM)	EM50
	MHOM/EC/90/EM52	El Moral	EM52
	MHOM/EC/90/PE130	Paraiso Escondido (PE)	PE130
	MHOM/EC/90/PQ91	Puerto Quito (PQ)	PQ91
	MHOM/EC/90/PR88	Paraiso (PR)	PR88
	MHOM/EC/90/PV1	Portoviejo (PV)	PV1
	MHOM/EC/90/PV10	Portoviejo	PV10
	MHOM/EC/90/PV12	Portoviejo	PV12
	MHOM/EC/90/PV19	Portoviejo	PV19
	MHOM/EC/90/PV156	Portoviejo	PV156
	MHOM/EC/90/PV158	Portoviejo	PV158
	MHOM/EC/90/PV159	Portoviejo	PV159
	MHOM/EC/90/SG13	San Gabriel (SG)	SG13
	MHOM/EC/90/SG16	San Gabriel	SG16
WHO reference strains			
L. (*V.*) *panamensis*	MHOM/PA/71/LS94	Canal Zone, Panama	LS94
L. (*V.*) *guyanensis*	MHOM/BR75/M4147	Para, Brazil	M4147
L. (*V.*) *braziliensis*	MHOM/BR/75/M2904	Para, Brazil	M2904

Pulsed field agarose gel electrophoresis (PFGE)

Pulsed field agarose gel electrophoresis was performed as described previously (Katakura *et al*., 1992, 1993). Cryopreserved promastigotes were thawed and grown in USAMRU medium. An agarose block containing 1 x 10^7 promastigotes was made and treated with 1 mg/ml proteinase K (Sigma, St. Louis, MO) and 1% sarcosyl (Sigma, St. Louis, MO) in 0.5 M EDTA, pH 8.0 at 50℃ for 2 days. The resultant agarose 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 60s for 36 hr using a turn-table type pulsed field gel electrophoresis apparatus (ATTO Corp., Tokyo, Japan). The gel was stained with 0.5 μg/ml ethidium bromide in 0.5 x TBE for 20 min, destained with 0.5 x TBE for 30 min and photographed. Yeast chromosomal DNAs of *Saccharomyces cerevisiae* (FMC, Rockland, ME) were used as the molecular standard. Diagrams of DNA karyotypes of the isolates were designed by analyzing photographs of PFGE gels.

Results

Chromosomal DNAs under 1000 kb were resolved in our pulsed field gel electrophoresis conditions. The PFGE revealed chromosomal DNAs of 14 *Leishmania* isolates at six endemic areas in the Pacific coastal regions; El Moral, Paraiso Escondido, Puerto Quito, Paraiso, Portoviejo and San Gabriel (Figs. 1.1.1 and 1.1.2). Diagrams of chromosomal DNA bands of a total of 19 *Leishmania* isolates including three Ecuadorian *L.* (*V.*) *panamensis* isolates (MHOM/EC /87/G05, G06 and G07) and three WHO reference strains such as *L.* (*V.*) *panamensis* (MHOM/PA/71 /LS94), *L.* (*V.*) *guyanensis* (MHOM/BR/75/M4147) and *L.* (*V.*) *braziliensis* (MHOM/BR/75/M2904) are presented in Figure 1.1.3. A diagram of DNA bands of the PV10 isolate was not shown because of poor separation (Fig. 1.1.2).

Small chromosomes under 400 kb were well separated and two chromosomal bands of approximately

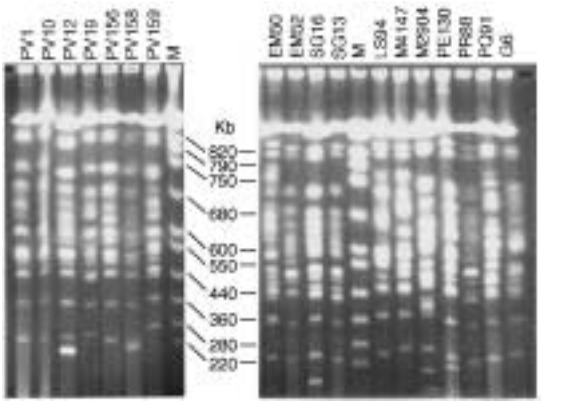

Figure 1.1.2. Separation of chromosomal DNAs of *Leishmania* isolates by pulsed field gel electrophoresis using 1.5% agarose gel run at 180 V with a 60-sec pulse time for 36 hr.
PV1, MHOM/EC/90/PV1; PV10, MHOM/EC/90/PV10; PV12, MHOM/EC/90/PV12; PV19, MHOM/EC/90/PV19; PV156, MHOM/EC/90 /PV156; PV158, MHOM/EC/90/PV158; PV159, MHOM/EC/90/PV159; EM50, MHOM/EC/90/EM50; EM52, MHOM/EC/90/EM52; SG16, MHOM/EC/90 /SG16; SG13, MHOM/EC/90/SG13; LS94, *L. (V.) panamensis* (MHOM/PA/71/LS94); M4147, *L. (V.) guyanensis* (MHOM/BR75/M4147); M2904, *L. (V.) braziliensis* (MHOM/BR/75/M2904); PE130, MHOM /EC/90/PE130; PQ91, MHOM/EC/90/PQ91; PR88, MHOM/EC/90/PR88; G6, L. (V.) panamensis (MHOM /EC/90/G06); M, *Saccharomyces cerevisiae* as the molecular standard.

230 and 350 kb appeared to be common among Ecuadorian isolates as well as *L.* (*V.*) *panamensis* and *L.* (*V.*) *guyanensis* reference stains (arrows in Fig. 1.1.3). A small DNA band under 200 kb was observed in four isolates of PV12, PE 130, EM50 and SG16 as well as *L.* (*V.*) *braziliensis* M2904. In *L.* (*V.*) *panamensis* G5 and G7, the smallest chromosomal band formed a doublet (Fig. 1.1.3; Katakura *et al*., 1993).

Medium-sized DNA bands of 400-800 kb were variable in size among isolates and showed the size complexity. However, the DNA banding patterns of the isolates from the same endemic area were much similar than those from different areas, as seen in iso-

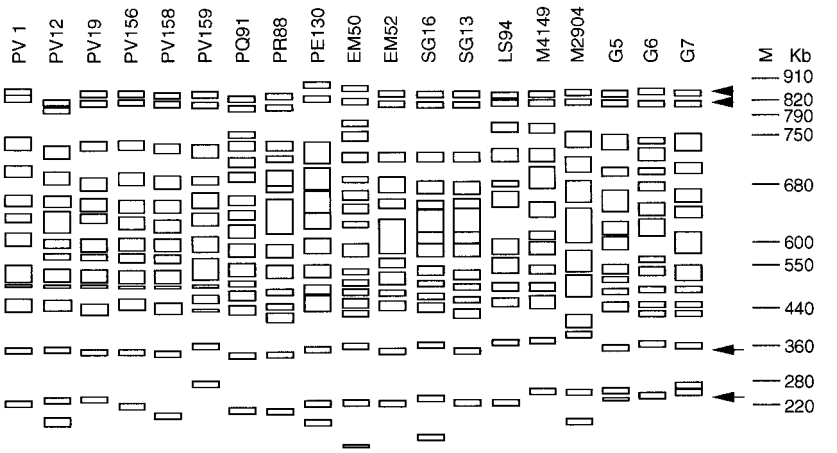

Figure 1.1.3. Diagrams of chromosomal DNAs of *Leishmania* isolates. For designations of each isolate, see Table 1.1.1 and Figure 1.1.2. M, the molecular standard.

lates of PV1, PV12, PV19, PV156, PV158 and PV159 at Portoviejo (Figs. 1.1.2 and 1.1.3). In addition, two DNA bands of approximately 800 and 850 kb were clearly separated and seem to be conserved in all isolates (arrow heads in Fig. 1.1.3).

Discussion

In this study we examined DNA karyotypes of a total of 17 *Leishmania* isolates from cutaneous leishmaniasis patients at 9 endemic areas of the central and northern part of the Pacific coastal regions in Ecuador (Fig. 1.1.1). The results revealed that DNA karyotypes of the isolates were variable but chromosomal DNA bands of approximately 230, 350, 800 and 850 kb seem to be common to all isolates (Fig. 1.1.3). Sizes of conserved chromosomal bands are somewhat smaller than those reported by Dujardin *et al.* (1993a), who analysed molecular karyotype of 45 reference strains and stocks representative of the New World *Leishmania* by pulsed field gel electrophoresis. They found a unique set of size-conserved chromosomal bands of 295, 405 and 900 kb in the *braziliensis* complex and of 350 and 930 kb in the *mexicana* complex. The DNA karyotypes of *Leishmania* isolates in this study were very similar to those of *L.* (*V.*) *panamensis* isolated from Panama and Ecuador, thereby these isolates appear to belong to *L.* (*V.*) *panamensis*. ELISA analysis of sera from the leishmaniasis patients also suggested that the patients were infected with *L.* (*V.*) *panamensis* (see Chapter 2-2 in this text). Furthermore, isoenzyme analysis was unable to distinguish *L.* (*V.*) *panamensis* from *L.* (*V.*) *guyanensis* isolates in Ecuador (unpublished data).

Intraspecific karyotype variation is a common feature in almost all *Leishmania* species including *L. (L.) major* (Giannini *et al.*, 1986), *L. (L.) infantum* (Bishop and Miles, 1987; Pages *et al.*, 1989), *L. (L.) donovani* (Bishop and Miles, 1987), *L. (L.) chagasi* (Bishop and Miles, 1987), *L. (V.) panamensis* (Giannini *et al.*, 1986), *L. (L.) amazonensis* (Scholler *et al.*, 1986) and *L. (V.) peruviana* (Dujardin *et al.*, 1993b). DNA karyotype variation of *L.* (*V.*) *panamensis* isolates in the Pacific coastal regions in Ecuador also indicated the genetic diversity of this species. A great diversity of mammalian reservoir hosts and possible sand fly vectors in the lowlands in Ecuador may be related to the diversity of the parasites (Hashiguchi and Gomez, 1991). However, mechanisms of polymorphisms on the size and number of the chromosomal bands between different strains are poorly understood

although DNA deletion and amplification may account for some of DNA size modification (Bastien *et al*., 1992).

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2. Identification of Species of Ecuadorian *Leishmania* Isolates by ELISA using Monoclonal Antibodies

ABSTRACT. One hundred twenty five Ecuadorian *Leishmania* stocks isolated from human, reservoir hosts and sandfly vectors at 40 different areas of the 13 provinces were classified into 7 groups with 9 species-specific monoclonal antibodies and ELISA technique. Eighty nine of 125 isolates were identified as *Leishmania* (*Viannia*) *panamensis*, 15 strains as *L.* (*Leishmania*) *mexicana*, 4 strains as *L.* (*L.*) *amazonensis*, 4 strains as *L.* (*V.*) *braziliensis*, 3 strains as *L.* (*L.*) *major*-like, and 3 strains as *L. (V.) equatorensis*. Clear-cut answer on the classification was not obtained from 7 isolates.

Introduction

In Ecuador, New World leishmaniases are widely distributed in areas from the Pacific coastal and Amazonian lowland regions to the Andean highland regions. In our consecutive survey of the disease, the first *Leishmania* strain was isolated from wild mammals in 1982 (Hashiguchi *et al*., 1985). During the past 14 years, 125 *Leishmania* strains have been isolated from patients, reservoir hosts and sandfly vectors in the 13 different provinces of this country. Among these stocks, 30 isolates have been already identified as *L.* (*Leishmania*) *amazonensis*, *L.* (*L.*) *mexicana*, *L.* (*Viannia*) *braziliensis*, *L.* (*V.*) *panamensis*, *L.* (*L.*) *major*-like, and *L.* (*V.*) *equatorensis* by zymodeme, schizodeme and serodeme analyses (Mimori *et al*., 1989; Hashiguchi *et al*., 1991; Grimaldi *et al*., 1992; Katakura *et al*., 1993, 1994). However, the classification of species of most isolates has been left unsolved.

Species- and/or subspecies-specific monoclonal antibodies have been developed for characterization and identification of *Leishmania* species (McMahon-Pratt and David, 1981; McMahon-Pratt *et al*., 1982, 1985; Anthony *et al*., 1985; Grimaldi *et al*., 1987). Thus, we took advantage of the serodeme analysis by ELISA for classification of Ecuadorian isolates. The present paper deals with the reactivities of 125 Ecuadorian stocks to species-specific monoclonal antibodies.

Materials and Methods

Parasites

As standard strains, 7 WHO reference strains of New World *Leishmania* and 2 Ecuadorian reference strains, *L. (V.) equatorensis* and *L. (L.) major*-like, were also used in this experiment (Table 2.2.1). Ecuadorian isolates used in this experiment were as follows: 111 isolates from patients; 6 from wild mammals and a domestic dog; and 8 from sand flies. The clinical and epidemiological data of these isolates are given in Tables 2.2.2 to 2.2.4.

Preparation of antigens for ELISA

The isolates were cultured with Schneider's medium containing 15% heat-inactivated fetal bovine serum. The harvested promastigotes were washed by centrifugation with phosphate buffered saline solution. The washed parasites were immediately suspended in lysis buffer (10 m*M* Tris-HCl, pH 7.5; containing 2 m*M* EDTA, 1.6 m*M* phenyl methyl sulfonyl fluoride, 2 m*M* iodoacetoamide, 100 μg/ml leupeptin). After sonication of the material for one min, the cell homogenate was centrifuged at 1600 x g for 10 min. The supernatant was adjusted to 10 μg protein concentration per ml with coating buffer.

Monoclonal antibodies

Eight monoclonal antibodies, namely B4, B11, B16, B18, B19, M8, T3/M13, and 7H9, were used in

this experiment. Furthermore, A1 monoclonal antibody was newly prepared in our laboratory according to the Köhler and Milstein method (1975), using P3U1 myeloma cells. The specificity and characterization of A1 monoclonal antibody will be described elsewhere.

ELISA

ELISA was carried out with the microwell ELISA mate® Kit for peroxidase conjugate (Kirkegaard & Perry Laboratory, USA). The wells of 96-well polystyrene microplate (Falcon, USA) were coated with 100 μl of the antigen solutions at 4℃ overnight. After blocking with BSA solution of the Kit, each well was incubated with 9 monoclonal antibodies at 37℃ for 1hr. The wells were then washed 5 times with wash solution of the Kit by microplate washer, automatically, and 100 μl of peroxidase conjugated rabbit anti-mouse immunoglobulins (Dako, Denmark) was applied each well. The plate was incubated for 1 hr at 37℃. After washing, the mixture of ABTS peroxidase substrate and H_2O_2 solution of the Kit was added to each well, and incubated 30 min at 37℃. The reaction was stopped by adding of stop solution of the Kit. The absorbent at 405 nm was measured using microplate reader and the relative ratio against negative control was calculated. Values over 10 were considered positive.

Results

In the first step of this study, the reactivities of 9 monoclonal antibodies in total to WHO and Ecuadorian reference strains were examined by ELISA. The results are shown in Table 2.2.1. The monoclonal antibodies used in this study showed a high and consistent qualitative specificity at species level of reference strains. For instant, B4 and B11 for *L. (V.) panamensis*; B16 and B18 for *L. (V.) braziliensis*; B19 for *L. (V.) guyanensis*; A1 for *L. (L.) amazonensis*; M8 for *L. (L.) mexicana*; 7H9 for *L. (V.) equatorensis*. On the other hand, all monoclonal antibodies, except for T3/M13, used in this study did not show a high and consistent qualitative specificity against *L. (L.) major*-like strains.

To check the availability of these monoclonal antibodies to Ecuadorian parasites, the reactivities of these monoclonal antibodies against 30 known Ecuadorian strains, which have been previously identified as *L. (L.) amazonensis*, *L. (L.) mexicana*, *L. (V.) braziliensis*, *L. (V.) panamensis*, *L. (L.) major*-like, and *L. (V.) equatorensis*, were also tested. The species-specificity of the present monoclonal antibodies against the Ecuadorian strains was very high as well as that against the reference stains, except for 5 *L. (L.) mexicana* strains.

Based on the reactive pattern of reference strains to these monoclonal antibodies, 125 isolates were classified by ELISA tests. As shown in Tables 2.2.1 to 2.2.4, 89 of 125 isolates were classified as *L. (V.) panamensis*, 15 strains as *L. (L.) mexicana*, 4 strains as *L. (L.) amazonensis*, 4 strains as *L. (V.) braziliensis*, 3 strains as *L. (L.) major*-like, and 3 strains as *L. (V.) equatorensis*. Other 7 isolates did not give a clear answer on the classification in this experiment.

In Ecuadorian *L. (V.) panamensis* strains, two reactive patterns were observed. One was very similar to that of the reference strain, the other reacted only with B4. Ratio of the latter reactive pattern was 6.7% (6/89). No or low reactivity of B18 monoclonal antibody was confirmed in 4 Ecuadorian *L. (V.) braziliensis* strains. B19 monoclonal antibody was very specific for *L. (V.) guyanensis* of reference strain (Table 2.2.1). However, no Ecuadorian isolates reacted with this antibody. Consistent qualitative specificity of 7H9 monoclonal antibody was very high against *L. (V.) equatorensis* of Ecuadorian reference strain. Three isolates reacted with this antibody. The two were isolated from reservoir hosts, and the other one from sandfly vector. G-02 strain of *L. (L.) amazonensis* reacted A1 and T3/M13 monoclonal antibodies. However, other 3 strains, G-03, G-04, and INH-690, reacted with both A1 and M8 monoclonal antibodies.

Distribution of *Leishmania* parasites isolated from

Table 2.2.1. Reactivities of *Leishmania* species-specific monoclonal antibodies to reference strains and selected Ecuadorian *Leishmania* isolates

Designation	Species	Monoclonal antibodies								
		B4	B11	B16	B18	B19	A1	M8	7H9	T3/M13
WHO reference strains										
MHOM/BR/75/M2904	*L. (V.) braziliensis*	0.9	0.4	48.8	44.7	1.4	0.4	1.3	8.2	8.1
MHOM/PA/71/LS94	*L. (V.) panamensis*	52.3	39.3	2.8	0.3	1.1	0.1	0.9	0.7	6.4
MHOM/BR/75/M4147	*L. (V.) guyanensis*	1.1	0.8	5.0	1.1	31.7	0.4	1.0	0.8	7.7
MHOM/BR/73/M2269	*L. (L.) amazonensis*	0.1	0.3	1.7	0.1	0.3	19.5	1.3	1.0	4.5
MHYC/BZ/62/M379	*L. (L.) mexicana*	0.1	0.1	0.8	0.1	0.1	0.3	25.2	0.1	31.0
MHOM/BR/74/PP75	*L. (L.) chagasi*	0.3	0.2	3.7	0.3	1.0	0.2	0.7	1.6	5.8
MHOM/SU/73/5ASKH	*L. (L.) major*	1.0	0.6	7.7	1.5	3.2	0.7	4.1	1.8	59.2
Ecuadorian reference stains										
MCHO/EC/82/Lsp-1	*L. (V.) equatorensis*	0.1	0.1	0.7	0.1	0.1	0.1	0.1	35.1	36.7
MHOM/EC/87/G-09	*L. (L.) major*-like	0.6	0.1	3.1	0.8	1.4	0.1	1.1	0.4	55.9
Ecuadorian isolates										
MHOM/EC/87/G-07	*L. (V.) panamensis*	56.7	51.2	4.4	1.5	2.5	0.7	1.6	2.1	6.4
MHOM/EC/90/SG-13	*L. (V.) panamensis*	53.5	28.1	5.4	0.8	1.8	0.2	0.9	1.4	5.5
MHOM/EC/90/PV-158	*L. (V.) panamensis*	30.5	6.1	5.7	1.3	2.3	0.2	0.8	1.2	3.7
MHOM/EC/88/TA-1	*L. (V.) braziliensis*	6.1	1.5	28.9	1.8	1.5	1.6	2.1	2.1	1.8
MHOM/EC/88/INH-03	*L. (V.) braziliensis*	5.8	1.8	27.3	8.0	2.1	1.3	1.4	2.2	2.5
MHOM/EC/90/EC-50	*L. (V.) braziliensis*	3.4	1.1	46.5	2.7	4.0	1.1	2.9	0.9	1.6
MSCI/EC/87/G-02	*L. (L.) amazonensis*	0.9	1.1	2.0	1.2	1.6	14.8	3.2	9.2	14.1
MPOT/EC/87/G-03	*L. (L.) amazonensis*	1.2	0.4	2.8	0.9	1.7	55.6	16.4	1.0	6.5
MHOM/EC/88/PT-001	*L. (L.) mexicana*	1.1	0.6	4.9	1.0	1.0	1.7	30.0	1.0	35.8
MHOM/EC/89/AL-1	*L. (L.) mexicana*	1.3	1.4	3.8	1.6	1.7	1.9	11.3	4.5	58.0
MHOM/EC/92/HU-01	*L. (L.) mexicana*	1.5	1.3	2.7	1.4	1.5	1.8	7.3	1.4	19.3
MHOM/EC/88/PT-115	*L. (L.) major*-like	1.2	1.4	3.1	1.5	1.7	2.0	3.1	1.8	24.3
IHAR/EC/93/OC-4	*L. (V.) equatorensis*	1.7	1.9	1.8	1.6	2.6	1.1	2.2	47.6	58.2

40 different endemic areas was summarized in Table 2.2.2. In Tables 2.2.3 and 2.2.4, furthermore, the summary of all the isolates obtained were arranged by the clinical and parasitological data of patients, and the species and isolation areas (locations) of reservoir hosts and sandfly vectors.

Discussion

A variety of molecular and biochemical methods have been used for characterization and identification of *Leishmania* species. Species- and/or subspecies-specific monoclonal antibodies have been developed to characterize and identify the New World species of *Leishmania* (McMahon-Pratt and David, 1981; McMahon-Pratt *et al*., 1982, 1985; Anthony *et al*., 1985; Grimaldi *et al*., 1987). In this study, 9 monoclonal antibodies were used to classify Ecuadorian *Leishmania* isolates by ELISA method. Among these monoclonal antibodies, the availability of B4, B11, B16, B18, B19 and M8 monoclonal antibodies against

Table 2.2.2. *Leishmania* species isolated from the 13 different provinces and its provenance details

Province	Species	Locality (No. of isolates)
ESMERALDAS		
	L. (L.) amazonensis	Muisne(1)
	L. (V.) panamensis	Quininde(1), Zapallo Grande(1), 5 de Junio(1)
	L. (L.) major-like	Quininde(1)
MANABI		
	L. (V.) panamensis	Calceta(3), Calderon(2), El Calmen(1), El Moral(13), H. Vasquez(1), Portoviejo(6), Progreso(7), San Gabriel(19), San Placido(1), San Sebastian(2)
LOS RIOS		
	L. (V.) panamensis	6 de Agosto(1), Quevedo(1)
	L. (L.) amazonensis	Palenque(2)
GUAYAS		
	L. (V.) panamensis	El Triunfo(1), Manglaralto(1), Milagro(1), Naranjal(6), Olon(4), Pascuales(1), Puerto Inca(1)
	L. (V.) equatorensis	Naranjal(2)
EL ORO		
	L. (V.) panamensis	Santa Rosa(1)
PICHINCHA		
	L. (V.) panamensis	Sto. Domingo(1), Puerto Quito(4), Paraiso(1), 24 de Mayo(4)
BOLIVAR		
	L. (L.) amazonensis	Echeandia(1)
	L. (V.) panamensis	Caluma(1)
CHIMBORAZO		
	L. (L.) mexicana	Alausi(2), Huigra(2)
	?	Alausi(1), Huigra(3)
CAÑAR		
	L. (V.) equatorensis	Ocaña(1)
	?	Ocaña(2)
AZUAY	*L. (L.) mexicana*	Dug Dug(1), Paute(10)
	L. (L.) major-like	Paute(2)
	?	Paute(1)
NAPO		
	L. (V.) braziliensis	El Coca(1)
MORONA SANTIAGO		
	L. (V.) braziliensis	Taisha(1)
ZAMORA CHINCHIPE		
	L. (V.) braziliensis	El Chorro(1), La Chonta(1)

L. (V.) panamensis, L. (L.) mexicana and *L. (L.) major*-like species of Ecuadorian strains has been demonstrated by Mimori *et al*. (1989) and Hashiguchi *et al*. (1991), using indirect radioimmune binding assay technique. Species specificity of 7H9 monoclonal antibody to *L. (V.) equatorensis* has been also reported by Terabe *et al*. (1994), using ELISA method. From our experiment used 30 known Ecuadorian strains which have been identified at species level by other methods, it was appeared that the present monoclonal antibodies were useful for classification of Ecuadorian *Leishmania* parasites. Based on the results obtained from ELISA-monoclonal antibodies technique, 119 of 125 isolates were classified as *L. (V.) panamensis*, *L. (L.) mexicana*, *L. (L.) amazonensis*, *L. (V.) braziliensis*, *L. (L.) major*-like, and *L. (V.) equatorensis*. On the other hand, only 7 isolates did not give a clear answer on the classification in this experiment. However, from the reactive pattern in ELISA and isolation area of these 7 organisms, it is assumed that they belong to *L. (L.) mexicana* or *L. (L.) major*-like. For further clarification of species of the isolates, the preparation of monoclonal antibody to *L. (L.) major*-like is now in progress.

Table 2.2.3. Summary of clinical and parasitological data of patients from whom *Leishmania* stocks were isolated at different endemic areas in Ecuador

Designation	Species by ELISA	Age	Sex	No. and size of lesions		Site of lesions	Type of lesions and lymphadenopathy	Duration time (months)	Skin test	Locality
				No.	size(mm)					
MHOM/EC/86/Paute	Lm-l	4	M	1	3x5	A	U, L:NF	5	+	Paute
MHOM/EC/87/G-05	Lp	20	F	2	10x8,15x12	A	U, L:generalized	4	+	Quininde
MHOM/EC/87/G-06	Lp	21	M	1	25x9	A	U, L:NF	2	+	Zapallo Grande
MHOM/EC/87/G-07	Lp	38	F	1	30x27	A	U, L:NF	3	+	Sto. Domingo
MHOM/EC/87/G-09	Lm-l	18	F	1	10x10	FO	U, L:NF	4	+	Quininde
MHOM/EC/88/INH-01	Lp	27	M	1	30x30	A	U, L:localized	2.5	+	Naranjal
MHOM/EC/88/INH-02	Lp	41	M	1	10x10	H	U, L:generalized (large)	4	+	Santa Rosa
MHOM/EC/88/INH-03	Lb	23	M	1	30x30	A	U, L:NF	3	+	El Coca
MHOM/EC/88/INH-04	Lp	17	M	7	40x40, 30x30, 10x10(4), 15x15	FA, S, BO(b), FO(b)	U L:NF	1.5	+	Puerto Inca
MHOM/EC/88/INH-08	Lp	25	F	1	30x17	A(w)	U, L:positive	3	+	5 de Junio
MHOM/EC/88/INH-10	Lp	19	M	1	25x20	A(w)	U, L:positive	3	ND	Olon
MHOM/EC/88/INH-11	Lp	22	M	1	30x30	A(w)	U, L:positive	4	ND	Olon
MHOM/EC/88/INH-12	Lp	21	M	1	20x15	A(w)	U, L:NF	4	ND	Olon
MHOM/EC/88/INH-13	Lp	21	M	4	20x30(3), 10x10	A, FO	U, L:NF	4	ND	Olon

In the column of "species by ELISA", Lm-l: *L. (L.) major*-like, Lp: *L. (V.) panamensis*, Lb: *L. (V.) braziliensis*, La: *L. (L.) amazonensis*, Lm: *L. (L.) mexicana*, Leq: *L. (V.) equatorensis*. In the column of "site of lesions", FA: face, A: arm, A(w): arm (wrist), A(b) : arm (back side), H: hand, FO: foot, FO(b): foot (back side), S: shoulder, BO: body, BO(b): body (back side), N: neck, N(b): neck (back side). In the column of "type of lesions", U: ulcer, N: nodule, E: erythema, P: papule, L: lymphadenopathy, NF: not found of lymphadenopathy, NM: not measured, ND: not done.

Table 2.2.3. continued

Designation	Species by ELISA	Age	Sex	No. and size of lesions		Site of lesions	Type of lesions and lymphadenopathy	Duration time (months)	Skin test	Locality
				No.	size(mm)					
MHOM/EC/88/INH-17	Lp	30	M	2	20x15, 15x15	BO	U, L:NF	3	ND	Puerto Quito
MHOM/EC/88/INH-21	Lp	16	M	5	10x10, 5x5(2), 3x3(2)	H	U, L:positive (axilla)	1	ND	El Triunfo
MHOM/EC/88/INH-23	LP	28	F	11	40x40, 20x30, 20x20, less than 5x5(8)	FA, BO, A(b)	U, E	2	ND	Pascuales
MHOM/EC/88/INH-29	LP	28	F	2	21x15, 15x15	FO U,	L:NF	4	ND	6 de Agosto
MHOM/EC/88/INH-30	Lp	22	M	2	35x25, 25x15	FO, FA	U, L:NF	3	ND	EL Carmen
MHOM/EC/88/INH-36	Lp	1	F	1	5x5	H	U, L:NF	1	ND	Milagro
MHOM/EC/88/INH-37	Lp	25	M	2	10x13, 8x5	H, FO	U, L:localized	2	ND	Quevedo
MHOM/EC/88/INH-38	Lp	29	M	7	5x5(7)	FA, H, FO	U, L:NF	3	ND	Caluma
MHOM/EC/88/PT-001	Lm	5M	M	2	4x3, 5x4	FA	N, L:NF	4	ND	Paute
MHOM/EC/88/PT-006	Lm	11M	M	1	15x10	FA	U, L:NF	7	ND	Paute
MHOM/EC/88/PT-007	Lm	11M	F	1	3x7	FA	P, L:NF	9	ND	Paute
MHOM/EC/88/PT-023	Lm	10M	M	4	5x5, 2x2, 1x1(2)	FA	P, L:NF	4	+	Paute
MHOM/EC/88/PT-024	Lm	11M	M	2	2x2(2)	FA	P, L:NF	9	ND	Paute
MHOM/EC/88/PT-025	Lm	5M	M	1	2x3	FA	P, L:NF	4	ND	Paute
MHOM/EC/88/PT-027	Lm	10M	F	3	3x3(3)	FA	P, L:NF	8	ND	Paute
MHOM/EC/88/PT-027	Lm	10M	F	3	3x3(3)	FA	P, L:NF	8	ND	Paute
MHOM/EC/88/PT-029	Lm	9M	F	5	4x4, 2x2(2), 1x1(2)	FA	P, L:NF	2	ND	Paute
MHOM/EC/88/PT-103	Lm	5	F	1	3x3	FA	P, L:NF	4	ND	Paute
MHOM/EC/88/PT-115	Lm-l	8	M	1	4x4	FA	P, L:NF	5	ND	Paute
MHOM/EC/88/TA-1	Lb	13	M	1	25x25	FA	U, L:localized	1	ND	Taisha
MHOM/EC/89/AL-1	Lm									Alausi
MHOM/EC/90/DD-35	Lm	1	M	1	4x3	FA	N, L:NF	6	ND	Dug Dug
MHOM/EC/90/EC-50	Lb	40	M	1	40x60	A(b)	N, L:NF	1	ND	El Chorro
MHOM/EC/90/EM-49	Lp	8	M	1	NM	FO	U, L:NF	2	ND	El Moral
MHOM/EC/90/EM-52	Lp	4	M	1	NM	FO	U, L:NF	3	ND	El Moral
MHOM/EC/90/EM-53	Lp	6	MM	3	NM	FA	U, L:NF	3	ND	El Moral
MHOM/EC/90/EM-54	Lp	18	F	10	NM	FA, A, E	U, L:NF	2	ND	El Moral
MHOM/EC/90/EM-55	Lp	15	F	5	NM	A, FO, BO, E	U, L:NF	1	ND	El Moral
MHOM/EC/90/EM-57	Lp	17	F	2	NM	A	U, L:NF	0.5	ND	El Moral
MHOM/EC/90/EM-58	Lp	15	F	2	NM	FO	U, L:NF	2	ND	El Moral
MHOM/EC/90/EM-59	Lp	30	F	2	NM	FA, BO(b)	U, L:NF	3	ND	El Moral
MHOM/EC/90/EM-60	Lp	19	F	2	NM	A, FO	U, L:NF	6	ND	El Moral

Table 2.2.3. continued

Designation	Species by ELISA	Age	Sex	No. and size of lesions		Site of lesions	Type of lesions and lymphadenopathy	Duration time (months)	Skin test	Locality
				No.	size(mm)					
MHOM/EC/90/EM-64	Lp	10	M	1	NM	FA	U, L:NF	1	ND	El Moral
MHOM/EC/90/INH-690	La	19	M	lot		all	U, N(diffuse)		-	Muisne
MHOM/EC/90/INH-785	Lp	23	M	3	13x13, 5x5, 3x3	FA, N, A(w)	U, L:NF	?	+	Manglaralto
MHOM/EC/90/INH-932	Lp									
MHOM/EC/90/LC-47	Lb	35	M	1	60x70	BO(b)	U, L:positive	5	ND	La Chonta
MHOM/EC/90/P0-02	Lp	7	M	2	10x5, 5x5	H	U, L:localized	2	ND	H. Vasquez
MHOM/EC/90/PE-130	Lp	4M	F	4	10x5(4)	A, FO	U, L:NF	2	ND	Puerto Quito
MHOM/EC/90/PQ-91	Lp	18	F	1	10x15	A	U, L:NF	3	+	Puerto Quito
MHOM/EC/90/PR-88	Lp	10	M	1	10x10	A	U, L:NF	8	ND	Paraiso
MHOM/EC/90/PV-001	Lp	13	F	2	15x10, 5x5	FA, FO	U, L:NF	2	ND	El Moral
MHOM/EC/90/PV-002	Lp	7	F	2	10x5, 5x5	H	U, L:localized	2	ND	El Moral
MHOM/EC/90/PV-004	Lp	1	M	3	10x15, 5x5, 2x3	FA, H	U, L:positive	3	ND	El Moral
MHOM/EC/90/PV-006	Lp	?	F	7	NM	FA, A	U, L:NF	0.5	ND	San Gabriel
MHOM/EC/90/PV-010	Lp	20	M	1	10x20	A	U, L:positive	3	ND	Calderon
MHOM/EC/90/PV-012	Lp	28	F	1	20x15	A	U, L:NF	1	ND	Progreso
MHOM/EC/90/PV-018	Lp	45	F	3	20x10, 1x1, 3x2	?	U, L:NF	0.5	ND	Calderon
MHOM/EC/90/PV-019	Lp	21	M	2	10x10, 6x5	?	U, L:NF	2	ND	San Placido
MHOM/EC/90/PV-156	Lp	55	F	1	5x3	A	U, L:NF	1	ND	Portoviejo
MHOM/EC/90/PV-157	Lp	20	M	6	10x4(3), 7x5, 5x3, 3x2	A, FO	U, L:NF	1	ND	Portoviejo
MHOM/EC/90/PV-158	Lp	20	F	2	20x10, 10x5	FA, BO(b)	U, L:NF	1	ND	Calceta
MHOM/EC/90/PV-159	Lp	25	F	1	5x7	FO	U, L:NF	4	ND	Portoviejo
MHOM/EC/90/PV-160	Lp	16	M	1	10x7	A	U, L:NF	2	ND	Portoviejo
MHOM/EC/90/PV-161	Lp	17	M	1	3x4	A	U, L:NF	1	ND	Calceta
MHOM/EC/90/PV-162	Lp	22	M	3	15x7, 5x3, 3x2	A	U, L:NF	3	ND	Calceta
MHOM/EC/90/SG-04	Lp	5	F	23	NM	FA, A, FO	U, L:NF	8	ND	San Gabriel
MHOM/EC/90/SG-13	Lp	21	F	1	NM	A	U, L:positive	3	ND	San Gabriel
MHOM/EC/90/SG-14	Lp	11	M	3	NM	FA, A	U, L:NF	2	ND	San Gabriel
MHOM/EC/90/SG-16	Lp	7	F	5	NM	FO, BO	U, L:NF	3	ND	San Gabriel
MHOM/EC/90/SG-17	Lp	14	M	3	NM	A, BO	U, L:NF	3	ND	San Gabriel
MHOM/EC/90/SG-23	Lp	48	F	2	NM	A(b)	U, L:NF	2	ND	San Gabriel
MHOM/EC/90/SG-24	Lp	15	F	3	NM	FA, A	U, L:NF	1	ND	San Gabriel
MHOM/EC/90/SG-26	Lp	9	MM	4	NM	FA, FO	U, L:NF	2	ND	San Gabriel
MHOM/EC/90/SG-27	Lp	10	F	1	NM	FA	U, L:NF	2	ND	San Gabriel
MHOM/EC/90/SG-33	Lp	9	F	2	NM	A	U, L:NF	5	ND	San Gabriel
MHOM/EC/90/SG-34	Lp	11	F	5	NM	FA, FO	U, L:NF	5	ND	San Gabriel

Table 2.2.3. continued

Designation	Species by ELISA	Age	Sex	No. and size of lesions		Site of lesions	Type of lesions and lymphadenopathy	Duration time (months)	Skin test	Locality
				No.	size(mm)					
MHOM/EC/90/SG-35	Lp	7	M	1	NM	H	U, L:NF	?	ND	San Gabriel
MHOM/EC/90/SG-37	Lp	26	F	3	NM	A(b)	U, L:NF	1	ND	San Gabriel
MHOM/EC/90/SG-39	Lp	1	F	1	NM	FA	U, L:NF	1	ND	San Gabriel
MHOM/EC/90/SG-40	Lp	32	F	1	NM	BO	U, L:NF	2	ND	San Gabriel
MHOM/EC/90/SG-44	Lp	35	M	4	NM	A	U, L:NF	3	ND	San Gabriel
MHOM/EC/90/SG-45	Lp	12	M	1	NM	FA	U, L:positive	1	ND	San Gabriel
MHOM/EC/90/SG-46	Lp	20	M	2	NM	A	U, L:NF	3	ND	San Gabriel
MHOM/EC/90/VC-096	Lp	37	M	1	10x10	A	U, L:NF	6	+	24 de Majo
MHOM/EC/90/VC-097	Lp	8	M	1	5x5	A	U, L:localized	12	+	24 de Majo
MHOM/EC/90/VC-098	Lp	1	2M	2	3x4, 4x4	A	U, L:NF	12	+	24 de Majo
MHOM/EC/90/VC-127	Lp	30	M	1	5x5	H	U, L:NF	1	ND	24 de Majo
MHOM/EC/91/PV-136	Lp	45	F	1	25x20	A	U, L:NF	3	+	Portoviejo
MHOM/EC/91/PV-141	Lp	8	M	1	13x15	BO(b)	U, L:NF	4	+	Progreso
MHOM/EC/91/PV-142	Lp	8	M	4	10x8, 8x8, 8x7, 5x5	FA, A	U, L:NF	3	+	Progreso
MHOM/EC/91/PV-143	Lp	36	F	1	35x30	FO	U, L:positive	2	+	San Sebastian
MHOM/EC/91/PV-144	Lp	18	F	2	25x15, 4x5	FA, A	U, L:NF	7	+	Portoviejo
MHOM/EC/91/PV-149	Lp	9	M	2	8x8, 3x3	FA	U, L:NF	1	+	Progreso
MHOM/EC/91/PV-158	Lp								ND	Progreso
MHOM/EC/91/PV-160	Lp								ND	Progreso
MHOM/EC/91/PV-172	Lp	7	M	2	12x10, 5x7	BO, FO	U, L:positive	5	ND	Progreso
MHOM/EC/91/PV-043	Lp	25	F	2	15x10, 10x10	A	U, L:localized	4	+	San Sebastian
MHOM/EC/91/PV-093	Lp	?	M	1	40x30	H	U, L:NF	2	+	San Sebastian
MHOM/EC/92/HU-01	Lm	1	F	2	10x10, 5x5	FA	U, L:NF	4	+	Huigra
MHOM/EC/92/HU-03	Lm	2	F	3	5x5, 1x1(2)	FA	U, L:NF	18	+	Huigra
MHOM/EC/92/HU-04	?	9	MM	4	5x5, 3x3(3)	FA	P, L:NF	3	+	Huigra
MHOM/EC/92/HU-06	?	2	M	1	3x2	A	U, L:NF	8	±	Huigra
MHOM/EC/95/NA-03	Lp	27	M	8	15x10, 5x5, 4x4, 3x3, 2x2(2), 1x1(2)	A(b), N(b), U, N, P, E	L:generalized	?	ND	Naranjal
MHOM/EC/95/NA-04	Lp	38	M	2	5x8, 20x15	A, N(b)	U, L:NF	?	ND	Naranjal
MHOM/EC/95/NA-06	LP	32	M	2	10x15, 10x10	A(b)	U, L:NF	?	ND	Naranjal
MHOM/EC/95/NA-08	Lp	28	M	4	15x10(2), NM(2)	A, A(b)	U, L:NF	?	ND	Naranjal
MHOM/EC/95/NA-09	Lp	25	M	1	10x5	A(b)	U, L:NF	?	ND	Naranjal

Table 2.2.4. Summary of *Leishmania* species isolated from reservoir hosts and sandfly vectors at different endemic areas in Ecuador

Designation	Species※ by ELISA	Reservoir hosts or sandfly vectors	Locality
MCHO/EC/82/Lsp-1	Leq	*Choloepus hoffmani didactylus*	Naranjal
MSCI/EC/82/Lsp-2	Leq	*Sciurus vulgaris*	Naranjal
MSCI/EC/87/G-02	La	*Sciurus vulgaris*	Palenque
MPOT/EC/87/G-03	La	*Potos flavus*	Palenque
MTAM/EC/87/G-04	La	*Tamandua tetradactyla*	Echeandia
MCAN/EC/88/INU-2	Lm	*Canis familiaris*	Paute
IAYA/EC/89/PAI-1	?	*Lutzomyia ayacuchensis*	Paute
ITRA/EC/90/PQI-1	Lp	*Lutzomyia trapidoi*	Puerto Quito
IAYA/EC/92/ALI-3	?	*Lutzomyia ayacuchensis*	Alausi
IAYA/EC/92/ALI-4	Lm	*Lutzomyia ayacuchensis*	Alausi
IAYA/EC/92/HUI-1	?	*Lutzomyia ayacuchensis*	Huigra
IHAR/EC/93/OC-2	?	*Lutzomyia hartmanni*	Ocaña
IHAR/EC/93/OC-3	?	*Lutzomyia hartmanni*	Ocaña
IHAR/EC/93/OC-4	Leq	*Lutzomyia hartmanni*	Ocaña

※La: *L. (L.) amazonensis*, Lm: *L. (L.) mexicana*, Lp: *L. (V.) panamensis*, Leq: *L. (V.) equatorensis.*

Until now, a little information on *Leishmania* species at every endemic foci has been available (Mimori *et al*., 1989; Hashiguchi *et al*., 1991; Katakura *et al*., 1994; Le Pont *et al*., 1994). Armijos and others (1991) have reported on *Leishmania* species obtained from 6 different provinces located at north of the country. From our results and other reports, it is suggested that there is a principal distribution pattern of Ecuadorian *Leishmania* species as follows. The major *Leishmania* species of the Pacific coastal lowland area is *L. (V.) panamensis*. *L. (L.) amazonensis*, *L. (V.) panamensis* and *L. (V.) equatorensis* distribute in the subtropical regions of the west Andean slopes extending from north to south of this country. In the Andean highland regions, *L. (L.) mexicana* and *L. (L.) major*-like parasites distribute. *L. (V.) braziliensis* is mainly found in the Amazonian regions.

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3. Nucleotide Sequence Variations in the Cytochrome C Oxidase Subunit I Gene within *Leishmania* Species

ABSTRACT. Determination of partial nucleotide sequences of COI genes of eight species of *Leishmania, i e., L. (Leishmania) amazonensis, L. (Viannia) equatorensis, L. (L.) major*-like, *L. (L.) mexicana, L. (L.) major, L. (V.) guyanensis, L. (V.) braziliensis* and *L. (V.) panamensis* was tried by a PCR method using primers designed for planarian COI genes. The present study showed that the primers are useful for the amplification of COI genes of four species, *i. e., L. (L.) major, L. (V.) guyanensis, L. (V.) braziliensis* and *L. (V.) panamensis.* A partial sequence of COI genes of the four species was determined.

Introduction

The species identification among the genus *Leishmania* had been performed by zymodeme, serodeme, schizodeme and karyodeme analyses. Recently, molecular biological techniques have been rapidly developed and the sequences of various genes have been used for molecular phylogenetic studies of different organisms. We have been trying to apply the nucleotide sequence of the cytochrome c oxidase subunit I gene for the identification of parasite and insect species, *i. e., Paragonimus, Dirofilaria, Anopheles*, and etc.

The mitochondrial DNA (mtDNA) of animals is a relatively small molecule, usually shorter than 16kb long and generally encodes 13 proteins, two ribosomal RNA and 22 tRNA. The small size, relatively rapid rate of evolutionary change, and maternal haploid inheritance of mtDNA make it suitable for examining population history and structure among closely related taxa. For example, cytochrome c oxidase subunit I and II (COI and COII) genes have been used to study the phylogenetic relationships of honeybee or the land snail (Crozier *et al*., 1989; Lecanidou *et al*., 1994). In the present study, we sequenced segments of the COI gene (391bp out of about 1500bp) to examine the intraspecific variation of the genus *Leishmania*.

Materials and Methods

Parasites

All the isolates of *Leishmania* were obtained from the collection of Kochi Medical School. Species name and designation of the isolates used are shown in Table 2.3.1. Eight species of *Leishmania* organisms were cultured in Schneider's drosophila medium containing 15% heat-inactivated fetal bovine serum. The harvested promastigotes were washed two times by centrifugation with phosphate buffered saline. The washed parasites were immediately fixed with and reserved in absolute ethanol. The materials were kept in refrigerator until used for DNA extraction.

Preparation of DNA

Total DNA was prepared from the materials preserved in absolute ethanol. Extraction buffer consisted of 50 mM Tris-HCl (pH 9.0) containing 10 mM EDTA, 200 mM NaCl, 0.5% (w/v) SDS and 200 μg/ml proteinase K. The resultant viscous solution was extracted several times with an equal volume of phenol that had been equilibrated with 1M Tris-HCl (pH 8.0) and then extracted 2 or 3 times with phenol/chloroform (1:1) and once with chloroform-isoamyl-alcohol (24:1). After an additional chloroform extraction, the nucleic acid was precipitated with 2.5 volumes of cold absolute ethanol containing 300 mM sodium acetate and then chilled at -80℃ overnight. After centrifugation at 14000 rpm for 30 min at 0℃, the pellet was dissolved in 200 μ1 of TE buffer (10 mM Tris-HCl (pH 7.6), 1 mM EDTA).

Sequencing strategies

PCR products were sequenced from both ends using a dye-primer cycle sequencing kit and a Model 377 DNA sequencer (Applied Biosystems). Double-stranded PCRs were performed to prepare insertions for clonings. The sequences of the primers used in the PCR were as follows: pri-a 5' TGG TTT TTT GTG CAT CCT GAG GTT TA3' and pri-b 5' AGA AAG AAC GTA ATG AAA ATG AGC AAC3'. These primers were originally designed for amplification of the planarian COI gene (Bessho *et al*., 1992a,b). PCR amplifications were performed according to the manufactures' instructions, with denaturation at 94℃ for 1 min (except for the first cycle for 4 min), annealing at 45℃ for 1 min and extension at 72℃ for 30 cycles, followed by final extension step of 7 min at 72℃. The PCR products were electrophoresed in 1% agarose 1600 gel in TAE buffer at 100 v for 30 min. Bands of about 450 bp were carried out by knife cutting under ultraviolet illumination. QIAquick spin PCR purification kit (Qiagen) was used for the extraction of PCR products. The extracted PCR products were ligated in pT7blue T-vector plasmid, and the vectors were transformed in NovaBlue competent cells with pT7blue T-vector kit (Novagen). The amplified plasmids were extracted with Wizard miniprep purification kit (Promega). Sequencing reactions were carried out with dye-primer cycle sequencing kit (Applied Biosystems and Amersham). The dye-primers used were T7 promoter primer and U-19 mer primer and the thermocycling was done at the condition of denaturation at 95℃ for 10 sec, annealing at 55℃ for 30 sec and extension at 70℃ for 1 min for 15 cycles followed with another 15 cycles of denaturation at 95℃ for 10 min and extension at 1 min. Sequence analysis was performed using a Model 377 DNA autosequencer (Applied Biosystems). DNA sequence data were aligned and analyzed using GENTYX computer software (Higgins *et al*., 1992). The nucleotide sequence of each species was basically determined with the accordance of three clone sequences read from both ends.

Results and Discussion

Eight species materials of *Leishmania* were used for PCR and only four showed a single PCR product with the expected size (about 450 bp) in ethidium-bromide stained agarose gel, *i. e., L. (V.) panamensis, L. (V.) guyanensis, L. (V.) braziliensis* and *L. (L.) major*. No PCR product was observed in other species. The reason why PCR products could not be found in the remaining species is not clear. The difference of these results may depend on the peculiar editing mechanism in trypanosomatid mitochondria. Clarifying the editing mechanism of each species may show the reason, but it was shown that the present primers are useful for PCR at least in the four species of *Leishmania* mentioned above.

Nucleotide sequences of 438 bp in the four species, in which whole sequences were amplified by the first PCR, were generally obtained (Fig 2.3.1). Some intraspecific differences in nucleotide sequences were observed in each species. Heterogeneity of cytochrome c oxidase subunit 1 gene sequences has been reported in the freshwater planarian having asexual reproduction (Bessho *et al*., 1992a,b). In planarians, not only interindividual heterogeneity but also heteroplasmy within an individual was reported. Further such a study will clarify the variation of the sequences in the genus *Leishmania*. Out of the 438 surveyed nucleotide positions, 42 variant nucleotide positions were found. The numbers of variant positions between two species are shown in Table 2.3.1. The closest possitive identical sequence of the present four species was that of kinetoplast of *L. tarentole* in the GeneBank nucleic acid sequence database. The poisson probabilities with *L. (V.) braziliensis, L. (V.) panamensis, L. (V.) guyanensis* and *L. (L.) major* were 1.7e-114, 1.0e-113, 7.0e-112 and 2.2e-103, respectively. The secondary identical sequence was that of *Trypanosoma brucei* mitochondrial maxicircle DNA. The poisson probabilities were: *L.(V.) braziliensis*, 2.8e-103; *L. (V.) panamensis*, 5.3e-102; *L. (V.) guyanensis*, 9.8e-99; and *L. (L.) major*, 4.5e-91. Further studies will be done to compare the nucleotide sequence of some pre-

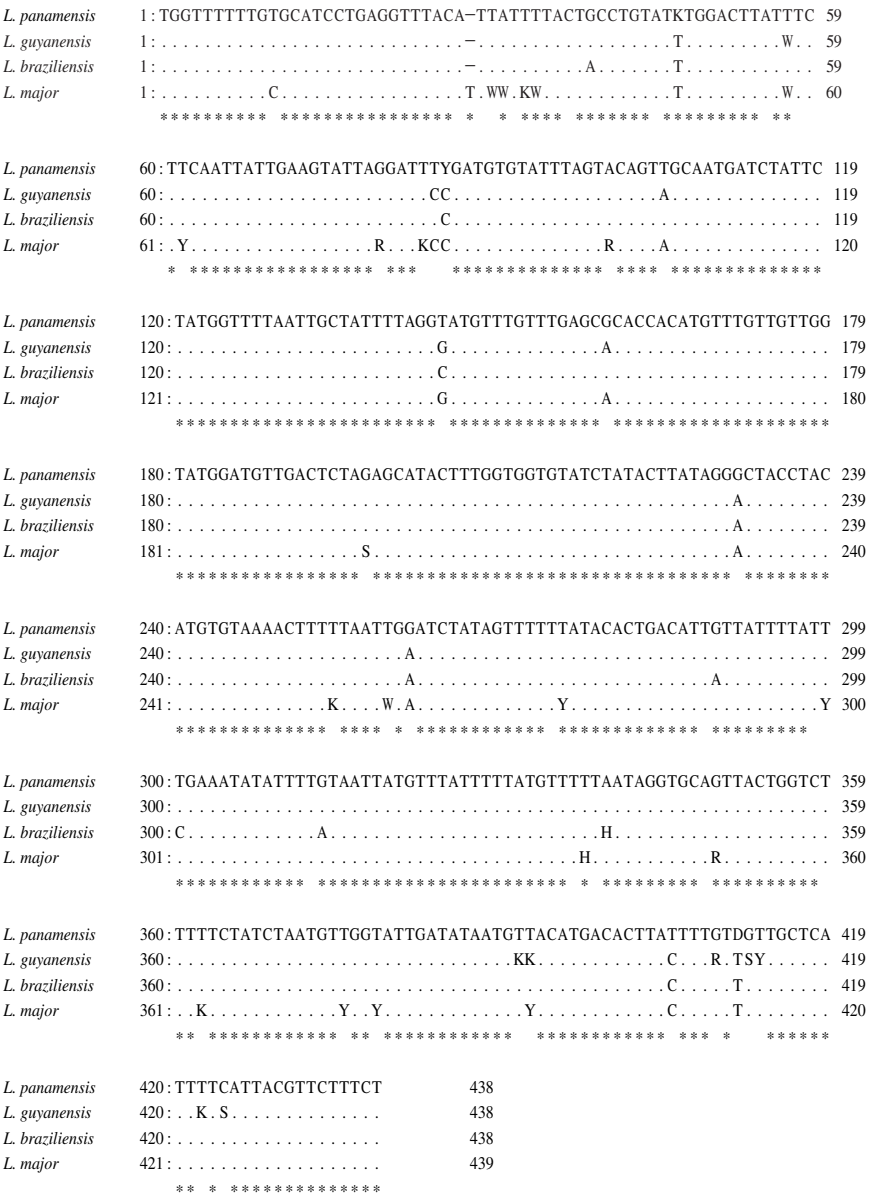

Figure 2.3.1. Nucleotide sequences of a 438 bp fragment of the mitochondrial COI gene from four species of *Leishmania* (W:A/T, R:A/G, M:A/C, K:T/G, Y:T/C, S:G/C. H:A/T/C).

Table 2.3.1. *Leishmania* isolates used in the present study

Species Name	Designation
Leishmania (L.) amazonensis	MHOM/BR/73/M2269
Leishmania (V.) equatorensis	MCHO/EC/82/Lsp1
Leishmania (L.) major-like	MHOM/EC/87/G-09
Leishmania (L.) mexicana	MHYC/BZ/62/M379
Leishmania (L.) major	MHOM/SU/73/5ASKH
Leishmania (V.) guyanensis	MHOM/BR/75/M4147
Leishmania (V.) braziliensis	MHOM/BR/75/M2904
Leishmania (V.) panamensis	MHOM/PA/71/LS94

Table 2.3.2. Numbers of variant positions of nucleotide sequences between two species of *Leishmania*

Species	*L. (V.) panamensis*	*L. (V.) guyanensis*	*L. (V.) braziliensis*	*L. (L.) major*
L. (V.) guyanensis	7	—	—	—
L. (V.) braziliensis	7	7	—	—
L. (L.) major	9	2	10	—

ferrable genes for the establishment of species identification in the genus *Leishmania*.

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4. A Preliminary Study on Nucleotide Sequence Variations of *Lutzomyia* spp. in the Cytochrome C Oxidase Subunit I Gene

ABSTRACT. Partial nucleotide sequences of COI genes of the following three species: *Lutzomyia ayacuchensis*, *L. hartmanni* and *L. trapidoi* were determined by PCR method using primers designed for planarian COI genes. The results obtained showed that the primers were useful for the amplification of COI genes of these sandfly species. In the current study, a partial sequences of COI genes of the three species (441 bp) was determined.

Introduction

Species identification among the genus *Lutzomyia* has been mainly done based on external and internal morphological features. Recently, methods using isozyme electrophoresis patterns and karyotype have been also introduced for the species characterization of the organisms. Moreover, the sequences of various genes have been used for molecular phylogenetic studies. We have been trying to apply the nucleotide sequence of the cytochorome c oxidase subunit I gene for the identification of parasite and insect species. In the present study, we sequenced segments of the COI gene (441 bp out of about 1500 bp) to examine the intraspecific variation of the genus *Lutzomyia*.

Materials and Methods

Insects

All the adult flies collected by a protected human-bait method in Ecuador were preserved in 100% ethanol and were brought back to Japan. The materials were kept in refrigerator until required for DNA extraction. Sandfly species and their collection sites are shown in Table 2.4.1.

Preparation of DNA

Total DNA was prepared from the materials preserved in absolute ethanol. Extraction buffer consisted of 10 mM Tris-HCl (pH8.0) containing 10 mM EDTA, 150 mM NaCl, 0.1% (w/v) SDS and 200 μg/ml proteinase K. Each sandfly was homogenated with the 200 μl of extraction buffer. The resultant viscous solution was extracted twice with equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). The phenol had been equilibrated with 1 M Tris-HCl (pH8.0). The nucleic acids were precipitated with 500 μl of cold absolute ethanol containing 20 μl of 3 M sodium acetate and then chilled at -80℃ over night. After centrifugation at 14000 rpm for 30 min at 4℃, the pellets were washed with 1000 μl of 70% ethanol and dissolved in 20 μl of TE buffer (10 mM Tris-HCl (pH7.6), 1 mM EDTA).

Sequencing strategies

See Chapter 2.3 in this text.

Results and Discussion

Three species of the genus *Lutzomyia*, *L. ayacuchensis*, *L. hartmanni* and *L. trapidoi* were used for PCR and all the species showed a single PCR product with the expected size (about 450 bp) in ethidium-bromide stained agarose gel. The same primers had been also useful in PCR of *Anopheles* mosquitoes. Therefore, these primers may be one of the most preferrable for PCR amplifications of insect COI genes. Nucleotide sequences of 441 bp in the three species, in which whole sequences were amplified by the first PCR, were generally obtained (Fig. 2.4.1). A few

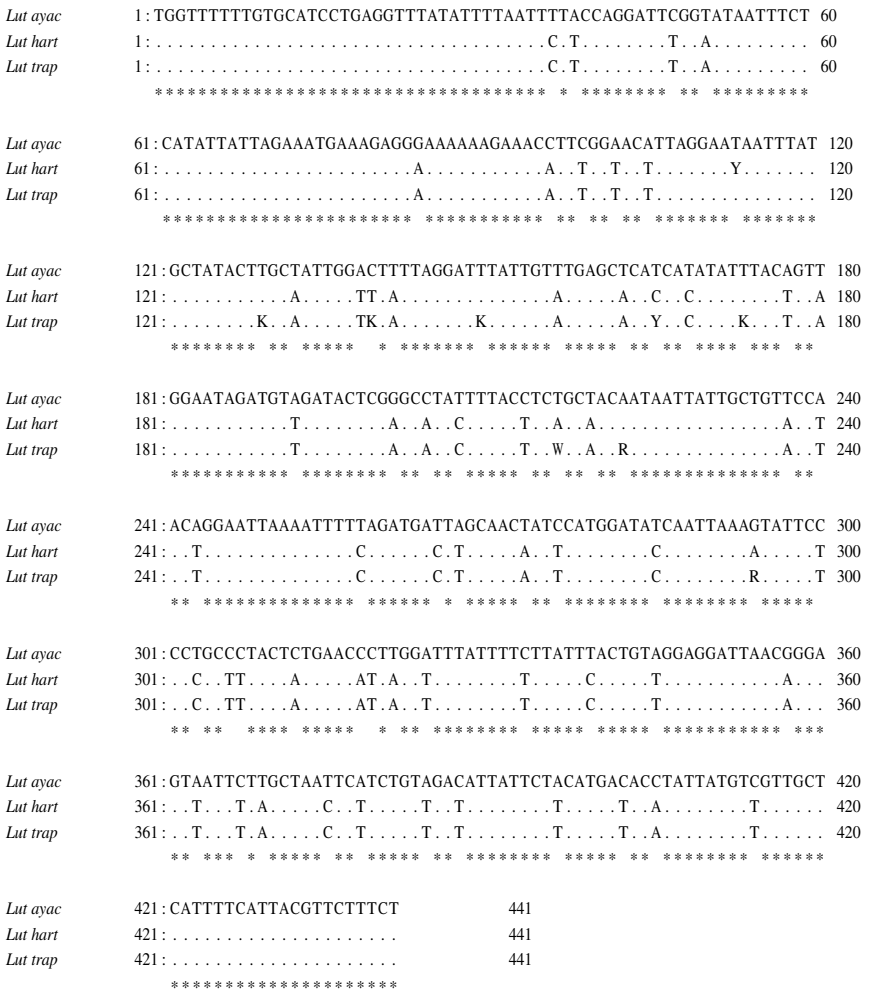

Figure 2.4.1. Nucleotide sequences of a 441 bp fragment of the mitochondrial COI gene from three species of *Lutzomyia*, *ayacuchensis* (*Lut ayac*), *hartmanni* (*Lut hart*) and *trapidoi* (*Lut trap*).

Table 2.4.1. Species and collected places of the sandflies used in the present study

Species	Place	Date (1996)
L. ayacuchensis	Huigra	August 8
L. hartmanni	Ocaña	July 27
L. trapidoi	Ocaña	July 27

Table 2.4.2. Numbers of variant positions of nucleotide sequences between two species of *Lutzomyia*

Species	*L. ayacuchensis*	*L. hartmanni*
L. hartmanni	60	—
L. trapidoi	56	8

intraspecific difference in nucleotide sequences were observed in each species. Out of the 441 surveyed nucleotide positions, 65 variant nucleotide positions were found. The numbers of variant positions between two species are shown in Table 2.4.1. The nearest positive identical sequence of *L. ayacuchensis* was that of *Drosophila melanogaster* mitochondria in the GeneBank nucleic acid sequence database with an identity of 83% (poisson probability: 4.2e-121). On the other hand, in the cases of *L. hartmanni* and *L. trapidoi*, the nearest positive identical sequence was that of *Feltia jaculifera* (dingy cutworm) COI gene; the identities were 85% and 84% with the poisson probabilities, 6.2e-127 and 5.4e-127, respectively. Further studies on other species of the genus *Lutzomyia* will be done. The differences of nucleotide sequences of genes will be applied for the establishment of species identification and phylogenetic studies of the genus *Lutzomyia* in the future.

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Chapter 3

Ultrastructural and Pathological Aspects

1. An Ultrastructural Study of Diffuse Cutaneous Leishmaniasis Caused by *Leishmania (Leishmania) mexicana* in Ecuador: the Role of Parasitophorous Vacuole in Macrophage-Parasite Interaction

ABSTRACT. In the current study, an electron microscopic observation was done on the skin biopsy materials taken from a diffuse cutaneous leishmaniasis (DCL) and a localized cutaneous leishmaniasis (LCL) patients in Ecuador. Large parasitophorous vacuoles (PVs) and disconnected cell membranes of *Leishmania* amastigotes were observed only in DCL. The margin of the cell membrane of disconnected site was bended outward. These findings support that proteo-high-molecular weight phosphoglycan (proteo-HMWPG) was released from the disconnected site of the membranes of the amastigotes, and that production of proteo-HMWPG was accelerated in DCL more than in LCL.

Introduction

Cutaneous leishmaniasis is a parasitic disease caused by various types of *Leishmania* spp. Man and domestic animals are infected by sandfly, a vector, with *Leishmania* parasites in their promastigote forms. Promastigotes are taken in the macrophages and then transform into the form of amastigotes. Amastigotes are reproduced by binary fission inside parasitophorous vacuoles (PVs) of the host macrophage. Light-microscopically, diffuse cutaneous leishmaniasis (DCL) shows numerous *Leishmania* parasites in specimens taken from the skin lesions of leishmaniasis. On the contrary, in localized cutaneous leishmaniasis (LCL), few *Leishmania* parasites can be seen in specimens taken from the skin lesions. Ultrastructural observation shows one parasitophorous vacuole in one *Leishmania* parasitized macrophage. Veress *et al.* (1994) pointed out the possibility that the presense or absense of a PV was the morphological sign of parasite survival. DCL is resistant to the traditional treatments, but LCL has a good response to them. LCL also shows natural healing. It may be difficult for *Leishmania* parasites to survive in LCL. To investigate difference in PV-parasite interaction between DCL and LCL, we performed the ultrastructural observation using the skin samples taken from DCL and LCL patients.

Materials and Methods

Patient history

A DCL patient, caused by *L.* (*Leishmania*) *mexicana,* was a 24-year-old male, having suffered from it for 8 years. The lesions were present almost all over the body, and revealed typical clinical features of DCL. The lesions showed papules, infiltrated plaques and nodules without ulcerations. The lesions immediately improved after treatments with Glucantime®, but relapsed intermittently with five or six months intervals after clinical improvement (Reyna *et al*., 1994).

A LCL patient, caused by *L.* (*Viannia*) *panamen-*

sis, was a 13-year-old male, having suffered from it for 3 months. The lesions showed single ulcer on the dorsum of the right hand, and revealed typical clinical features of LCL. (see Chapter 3. 2 about the clinical photographs of DCL and LCL cases in this text).

Electron microscopy

The tissue samples were biopsied from a nodule of the DCL patient and from the margin of the ulcer of the LCL patient. The samples were cut into small pieces, and fixed in phosphate-buffered 2% glutaraldehyde solution for electron microscopy. The samples were washed with phosphate-buffered 1% osmium tetroxide, and then dehydrated with an ethanol series and propylene oxide, and embedded in Epon 812 resin. Ultrathin sections of the samples were stained with uranyl acetate and lead citrate, then observed by JEOL 2000 electron microscopy.

Results

DCL

Macrophages attached closely with each other. A few lymphocytes and plasma cells were observed among the macrophages. Most of the macrophages showed various sized PVs. One large PV contained several *Leishmania* amastigotes, while another small PV contained one or two (Fig. 3.1.1A) . The shapes of *Leishmania* amastigotes were round and their diameters ranged from 2.1 to 3.5 μm. Each amastigote had a nucleus. The cell organells of the parasites showed well developed (Fig. 3.1.1B). The PV was filled with electron dense granules and a notch like structure was found in the PV (Fig. 3.1.1C). The cell membranes of *Leishmania* amastigotes in the PV disconnected at some places. The margin of the cell membrane at the disconnected site was bended outward of the amastigotes (Fig. 3.1.1D, E).

LCL

Amastigotes-phagocyted macrophages were scatterezd in the dermis. Several lymphocytes were observed gathering around the macrophage (Fig. 3.1. 2A). Amastigotes showed round or oval shape and their diameters were 2.1 to 2.8 μm. The cell organells of the amastigotes were well developed. No disconnecected site of the cell membrane of the amastigotes was found. Rough endoplasmic reticulums (RERs) of the macrophages in LCL were enlarged more than those in DCL (Fig. 3.1.2B). The cytoplasms of some macrophages showed degenerative appearances (Fig. 3.1.2C). No large PV could be observed (see Chapter 3. 2 about H. E. staining findings of DCL and LCL in this text).

Discussion

The mechanism(s) of survival of *Leishmania* amastigote is still unknown though PV is thought to be a morphological sign of parasite survival. It was reported that PV was common structure of parasitized macrophage not in human but also in hamster (Veress *et al*., 1981), and that no large PVs had been observed in human cutaneous leishmaniasis by any investigators. Nor we could find the papers describing large PV in LCL cases to our knowledge. However, Schurr *et al*. (1987) reported that the macrophages of DCL showed larger PVs than those of LCL. In our DCL case, large PVs were observed in numerous number of the macrophages, light- and electron-microscopically. Several experiments showed that large PVs of macrophages were found in animal models treated with inoculation of *Leishmania* parasites derived from human cutaneous leishmaniasis including DCL and LCL patients (Chang *et al*., 1987; Bretana *et al*., 1983; Bhutto *et al*., 1994). Thus, large PV formation may be resulted not to the factors in *Leishmania* parasite side but in host side.

In *Leishmania* (*L*.) *mexicana*, the amasigotes secrete proteo-phosphoglycan from the flagellar pockets (Fig. 3.1.3). Proteo-high-molecular weight phosphoglycan (proteo-HMWPG) appears to be a characteristic secretory product of *L*. (*L*.) *mexicana* amastigotes, which is released into the PV of the macrophages (Ilg

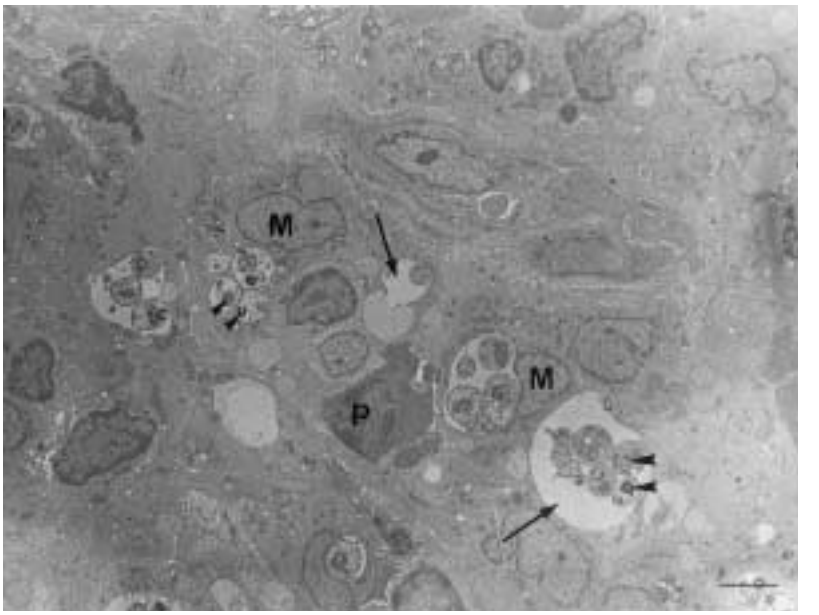

Figure 3.1.1A. Various sized PVs are found in macrophages in DCL. Each PV contains several numbers of *Leishmania* amastigotes. Arrow, PV; arrow head, *Leishmania* amastigotes; M, macrophage; P, plasma cell. Bar = 5 μm.

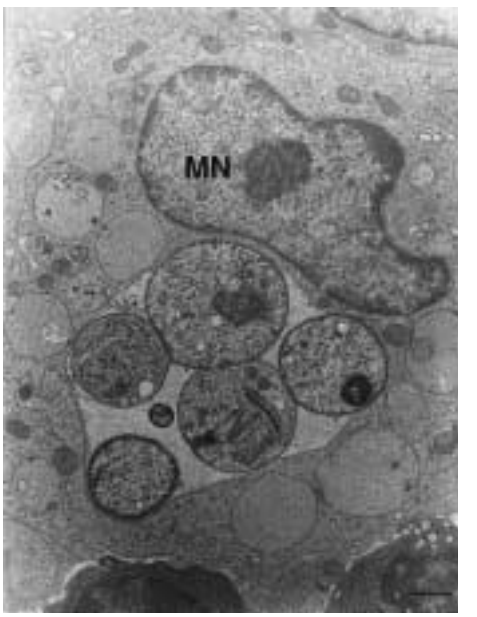

Figure 3.1.1B. *Leishmania* amastigotes show round shapes and their diameters ranged from 2.1 to 3.5 μm. MN, Nucleus of macrophage. Bar = 1 μm.

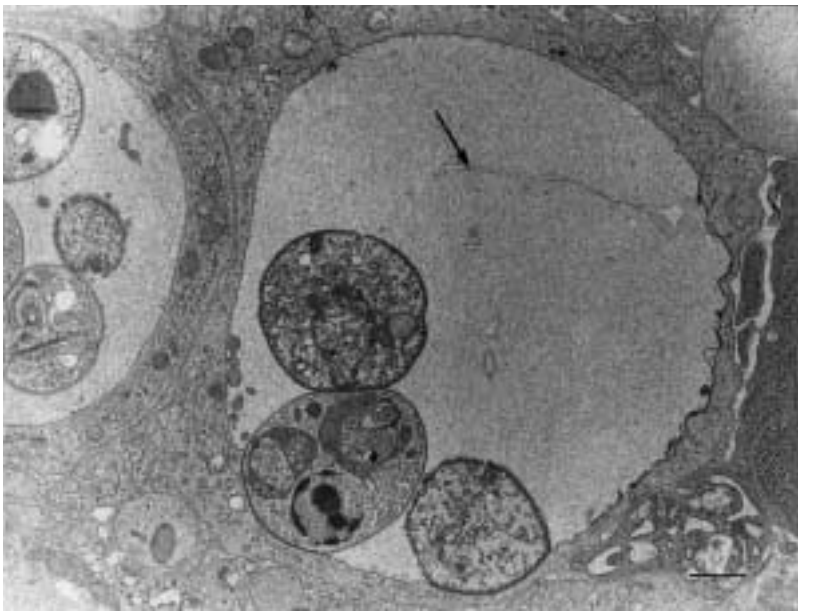

Figure 3.1.1C. The PV was filled with electron dense granules and a notch-like structure (arrow) was found in PV. Bar = 1 μm.

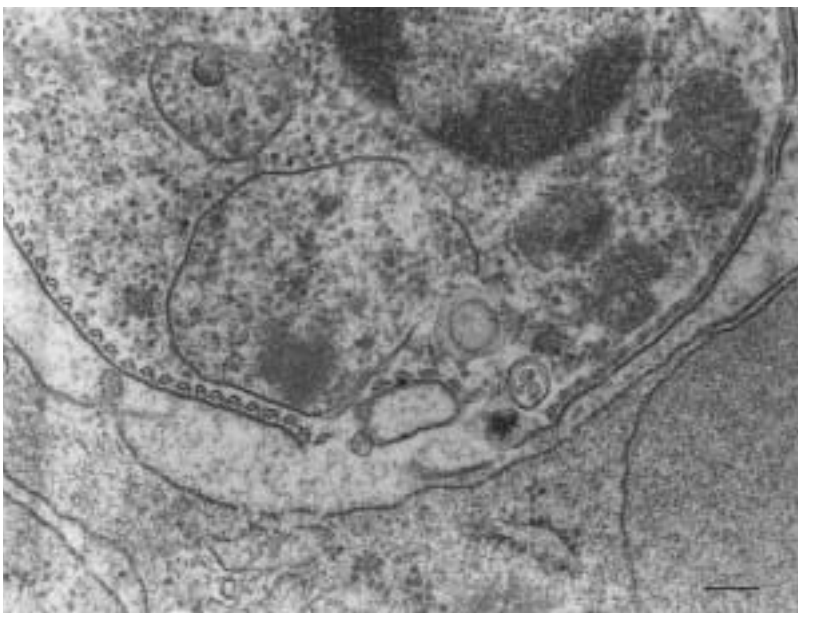

Figure 3.1.1D. The cell membranes of *Leishmania* amastigotes in PV were disconnected at some places. The margin of the cell membrane at the site was bended outward. Bar = 200 nm.

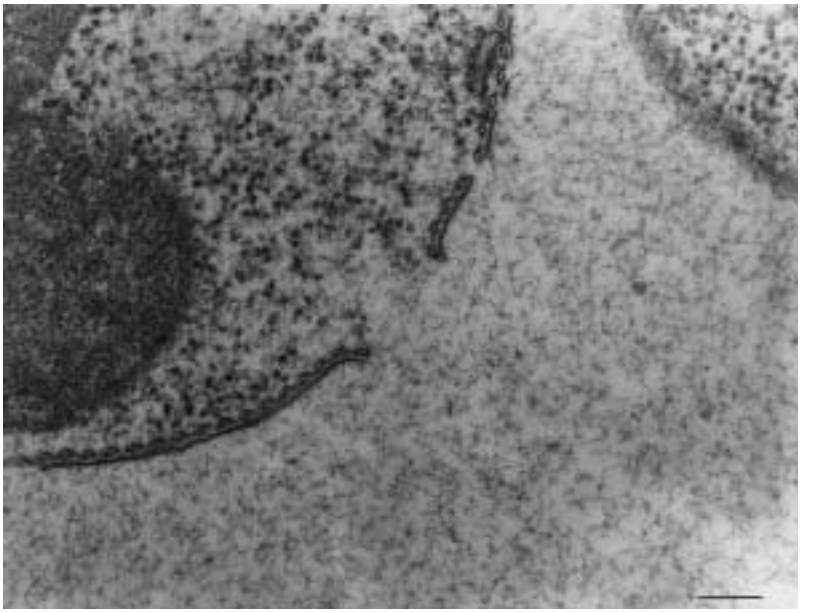

Figure 3.1.1E. See Figure 3.1.1D.

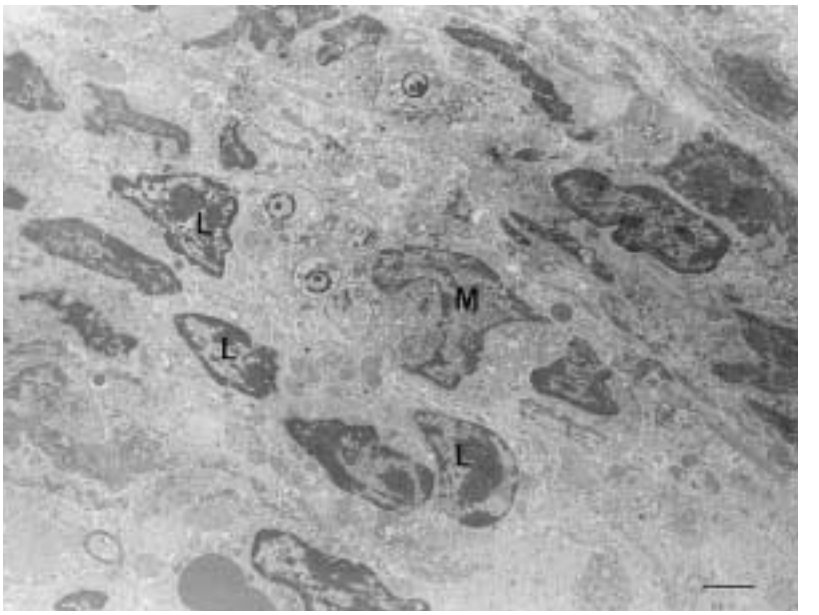

Figure 3.1.2A. Amastigotes-phagocyted macrophages are found in LCL. Several lymphocytes gather around the macrophage. M, macrophage; L, lymphocyte. Bar = 2 μm.

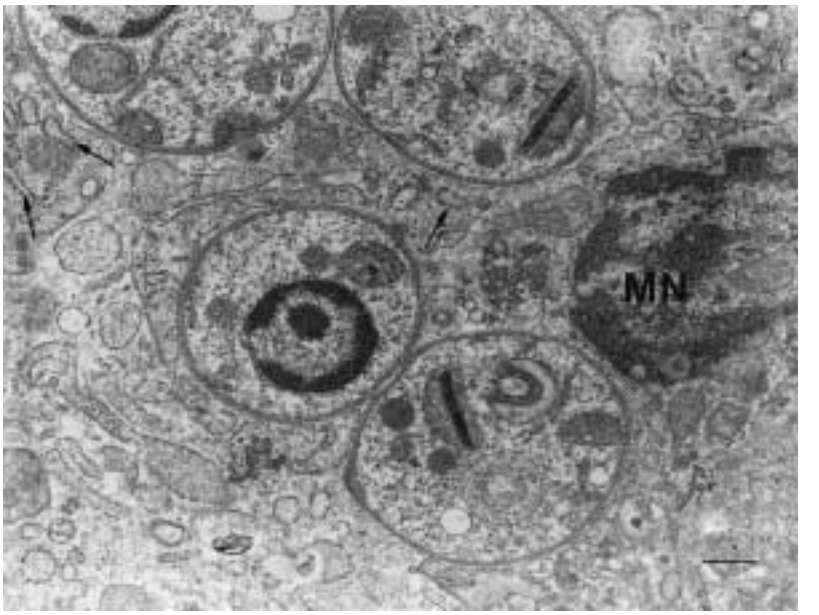

Figure 3.1.2B. Amastigotes show round or oval shape and their diameters are 2.1 to 2.8 μm. The cell organells of amastigotes are well developed. RERs of the macrophages are enlarged. MN, nucleus of macrophage; arrow, RER. Bar = 500 nm.

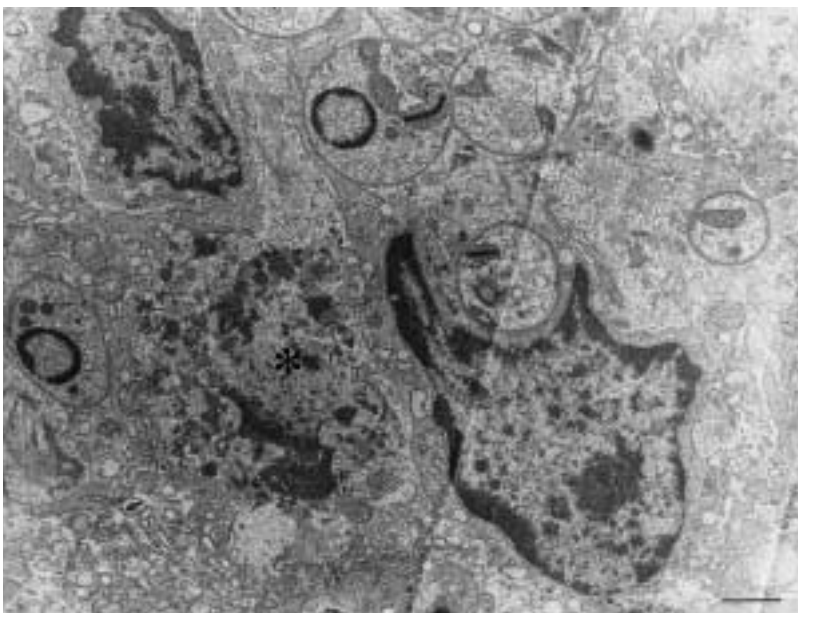

Figure 3.1.2C. Asterisk shows degenerated macrophage. Bar = 1 μm.

et al., 1994). Our electron microscopic observations revealed that the cell membranes of amastigotes in large PV of DCL were disconnected and the margin of the membrane was bended outward. Furthermore, no large PV or disconnected cell membrane of amastigotes were found in LCL. These findings support that the secretion of proteo-HMWPG from *Leishmania* amastigotes is accelerated in DCL more than in LCL.

DCL is considered to be manifestations of specific T cell anergy, and also known as split tolerance (Ulrich *et al*., 1995). It is well known that IL-1 produced by T cells activates macrophages. Our previous immunohistochemical study using anti-T cell antibody revealed that T cells gathered not around *Leishmania* parasitized macrophages in DCL but around those in LCL, and that the macrophages in DCL were activated less than those in LCL (see Chapter 3. 2 about the immunohistochemical study in this text). It is unknown whether large PV formation is inhibited by activation of macrophages or not. C3H/HeN mice are naturally resistant to cutaneous and systemic infections with the protozoan parasites. In previous experiment using *Leishmania* parasitized mice, anti-IFN-γ -treated C3H/HeN mice developed cutaneous lesions, and the parasites spreaded to the regional lymph nodes, and then metastasized to the spleens and livers (Belosevic *et al*., 1989). As a further experiment, relationship between PV formation and macrophage activating factor, such as IFN-γ, should be investigated.

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2. Diffuse Cutaneous Leishmaniasis: the Pathological Difference Between Localized and Diffuse Forms

ABSTRACT. A case of diffuse cutaneous leishmaniasis (DCL) and two cases of localized cutaneous leishmaniasis (LCL) were examined immunohistochemically by using anti-T cell, CD45RO antibody and anti-lysozyme antibody. T-lymphocytes infiltrated throughout the dermis and were observed surrounding the macrophages in the LCL lesions. In contrast to this, the macrophages in the DCL lesions were not activated in comparison with those in the LCL lesions. It is suggested that the macrophages may not play their role as antigen presenting cells in the DCL lesions.

Introduction

Cutaneous leishmaniasis is clinically divided into classical, diffuse and disseminated types. Clinical manifestations of diffuse cutaneous leishmaniasis (DCL) show so many nodules and papules on the whole body, but no ulcer. Those of localized cutaneous leishmaniasis (LCL), on the contrary, show one or several papules, nodules and ulcers, of which margin elevates like bank (Hashiguchi, 1996). *Leishmania* parasites are seen countlessly in DCL, in contrast to this, very few *Leishmania* parasites can be seen in LCL (Carvalho *et al*., 1994). *Leishmania* parasites proliferate in the cytoplasm of the host macrophage, but *Leishmania* parasite-macrophage interaction is not well known. We performed immunohistochemical study with anti-lysozyme antibody to investigate the activities of the macrophages in DCL and LCL specimens and also performed immunohistochemical study with anti-T cell and B cell antibodies to investigate their relationship with macrophages. We compared the macrophages in both cases electron-microscopically.

Materials and Methods

The tissue samples were biopsied from one case of the DCL patient (Fig. 3.2.1A, B) and two cases of the LCL patients (Fig. 3.2.1C) in Ecuador, South America. The samples were taken from the center of the nodule in the DCL patient and taken from the margin of the ulcer in the LCL patients. The samples were cut into two pieces. One was fixed in 10% formalin solution for light microscopy. The other was cut into small pieces, and fixed in phosphate-buffered 2% glutaraldehyde solution for electron microscopic examination.

Light microscopy

The tissue samples were dehydrated in an ethanol series, and embedded in paraffin. The samples were sectioned 4 μm thickness for H. E. staining and immunohistochemical staining with anti-lysozyme antibody, anti-human T cell, CD45R0 antibody and anti-human B cell, CD20 antibody (Dako, Kyoto, Japan). Immunohistochemical staining was performed with DAKO LSAB kit (Dako, Kyoto, Japan). Steps in staining of dehydrated sections were as follows: 1) blocking with 3% hydrogen peroxidase; 2) incubation with diluted normal bovine serum for 5 min to block nonspecific reactions; 3) incubation with each first antibody for overnight at 4℃; 4) incubation with biotinylated anti-mouse IgG for 10 min at room temperature; 5) incubation with avidine-biotine-peroxidase complex for 10 min at room temperature; 6) placement in streptavidine in Tris-HCl buffer; 7) color development by immersion in the AEC solution containing 0.005% H_2O_2 for 30 sec at room temperature; 8) counterstaining of sections with hematoxyline; 9) coverslipping with AQUATEX solution (Merck Co., Tokyo, Japan).

Electron microscopy

The samples were washed with phosphate-buffered 1% osmium tetroxide, and then dehydrated with an ethanol series and propylene oxide, and embedded in Epon 812 resin. Ultrathin sections of the samples were stained with uranyl acetate and lead citrate, then observed by JEOL 2000 EX electron microscopy.

Results

H.E. staining

DCL

No cell infiltration into the epidermis was seen, though the epidermis was slightly thin. Dense cell infiltration was observed down to the lower dermis through clear zone which existed just beneath the epidermis (Fig. 3.2.2A). Dense cell infiltration consisted of lymphocytes, a few plasma cells and numerous macrophages with big vacuoles. Most of the macrophages contained one or several parasites within their vacuoles (Fig. 3.2.2B).

LCL

The epidermis showed parakeratosis, hyperkeratosis and cell infiltration of lymphocytes. Dense cell infiltration was observed throughout the dermis without clear zone as seen in DCL (Fig. 3.2.2C). Dense cell infiltration consisted of lymphocytes, plasma cells and macrophages without big vacuoles. Parasites were observed only in a few macrophages.

Immunohistochemical staining

DCL

Anti-lysozyme positive cells were observed throughout the dermis (Fig. 3.2.3A), but each macrophage was weakly stained. By the both anti-B cell and anti-T cell antibody staining, the majority of positive cells existed at the lower dermis but a few among the group of the macrophages (Fig. 3.2.3B).

LCL

Anti-lysozyme positive cells were scattered throughout the dermis, and each macrophage was stained more strongly than that of DCL (Fig. 3.2.3C). Positive cells to anti-B cell and anti-T cell antibody were observed throughout the dermis and T lymphocytes infiltrated to the epidermis (Fig. 3.2.3D, E) .

Electron microscopy

DCL

Macrophages attached closely with each other. Lymphocyte and plasma cell were observed between the macrophages. Most of the macrophages showed various sized parasitophorous vacuoles (PVs). The big PV contained several *Leishmania* parasites, while the small PV contained one or two. The PV was filled with low electron dense granules and the space was observed at some area. The lack of cell membrane of *Leishmania* parasite in PV was observed.

LCL

Leishmania parasites could be seen in the macrophages, but the number of parasites was too less than those of DCL. In addition, the PVs were so small to be seen clearly.

Discussion

Histopathologically in cutaneous leishmaniasis, varying numbers of lymphocytes, plasma cells and macrophages infiltrate the lesions. Eventually the lesions become granulomatous. Numerous numbers of *Leishmania* parasites are present in DCL lesions, but few in LCL tissue. The epidermis have a normal appearance in DCL nodule (Bittencourt *et al*., 1992). Our DCL epidermis was also showed almost normal appearance but cell infiltration was shown in LCL epidermis. DCL is considered to be manifestations of specific T cell anergy, also known as split tolerance (Bittencourt *et al*., 1992). The previous report has described that IL-1 β mRNA was expressed in LCL epidermis but abscent in most DCL samples using the RT-PCR, and has also described that DCL epi-

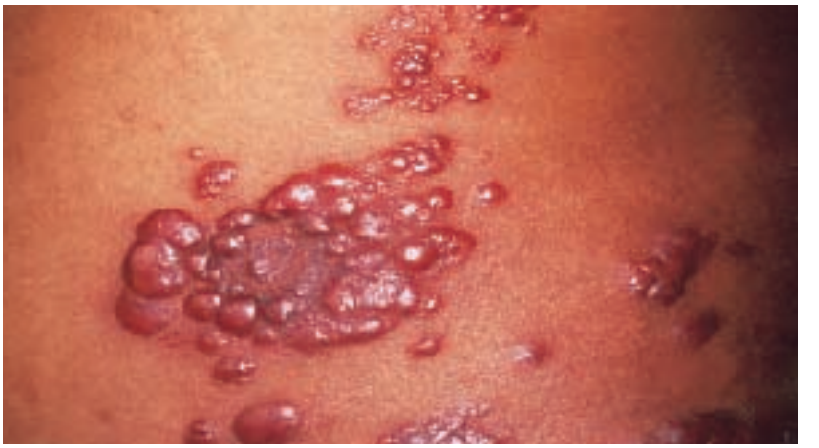

Figure 3.2.1A. Clinical appearance of the DCL patient's body.

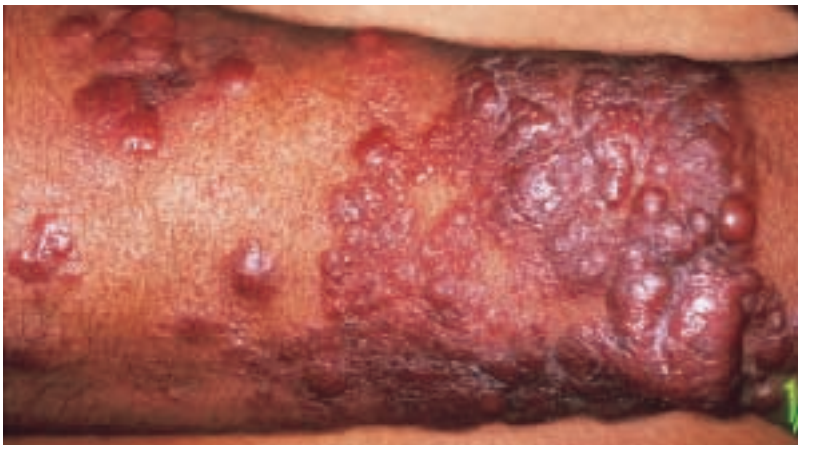

Figure 3.2.1B. Clinical appearance of the DCL patient's foot.

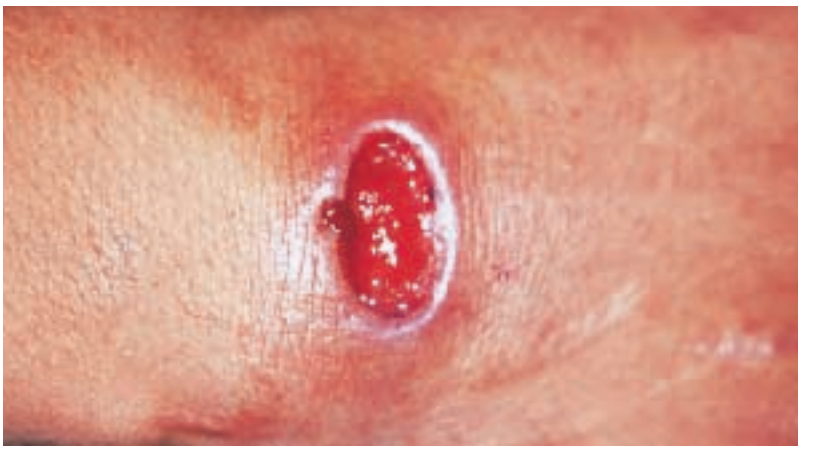

Figure 3.2.1C. Clinical appearance of the LCL patient' forearm patient's forearm.

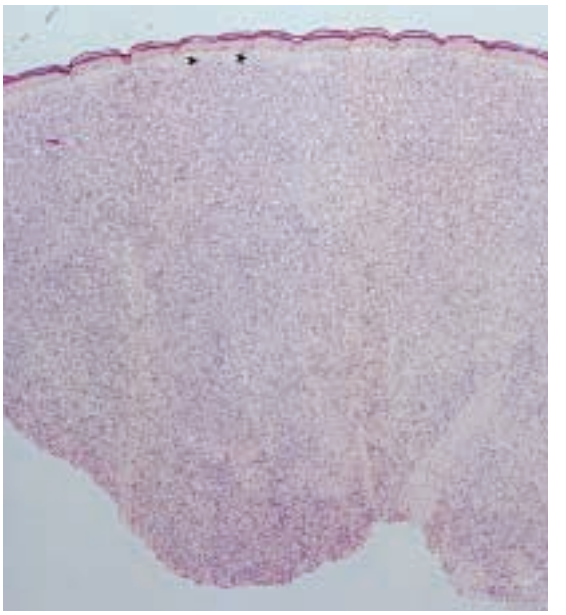

Figure 3.2.2A. H. E. staining of the DCL lesion, asterisk: clear zone (×40).

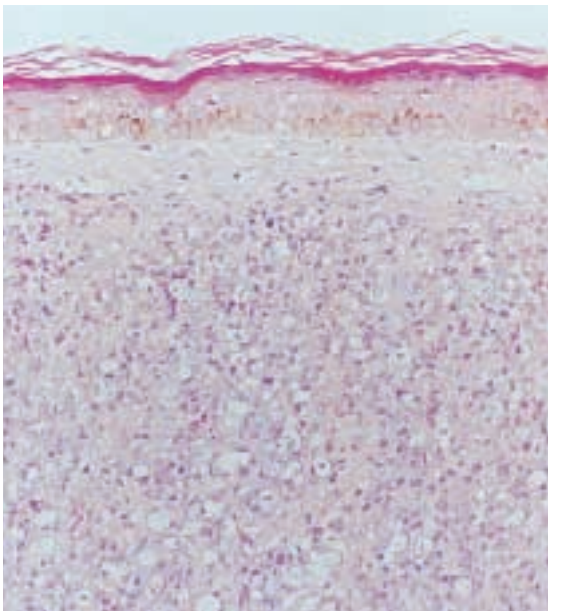

Figure 3.2.2B. H. E. staining of the DCL lesion. *Leishmania* parasites were observed in the vacuole of the macrophages (×200).

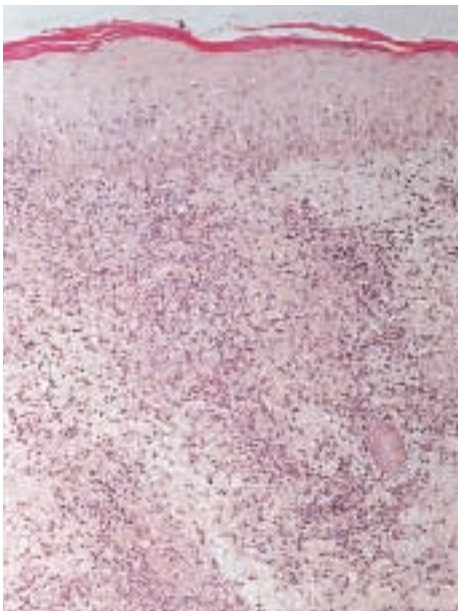

Figure 3.2.2C. H. E. staining of the LCL lesion (×100).

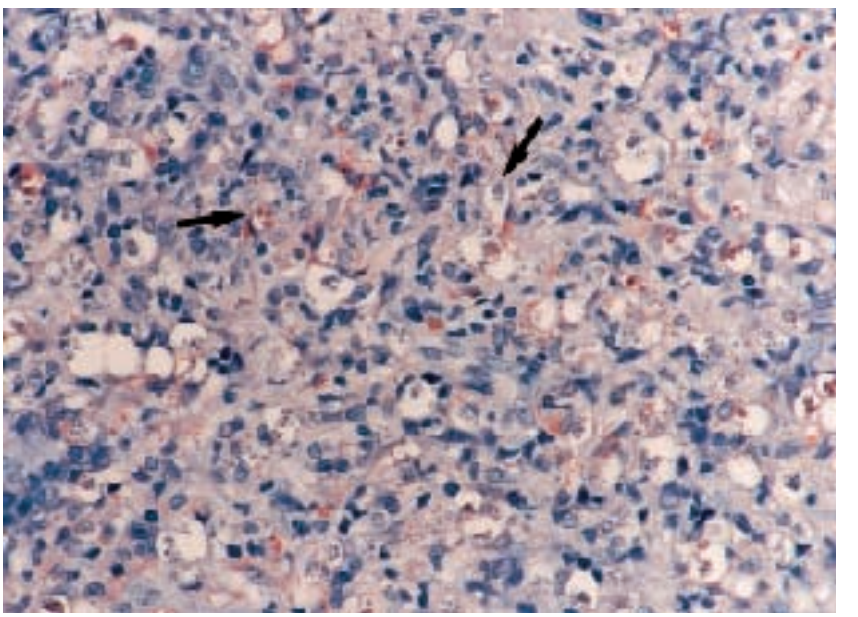

Figure 3.2.3A. Anti-lysozyme antibody staining of the DCL lesion, arrow: *Leishmania* parasites (×400).

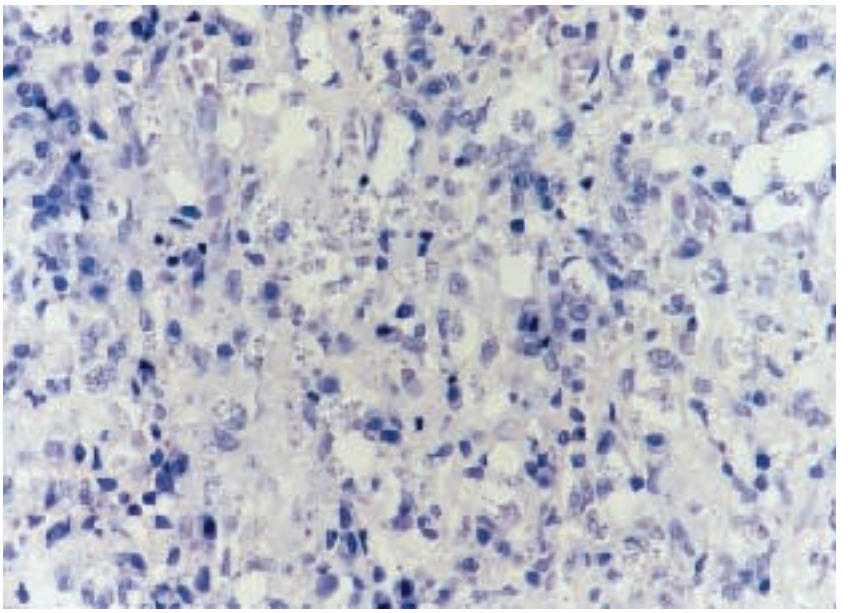

Figure 3.2.3B. Anti-T lymphocyte antibody staining of the DCL lesion (×400).

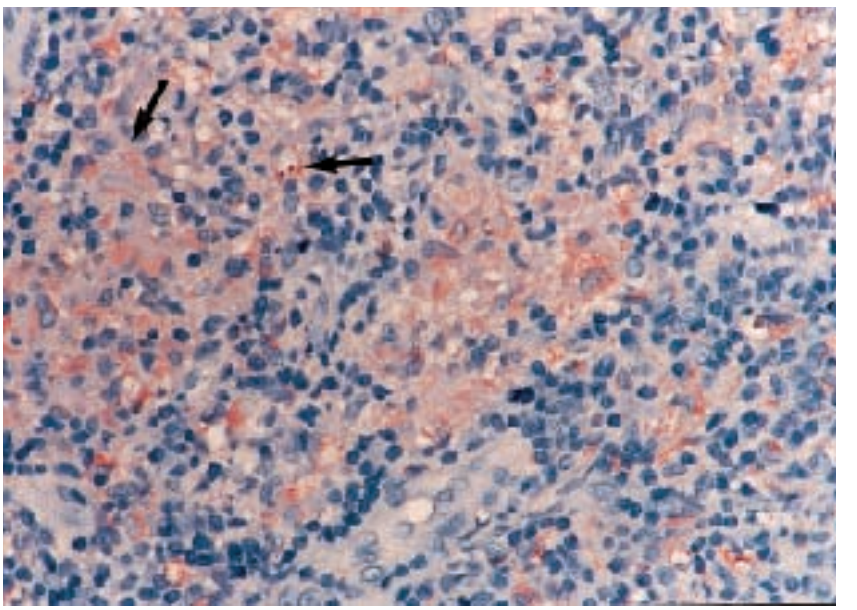

Figure 3.2.3C. Anti-lysozyme antibody staining of the LCL lesion, arrow: *Leishmania* parasites. Note the intensity of the staining, compared with Figure 3.2.3A (×400).

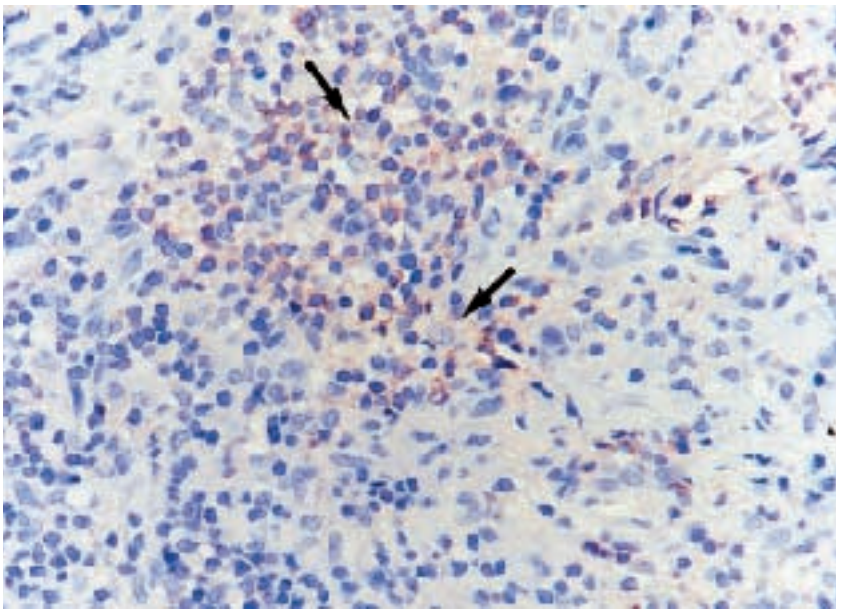

Figure 3.2.3D. Anti-T lymphocyte antibody staining of the LCL lesion, arrow: macrophage. T lymphocytes were observed surrounding the macrophage (×400).

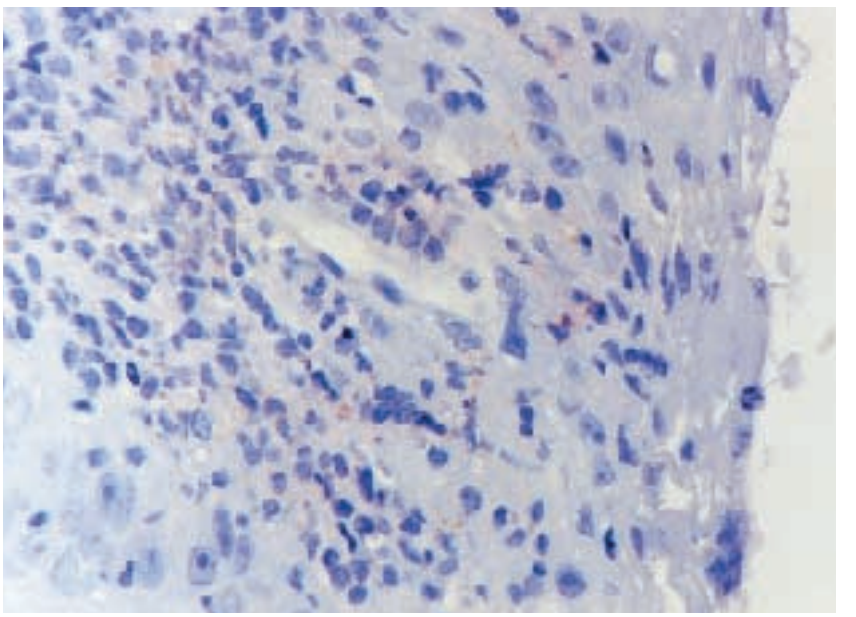

Figure 3.2.3E. Anti-T lymphocyte antibody staining of the LCL lesion (×400).

dermis showed fewer Langerhans cells than LCL epidermis (Caceres-Dittmar *et al*., 1992, 1993). In this immunohistochemical study of anti-T cell antibody, T lymphocytes infiltrated into the epidermis only in the LCL lesions. Thus, it is considered that DCL epidermis does not function fully as an immune response place.

In our DCL lesion, a few T lymphocytes infitrated into the group of the macrophages and the majority of those cells existed at the lower dermis. In comparison with anti-lysozyme staining, the macrophages in LCL lesions were more strongly stained than DCL lesions. These results suggest that the macrophages in DCL lesions are less activated than those in LCL lesions because few T cells exist near the DCL macrophages.

Numerous *Leishmania* parasites were observed in the DCL patient. *Leishmania* parasites proliferate in host macrophage and are present at amastigote form. The another difference in H.E. section between our DCL lesions and LCL lesions was that the clear zone just beneath the epidermis was present in DCL lesions.

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3. Light Microscopic and Electron Microscopic Study on Patients with Cutaneous Leishmaniasis Treated with Mefloquine (Mephaquin®)

ABSTRACT. A 41-year-old man with cutaneous leishmaniasis was treated with a dose of 4.2 mg/kg/day for 6 days of mefloquine (Mephaquin®). His ulcer was completely cured within 6 weeks of the medication. The specimens taken from the patient treated with mefloquine were observed light-microscopically and electron-microscopically. After the treatment, it was found that in the specimens with H. E. staining, inflammatory cell infiltration was remarkably decreased, that no leishmanial parasite was observed, and also that in the specimen with anti-asialo GM1 antibody staining, the activity and cytotoxicity of macrophages were remarkably diminished. Electron microscopic study which revealed degeneration of macrophages after the treatment supported the light microscopic findings. The leishmanicidal effectiveness of mefloquine may be related to macrophage-mefloquine interactions.

Introduction

Many drugs are used for treatments of cutaneous leishmaniasis. However, each treatment contains some problems such as adverse reactions and economical costs. Mefloquine, an anti-malarial drug for multiple drug-resistant malaria, is schizonticidal, and destroys the erythrocytic, asexual forms of the *Plasmodium* parasites in man (Desjardins *et al.,* 1979). Caillard *et al*. (1995) reported that mefloquine acted on the midterm trophozoites in malaria and that *Plasmodium* parasites at this stage in mice were immediately destroyed by the drug. If mefloquine is effctive on *Leishmania* parasites, we could obtain one more alternative for the treatment of cutaneous leishmaniasis.

Sixteen patients with cutaneous leishmaniasis were treated with the administration of mefloquine in Ecuador, South America by Gomez *et al*. (1995). As a result, nine of them had been clinically cured within 3 weeks of the drug administration; six of them within 4-6 weeks; one patient after 8 weeks. No specific adverse reactions were observed during the treatment. The mechanism how mefloquine affects *Leishmania* parasites is unknown. It is, however, speculated that mefloquine may inhibit amastigote-macrophage interactions and/or directly act on the parasite in leishmaniasis with the same manner as in malaria. Electron microscopic study on a patient with cutaneous leishmaniasis was performed to investigate how *Leishmania* parasites and macrophages would change before and after the treatment.

Materials and Methods

Mefloquine (Mephaquin®, Mepha Ltd., Switzerland) was administered orally with the dose of 4.2 mg/kg/day for 6 days to cutaneous leishmanial patients. The administration was repeated with 3 weeks intervals depending on the degree of recovery of each patient. The specimens used in this study were taken from a 41-year-old male cutaneous leishmanial patient treated with this method. A total of four specimens were taken from the margin of the ulcer sized 35×25 mm with one specimen each at a time, at four different times: before the oral treatment, 2 weeks, 4 weeks and 6 weeks after the treatment. Each specimen was cut into two pieces: one for light microscopic examination and the other for electron microscopic one. The former was stained with two different methods: hematoxilin-eosine (H. E.) staining and immunohistochemical staining with anti-asialo GM1 antibody.

Light microscopy

The tissue samples were fixed in 10% formalin solution, and embedded in paraffin. The samples were sectioned for both H. E. staining and immunohistochemical staining with anti-asialo GM1 antibody. Immunohistochemical staining was performed with DAKO LSAB kit (Dako Co., Kyoto, Japan). Steps in staining of dehydrated sections were as follows: 1) blocking with 3% hydrogen peroxidase; 2) incubation with diluted normal bovine serum for 5 min to block non-specific reactions; 3) incubation with rabbit anti-asialo GM1 antibody (Wako Chemicals, Osaka, Japan) for overnight at 4℃; 4) incubation with biotinylated anti-mouse IgG for 10 min at room temperature; 5) incubation with avidine-biotine-peroxidase complex for 10 min at room temperature; 6) placement in streptavidine in Tris-HCl buffer; 7) color development by immersion in the AEC solution containing 0.005% H_2O_2 for 30 sec at room temperature; 8) counterstaining of sections with hematoxyline; 9) coverslipping with AQUATEX solution (Merck Co., Tokyo, Japan).

Electron microscopy

The tissue samples were cut into small pieces, fixed in phosphate-buffered 2% glutaraldehyde solution, washed with phosphate-buffered 1% osmium tetroxide. The samples were then dehydrated with an ethanol series and propylene oxide, and embedded in Epon 812 resin. Ultrathin sections of the samples were stained with uranyl acetate and lead citrate, then observed by JEOL 2000 EX electron microscopy.

Results

H.E. staining

The specimens taken from the patient before the oral treatment with Mephaquin® showed epidermal hyperplasia and mild exocytosis of lymphoid cells, while those taken 6 weeks after the treatment revealed the almost normal epidermis (Fig. 3.3.1A, B). The former showed dense infiltration of lymphocytes, plasma cells and macrophages throughout the dermis together with *Leishmania* parasites both inside and outside of the macrophages (Fig. 3.3.2A). In contrast to this, the latter showed localized infiltration of lymphocytes, plasma cells and giant cells around the vessels without *Leishmania* parasites (Fig. 3.3.2B).

Immunohistochemical staining

Immunohistochemical study revealed the following: in the specimens before the treatment, the anti-asialo GM1 antibody positive, light-brownish cells were observed throughout the dermis. Each positive cell was larger than lymphocyte which was not stained by the anti-asialo GM1 antibody (Fig. 3.3.3A). These findings in the specimens before the treatment indicated that most of macrophages were activated and cytotoxic. On the other hand, the specimens taken 6 weeks after the treatment showed only the anti-asialo GM1 antibody negative cells which were present only around the vessels. These cells consisted of macrophages, plasma cells and lymphocytes (Fig. 3.3.3B). It was speculated that macrophages in the specimen 6 weeks after the treatment lost their activities and cytotoxicity.

Electron microscopy

Electron microscopic study revealed the following: in the specimen before the treatment, the macrophages attached with each other. The cell organells within those were well developped (Fig. 3.3.4A). In the specimen taken from 6 weeks after the treatment, however, the intercellular spaces between the macrophages were enlarged. The cytoplasms of these cells showed vacuolar degeneration and the cell membranes appeared degenerated (Fig. 3.3.4B). The rough endoplasmic reticulums (RERs) of the plasma cells were significantly enlarged in the specimen before the treatment. This indicated that plasma cells were active making proteins (Fig. 3.3.5A).

On the contrary, RERs of the plasma cells in the specimen 6 weeks after the treatment were almost normalized, which indicated that the activities found in the specimens before the treatment diminished (Fig.

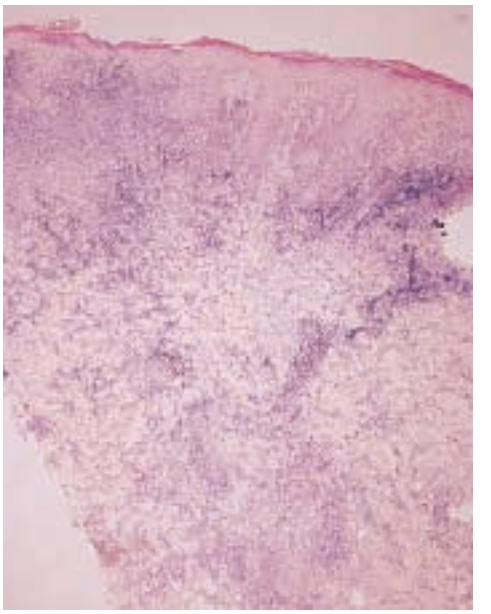

Figure 3.3.1A. H. E. stained features before the treatment. Epidermal hyperplasia and mild exocytosis of lymphoid cell are shown. Dense cellular infiltration is remarkable throughout the dermis.

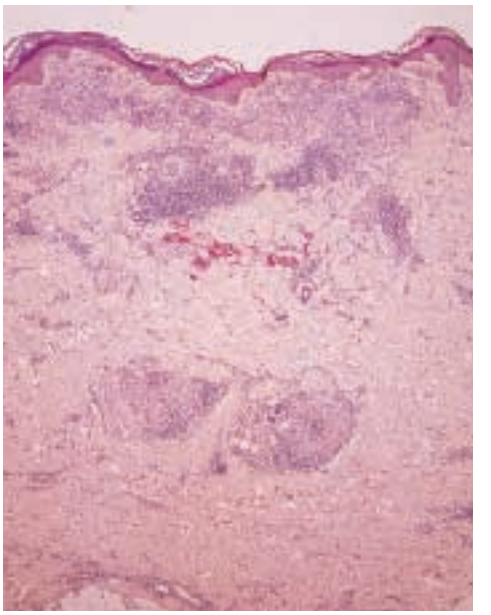

Figure 3.3.1B. H. E. stained features 6 weeks after the treatment. Epidermis is almost normalized. Cellular infiltration is localized around the vessels.

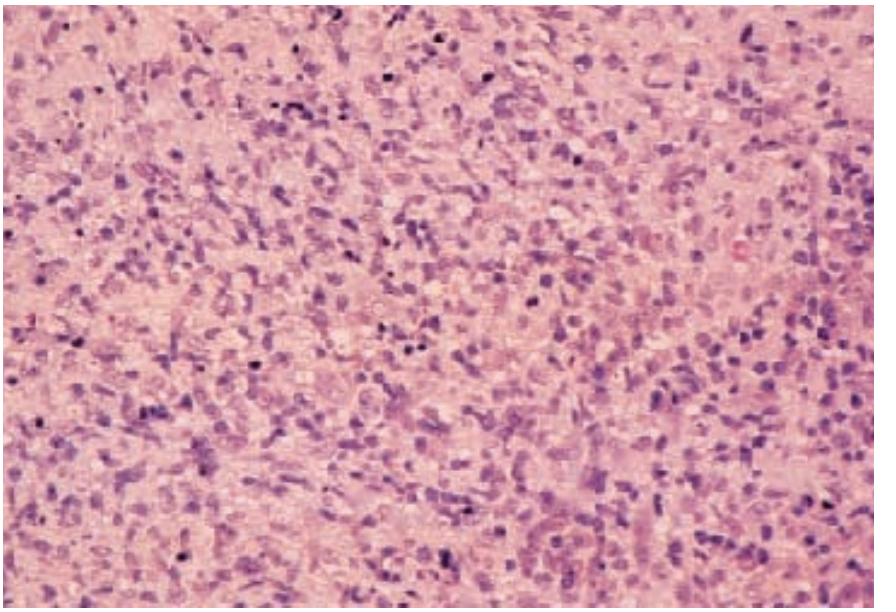

Figure 3.3.2A. High magnification view stained by H. E. before the treatment. Dense cellular infiltration consists of lymphocytes, plasma cells and macrophages. *Leishmania* parasites are observed both inside and outside the macrophages.

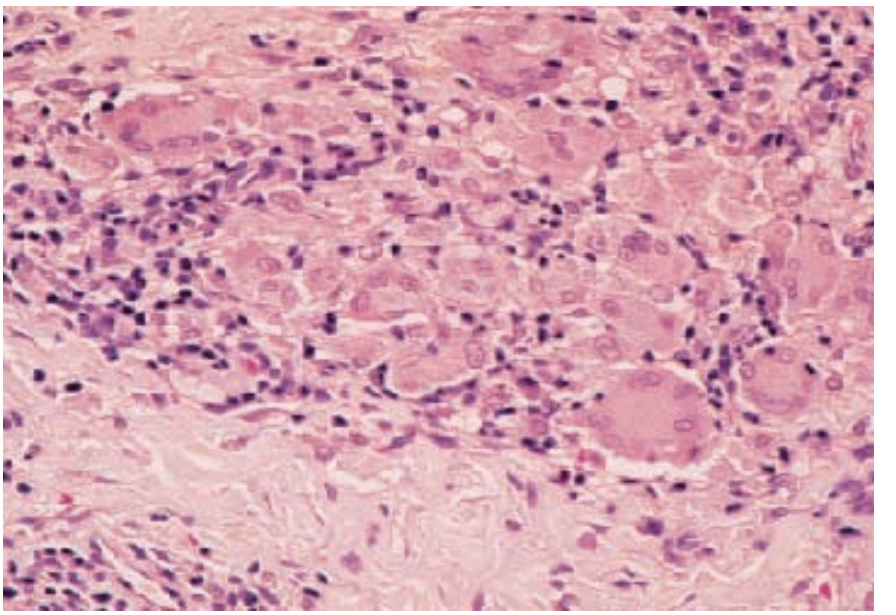

Figure 3.3.2B. High magnification view stained by H.E. 6 weeks after the treatment. *Leishmania* parasites are not observed.

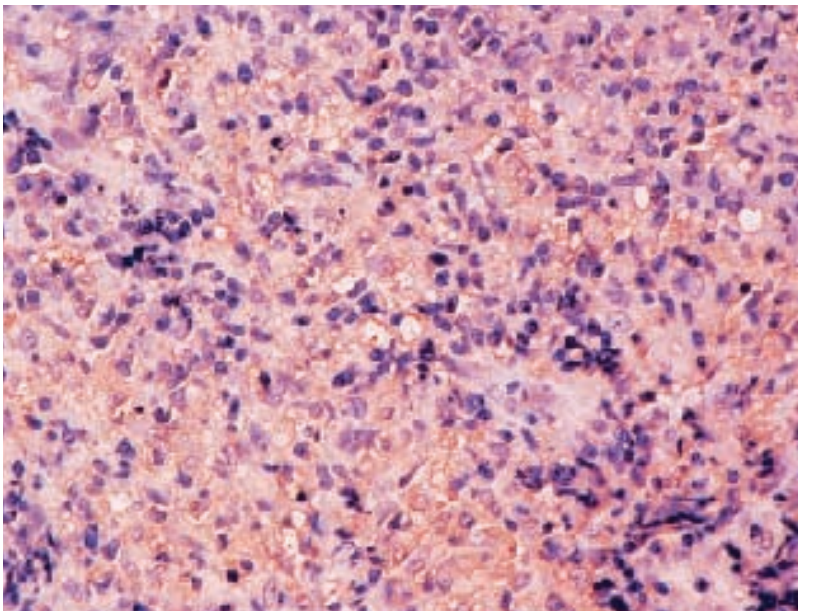

Figure 3.3.3A. Anti-asialo GM1 antibody stained features before the treatment. Positive cells are shown throughout the dermis.

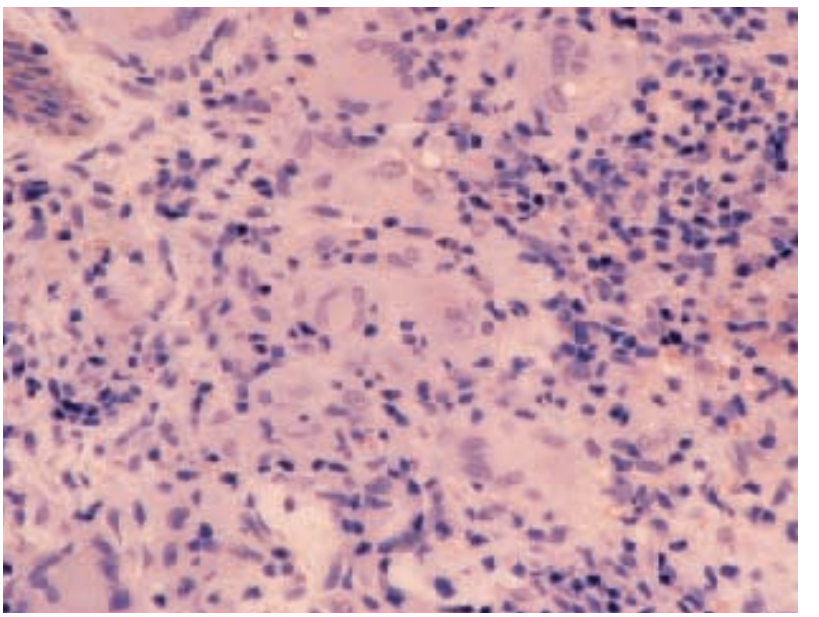

Figure 3.3.3B. Anti-asialo GM1 antibody stained features 6 weeks after the treatment. Positive cells are seen only around the blood vessels.

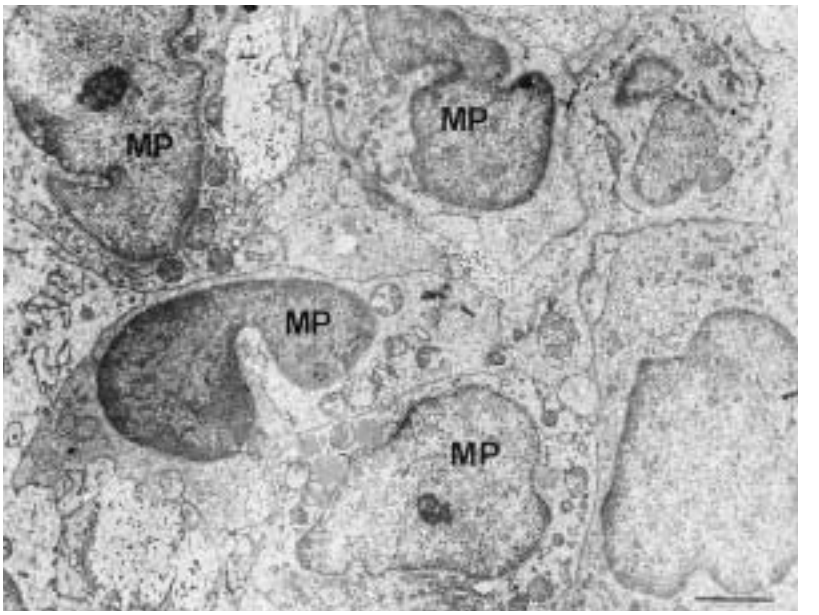

Figure 3.3.4A. Electron microscopic features before the treatment. Macrophages attach with each other and the cell-organells are well developped. (MP: macrophage, Bar=2 μm)

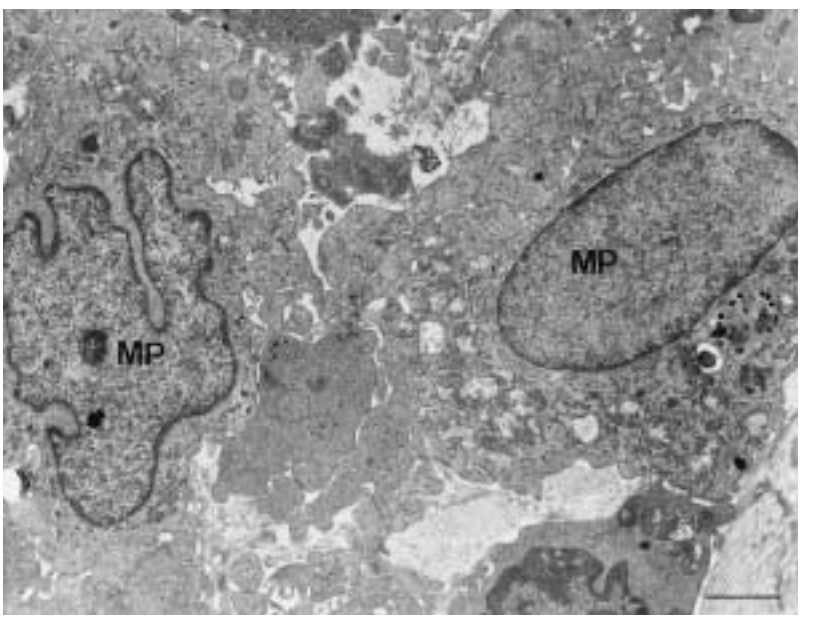

Figure 3.3.4B. Electron microscopic features 6 weeks after the treatment. Intercellular spaces between the macrophages are enlarged. Macrophages show vacuolar degeneration. (MP: macrophage, Bar=2 μm)

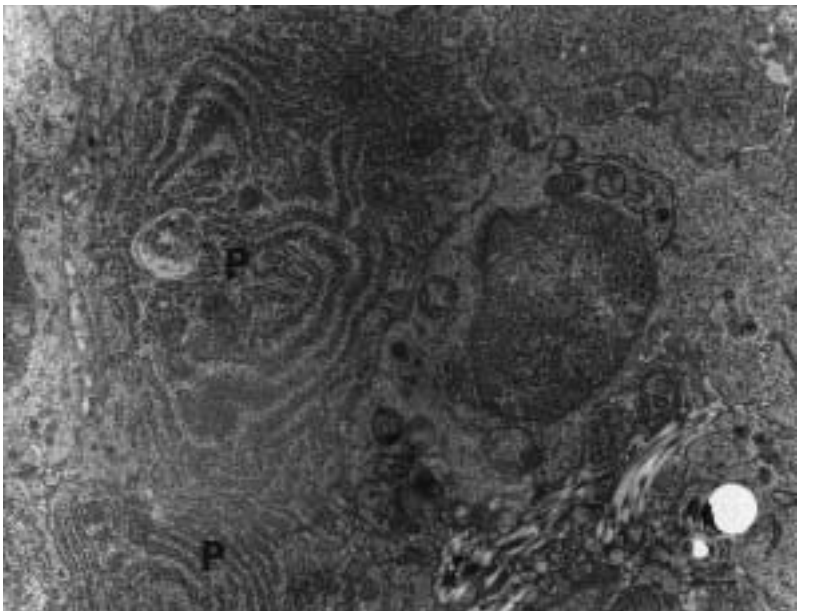

Figure 3.3.5A. Electron microscopic features before the treatment. RERs of the plasma cells are significantly enlarged. (P: plasma cell, Bar=2 μm)

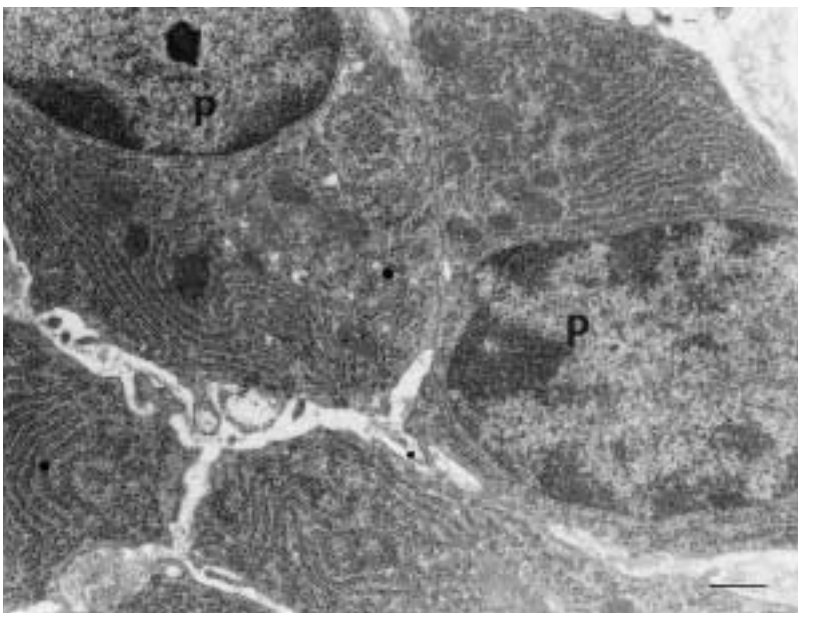

Figure 3.3.5B. Electron microscopic features 6 weeks after the treatment. RERs of the plasma cells are almost normalized. The intercellular spaces between the plasma cells are enlarged. (P: plasma cell, Bar=2 μm)

3.3.5B). The plasma cells attached with each other before the treatment, while the intercellular spaces between plasma cells were enlarged in the specimen 6 weeks after the treatment, the similar findings as in the macrophages (Fig. 3.3.5B). No *Leishmania* parasites were observed in macrophages 6 weeks after the treatment.

Discussion

The results of the treatment with mefloquine (Mephaquin®) against cutaneous leishmaniasis patients in Ecuador showed 100% cure rate (Gomez *et al*., 1995). In this study, macrophages, plasma cells and *Leishmania* parasites were observed light-microscopically and electron-microscopically to investigate the effect of mefloquine against cutaneous leishmaniasis. The findings such as decreased cellular infiltration and disapperance of *Leishmania* parasites in the specimen after the treatment did not contradict the clinical healing of cutaneous leishmaniasis.

Previous reports showed that macrophages in cutaneous leishmaniasis were activated (Ridley, 1987; Gaafar *et al*., 1995). Those reports hypothesized two mechanisms for the elimination of the parasites: 1) intact macrophages are activated and destroy the parasitized macrophages; 2) the parasitized macrophages are destroyed by a necrotizing process. The latter is believed to be more efficient for parasite elimination. Anti-asialo GM1 antibody positive cells are thought to be cytotoxic macrophages from the results that the cytotoxicity of the *in vitro* activated macrophages was reduced by the treatment with anti-asialo GM1 plus complement (Akagawa *et al.*, 1982). So we performed immunohistochemical staining with anti-asialo GM1 antibody as a marker for activation of the macrophages. The presence of anti-asialo GM1 antibody positive macrophages indicates that the conditions to eliminate *Leishmania* parasites are in active phase. The finding that only anti-asialo GM1 antibody negative cells were observed in the specimen after the treatment, may suggest that *Leishmania* parasites were eliminated after the treatment with mefloquine and that it was no longer necessary for macrophages to become activated.

Some reporters have described about the ultrastructural morphology of parasitized macrophages in leishmaniasis (Akiyama *et al.*, 1972; Alexander *et al.*, 1975; Chang *et al.*, 1976, 1978; Veress *et al.*, 1981). According to these reports, the morphological sign of parasite survival might be considered as the presence or abscence of parasitophorous vacuole (PV). PVs are common structures not in human parasitized macrophages but in hamster parasitized macrophages. No large PVs have been observed in human cutaneous leishmaniasis by any investigators. This difference between human and hamster parasitized macrophages may be related to the speculation that the PV results from the inability of the macrophages to kill the parasite. In our study, we could not observe the macrophages containing *Leishmania* parasites in the specimens after the treatment. That is why we compared electron-microscopically parasite-abscence macrophages in the specimens before and after the treatment. The degenerative appearance of the cytoplasm and the cell membrane suggested inactivity of the macrophage in the specimens after the treatment. Enlargement of the intercellular space between the macrophages may also suggest inactivity of those. In addition, the changes of the plasma cells after the treatment, such as normalization of the RER and enlargement of intercellular space between plasma cells, suggested inactivity of those plasma cells. We consider that the changes shown in the macrophages and the plasma cells before and after the treatment may be a result of cure of leishmaniasis. Thus, mefloquine is expected to be an effective anti-leishmanial drug. As a further investigation, we are planning to test mefloquine on leishmanial hamster and to examine electron-microscopically the stage sensitivity of *Leishmania* parasites against the drug *in vitro*.

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Chapter 4

Diagnosis using Molecular Techniques

1. A Trial to Detect *Leishmania* Parasites in Paraffin-Embedded Skin Biopsies using Polymerase Chain Reaction

ABSTRACT. We tried to detect *Leishmania* amastigotes in 11 skin biopsies, ordinarily formalin-fixed and paraffin-embedded, from patients with cutaneous leishmaniasis in Ecuador by polymerase chain reaction (PCR) using specific primers of *Leishmania* organisms that had been reported so far. In the current study, it was not able to confirm the specifically amplified DNA by PCR using the genomic DNA extracted from *Leishmania* protozoans. However, we could only detect the specific DNA in some of the formalin-fixed and paraffin-embedded skin specimens. By modifying such a technique, it would be necessary to establish more convenient and reliable methods to detect *Leishmania* amastigotes by PCR using ordinarily formalin-fixed and paraffin-embedded skin specimens in further investigations.

Introduction

Leishmaniasis is divided into four broad divisions: cutaneous (CL), diffuse cutaneous (DCL), mucocutaneous (MCL), and visceral forms (VL). These clinical types are mainly caused by the different species of the genus *Leishmania* as follows: CL is caused by *L. (Leishmania) mexicana* complex, *L. (L.) mexicana, L. (L.) amazonensis, L. (L.) pifanoi, L. (L.) garnhami* and *L. (L.) venezuelensis*; CL and/or MCL are caused by *L. (Viannia) braziliensis* complex, *L. (V.) braziliensis, L. (V.) guyanensis* and L. *(V.) panamensis*; and VL is caused by *Leishmania (L.) donovani* complex, *L. (L.) donovani, L. (L.) infantum* and *L. (L.) chagasi*. Traditional methods to detect these *Leishmania* protozoans include direct visualization by Giemza or Wright staining and/or culture of the tissue materials. Recently, polymerase chain reaction (PCR) has been established as a reliable method to detect infectious agents such as protozoans, helminths, bacilli, and etc. It is essentially important for dermatologists to detect *Leishmania* protozoans in skin lesions not only for differential diagnoses of ulcer-forming skin diseases but also for investigating relationships between the clinical manifestations of the disease and the causative species. The purpose of this study is to detect *Leishmania* amastigotes in skin lesions by PCR using specific primers of *Leishmania* that have been reported so far. In this study, we report the results of preliminary trials, 1) to detect four *Leishmania* species mentioned above by PCR using the specific primers for their characterization, and 2) to detect *Leishmania* amastigotes from formalin-fixed and paraffin-embedded skin specimens by PCR using these primers.

Materials and Methods

Following four species of the genus *Leishmania* were used for genomic DNA extraction in this study: *L. (L.) mexicana* (MHOM/EC/88/PT-007), *L. (L.) amazonensis* (MPOT/EC/87/G-02), *L (V.) braziliensis* (MHOM/EC/88/INH-03), and *L. (V.) panamensis* (MHOM/EC/88/INH-04). The biopsy materials used were ordinarily formalin-fixed and paraffin-embedded skin specimens taken from 11 patients with cutaneous leishmaniasis in Ecuador.

Extraction of genomic DNA from Leishmania *parasites*

The genomic DNA extractions from *L. (L.) mexicana, L. (L.) amazonensis, L. (V.) braziliensis* and *L. (V.) panamensis* were performed as follows: promastigotes from *in vitro* culture of each *Leishmania* species were centrifuged with 3000 r.p.m. for 5 min. The pellet was incubated in lysis buffer (2% SDS, 10 mM Tris-HCl (pH8.0), 150 mM NaCl, 10 mM EDTA) which contained proteinase K 500 μg/ml at 55℃ for 24 hrs. Ethanol precipitation was performed with each pellet after phenol-chloroform extraction and chloroform-isoamyl-alcohol extraction, respectively. The product was used as a template after having been adjusted to 1 μg/ μl using dH_2O.

Extraction of DNA from paraffin slices

Six or seven paraffin slices, 10 μm each in thickness, were cut off from blocks of the ordinarily formalin-fixed and paraffin-embedded skin biopsy specimens. After deparaffinization with xylene-alcohol and air dry, the tissues were dissolved in 400 μl of TE (10 mM Tris-1 mM EDTA (pH 7.5) and the same amount of InstaGeneTMMatrix (BIO-RAD, USA). They were incubated at 56℃ for 2 hrs, and additionally heated at 100℃ for 5 min. Then, ethanol precipitation was performed after phenol-chloroform treatment to extract genomic DNA, which was dissolved in 20 μl of TE.

PCR for Leishmania *parasites*

The primers used in this study were prepared as follows: L1 (5'-GAACGGGGTTTCTGTATGG-3') and L2 (5'-TACTCCCCGACATGCCTCTG-3') for *L. (V.) braziliensis* complex; US1 (5'-TTGGCCTGAAAAATGGACCCG-3') and LS1 (5'-ACACCCAAACACAACCAAACC-3') for *L. (L.) mexicana* complex; US2 (5'-GTGGGGGAGGGGCGTTCT-3') and LS2 (5'-ATTTTACACCAACCCCCAGTT-3'), which are primers for a common portion of the two species complexes (Eshita *et al*., 1995); 13A (5'-TTGACCCCCAACCACATTATA-3') and 13B (5'-ATATTACACCAACCCCCAGTT-3'), which are primers for a common portion of the two species complexes, *L. (V.) braziliensis* and *L. (L.) mexicana* (Rodrigues *et al*., 1994). Figure 4.1.1 shows the homologies of the primers and the *Leishmania* isolates and species used in this study. As for the reaction conditions for PCR, we followed those reported by the respective authors (Eshita *et al*., 1995; Rodriguez *et al*., 1994; Lopez *et al*., 1993). The reaction buffer for PCR consisted of 10 mM Tris-HCl (pH8.9), 1.5 mM $MgCl_2$, 80 mM KCl, 500 μg/ml BSA, 0.1% Sodium Cholate, and 0.1% Triton X-100. The enzyme dilution buffer contained 10 mM Tris-HCl (pH7.5), 300 mM KCl, 1 mM DTT, 0.1mM EDTA, 500 μg/ml BSA, 50% Glycerol, and 0.1% Triton X-100. The reaction mixture of those was a total of 100 μl consisting of 1 μl of genomic DNA, primer 2 μl, 10x buffer 10 μl, 5 mM dNTP 4 μl, DW 78 μl, and Tth DNA polymerase (TOYOBO Co., Ltd., Japan) 1U/ μl.

Method for PCR using paraffin slice specimens

We used the primers, US2/LS2, under the following conditions: denature 94℃ 1 min, annealing 47℃2 min, extention 72℃ 3 min, with the number of cycles of 30 times. The PCR product obtained shows 114 basepairs. As for negative controls, we used genomic DNA from paraffin slices of a skin specimen of a healthy individual from Okinawa and dH_2O. In total, two trials of PCR were performed using different annealing conditions and different templates. In the second trial, the annealing conditions were changed from 47℃ for 2 min to 43℃ for 2 min, and the template was replaced with 10 μl of the first PCR product.

We performed nested PCR because the amount of genomic DNA obtained from the paraffin embedded specimens was little. The primer used for the nested PCR was designed choosing the outer base sequences of US2/LS2 primer. That is, 5'-TGGTGGAAATTGGTGGGAAA-3' for US2 outer primer, and 5'-GGCGGCTGCTCTGGATTAT-3' for LS2 outer primer were used. After having performed PCR with

File name: X54470/*L.(V.) guyanesis* kinetoplast minicircle DNA, conserved region
Sequence size: 222

L1 primer; sequence size: 19
L2 primer; sequence size: 20

[Accession No.: X54470/222 bp]

```
  1' AATTCTAAATTTCAAGCCTCTTAGAGGCCTATTTCACGGCATTCTAACTCTAGCCGGAGT

 61' GCGCGGCCCACTATATTACACCAACCCCTAATTGTGCACGGGGAGGCCAAAAAACGTGAT

121' TTTCGGGGGATTTTTGAACGGGGTTTCTGTATGCCATTTTTCGATTTGCAGAACGCCCCT
                     ********************----------------------------
           (L1 primr)     GAACGGGGTTTCTGTATGC
181' ACCCAGAGGCATGTCGGGGAGTAAAATTTGGTCGCTATTTTT
     ---*******************
        CAGAGGCATGTCGGGGAGTA    (L2 primer)
```

PCR

incubation	94℃	2 min.
denature	94℃	1 min.
annealing	54℃	2 min.
extention	72℃	3 min.
cycles	30 cycles	
PCR product	72 bp	

Figure 4.1.1. The primers and their homologies of *Leishmania* isolates and species used in this study.

these primers, we used 1/10 (10 μl) of the amplified DNA as a template. Further PCR was performed using US2/LS2 primer and the template which had been adjusted to 100 μl adding fresh PCR mixture to it. The PCR conditions for this time was as follows: incubation 94℃ for 2 min, denature 94℃ for 1 min, annealing 47℃ for 2 min, and extention 77℃ for 3 min. The expected amplified DNA size was 184 bp.

Results

The results of PCR using Leishmania *parasites*

The amplified DNA products were found at the site of approximately 72 bp in INH-03 and INH-04 with the primers of L1/L2. No positive bands were found in PT-007 and G-02 with these primers. Weakly positive bands were seen at the site of approximately 65 bp in PT-007 and G-02 using the primers of US1/LS1. However, no bands were observed at the site of 341 bp, which had been reported previously (Eshita *et al.*, 1995). The amplified DNA products were found at the site of approximately 114 bp in all the *Leishmania* parasites used with the primers of US2/LS2. A small amount of extrabands were seen at the site of 200 bp in PT-007 and G-02 with these primers. dH_2O used as a control showed no bands (Fig. 4.1.2). The amplified DNA products were recognized at the site of approximately120 bp with the primers of 13A/13B in all the four *Leishmania* species used as seen with the primers of US2/LS2 (Fig. 4.1. 3).

File name: M21325/ *L.(L.) amazonensis* (IFLA/BR/67/PH8) kinetoplast DNA, clone pMAT38
Sequence size: 716

US1 primer; sequence size: 21
LS1 primer; sequence size: 21

[Accession No.: M21325/716 bp]

```
  1' GTGGTTTTTGGCCTGAAAAATGGACCCGCCCAAACTTTCTGCCCGTGGGGGAGGGGCGTT
           ********************----------------------------------
   [US1 primer] TTGGCCTGAAAAATGGACCCG
 61' CTCAAAATGGCAAAAATGGGTGCAGAAACCCCGTCATATTTTCGGGGAATTCGGGGAATT
     ----------------------------------------------------------
121' TCGGCTCGGGCGGTGAAAACTGGGGGTTGGTGTAAAATAGGGGCGGCTGCTTCGGGCTTG
     ----------------------------------------------------------
181' TTTCTTTTTGAGGTCGCGTTTGTTTGGGTATGTACTTGGTGGGTGGTAGTCGAGTTGGTT
     ----------------------------------------------------------
241' TGTGCCTTGAAAATTGTTTTTATTTTAACTTTATTTTAAAAGTTTGGATTCGATGGATTA
     ----------------------------------------------------------
301' TAATGTTTGTTTTGCTGTTGGAATGGTGGTTTGGTTGTGTTTGGGTGTTATATGACGTGA
     ----------**********
                GGTTTGGTTGTGTTTGGGTGT    [LS1 primer]

361' TGGTTGCTCGTCTGATTGGTATTGTCTTTGGGGGTCGGAAGTTGGTGTATTTGTGCGGAT

421' GATGAGGCTTACAGTTATAATATAAGTTTATATTGTATTTAATGCCATTTAATGATGTTA

481' TCTGGTTGTATGTAATCTCTTGGGGTTTTTGGTGTGGCCTTGTAGTGGACGGGAATGATT

541' TTTGGATATGATTATAATTTTATTTGAAATGTACATATATTCAATTTCGTTGTTATAATG

601' TTTTTGTTATGTGTGTTTAATTGTTAGATTTATGATGACGTGACAGTACTGTTAGTTAAG

661' GATAGACATGTGTGAGGTAATAGATAGTAGGATTAGGGTGGTGTGAGAGGTGGTTT
```

PCR

incubation	94℃	2 min.
denature	94℃	1 min.
annealing	50℃	2 min.
extention	72℃	3 min.
cycles	30 cycles	
PCR product	341 bp	

Figure 4.1.1. continued.

The results of PCR using the extracted DNA from the paraffin slice specimens of the patients with leishmaniasis

The first trial of PCR showed band only in lane 6 (EC/7/96/8/13) (Fig. 4.1.4). However, the second trial showed no positive bands in any lane (Fig. 4.1.5). We performed nested PCR (Blikaytis *et al*., 1990) using an outer primer which we designed outside the primers of US2/LS2. The first step using our own primers revealed positive band in only one specimen

File name: U19811/*L.(V.) panamensis* kinetoplast minicircle, partial sequence, fragment LPAN
Sequence size: 623

US2 primer; sequence size: 18
LS2 primer; sequence size: 21

[Accession No.: U19811/623 bp]

```
001' AAATTTATTGATATTAATAGTATACTATATGGTGCTGTTATTGTTATCTATTACTGTTCT

 61' CGGTTAGCTTTNATATAATATTTACTATATTAGCATTTTATACTTGTATGGTTACGTTGG

121' CTGATTGTGATGGATTTAATAACTTTATTGACTGTAACATTAGCTAAACCTTATTACTAC

181' TCTGTTATGCTCTGTTAGAAATAATATTTTTGTTTATAAAATTCTAAAATTGGGCATAAA

241' ATTCTAAAATTGGGCATAAAATCGTACCACCCGACATGCCTCTGGGTAGGGGCGTTCTGC
                                                  ******************--
                                      [US2 primer]          GTGGGGGAGGGGCGTTCT
301' GGAAAACCGAAAAATGGCATACAGAAACCCCGTTCAAAAATAGCCCGAAAAATCGCGGTT
     ------------------------------------------------------------
361' TTTTGGGCCTCCCCGTGCACAATTAGGGGTTGGTGTAATATAGTGGGCCGNGCACCCCGA
     ----------------------** * ************ **
                          AACTGGGGGTTGGTGTAAAAT    [LS2 primer]
421' CTCGGGGCTAGCTCTCGTGGAACTANGGNCNCTTAGAGGCTTGATTTATAAATTAAATAT

481' TTTGGATAGATTGTTATTAGCTAATAAAGTTAAATAGTTGTTTGATTAATTGCATTACTA

541' TGTTGCTTACAGTGAATAATTTGGTTATAATTAATTTATTATTACGTTGAATAAATACTC

601' GATAGATACATCATTGAATAATA
```

PCR

incubation	94℃	2 min.
denature	94℃	1 min.
annealing	47℃	2 min.
extention	72℃	3 min.
cycles	30 cycles	
PCR product	114 bp	

Figure 4.1.1. continued.

(DCL) not at the expected site of 184 bp but at the site of 480 bp (Fig. 4.1.6). However, the second step of nested PCR showed no positive reactions.

Discussion

Fast and accurate diagnosis is the first step for a proper treatment. Traditional culture methods require long times for detecting *Leishmania* parasites, for example, with a low rate of successful detection of the organisms. It is well-known that recently PCR has been established as a time-saving and reliable method for the diagnosis of various infectious diseases.

File name: U19805/*L. (V.) braziliensis* kinetoplast minicircle, partial sequence, fragment MCLB06
Sequence size: 774

US2 primer; sequence size: 18
LS2 primer; sequence size: 21

[Accession No.: U19805/774 bp]

```
   1' GAAAACCCCCGGACACCAGCCGGTNCCCAAACCNGANCCGCGGGCGNCGGAGTAGANGAA

  61' AAAGGGGCCCANGCCCTCCNNNTAAAAAANGGNNGGAAGGGNGGCAGAGAAAANAGNAAC

 121' ACANCCCAANCCGCCNACGGGCCCCCAAACNAGACCGGAACANGNACANCCAGGAAAGAG

 181' GCGGCACANNAANAGGGAGNCNAAAANACTANNCANGTTANACAACNNCGANACCTAGGA

 241' AACTAAGGATATNGCCCATNNAGCTCCNAGNGCTAGATNNCCATAACAGCGCNAGNTTAT

 301' TACAAGGNTGNAACCATTATATTANACNAGATACTANTACTAGNCTANTANTTAATNTAA

 361' TAATGNTACTTACTCGATACAGCNNTANTATATAACTATGAATAATTGAACTAGTTTGAT

 421' TACTAGTATTACATTGATTATAATGAGNTTAANTATATATAANTTTATTTATACTTTACT

 481' CAATTATTACACGAGTANTTTTATTATATAGGCTCTCTAATGCACAGTTTAATAATATTG

 541' TTAGTAAATAATTTTTAGAAATAATAATTTAATAAAATTTAGTTTTTACTGCGTTATGGT

 601' CTAGAAAATTACGATAAAATCGTACTTCCCCGACATGCCTCTGGGTAGGGGCGTTCTGCG
                                                  ***  **********
                                       [US2 primer]     GTGGGGGAGGGGCGTTCT---
 661' AAATTCGATTTTTGGCATACAGAAACCCCGTTCAAAAAATGCCCAATTTTCACGATTTTT
      ------------------------------------------------------------
 721' GGCCTCCCCGTGCACAATTAGGGGTTGGTGTAATATAGTGGGCCGCGCACCCCG
      ----------------**  *  ************  **
                        AACTGGGGGTTGGTGTAAAAT    [LS2 primer]
```

PCR

incubation	94℃	2 min.
denature	94℃	1 min.
annealing	47℃	2 min.
extention	72℃	3 min.
cycles	30 cycles	
PCR product	114 bp	

Figure 4.1.1. continued.

The total number of reported cases of leishmaniasis in Japan is yet small (about 20 cases) (Nonaka, 1995). However, a large number of people are suffering from leishmaniasis as WHO has designated it as one of 6 TDR's major diseases in the world. Therefore, it is not difficult to imagine that possibilities we encounter it in the near future will increase gradually because of the accelerated intercommunication in the current world.

Leishmaniasis is caused by protozoan parasites that

File name: U19810/*L.(L) amazonensis* kinetoplast minicircle, partial sequence, fragment LAMAZ
Sequence size: 599

US2 primer; sequence size: 18
LS2 primer; sequence size: 21

[Accession No.: U19810/599 bp]

```
  1' TTTTTGATTTAGTAGTTAATAGCTGTGTTTATTTGTGTGGGTGGTGGTATGGTGTTACAG

 61' AGTTTGGTGGAAATTGGTGGGAAAATGGTCCCGGCCCAAACTTTTCTGCCGTGGGGAGGG
               US2 outer primer                                  **********
     TGGTGGAAATTGGTGGGAAA            [US2 primer]                GTGGGGGAGGG

121' GCGTTCTGCGGATTCGGGAAAAATGAGTGCGAAACCCCGGTTCATAATTTGGGGGATTTT
     *******----------------------------------------------------
     GCGTTCT
181' GGAGAATTCCGGCTCCGAGGCTCGAAACTGGGGGTTGGTGTAAAATAGGGGCGGCTGCTC
     -------------------------**********************  US2 outer primer
                              AAACTGGGGGTTGGTGTAAAATA  GGCGGCTGCTC
                                    [LS2 primer]
241' TGGATTATTNATTTTAAAATTAATTATTTTGTTAAAATATATTTTGGTTAGGAATATGTT
     ********
     TGGATTAT
301' TTTATTGCTTCTGCATTTGTTAGATTGGCTGATGCTCTGTAGGTTGTTTGTTTTAGGCTC

361' TAGTGGTCTTTTGATTGCGTTCTTTTTCCTTACAATTTGTATTTATATTACATATATTAC

421' TTAGGGGTTATTTGTTTGGTATGAGGTAGGCTGTGTTATGCTCATGAGTTTGTGATATTG

481' TTTAGATTTATAATGTTAGATCGGATTATATGTTCCTCGTTTACTATTTGTGATGTTTGT

541' GCTAATTTGNAATNAATAGTTGGGACGGTTGGACCGGTTGTTNTAAGGATTACATGNGT
```

PCR

incubation	94℃	2 min.
denature	94℃	1 min.
annealing	47℃	2 min.
extention	72℃	3 min.
cycles	30 cycles	
PCR product	114 bp (US2/LS2 primer)	
	184 bp (US2/LS2 outer primer)	

Figure 4.1.1. continued.

belong to the Kinetoplastidae that has kinetoplast DNA. It is transmitted by the arthropod vectors, sandflies. The Kinetoplastidae is subdivided into the genera *Trypanosoma* and *Leishmania*. The two genera have different vectors and different clinical manifestations, but are common in that both have nucleus and kinetoplast. The kinetoplast DNA consists of maxicircle and minicircle; the latter occupies most of the DNA. The number of minicircles is approximately 300 in the genus

File name: Z11552/*L.(L.) mexicana* kinetoplast minicircle DNA
Sequence size: 660

US2/primer; sequence size: 18
LS2/primer; sequence size: 21

[Accession No.: Z11552/660 bp]

```
  1' GGACCTTTGATATATGGGCAGGTTCATTGTCTTTTGATATGCGATTTTTGTTTGTTGCTT

 61' TTCTGGTGGACGTTGTTGTTGGTTGTGCTGGATTGAGATTGGGTGCCTTACAGAATAAAA

121' GGCCTTTAATTAGGTAAGTGAGGGGTGTGTAATGGTAAGTTTTTGATTCTTGACGTTATA

181' TTTTGGTATACTTTGTTTTAATGTCAGTGATACATGTTACTATTACGATGAATTAATTTC

241' TCTTTATATAAACTGTATACATTGCCTGGTTAAGTGTTGAATGTTTGTGTTAAGGAATGT

301' AATTGTTTTGTACGAGTTAGATGTGGATTTAGTGTGGGTGATGTGTTGGTGGTGATGATT

361' TGGGTTGGGAGTGGGCTTGAAAATAGGGACAAAAAACGAAACTTTTCTGCCCGTGGGGGA
                                                          ********
                                                  [US2 primer]      GTGGGGGA
421' GGGGCGTTCTGCGAAAATGGCAAAAATGAGTGCAGAAACCCCGTTCATAATTTAGGGGAA
     **********----------------------------------------------------
     GGGGCGTTCT
481' TTCCTGGAATTTCAGCTCCGGCGGTGGAAACTGGGGGTTGGTGTAAAATAGTGGGGGTGG
     ----------------------------**********
                                  AACTGGGGGTTGGTGTAAAAT     [LS2 primer]
541' TGGGGATTTTTATTTTATTTGGAATTGTGAGGGCTTTGTAGGCGTTGTTACACGGCATTC

601' TATTTGTTTTGTTTAGCTGTTCTTTTGTGTGGTTACGTGCGGCTTTGTGTGTTATGAAGG
```

PCR

incubation	94℃	2 min.
denature	94℃	1 min.
annealing	47℃	2 min.
extention	72℃	3 min.
cycles	30 cycles	
PCR product	114 bp	

Figure 4.1.1. continued.

Trypanosoma, while that in the genus *Leishmania* is less than 10. This fact naturally supports an idea and possibility to identify and classify the *Leishmania* parasites on the basis of kinetoplast DNA. The kinetoplast DNA has 2500 basepairs, and the homologies are less in the genus *Leishmania* than in the genus *Trypanosoma*. It is expected from these facts that the *Leishmania* parasites will be classified according to differences in base sequences in the near future (Sugane, 1989).

The *Leishmania* parasites have been classified into four groups: *L. (L.) donovani* complex, *L. (L.) tropica* complex, *L. (L.) mexicana* complex, and *L. (V.) braziliensis* complex. All the *Leishmania* parasites

File name : M21326/ *L.(L.) amazonensis* (MHOM/BR/00/Raimundo) kinetoplast DNA, clone pMAT170
Sequence size: 902

US2 primer; sequence size: 18
LS2 primer; sequence size: 21

[Accssion No.: M21326/902 bp]

```
  1' ATGGTTTTTGGCCTGGAAAAATGGTCCCGGCCCAAACTTTTCTGCCCCGTGGGGGAGGGG
                                                  ************
                                          (US2 primer)       GTGGGGGAGGGG
 61' CGTTCTGCGGAAACCTCAAAAATGAGTGCAGAAACCCCGTTCATATTTTGGGGGATTTTT
     ******----------------------------------------------------------
     CGTTCT
121' GGGAATTTCGGTTCGGACGGTGGAAACTGGGGGTTGGTGTAAAATAGGGGCGGCTGCTTC
     ----------------------**********
                           AACTGGGGGTTGGTGTAAAAT    (LS2 primer)
181' GGGCTTGTTCCTTTTTGAGGTCGCGTTTGTTTGGGTATGTACTTGGTGGTTGGTAGTCGA
241' GTTGGTTTGTGCCTTGAAATGTTTTTATTTTAACTTTATTTTAAAAGTTTGGATTCAATG
301' GATTATAATGTTTGTTTTGCTGTTGAAATGGTGGTTTGGCTGTGTTTGGGTGTTATATGA
361' CGTGATGGTTGTTCGTCTGATTGGTATTGTCTTTGGGGATCGGAAGTTGGTGTATTTGTG
421' CGGATGGTGAGGCTTACAGTTATAATATAAGTTTATATTGTATTTAATGTCATTTAATGA
481' TGTTATCTGGTTGTGTGTAATCTCTTGGGGTTTTTGGTGTGGCCTTTGTAGTGGGCGGAC
541' TGATTTTTGGATATGATTATAATTTTATTTAAATGTGCATATATTCAATTTCGTTGTTAT
601' AATGTTTTTGTTATGTGTGTTTAATTGTTAGATTTGTGATGACGTGACAGTACTGTTAGT
661' TAAGGATAGACATATGTGAGGTAATAGATAGTAGGATTAGGGTGGTATGAGAGATGGTTT
721' ACTGATTTTTGGATATGATTATAATTTTATTTAAATGTGCATATATTCAATTTCGTTGTT
781' ATAATGTTTTTGTTATGTGTGTTTAATTGTTAGATTTGTGATGACGTGACAGTACTGTTA
841' GTTAAGGATAGACATATGTGAGGTAATAGATAGTAGGATTAGGGTGGTATGAGAGATGGT
901' TT
```

PCR

incubation	94℃	2 min.
denature	94℃	1 min.
annealing	47℃	2 min.
extention	72℃	3 min.
cycles	30 cycles	
PCR product	114 bp	

Figure 4.1.1. continued.

cause skin lesions, but leishmaniasis, which manifests skin lesions mostly, is caused by the *Leishmania* parasites other than *L. (L.) donovani* copmlex that mainly causes visceral leishmaniasis (VL). Major localities for *L. (L.) tropica* complex are Middle East, the Mediterranean basin, and Africa, while those for *L. (L.) mexicana* complex and *L. (V.) braziliensis* complex are only the New World. *L. (L.) mexicana* complex mainly causes cutaneous leishmaniasis (CL), and *L. (V.) braziliensis* complex causes CL and mucocutaneous leishmaniasis (MCL) (Hashiguchi, 1996). Therefore, we, dermatologists, are mostly concerned with *L. (L.) tropica* complex, *L. (L.) mexicana* complex and *L. (V.) braziliensis* complex. Parts of the base sequences of kinetoplast DNA of those have been already reported (Rogers, 1988; Samuelson, 1991).

File name: U19811/*L.(V.) panamensis* kinetoplast minicircle, partial sequence, fragment LPAN
Sequence size: 623

13B foward primer; sequence size: 18
13A reverse primer; sequence size: 21

[accession No.: U19811/ 623 bp]

```
  1' AAATTTATTGATATTAATAGTATACTATATGGTGCTGTTATTGTTATCTATTACTGTTCT

 61' CGGTTAGCTTTNATATAATATTTACTATATTAGCATTTTATACTTGTATGGTTACGTTGG

121' CTGATTGTGATGGATTTAATAACTTTATTGACTGTAACATTAGCTAAACCTTATTACTAC

181' TCTGTTATGCTCTGTTAGAAATAATATTTTTGTTTATAAAATTCTAAAATTGGGCATAAA

241' ATTCTAAAATTGGGCATAAAATCGTACCACCCGACATGCCTCTGGGTAGGGGCGTTCTGC
                                                  ***  ***********--
                              [13B foward primer]              GTGGGGGAGGGGCGTTCT
301' GGAAAACCGAAAAATGGCATACAGAAACCCCGTTCAAAAATAGCCCGAAAAATCGCGGTT
     ------------------------------------------------------------
361' TTTTGGGCCTCCCCGTGCACAATTAGGGGTTGGTGTAATATAGTGGGCCGNGCACCCCGA
     ----------------------**  *  **************  **
                              AACTGGGGGTTGGTGTAAAAT
421' CTCGGGGCTAGCTCTCGTGGAACTANGGNCNCTTAGAGGCTTGATTTATAAATTAAATAT

481' TTTGGATAGATTGTTATTAGCTAATAAAGTTAAATAGTTGTTTGATTAATTGCATTACTA

541' TGTTGCTTACAGTGAATAATTTGGTTATAATTAATTTATTATTACGTTGAATAAATACTC

601' GATAGATACATCATTGAATAATA
```

PCR

incubation	94℃	2 min.
denature	94℃	1 min.
annealing	50℃	2 min.
extention	72℃	3 min.
cycles	25 cycles	
PCR product	120 bp	

Figure 4.1.1. continued.

Traditional methods to detect the *Leishmania* parasites include direct visualization of the organisms by Giemsa staining and/or culture of tissue smears, which were reported by Gomez *et al*. (1987). Skin biopsy is also utilized for this purpose to detect the parasites within the macrophages in the dermis. Methods to indirectly detect leishmaniasis cases include Montenegro skin test, indirect immunofluorescent antibody test, enzyme-linked immunosorbent assay (ELISA). However, those methods are not so reliable because they often show negative results when performed at early stages of infection, or cross reactions sometimes occur with other pathogens (Manson-Bahr, 1987). Recently, many reports have appeared to show

File name : U19805/*L.(V.) braziliensis* kinetoplast minicircle, partial sequence, fragment MCLB06
Sequence size: 774

13B foward primer; sequence size: 18
13A reverse primer; sequence size: 21

[Accession No.; U19805/774 bp]

```
  1' GAAAACCCCCGGACACCAGCCGGTNCCCAAACCNGANCCGCGGGCGNCGGAGTAGANGAA
 61' AAAGGGGCCCANGCCCTCCNNNTAAAAAANGGNNGGAAGGGNGGCAGAGAAAANAGNAAC
121' ACANCCCAANCCGCCNACGGGCCCCCAAACNAGACCGGAACANGNACANCCAGGAAAGAG
181' GCGGCACANNAANAGGGAGNCNAAAANACTANNCANGTTANACAACNNCGANACCTAGGA
241' AACTAAGGATATNGCCCATNNAGCTCCNAGNGCTAGATNNCCATAACAGCGCNAGNTTAT
301' TACAAGGNTGNAACCATTATATTANACNAGATACTANTACTAGNCTANTANTTAATNTAA
361' TAATGNTACTTACTCGATACAGCNNTANTATATAACTATGAATAATTGAACTAGTTTGAT
421' TACTAGTATTACATTGATTATAATGAGNTTAANTATATATAANTTTATTTATACTTTACT
481' CAATTATTACACGAGTANTTTTATTATATAGGCTCTCTAATGCACAGTTTAATAATATTG
541' TTAGTAAATAATTTTTAGAAATAATAATTTAATAAAATTTAGTTTTTACTGCGTTATGGT
601' CTAGAAAATTACGATAAAATCGTACTTCCCCGACATGCCTCTGGGTAGGGGCGTTCTGCG
                                                    **** **********---
                [13B foward primer]                   GTGGGGGAGGGGCGTTCT
661' AAATTCGATTTTTGGCATACAGAAACCCCGTTCAAAAAATGCCCAATTTTCACGATTTTT
  1'-------------------------------------------------------------
721' GGCCTCCCCGTGCACAATTAGGGGTTGGTGTAATATAGTGGGCCGCGCACCCCG
     ----------------** * ************ **
                     AACTGGGGGTTGGTGTAAAAT      [13A reverse primer]
```

PCR

incubation	94℃	2 min.
denature	94℃	1 min.
annealing	50℃	2 min.
extention	72℃	3 min.
cycles	25 cycles	
PCR product	120 bp	

Figure 4.1.1. continued.

efficacy of PCR to detect the *Leishmania* parasites (Rodriguez *et al*., 1994; Lopez *et al*., 1993; Brujin *et al*., 1993)

As a preliminary trial, we performed the PCR method for detecting *Leishmania*, using four species of the genus *Leishmania* with a hope of its clinical application for the diagnosis of leishmaniasis. The results showed bands in MHOM/EC/88/INH-03 (*L. (V.) braziliensis*) and MHOM/EC/88/INH-04 (*L. (V.) panamensis*), but not in MHOM/EC/88/PT-007 (*L. (L.) mexicana*) and MSCI/EC/87/G-02 (*L. (L.) amazonensis*) with the primers of L1/L2. Negative reactions were obtained in all the four species with the primers of US1/LS1, contrary to our expectation that bands would appear at the site of 341 bp in PT-007. We thought that PCR didn't work well for some unknown reasons. The amplified DNA products were obtained in all the four kinds of genomic DNA at the site of 114 bp with the primers of US2/LS2. Therefore, we considered that it would be possible to detect leishmaniasis cases caused by *L. (L.) mexicana* complex and *L. (V.) braziliensis* complex with the primers of

File name: U19810/*L.(L) amazonensis* kinetoplast minicircle, partial sequence, fragment LAMAZ
Sequence size: 599

13B foward primer; sequence size: 18
13A reverse primer; sequence size: 21

[Accession No.: U19810/599 bp]

```
  1' TTTTTGATTTAGTAGTTAATAGCTGTGTTTATTTGTGTGGGTGGTGGTATGGTGTTACAG

 61' AGTTTGGTGGAAATTGGTGGGAAAATGGTCCCGGCCCAAACTTTTCTGCCGTGGGGAGGG
                                                [13B foward primer]
                                                GTGGGGGAGGG
121' GCGTTCTGCGGATTCGGGAAAAATGAGTGCGAAACCCCGGTTCATAATTTGGGGGATTTT
     GCGTTCT          [13A reverse primer]
181' GGAGAATTCCGGCTCCGAGGCTCGAAACTGGGGGTTGGTGTAAAATAGGGGCGGCTGCTC
                                        AACTGGGGGTTGGTGTAATAT    [13A reverse primer]
241' TGGATTATTNATTTTAAAATTAATTATTTTGTTAAAATATATTTTGGTTAGGAATATGTT

301' TTTATTGCTTCTGCATTTGTTAGATTGGCTGATGCTCTGTAGGTTGTTTGTTTTAGGCTC

361' TAGTGGTCTTTTGATTGCGTTCTTTTTCCTTACAATTTGTATTTATATTACATATATTAC

421' TTAGGGGTTATTTGTTTGGTATGAGGTAGGCTGTGTTATGCTCATGAGTTTGTGATATTG

481' TTTAGATTTATAATGTTAGATCGGATTATATGTTCCTCGTTTACTATTTGTGATGTTTGT

541' GCTAATTTGNAATNAATAGTTGGGACGGTTGGACCGGTTGTTNTAAGGATTACATGNGT
```

PCR

incubation	94℃	2 min.
denature	94℃	1 min.
annealing	50℃	2 min.
extention	72℃	3 min.
cycles	25 cycles	
PCR product	120 bp	

Figure 4.1.1. continued.

US2/LS2 if target *Leishmania* species were limited.

Laskay *et al*. (1995) reported that 7 out of 40 cases with suspect of leishmaniasis but with negative results of *Leishmania* culture showed positive results by PCR, and stated that it was worth while diagnostically performing PCR especially for the chronic cases. Our results with the paraffin-embedded specimens from the patients with leishmaniasis showed no PCR products except from EC/7/96/8/13. The reasons for this might be that the amount of DNA collected from the paraffin slices was insufficient, or that PCR didn't work well probably due to paraffin and/or formalin that might have obstructed the entire courses of PCR, or that little amount of PCR products might have lead to false negative results. Considering these possible reasons, we retried PCR using TAKARA Ex Taq with

File name: Z11555/*L.(L.) mexicana* kinetoplast minicircle DNA
Sequence size : 699

13B foward primer; sequence size: 18
13A reverse primer; sequence size: 21

[Accession No.; Z11555/699 bp]

```
  1' GGTCCCGGCCCAAACTTTTCTGCCCCGTGGGGGAGGGGCGTTCTGCGAATTTGGGAAAAA
                                  ******************----------------
        [13B foward primer]                GTGGGGGAGGGGCGTTCT

 61' TGAGTGCAGAAACCCCGTTCATATTTTGGGGAATTTTGGGGAATTCCGGCTCCGGCGGTC
     ------------------------------------------------------------
121' ACAACTGGGGGTTGGTGTAAAATACCCGGTGCGTGGGTATGGTTAGTGGTAGAGCTTTGT
       ****************** **
       AACTGGGGGTTGGTGTAATAT      [13A reverse primer]

181' CGTAGGCTTGGTCTTGATGCTTTAGATTAGTTTTGGGCTGGTGAGTTTTGTTTGGTAGAT
241' TTGGTATACATTTTAAATTGTCTGTTATTTGATTTTAATAATCTCTTTGGTATAGTTATT
301' ATTCTATGTGGTATTAGTTTGTTAGGATGTTGTGTGTTGGATTTGGTGTCTTGGATTTGG
361' GCTGCCCGTGGGTTTATATATTTATGGTTGAATAATGGAGATAATATAGAATACTGATAA
421' GTTATATTATATATTATTAGGTGGTAGTGGATTGGATATGGTAATGTGGGGGTTTGTTGC
481' ATTATGTGGTACGTGGATGTTTATGTTGTCTTTCGTATTTATATTTAGCTTTTCGTTTTG
541' AATTTCTGTTTATGTTATTATATCGTTGTGGTCATTGTGATATTTGGGTTATTTGTATGT
601' TCTGGTTTTGAGATATGTGTTTGTTAATTAAAAGATGTTTTGAGGGGATTAGACTTGGTT
661' TGGATATGCTGATTTGGTTAGGAGTTTCTGGGGAAAGTG
```

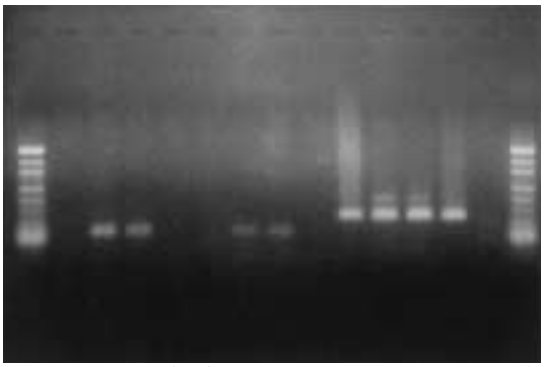
PCR

incubation	94℃	2 min.
denature	94℃	1 min.
annealing	50℃	2 min.
extention	72℃	3 min.
cycles	25 cycles	
PCR product	120 bp	

Figure 4.1.1. continued.

TAKARA RN easy Mini Kit (50) to collect DNA, but no expected PCR products were obtained.

It is necessary for clinicians without a thorough knowledge of molecular biology to be able to judge easily the results of PCR to detect *Leishmania* parasites in skin lesions. In order to attain this purpose, it is desirable that the primers should amplify specific portions of *Leishmania* parasites and that the PCR products should be located at such a site as the clinicians can easily detect them. Because of their short base sequences in DNA, it is sometimes difficult to recognize PCR products with the size less than 100 bp when compared with a size marker or a primer. Among the primers we used in this study, US2/LS2 that form bands at the site of 114 bp is the most useful for clinicians to detect *Leishmania* infections by PCR. However, an ideal method for clinicians is to detect *Leishmania* parasites from paraffin slice specimens. However, in the present trials, we have not been able to establish a method to extract DNA from paraffin slice specimens yet, and, this will be done in the future.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

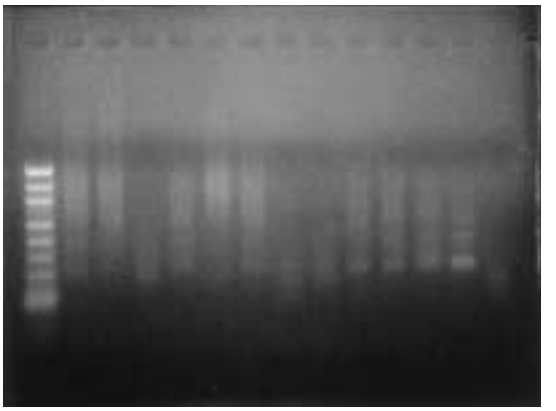
Figure 4.1.2. The results of PCR using *Leishmania* parasites.
lane 1: size marker; pUC/HapII
primer L1/L2
lane 2: MHOM/EC/88/PT-007(*L. (L.) mexicana*)
lane 3: MHOM/EC/88/INH-03(*L. (V.) braziliensis*)
lane 4: MHOM/EC/88/INH-04(*L. (V.) panamensis*)
lane 5: MPOT/EC/87/G-03(*L. (L.) amazonensis*)
primer US1/LS1
lane 6: MHOM/EC/88/PT-007(*L. (L.) mexicana*)
lane 7: MHOM/EC/88/INH-03(*L.(V.) braziliensis*)
lane 8: MHOM/EC/88/INH-04(*L.(V.) panamensis*)
lane 9: MSCI/EC/87/G-03(*L.(L.) amazonensis*)
primer US2/LS2
lane 10; MHOM/EC/88/PT-007(*L. (L.) mexicana*)
lane 11: MHOM/EC/88/INH-03(*L. (V.) braziliensis*)
lane 12: MHOM/EC/88/INH-04(*L. (V.) panamensis*)
lane 13: MSCI/EC/87/G-03(*L. (L.) amazonensis*)
lane 14: dH_2O
lane 15: size marker; pUC/HapII

1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 4.1.4. The results of PCR on paraffin-embeded skin tissue from the patients with leishmaniasis. Lane 6 showed a band (positive).
primer US2/LS2
lane 1: size marker; pUC/HapII
lane 2: EC/3/96/8/13
lane 3: EC/4/96/8/13
lane 4: EC/5/96/8/13
lane 5: EC/6/96/8/13
lane 6: EC/7/96/8/13
lane 7: EC/9/96/8/13
lane 8: EC/11/96/8/13
lane 9: EC/14/96/8/13
lane 10: EC/15/96/8/13
lane 11: EC/16/96/8/13
lane 12: DCL
lane 13: normal skin (Okinawa)
lane 14: dH_2O

1 2 3 4 5

Figure 4.1.3. The results with the primers, 13A/13B.
lane 1: size marker; pUC/ HapII
primer 13A/13B
lane 2: MHOM/EC/88/ PT-007 (*L. (L.) mexicana*)
lane 3: MHOM/EC/88/ INH-03 (*L. (V.) brazilien*sis)
lane 4: MHOM/EC/88/ INH-04 (*L. (V.) panamensis*)
lane 5: MSCI/EC/87/G-03 (*L. (L.) amazonensis*)



1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 4.1.5. The second PCR trial on paraffin-embedded skin tissue.
primer US2/LS2
lane 1: size marker; pUC/HapII
lane 2: EC/3/96/8/13
lane 3: EC/4/96/8/13
lane 4: EC/5/96/8/13
lane 5: EC/6/96/8/13
lane 6: EC/7/96/8/13
lane 7: EC/9/96/8/13
lane 8: EC/11/96/8/13
lane 9: EC/14/96/8/13
lane 10: EC/15/96/8/13
lane 11: EC/16/96/8/13
lane 12: DCL
lane 13: normal skin (Okinawa)
lane 14: dH_2O

1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 4.1.6. Nested PCR on paraffin-embeded skin tissue. Lane 12 (DCL) showed a band (positive).
primer US2/LS2 outer primer
lane 1: size marker; pUC/HapII
lane 2: EC/3/96/8/13
lane 3: EC/4/96/8/13
lane 4: EC/5/96/8/13
lane 5: EC/6/96/8/13
lane 6: EC/7/96/8/13
lane 7: EC/9/96/8/13
lane 8: EC/11/96/8/13
lane 9: EC/14/96/8/13
lane 10: EC/15/96/8/13
lane 11: EC/16/96/8/13
lane 12: DCL
lane 13: normal skin (Okinawa)
lane 14: dH_2O

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2. A Comparative Study of Conventional and PCR-Based Diagnosis of Cutaneous Leishmaniasis in Ecuador

ABSTRACT. The results of a preliminary trial carried out in an endemic area for cutaneous leishmaniasis in Ecuador, are reported here. The diagnostic value of a polymerase chain reaction (PCR) specific for *Leishmania (V.) braziliensis* complex was compared with that of currently recommended 3 methods: microscopic examination of dermal scrapings, *in vitro* culture of patient tissue biopsy and histopathologic examination. The outcome of this trial was that PCR was consistently more sensitive than any of the three currently recommended methods of diagnosis. PCR results were much faster than the culture and histopathologic methods. The sensitivity of visualizing amastigotes in scraping samples, which is simple, cheap and conventional method in use reached to only 37.5%. The current results showed that the PCR technique can provide the means for early and rapid diagnosis of leishmaniasis in Ecuador. This should reduce morbidity and treatment costs of cutaneous leishmaniasis.

Introduction

Leishmaniasis is endemic in many areas of tropical and subtropical America. At least 24 countries are affected and it constitutes an important public health problem in the New World (Grimaldi and Tesh, 1993). The number of reported human cases of New World leishmaniasis has increased dramatically in recent years (Walton *et al*., 1988). Although *Leishmania (Viannia) braziliensis* and *L. (Leishmania) mexicana* complexes, are responsible for most of the cases in Latin American countries; the former is by far the most prevalent (Lopez *et al*., 1993).

Diagnosis of leishmaniasis is often complicated, in particular with *L. (V.) braziliensis* complex, the number of parasites present in lesions is scarce (Marsden, 1985). In endemic Latin American countries the diagnosis of cutaneous leishmaniasis is usually made on the basis of clinical manifestations. The serological tests are not able to discriminate between past or present infections. Furthermore, serologic tests show cross-reactivities with sera from individuals infected with other diseases such as Chagas disease, malaria, brucellosis, leprosy and tuberculosis which co-exist in leishmaniasis endemic regions in the New World (Guevara *et al*., 1989).

The laboratory procedures used for diagnosis of leishmaniasis have been extensively reviewed (Grimaldi and McMahon-Pratt, 1991; Manson-Bahr, 1987; Palma and Gutierrez, 1991). The classical methods used for direct demonstration of the parasite in tissues or skin lesions include the following: (i) examination of stained smears or histologic sections; (ii) inoculations of hamsters with aspirates from infected tissues or with triturated tissue fragments; (iii) *in vitro* culture of tissue homogenates or aspirates in biphasic media. A combination of these direct techniques is still the most commonly used method for the diagnosis of leishmaniasis, especially the cutaneous form (Grimaldi and McMahon-Pratt, 1991; Navin *et al*., 1990). New approaches for the detection of parasites, such as standard DNA hybridization procedures have been attempted since the early 1980s. In spite of their considerable sensitivity, detecting as few as 50-100 parasites (Lopez *et al*., 1988; Wirth *et al*., 1986), and the incorporation of nonradioactive detection systems (Lopez *et al*., 1988; Barker, 1989), their potential use in routine diagnosis has been hampered by the multiple step nature of standard hybridization procedures. The development of the polymerase chain reaction (PCR) (Saiki *et al*., 1985) has provided a more

powerful approach to the application of molecular biologic techniques for diagnosis. Extension primers were designed to amplify conserved sequences found in minicircles of kinetoplast DNA (kDNA) of *Leishmania* parasites. Such a target is eminently suitable because the kinetoplast is known to posses thousands of copies of the minicircular DNA (Simpson, 1987) thereby enhancing the likelihood of detection of the parasite.

In Ecuador, leishmaniasis is endemic in many regions including coastal, east jungle and Andean valleys (Amunarriz, 1991; Hashiguchi and Gomez, 1992; Barrera *et al*., 1994). In the present study, we selected *Leishmania* infected patients from El Carmen, Province of Manabi, Ecuador and compared between the conventionally recommended methods of visualizing amastigotes or promastigotes from human tissue samples, and the PCR procedure. The finding provides guidelines for the selection of the most sensitive and appropriate methods for the diagnosis of cutaneous leishmaniasis in endemic areas of Ecuador.

Materials and Methods

Patients and study area

A total of 25 patients with skin lesions compatible with cutaneous leishmaniasis were evaluated between January and May 1997. Patients were enrolled if the ulcerative, papular, or nodular skin lesions, lasting for two or more weeks, were not attributable to trauma. All patients are residents in the canton El Carmen, small town located in the Pacific region, Province of Manabi, Ecuador at 600 m above sea level. It has approximately 68,000 inhabitants distributed into 24 communities. The samples were taken in a local hospital and patients were noticed of the purpose of the study.

Diagnostic procedures

Lesion margins were cleaned and debrided with 70% alcohol and furacin detergent. The dermal scrapings and smears were Giemsa-stained and then examined for amastigotes at x100 magnification. After injecting 2% xylocaine, a biopsy was taken at the margin of the lesion with a 4-mm diameter disposable punch (Kai, Japan). The biopsy was divided into three portions, one was fixed in 10% formalin for histopathologic examination. Another part was sectioned in various parts and inoculated into two NNN culture medium tubes with penicilin and streptomycin, maintained for 24 hrs at 4℃, and then incubated at 27℃, were examined every 2-3 days looking for promastigotes growth under an inverted microscope for a period of one month, or until positive. The last portion was maintained in 200 ml of TE buffer (Tris-EDTA) in congelation until DNA extraction.

DNA preparation and PCR assay

Approximately 1 mm^3 of the biopsy specimen was immersed in 100 μl of lysis buffer (10 mM Tris-HCl, pH8.0; 10 mM EDTA; 1 μg/μl of proteinase K) and incubated at 55℃ for 2 hrs. The digested tissue was treated with phenol/chloroform for DNA extraction and precipitated with sodium acetate. DNA was stored at -20℃ until use.

The PCR was performed as described previously (Lopez *et al*., 1993). Briefly, 3 μl of purified DNA samples were added in final volume of a 20 μl reaction mixture containing 1 μM of each *L. (V.) braziliensis* complex specific primers (MP1L; 5'-TACTCCCC GACATGCCTCTG-3' and MP3H; 5'-GAACGGGGT TTCTGTATGC-3'), and 1 unit of Taq DNA polymerase (Perkin-Elmer-Cetus, Norwalk, CT). In the first cycle the temperature steps were 3 min at 94℃, followed by 29 cycles each one consisting of 1 min at 94℃ (denaturation), 1 min at 54℃ (annealing) and 30 seconds at 72℃ (elongation). All tests were carried out with the following controls: positive control (biopsy patients with parasitolgically proven leishmaniasis) and negative control (skin biopsies from individuals with ulcerations other than leishmaniasis). DNAs from both positive and negative controls were added in every PCR reaction. The amplified products were visualized by ethidium bromide staining, after 1% agarose gel electrophoresis, under UV light (Fig. 4.2.1).

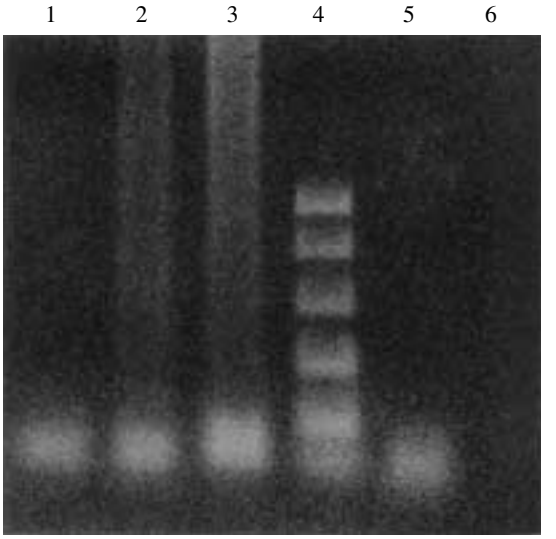

Figure 4.2.1. A respective example of the results obtained using the PCR assay in skin biopsy samples from Ecuadorian patients suspected of leishmaniasis are shown. Lane 1, 2 and 3: PCR products from leishmaniasis-infected Ecuadorian patients. Lane 4: DNA size marker. Lane 5: positive control of *Leishmania (Viannia) braziliensis* complex. Lane 6: negative control. One fifth of the PCR product was electrophoresed in 1% agarose gels, and visualized by ethidium bromide staining.

Table 4.2.1. Pairwise comparison between PCR method and three standard methods

Patient number	Stained smear	Culture	Histopathologic examination	PCR
1	positive	positive	negative	positive
2	positive	positive	negative	positive
3	positive	positive	negative	ND
4	negative	negative	negative	negative
5	positive	positive	negative	positive
6	positive	positive	negative	positive
7	positive	positive	negative	positive
8	ND※	positive	negative	ND
9	negative	negative	negative	negative
10	negative	negative	negative	positive
11	negative	positive	positive	positive
12	negative	positive	negative	positive
13	negative	negative	negative	negative
14	negative	positive	negative	positive
15	negative	positive	positive	positive
16	negative	negative	negative	positive
17	negative	negative	negative	negative
18	negative	negative	negative	negative
19	positive	positive	negative	positive
20	negative	positive	positive	positive
21	negative	negative	positive	positive
22	negative	negative	negative	positive
23	positive	negative	negative	positive
24	positive	negative	positive	positive
25	positive	positive	positive	positive

※ND = not done.

Results

The three standard diagnostic methods for leishmaniasis with which the PCR method is compared were carried out in an endemic area of cutaneous leishmaniasis in Ecuador, under field conditions. The pairwise comparison between the PCR method and the three standard diagnostic methods is given in Table 4.2.1. PCR was consistently more sensitive than smear examination, histopathologic examination and culture in specific medium. PCR detected *Leishmania* kDNA in two samples negative for the three other procedures.

Discussion

The three recomended standard diagnostic methods used in this study are the best currently available. Hamster inoculation requires animal facilities and this service is not always available, especially in field conditions. The limited sensitivity of histopathology (20%) was probably due to the scarcity of organisms or shrinkage and/or distortion of amastigotes during fixing which is consistent with previous reports (Cuba-Cuba *et al*., 1984; Salinas *et al*., 1989; De Brujin *et al*., 1993) showing that histopathology has limited sensitivity and, given its low utility in relation to cost, it is best to use it as a complementary test rather than as primary diagnostic procedure.

The procedure for immediate diagnosis, dermal scraping, demonstrated only 37.5% (9/24) of sensitivity. The dermal scraping method is recommended, however, for its simplicity and low cost.

Otherwise, 13 (52%) out of 25 biopsy samples were positive after culture in especific media. However, there is a high risk of bacterial and/or fungal contamination working in field conditions which decrease the sensitivity of the test. Also, cultures require at least one week for parasite growth. Although parasite isolation is the gold standard for leishmaniasis diagnosis, its limitations clearly indicate that more sensitive methods are needed to increase sensitivity.

In the contrary, PCR detected *Leishmania* DNA in 17 (74%) of the 23 samples tested so far. These results show PCR to be consistently more sensitive than the three recommended methods. The confirmed specificity for *Leishmania (Viannia)* species, when crude samples are used, enhances the utility of PCR for diagnosis of cutaneous leishmaniasis in endemic areas. Also, PCR results are available much faster than those of the culture-methods. Further studies to evaluate the usefulness of PCR to monitor treatment of *Leishmania* infected patients are ongoing.

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3. Preliminary Studies on Diagnosis of Cutaneous Leishmaniasis using Skin Biopsy Specimens by Polymerase Chain Reaction (PCR)

ABSTRACT. Diagnostic methods of cutaneous leishmaniasis by polymerase chain reaction (PCR) were studied using cultured parasites and human skin biopsy specimens from Ecuador. Template DNAs were prepared by boiling for 10 min in 5 % Chelex solutions. *Leishmania* were detected by PCR using primers designed from minicircle (13A and 13B) and mini-exon gene (S-1629 and S-1630). The former primers amplified non-specific products in human DNA and sensitivity of the reaction was low. The latter ones never amplified non-specific products even in human template and enabled the subgenus level identification. When sensitivity was increased in the latter system, the results well coincided with those of identifications by smear or culture in the field survey. Moreover, three unknown samples by morphological examination were diagnosed to be parasites belonging to subgenus *Viannia* by the present PCR method.

Introduction

The diagnosis of cutaneous leishmaniasis depends mainly on the microscopical demonstration of parasites from smear specimens or culture. However, in the case of few parasites in the lesions and for the estimation of successful treatment, more sensitive methods are required.

Since Rodgers *et al*. (1990) reported the PCR method using 13A and 13B primers designed from kinetoplast minicircle, many methods based on PCR have been reported. The main target regions to amplify are kinetoplast minicircle DNA (Smyth *et al*., 1992; Lopez *et al*., 1993; Bhattacharyya *et al*., 1993; Rodriguez *et al*., 1994; Laskay *et al*., 1995; Bhattacharyya *et al*., 1996), small-subunit rRNA gene (Guevara *et al*., 1992; Meredith *et al*., 1993; Mathis and Deplazes, 1995) and the mini-exon genes repeat (Hassan *et al*., 1993; Fernandes *et al*., 1994; Ramos and Simpson, 1996). RAPD-PCR (Noyes *et al*., 1996; Motazedian *et al*., 1996) and AP-PCR (Pogue *et al*., 1995a, b) were also developed. However, Dobner *et al*. (1994) reported the presence of intraspecific variation by the analysis of kinetoplast minicircle DNA. As the method with high sensitivity and convenience to mass treatment, enzyme-linked immunosorbent assay (ELISA) after PCR (Costa *et al*., 1996) and PCR-SHELA (Qiao *et al*., 1995) were reported, but those methods require each species specific probe to identify species. Among the PCR methods of *Leishmania* diagnosis, the detection by PCR from patient's materials was regarded as the most reliable one (Ravel *et al*., 1995; Nuzum *et al*., 1995). Recently Ramos and Simpson (1996) proposed the possibility to identify *Leishmania* species by the PCR products of conserved region of the mini-exon repeat using S-1629 and S-1630 primers. We report here the results of our preliminary trial on species identification of *Leishmania* by PCR using two sets of primers 13A - 13B and S-1629- S-1630.

Materials and Methods

Template DNAs were prepared from eight *Leishmania* species, *L. (Leishmania) major, L. (L.) major*-like, *L. (L.) mexicana, L. (L.) amazonensis, L. (V.) braziliensis, L. (V.) guyanensis, L. (V.) panamensis* and *L. (V.) equatorensis* maintained by culture in Kochi Medical School. Cultures containing parasites were washed by physiological saline and fixed in 100% ethanol. A total of 40 ml of suspension were cen-

Table 4.3.1. Comparison of PCR results using two primer-sets, 13A-13B and S-1629-S1630

Specimen	Smear or culture	Polymerase chain reaction		
		13A-13B	S-1629	S-1630
Huigra 16	−	−	−	
La Moya 2	+	−	−	±※※
Alausi 8	+	+	*L. (L.) mexicana*	
Alausi 21	+	−	−	−
Mil 1	+	−	−	−
Mil 3	+	+	*L. (V.)*※	
Mil 4	+	+	*L. (V.)*	
Mil 5	+	−	−	+
Mil 6	+	+	*L. (V.)*	
Mil 7	+	+	*L. (V.)*	
Mil 8	+	+	*L. (V.)*	
Mil 9	+	+	*L. (V.)*	
Mil 10	+	−	−	+
Mil 11	?	−	−	+
Mil 12	+	+	*L. (V.)*	
EI Mamey 6	?	−	−	+
EI Mamey 7	?	−	−	−
La Moya 1	−	−	−	−
Zhucay 21	−	−	−	−
Zhucay 22	?	−	−	+
Zhucay 23	?	−	−	±
Zhucay 24	?	−	−	−
Zhucay 26	?	−	−	−
Zhucay 27		−		

※*L. (V.)* represents one or more species belonging to subgenus *Viannia*.
※※±; very weak band was observed.

In subgenus *Leishmania* group, distinct products were gained in three species; 460 bp and 1000 bp in *L. (L.) major*, 360 bp in *L. (L.) mexicana* and 320 bp and 800 bp in *L. (L.) amazonensis* although the same products pattern was observed in *L. (L.) major* and *L. (L.) major*-like strain. However almost the same products (290 bp and 620 bp) were obtained in three species belonging to subgenus *Viannia, L. (V.) braziliensis, L. (V.) guyanensis* and *L. (V.) panamensis*, except for 70 bp and 140 bp in *L. (V.) equatorensis* (Fig. 4.3.3). In this system no products were amplified in human DNA. From these results, it would be possible to identify species at subgenus level by the present PCR.

Field samples were also analysed by PCR using mini-exon primers. Parasites were determined as *L. (L.) mexicana* in Alausi 8 and it was thought to be infected by parasites belonging to subgenus *Viannia* in Mil 3, Mil 4, Mil 6 Mil 7, Mil 8, Mil 9 and Mil 12 (Fig. 4.3. 4 and Table 4.3.1). In Huigra 16, La Moya 2, Alausi 21, Mil 1, Mil 5, Mil 10, Mil 11, El Mamey 6, El Mamey 7, La Moya 1, Zhucay 21, Zhucay 22, Zhucay 23, Zhucay 24, Zhucay 26 and Zhucay 27 PCR

trifuged at 10000 rpm for 5 min and after the removal of supernatant precipitates were air-dried in refrigerator. Then 200 ml of 5% Chelex 100 resin (Bio Rad Laboratories, Hercules, CA, USA) solution were added and mixed to resuspend parasites in the solution. They were boiled for 10 min and used as a template.

Human skin smear specimens (field samples) were obtained from inhabitants during field survey, 1992-1993, in Ecuador. Samples were smeared on the slide glasses and fixed with 100% ethanol after dry. Every specimens on slide glass were removed by a sterile cutter knife with 20 ml of re-distilled sterile water and transferred into a microtube. After the addition of 240 ml of 5% Chelex 100 resin solution, the tubes were boiled for 10 min and 1 ml of supernatant was used as the template.

Polymerase chain reaction (PCR) was performed using S-1629 and S-1630 primers based on the method of Ramos and Simpson (1996). PCR mixture (total 50 ml) contains 200 mM dNTP, Taq polymerase (Amplitaq, Perkin-Elmer Cetus, Norwalk, CT) 1.25 unit, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 10 % (0 and 5%) DMSO (dimethylsulfoxide), each 200 nM primer and 1 ml template DNA. After 5 cycles of 95℃ for 1.5 min, 45℃ for 1 min and 65℃ for 1.5 min, 35 cycles of reaction were performed under the condition of 95℃ for 1.5 min, 50℃ for 1 min and 72℃ for 2 min and followed by a cycle of 72℃ for 7 min and stored at 4℃. In another experiments, six cycles were increased; PCR was performed under the condition after first step of 6 cycles of 95℃ for 1.5 min, 45℃ for 1 min and 65℃ for 1.5 min, and second step of 40 cycles of 95℃ for 1.5 min, 50℃ for 1 min and 72℃ for 2 min.

In the case of PCR using 13A and 13B primers, 28 cycles of reaction were performed under the conditions of 94℃ for 1.5 min, 64℃ for 1.5 min and 72℃ for 1.5 min in the same mixtures without DMSO. Products were electrophoresed in agarose gel, stained with ethidium bromide and analysed.

Results

The system using 13A and 13B primers amplified 120 bp products from every *Leishmania* species, but products of the same size were obtained in human template DNA. After the examination of PCR condition to distinguish *Leishmania* from human, annealing temperature and cycles were determined to 64℃ and 28 cycles, respectively. In this condition there are no non-specific amplification when 10 ng human DNA was used as template (Fig. 4.3.1). Field samples were tested by this method. PCR products using 13A-13B primers were detected from samples of Alausi 8, Mil 3, Mil 4, Mil 6, Mil 7, Mil 8, Mil 9 and Mil 12. The samples of Huigra 16, La Moya 2, Alausi 21, Mil 1, Mil 5, Mil 10, Mil 11, El Mamay 6, El Mamay 7, La Moya 1, Zhucay 21, Zhucay 22, Zhucay 23, Zhucay 24, Zhucay 26 and Zhucay 27 were considered to be negative (Fig. 4.3. 2 and Table 4.3.1).

PCR were performed using the mini-exon primers (S-1629 and S-1630), 10% DMSO and template DNAs of *L. (L.) major, L. (L.) major*-like, *L. (L.) mexicana, L. (L.) amazonensis, L. (V.) braziliensis, L. (V.) guyanensis, L. (V.) panamensis* and *L. (V.) equatorensis*.

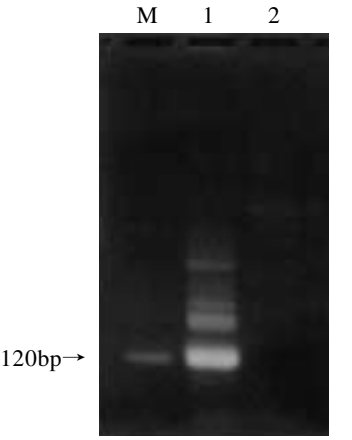

Figure 4.3.1. PCR products using 13 A - 13 B primers stained with ethidium bromide after agarose gel electrophoresis. M; 120 bp marker: Lane 1; *L. (Leishmania) mexicana* DNA: Lane 2; human DNA (10 ng).

Table 4.3.2. Differences of PCR products according to the difference of DMSO concentration; numbers represent products size in basepairs

Species	DMSO concentration			
	0-4%	5%	8%	10%
L. (L.) major	290 (very weak)	290, 500		460, 1,000
L. (L.) major-like				470
L. (L.) mexicana		500		360
L. (L.) amazonensis		320, 800	320, 800	
L. (V.) braziliensis	290	290, 600		290, 620
L. (V.) guyanensis	290	290, 600		290, 620
L. (V.) panamensis	290	290, 600		290, 620
L. (V.) equatorensis	100	100	100, 140	70, 140
Human	no products	no products	no products	no products

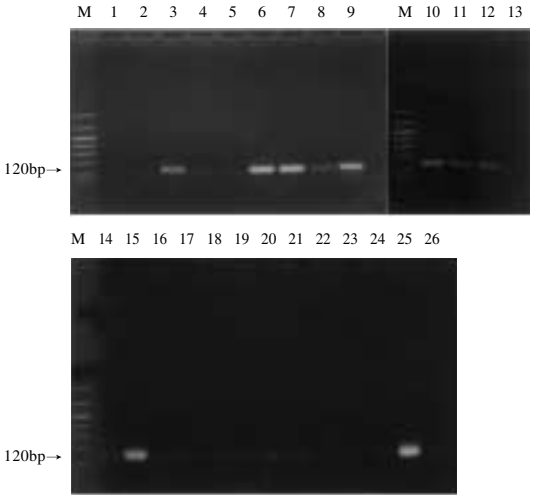

Figure 4.3.2. PCR products of skin smear specimens using 13A-13B primers stained with ethidium bromide after agarose gel electrophoresis. M; marker, Lane 1; Huigra 16, Lane 2; La Moya 2, Lane 3; Alausi 8, Lane 4; Alausi 21, Lane 5; Mil 1, Lane 6; Mil 3, Lane 7; Mil 4, Lane 8; Mil 5, Lane 9; Mil 6, Lane 10; Mil 7, Lane 11; Mil 8, Lane 12; Mil 9, Lane 13; Mil 10, Lane 14; Mil 11, Lane 15; Mil 12, Lane16 ; El Mamay 6, Lane 17; El Mamay 7, Lane 18; La Moya 1, Lane 19; Zhucay 21, Lane 20; Zhucay 22, Lane 21; Zhucay 23, Lane 22; Zhucay 24, Lane 23; Zhucay 26, Lane 24; Zhucay 27, Lane 25; *L. (L.) mexicana* DNA, Lane 26; human DNA (1 ng).

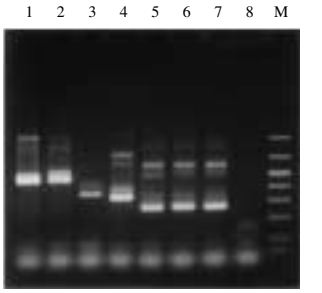

Figure 4.3.3. Results of PCR with 10 % DMSO using S-1629 and S-1630 mini-exon primers. Stained agarose gel with ethidium bromide after electrophoresis. M; marker (1,000, 700, 525 + 500, 400, 300, 200, 100 and 50 bp), Lane 1; *L. (L.) major*, Lane 2; *L. (L.) major*-like, Lane 3; *L. (L.) mexicana*, Lane 4; *L. (L.) amazoensis*, Lane 5; *L. (V.) braziliensis*, Lane 6; *L. (V.) guyanensis*, Lane 7; *L. (V.) panamensis*, Lane 8; *L. (V) equatorensis*.

products were not detected. Samples of PCR negative but diagnosed positive or unknown in *Leishmania* infection by smear or culture method were re-examined by PCR 6 cycles added. After the modified PCR, five samples (Mil 5, Mil 10, Mil 11, El Mamey 6 and Zhucay 22) proved to be positive (parasites belonging to *Viannia*) and two samples (La Moya 2 and Zhucay 23) showed very weak band (±) (belonging to *Viannia*). In samples of Mil 11, El Mamey 6 and

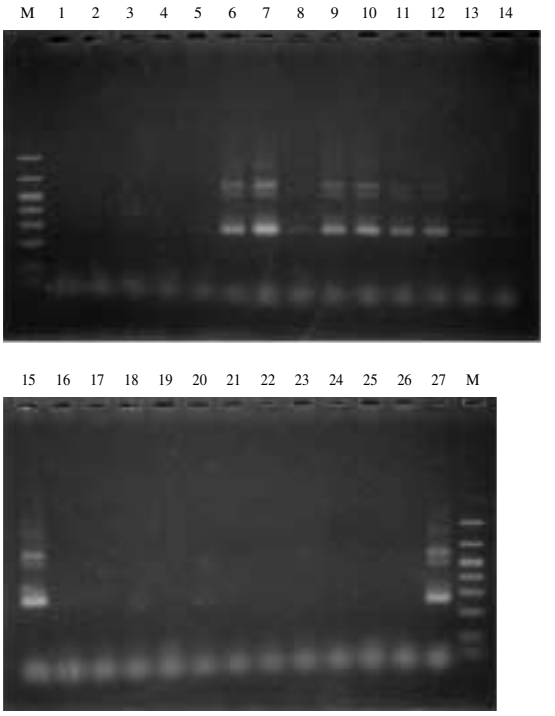

Figure 4.3.4. Results of PCR with 10% DMSO using S-1629 and S-1630 mini-exon primers. Stained agarose gel with ethidium bromide after electrophoresis. M; marker (1,000, 700, 525 + 500, 400, 300, 200, 100 and 50 bp), Lane 1; Huigra 16, Lane 2; La Moya 2, Lane 3; Alausi 8, Lane 4; Alausi 21, Lane 5; Mil 1, Lane 6; Mil 3, Lane 7; Mil 4, Lane 8; Mil 5, Lane 9; Mil 6, Lane 10; Mil 7, Lane 11; Mil 8, Lane 12; Mil 9, Lane 13; Mil 10, Lane 14; Mil 11, Lane 15; Mil 12, Lane16; El Mamay 6, Lane 17; El Mamay 7, Lane 18; La Moya 1, Lane 19; Zhucay 21, Lane 20; Zhucay 22, Lane 21; Zhucay 23,Lane 22; Zhucay 24, Lane 23; Zhucay 26, Lane 24; Zhucay 27, Lane 25; No DNA, Lane 26; human DNA (10 ng), Lane 27; *L. (V.) brasiliensis* DNA.

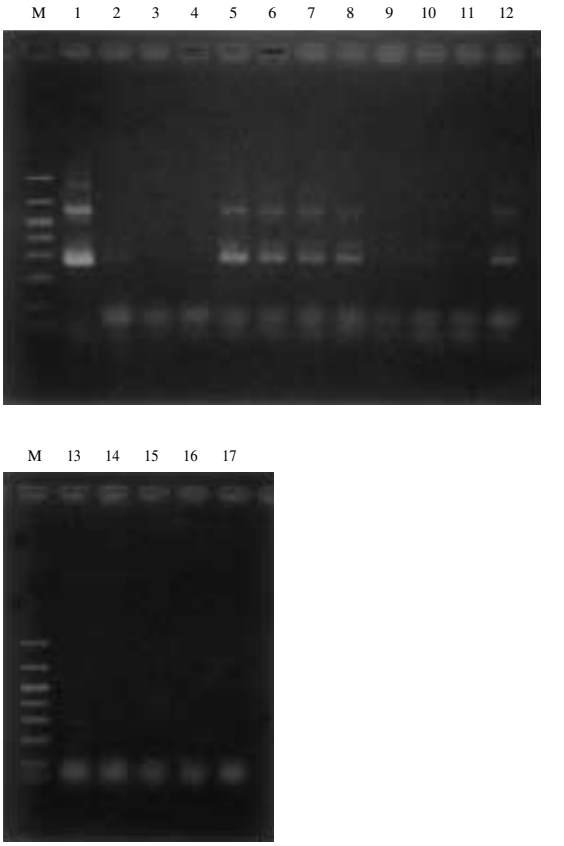

Figure 4.3.5. Results of PCR with 10 % DMSO using S-1629 and S-1630 mini-exon primers. Stained agarose gel with ethidium bromide after electrophoresis. M; marker (1,000, 700, 525 + 500, 400, 300, 200, 100 and 50 bp), Lane 1; *L. (V.) brasiliensis* DNA, Lane 2; La Moya 2, Lane 3; Alausi 21, Lane 4; Mil 1, Lane 5; Mil 5, Lane 6; Mil 10, Lane 7; Mil 11, Lane 8; El Mamay 6, Lane 9; El Mamay 7, Lane 10; La Moya 1, Lane 11; Zhucay 21, Lane 12; Zhucay 22, Lane 13; Zhucay 23, Lane 14; Zhucay 24, Lane 15; Zhucay 26, Lane16; Zhucay 27, Lane 17; No DNA.

Zhucay 22, infections were undetermined by smear or culture method, but they were probably thought to be positive (parasites belonging to *Viannia*) by the results of PCR. However, samples of Alausi 21 and Mil 1 were still negative (Fig. 4.3.5 and Table 4.3.1).

Discussion

Results of PCR using primers designed from mini-exon repeat have well coincided with that of histological or microscopical diagnosis as a whole. Final accordance rate was 87.5 % (14/16). As no fales-positive reactions were observed, positive samples in PCR were thought to contain *Leishmania* without doubt. Accordingly, when samples undetermined by smear examination or culture method turned positive by PCR, they should be diagnosed *Leishmania* positive.

As 13A-13B primers amplified non-specific products in human DNA, the cycles were decreased not to produce the bands. So the sensitivity was lowered, however the specimens diagnosed positive by this system were apparently positive ones. Non-specific products were never amplified in PCR systems using S-1629 and S-1630 primers. The minimal sensitivity including whole process was not determined in both systems using 13A and 13B primers and S-1629 and S-1630 primers because of the technical difficulties.

In the process of this study using S-1629 and S-1630 primers, each species has different reactivity to the concentrations of DMSO. Namely in subgenus *Leishmania*, the lower concentration of DMSO, the less products were detected. In contrast, in subgenus *Viannia*, clear bands were detected even in mixture without DMSO. But *L. (V.) equatorensis* had the peculiar reaction mode that the most abundant products were obtained in 0% DMSO condition (Table 4.3.2). So the role of DMSO is not the same in each species although its function is not clear in PCR. As the small sized products of PCR using 10% DMSO in *L. (V.) equatorensis* were detectable in agarose gel, we applied that concentration of DMSO in further analysis.

Variations in both the length and primary sequence of the non-transcribed spacers have been reported in *Leishmania*. *L. (V.) lainsoni* has 157 bp deletions in non-transcribed spacer of mini-exon gene and showed 32 bp deletion during polymerizing process in PCR (Fernandes *et al*., 1994). And Cupolillo *et al*. (1995) reported from the analysis of internal transcribed sequences (ITS) of rRNA gene that *L. (V.) lainsoni* and *L. (V.) equatorensis* were quite separate from other species and some association in phylogenic position. So our results in *L. (V.) equatorensis* suggest the presence of some deletion in mini-exon genes and artifact in PCR.

At present the identification of *Leishmania* by PCR has not been perfectly established because species in subgenus cannot be discriminated each other in any systems. The efforts to sequence non-transcribed spacer in mini-exon gene repeats may enable the complete identification at species level by simple PCR method. However, even in the present situation PCR is useful in diagnosis of *Leishmania* infection and the evaluation of treatments.

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Chapter 5

Clinical and Epidemiological Aspects

1. Clinical Survey of Cutaneous Leishmaniasis in Ecuador for 5 Years (1991-1995)

ABSTRACT. In this study, we analyzed clinical and epidemiological data on cutaneous changes of leishmaniasis recorded in the period from 1991 to 1995 in Ecuador. A total of 348 cutaneous leishmaniasis patients living in Ecuador were examined for this study. The study areas were included Provinces of Manabi, Los Rios, Azuay, and Esmeraldas. These areas are located in the Pacific coast and the slope of Andean mountains. Each patient was thoroughly examined clinically and parasitologically. The total 348 patients in this study consisted of 204 males and 144 females. The mean age of patients was 21.2 years (± 1.2 s.e.) in males, 18.9 years (± 1.4 s.e.) in females and 20.2 years (± 0.9 s.e.) in total. Patients less than 20-year-old occupied more than half of all the patients examined. However, senior patients more than 50-year-old were seen, namely 15 cases (7.4%) in males, 11 cases (7.7%) in females and 26 cases (7.5%) in total. The mean duration period of cutaneous lesions was 5.3 months (± 0.4 s.e.) in males, 6.3 months (± 0.7 s.e.) in females and 5.7 months (± 0.4 s.e.) in total. The number of the patients with the onset of lesions less than 1 month was 18 cases (10.5%) in males, 8 cases (7.0%) in females and 26 cases (9.1%) in total. However, there were 8 cases (4.7%) in males, 7 cases (6.1%) in females and 15 cases (5.2%) in total that showed the duration period of lesions more than 13 months. The popular types of lesions included ulcer, nodule, erythematous plaque and papule. The most polular type was ulcer formation. The patients with ulcer occupied more than 50% in total. More than half of the lesions were located on the face and the extremities. The lesions on the trunk were seen more frequently in male than in female. On the other hand, the lesions on the legs were more popular in female. Almost half of the lesions were solitary, but the remainder were multiple. The cases with more than ten multiple lesions were seen in five of the male patients, two of the female patients and seven of the total patients. Several species of *Leishmania* parasites and vectors were reported in the areas examined in this study. Cutaneous manifestations of leishmaniasis in Ecuador may differ delicately from those in other areas because of the difference of species of the parasites, various behaviors of the vectors and the different life style of the inhabitants. Therefore. medical doctors assigned to the treatments for cutaneous leishmaniasis in Ecuador have not only medical knowledge but also entomological, ecological, environmental and anthropological knowledge.

Introduction

There are three types of leishmaniasis, that is, cutanenous, mucocutaneous and visceral leishmaniasis. Cutaneous leishmaniases are classified into four types, that is, localized, diffuse, recidivans and post kala-azar (Grevelink *et al*., 1996). Previously, we reported several findings on cutaneous manifestations of leishmaniasis in Ecuador (Nonaka *et al*., 1990a, b; Hashiguchi *et al*., 1992; Reyna *et al*., 1994). That is, there were marked differences in clinical findings of cutaneous leishmaniasis between the low-

land and highland of Ecuador. The most popular cutaneous change was ulcer, but non-ulcered cutaneous changes such as papules, plaques and nodules were also seen frequently. Big ulcers more than 900 mm^2 were also seen in 21 (14.7%) of 143 cases of cutaneous leishmaniasis. Lymphnode swellings were also seen in half of the patients examined, more frequently in males than in females. One case of generalized cutaneous leishmaniasis was reported in Ecuador. In this study, we analyzed data on cutaneous changes of patients with leishmaniasis recorded from 1991 to 1995 in Ecuador.

Materials and Methods

A total of 348 cutaneous leishmaniasis patients living in Ecuador were analyzed for this study. The study areas included Provinces of Manabi, Los Rios, Azuay and Esmeraldas. These areas are located in the Pacific coast and the slope of Andean mountains. The period of observation was 5 years from 1991 to 1995. Before each examination, all the necessary information on the patients was recorded on registration cards by the members of the public health care centers. Onset of the lesions was recorded by asking the time when the lesions started. Each patient was thoroughly examined clinically and parasitologically. Smears were taken from the edge of ulcers and stained with Giemsa and then examined microscopically using oil emulsion. Skin changes of the patients with cutaneous leishmaniasis were thoroughly examined for characteristics, size, site and number of the lesions. Skin test by using *Leishmania* promastigote antigens prepared by Furuya *et al*. (1991) was also performed on a part of patients as an immunological examination.

Results

The results obtained are summerized in Table 5.1.1 to 5.1.5. The total patients in this study were 348, 204 males and 144 females (Table 5.1.1). The mean age of patients was 21.2 years ($\pm$ 1.2 s.e.) in males, 18.9 years ($\pm$ 1.4 s.e.) in females and 20.2 years ($\pm$ 0.9 s.e.) in total. Young patients less than 20-year-old occupied more than half of all the patients examined. However, senior patients, more than 50-year-old were seen with 15 cases (7.4%) in males, 11 cases (7.7%) in females and 26 cases (7.5%) in total. The mean duration period of cutaneous lesions was 5.3 months ($\pm$ 0.4 s.e.) in males, 6.3 months ($\pm$ 0.7 s.e.) in female and 5.7 months ($\pm$ 0.4 s.e.) in total (Table 5.1.2). The number of the patients with the onset of lesions less than 1 month was 18 cases (10.5%) in males, 8 cases (7.0%) in females and 26 cases (9.1%) in total. However, there were 8 cases (4.7%) in males, 7 cases (6.1%) in females and 15 cases (5.2%) in total that showed the duration time of lesions more than 13 months. The popular types of lesions included ulcer, nodule, erythematous plaque and papule (Table 5.1.3). The most polular type was ulcer formation. The patients with ulcer occupied more than 50% in total. Distribution of the lesions is shown in Table 5.1.4. More than half of the lesions were located on the face and the extremities. The lesions on the trunk were seen more frequently in male than in female. On the other hand, the lesions on the legs were more popular in female. Almost half of the lesions were solitary, but the remainder were multiple (Table 5.1.5). The cases more than ten multiple lesions were seen in five of the male patients, two of the female patients and seven of the total patients.

Discussion

In 1990, we performed a comparative study of skin changes of cutaneous leishmaniasis between highland and lowland in Ecuador (Nonaka *et al*., 1990a). There were marked differences between the two areas. The mean age of the patients in lowland was 20 years, but that in highland was low (2 years). The number of the lesions was more in lowland than in highland. Large ulcers were frequently seen in lowland, but those

Table 5.1.1. Summary of patients with cutaneous leishmaniasis

Age (years)	Male (%)	Female (%)	Total (%)
− 5	31 (15.2)	29 (20.1)	60 (17.2)
6 − 9	28 (13.7)	25 (17.4)	53 (15.2)
10 − 19	49 (24.0)	36 (25.0)	85 (24.5)
20 − 29	51 (25.0)	22 (15.3)	73 (21.0)
30 − 39	19 (9.3)	11 (7.6)	30 (8.6)
40 − 49	11 (5.4)	10 (6.9)	21 (6.0)
50 − 59	4 (2.0)	6 (4.2)	10 (2.9)
60 −	11 (5.4)	5 (3.5)	16 (4.6)
Tolal	204 (100.0)	144 (100.0)	348 (100.0)

Table 5.1.2. The duration time of cutaneous leishmaniasis lesions

Time (months)	Male (%)	Female (%)	Total (%)
− 1	18 (10.2)	8 (7.0)	26 (9.1)
2 − 3	66 (38.3)	39 (33.9)	53 (36.5)
4 − 6	51 (29.6)	35 (30.4)	85 (30.0)
7 − 12	29 (16.9)	26 (22.6)	73 (19.2)
13 −	8 (4.7)	7 (6.1)	30 (5.2)
Tolal	172 (100.0)	115 (100.0)	348 (100.0)

Table 5.1.3. The clinical forms of cutaneous leishmaniasis lesions

Type	Male (%)	Female (%)	Total (%)
Erythema	0 (0)	1 (0.7)	1 (0.3)
Papule	18 (8.9)	14 (9.8)	32 (9.3)
Nodule	26 (12.8)	10 (7.0)	36 (10.4)
Plaque	25 (12.4)	17 (11.9)	42 (12.2)
Scar	9 (4.6)	19 (13.3)	28 (8.1)
Scar with erythema	0 (0)	2 (1.4)	2 (0.6)
Ulcer	123 (60.8)	80 (55.9)	203 (58.8)
Granuloma	1 (0.5)	0 (0)	1 (0.3)
Tolal	202 (100.0)	143 (100.0)	345 (100.0)

in highland were very small. The lesions were wet in lowland, however dry in highland. Mucocutaneous leishmaniasis was seen only in lowland and never seen in highland. In 1991, we reported Andean leishmaniasis in Ecuador caused by infection with *Leishmania (Leishania) mexicana* and *L. (L.) major*-like parasites (Hashiguchi *et al*., 1991). Cutaneous leishmaniasis in highland was similar to "Uta", a cutaneous form of the disease described in Peru. "Uta" is prevalent among the inhabitants to the western slopes and valley of the Peruvian Andes at the elevations between 600 and 3000 meters above sea level. This disease is thought to be caused by *L. (Viannia) peruviana* (or a variant of *L. (V.) braziliensis*). Therefore, the parasites of Andean cutaneous leishmaniasis in Ecuador is different from those in Peru. It is very

Table 5.1.4. The site of 391 lesions

Body area	Male (%)	Female (%)	Total (%)
Face	52 (21.7)	37 (24.6)	89 (22.8)
Ear	9 (3.7)	2 (1.3)	11 (2.8)
Neck	6 (2.5)	0 (0)	6 (1.5)
Back	10 (4.1)	4 (2.7)	14 (3.6)
Chest	2 (0.8)	0 (0)	2 (0.5)
Abdomen	1 (0.4)	0 (0)	1 (0.3)
Buttock	1 (0.4)	1 (0.7)	2 (0.5)
Trunk	3 (1.2)	0 (0)	3 (0.8)
Shoulder	1 (0.4)	1 (0.7)	2 (0.5)
Arm	21 (8.7)	5 (10.0)	36 (9.2)
Elbow	5 (2.1)	3 (2.0)	8 (2.0)
Forearm	53 (22.1)	22 (14.7)	75 (19.2)
Hand	8 (3.3)	2 (1.3)	10 (2.6)
Upper extremity	11 (4.6)	6 (4.0)	17 (4.3)
Thigh	10 (4.1)	3 (2.0)	13 (3.3)
Leg	44 (18.3)	51 (34.0)	95 (24.3)
Foot	1 (0.4)	0 (0)	1 (0.3)
Extremities	3 (1.2)	3 (2.0)	6 (1.5)
Tolal	241 (100.0)	150 (100.0)	391 (100.0)

Table 5.1.5. The number of lesions per patient

Number	Male (%)	Female (%)	Total (%)
1	100 (50.9)	65 (49.3)	165 (50.3)
2	41 (20.9)	25 (19.0)	66 (20.1)
3	25 (12.8)	18 (13.6)	43 (13.1)
4	10 (5.1)	9 (6.8)	19 (5.8)
5	6 (3.1)	7 (5.3)	13 (4.0)
6−9	9 (4.6)	6 (4.5)	15 (4.6)
10−	5 (2.6)	2 (1.5)	7 (2.1)
Tolals	196 (100.0)	132 (100.0)	328 (100.0)

interesting that clinical features of both types of leishmaniasis are almost similar disregarding different species of parasites and similar environmental conditions of inhabitants. Furthermore, we performed dermatological and parasitological examinations of cutaneous leishmaniasis in the Pacific coast of Ecuador (Nonaka *et al.*, 1992). The most frequent duration time of lesions was three months. Approximately 25% of the lesions persisted for more than five months. The mean number of cutaneous lesions was 2.7 in both sexes. The subjects with single lesion were most frequent, and 40% possessed multiple lesions. There were three cases with more than 10 cutaneous lesions out of 143 patients. The most frequent sites of lesions were extremities. The most popular cutaneous change was ulcer formation, but non-ulcered cutaneous

Table 5.1.6. The size of lesions

Size of lesions (mm^2)	Male (%)	Female (%)	Total (%)
− 9	30 (8.2)	13 (4.8)	43 (6.8)
10 − 25	70 (19.2)	33 (12.2)	103 (16.2)
26 − 100	92 (25.2)	61 (22.5)	153 (24.1)
101 − 400	114 (31.3)	89 (32.8)	203 (31.8)
401 − 1600	42 (11.5)	56 (20.7)	98 (15.4)
1601 −	6 (1.6)	4 (1.5)	10 (1.6)
unknown	11 (3.0)	15 (5.5)	26 (4.1)
Tolal	365 (100.0)	271 (100.0)	636 (100.0)

changes were also seen. The lesions with the size of less than 400 mm^2 were frequent. There were 21 cases with lesions of more than 900 mm^2 and all of them were ulcerated. In this study, we analyzed 348 patients in total. The mean age of the patients was 2 years older than that in the previous study (Nonaka *et al*., 1990a). Senior patients with more than 50 years of age were more frequent than those in the previous study. The mean duration period of cutaneous lesion was 1.5 months longer than that in the previous study. The mean number of cutaneous lesions was 2.7. Seven cases possessed more than 10 lesions. This datum showed a similar tendency to that in the previous report (Nonaka *et al*., 1990a). The most frequented site was the face following the extremities, that is, the exposed areas with various stimuli. This means that it may be possible for a sand fly to bite for aspiration of blood from exposed sites of humans.

Several species of parasites and vectors are reported in the areas examined in this study. Cutaneous manifestations of leishmaniasis in Ecuador will differ delicately from those in other areas because of the difference of species of the parasites, various behaviors of the vectors and different life style of the inhabitants. Therefore, medical doctors assigned to the treatments for cutaneous leishmaniasis should have not only medical knowledge but also entomological, ecological, environmental and anthropological knowledge.

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2. Cutaneous Leishmaniasis in El Carmen, Province of Manabi, Ecuador

ABSTRACT. Clinical cases of leishmaniasis registered during the period from 1985 to 1996 in a public hospital in El Carmen, Province of Manabi, Ecuador were briefly evaluated, in order to get an information on the global situation of the disease in the area. In the hospital a therapeutic trial was done in 1988-1989 using meglumine antimonate (Glucantime®) with different doses. The results on the therapy were also analysed and evaluated. The methodology of treatment used and the possibility of eventual misdiagnosis in such a rural hospital were taken into consideration, with the intention of improving medical care system in the endemic area of cutaneous leishmaniasis.

Introduction

Cutaneous leishmaniasis is an acute or chronic infection affecting tissues of mammmals, including man, caused by different species of protozoa of the genus *Leishmania*. The cutaneous form exists in different endemic areas of the world. In Latin America it is common in sylvatic focus of all countries with exception of Chile and Uruguay in South America. It is endemic in Central and South America reaching the estuary of Parana river in the east, and occidental Andean slopes of Peru in the west. In Ecuador cutaneous form is the most frequent clinical feature of the disease, but several cases of the mucocutaneous leishmaniasis are also seen throughout the year. Cutaneous leishmaniasis was reported for the first time by Valenzuela in 1920 (Rodriguez, 1974). The first case of mucocutaneous forms was described in 1924 by Heinert (Rodriguez, 1974). Since then, many cases of cutaneous and some of mucocutaneous leishmaniasis have been reported every year, and studies on transmision of the disease with reference to vectors and reservoirs started in 1982 and continued to date (Hashiguchi *et al*., 1987, 1990, 1992, 1994).

The lack of a well structured information system has not allowed epidemiologists to elaborate on a good and representative statistic feature of the disease in Ecuador. However, there is an important information that gives an idea on the incidence of leishmaniasis in the country. For example, case reports were made from Ecuador in different years as follows: 1981, 103 cases; 1982, 263 cases; 1983, 284 cases; and 1984, 1316 cases (PAHO, 1984).

In El Carmen Hospital, first case was reported in 1983, and since then, many cases have been registered every year. In this paper we reviewed the incidence of cutaneous leishmaniasis recorded to date in the hospital, and analyzed the results of a therapeutic trial with five groups of patients who received meglumine antimonate Glucantime® between 1988 and 1989.

Materials and Methods

The study area, Canton El Carmen, Province of Manabi was located at the north-western regions of the Pacific coast of Ecuador (Fig. 5.2.1). The area includes many small towns, villages, settlement and spread farms or field houses at mountainous parts of the cordillera between Andean slopes and the coast of the country. All patients diagnosed with leishmaniasis during 1988 and 1996 were included in the evaluation of general incidence, and a clinical trial of treatment done in 1988 - 1989 (Garcia and Bastidas, 1991) is reviewed and analyzed.

The used method for diagnosis has been the direct examination, that is the stained smear of material taken from the lesions. Smears were fixed with pure ethanol and stained with Giemsa or Wright colouring. We reviewed data on patients studied in the trial 1988 - 1989 which included procedence, sex, age and living

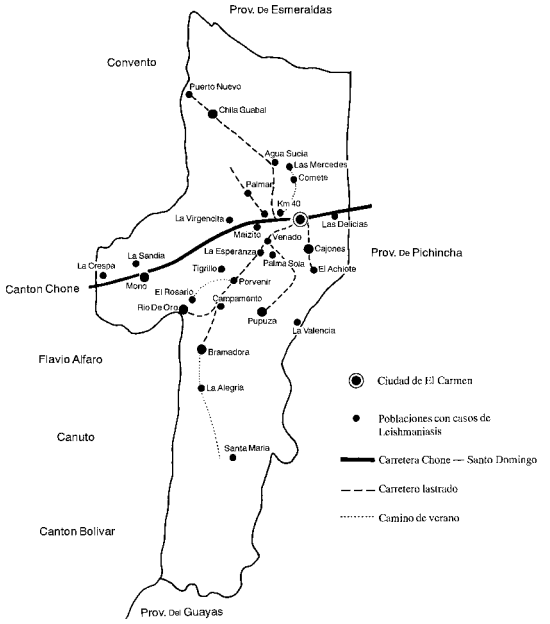

Fig. 5.2.1. Map of the study areas.

activity of patients; lesions evaluation by type, location, size and total number, time of evolution; and treatment, healing time and cure rate of lesions. Probable side effects were also taken into consideration. Treatment scheme used in the hospital was carefully evaluated. The scheme of treatment was structured using very low doses for some patients, because in that time there was no information on the species of *Leishmania* existing in the area, and there was always the possibility of benign leishmaniasis caused by not so virulent strains.

Treatment trial using meglumine antimonate was done during 1988 and 1989 as the following schedules in each group, Group A, 5cc (1 ampoule) each day for 10 days; Group B, 5cc everyday, during 14 days; Group C, 5cc everyday, during 20 days; Group D, Glucantime®, 60 mg/kg/day, for 10 days; Group E, Glucantime®, 60 mg/kg/day, for 14 days.

Results

Retrospective epidemiological data analysis

The total numbers of cases of leishmaniasis registered from 1985 to 1996 in El Carmen Hospital are shown in Table 5.2.1. All patients were parasitologically confirmed by microscopical examination of smear specimens taken from clinically suspected leishmanial lesions. A preliminary trial of treatment with Glucantime® was done in 1988 - 1989 with 241 patients treated in El Carmen Hospital, Manabi, Ecuador. These data are analyzed further as follows.

Age distribution of subjects

Ages of 134 patients (55%) were within 11 and 30 years old, with an age peak within 21 and 30 (69 patients; 28.6%) as shown in Table 5.2.2.

Sex distribution of subjects

Of the total subjects in the present retrospective study, 144 (59.8%) were male and 97 (40.2%) were female, as shown in Table 5.2.3.

Occupation of subjects

Distribution of life activities of patients studied in 1988-1989, is shown in Table 5.2.4. It is clear that the highest incidence is seen in those persons with some kind of field activities, which expose them to the bite of sandflies, in the forest (Table 5.2.4).

Residence of subjects

Among 241 persons diagnosed as cutaneous leishmaniasis in El Carmen Hospital, 61 (25.3%) were from same town (El Carmen) where the hospital is located (Fig. 5.2.1); 18 (7.4%) came from Rio de Oro;

Table 5.2.1. Parasitologically confirmed leishmaniasis cases in Hospital El Carmen, Province of Manabi, Ecuador during 1985 and 1996

Year	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996
No. of case	10	9	6	89	232	349	124	24	54	104	64	70+

Table 5.2.2. Age distribution of subjects registered at the hospital

Age groups	No. of patients	Percentage (%)
<1 year	3	1.2
1 - 10	54	22.4
11 - 20	65	26.9
21 - 30	69	28.6
31 - 40	24	9.9
41 - 50	15	6.2
> - 50	11	4.5
Total	241	100.0

Table 5.2.3. Sex distribution of subjects in this study

Sex	No. of patients	Percentage (%)
Male	144	59.8
Female	97	40.2

Table 5.2.4. Occupation of subjects found in this study

Occupation	No. of patients	Percentage (%)
Agriculture	68	28.2
Not specified	60	24.8
Students	50	20.7
House keeping	40	16.5
Car driver	3	1.2
Business man	3	1.2
Bricklayer	2	0.8
Public employee	2	0.8
Mechanical technician	2	0.8
Teachers	2	0.8
Dressmaker	3	1.2
Tapster	1	0.4
Wood-cutter	1	0.4
Fisher	1	0.4
Hair-dresser	1	0.4
Nursering	1	0.4
Total	241	100.0

17 (7%) from Pupuza; 15 (6.2%) from Bramadora; 8 (3.3%) from Cajones; 5 (2%) from Chila Guabal; 5 (2%) from Porvenir and 5 (2%) from Mono. From each one: EL Cedro Chila, Palma Sola and Las Mercedes

Table 5.2.5. Frequency distribution of number of lesions observed in subjects in this study

No. of lesions	No. of patients	Percentage (%)
1	134	55.6
2	47	19.5
3	28	11.6
4	1	56.2
5	7	2.9
6	3	1.2
7	2	0.8
9	1	0.4
10	3	1.2
11	1	0.4
Total	241	100.0

Table 5. 2. 6. Number of lesions observed on the body surface of subjects

Body area	No. of lesions	Percentage (%)
Upper extrem.	148	30.2
Lower extrem.	238	48.6
Face	72	14.7
Thorax	23	4.7
Neck	8	1.6
Total	489	100.0

4 patients (4.9%) came; from each one: La Alegria, Tres Ranchos, Tigre, Maicito, Venado and Mongoya 3 patients (7.4%) came; from each one: El Rocio, Puerto Nuevo, La Esperanza, Agua Sucia, Las Delicias, Km 26, La Crespa, La virgencita and Colonape 2 patients came; from each one: Tripa de Pollo, Las Vainas, Km 62, Campeche, El Rosario, Limones, Km 58, Piojito, Cohete, La Valencia, Palanca, Tinaja, Canali, Pita, El Guayabo, Km 40, Km 38, Chontillal, Campamento, El Achote, La Sandia, La Salvacion, El Palmar, Km 21, Km 44, Tigrillo, Santa Maria, Chiparo, Toquilla, Km 32 and La Mediana, 1 patient (12.4%) came. Some patients also came from other areas besides Canton El Carmen:10 (4.1%) from Esmeraldas Province; from each one: Canton Flavio Alfaro, Pedernales and Santo Domingo de Los Colorados 2 patients (total: 2.4%) came; from each one: Amazonia,

Table 5.2.7. Duration time of lesions as of the examination at El Carmen Hospital

Duration time (month)	No. of patients	Percentages (%)
< 1	19	7.8
1	74	30.7
2	80	33.1
3	29	12.0
4	18	7.4
5	4	1.6
6	5	2.0
7	6	2.4
8	1	0.4
9	2	0.8
12	1	0.4
18	1	0.4
48	1	0.4
Total	241	100.0

Table 5.2.8. Side effects of subjects treated with Glucantime® injection

Symptoms	No. of patients	Percentages (%)
Fever	8	5.4
Dizziness	4	2.7
Rash	2	1.3
Nausea	1	0.6
Headache	1	0.6
Parestesia	1	0.6
Vomiting	1	0.6
Cough	1	0.6
Weakness	1	0.6
Malaise	1	0.6
None	126	85.5
Total	147	100.0

Calceta, Convento, Quinonez, and Chone 1 patient (total: 2%) came.

Number of lesions found in subjects

Number of lesions observed in patients ranged from one to 11. The distribution of total number of leishmanial lesions is shown in Table 5.2.5.

Anatomical distribution of lesions

The distribution of cutaneous leishmaniasis lesions on the body surface of subjects registered in the hospital is shown in Table 5.2.6.

Size of lesions

A total of 489 ulcerous lesions were observed in the present subjects of cutaneous leishmaniasis. Of these, 157 (32.2%) had a diameter of 2 cm; 133 (27.4%), 1 cm; 112 (23%), 3 cm; the remainings (17.4%), between 5 and 10 cm.

Duration time of lesions

The majority of duration time of lesions ranged from 1 month to 5 months at the time of examination of subjects, before treatment and registered in 1988-1989, as shown in Table 5.2.7.

Results of treatment with Glucantime®

Among 119 patients treated with 5cc (1.5g) of Glucantime®/day, during 10 days, only 48 (40.2%) accomplished periodic control attendance to the hospital during three months. 42 were confirmed with healed lesions, of which one presented reactivation 8 months later. The other six did not heal lesions during observation of three months. Failure rate in this group is 14.5% (7 patients). Cure rate was 85.5%.

Among 49 patients treated with 5cc (1.5g) of Glucantime®/day, for 14 days, only 15 completed routinary controls for three months. Fourteen healed lesions, with one reactivation 1 month later. One patient never healed during the treatment. Failure rate was 13.3% (2 patients). Cure rate was 86.7%.

Among 19 patients treated with 5cc (1.5g) of Glucantime®/day, for 20 days, only 6 completed three months control. One (16.6%) of the six never healed lesions. Failure rate was 16.6% (1 patient). Cure rate was 83.4%.

Among 40 patients treated with Glucantime®, 60 mg/kg/day for 10 days, only 20 came for controls during three months. All of them healed lesions without reactivation within three months. Cure rate was 100%.

Among 12 patients treated with Glucantime®, 60

mg/kg/day for 14 days, only 4 patients completed three months control. All of them healed completely. Cure rate for 4 patients was 100%.

Probable side effects observed

Only 147 patients could be evaluated for probable side effects. Of them, 21 (14.2%) showed slight clinical symptoms as shown in Table 5.2.8.

Discussion

Leishmaniasis was recognized for the first time as a public health problem in Province of Manabi in 1984 (unpublished data), when a big number of cases appeared in some areas of the province. Since then, epidemiological studies have been done in order to understand the epidemiological aspects of the disease (Coronel *et al*., 1986; Alava *et al*., 1990).

The clinical cases registered at the El Carmen Hospital, in our opinion, could be directly correlated with the reservation of antimonial drugs existing in the hospital at different seasons. Because people tends to avoid looking for medical care, when they know there is no treatment avaliable. So, after the breakout of leishmaniasis in 1984, many cases were not registered because of the lack of chemotherapy. And then, when people realized that medical care was just diagnosis and not treatment because of the lack of opportune provision of drugs, they quiet attendance to hospitals and try any other ways, such as natural medicine, witchery, religious ceremonies etc. We know there were hundreds of cases in each of the Cantons of Manabi Province, but the incidence registered until 1987 does not represent the actual incidence. In 1988 - 1991, a reasonable amount of drugs was sent to every medical center in Manabi, and people attending those centers were so registered, and incidence is more like reality in this period. In 1992, the lack of antimonial drugs was again the problem, and it persists to date. Registered cases are few in the hospital, but we have found so many cases by active field research during 1986 and 1992 (Nonaka *et al*., 1992). About clinical therapeutical trial done in 1988-1989, we can say that age and sex of patients does not have any significance as transmission aspect, but these factors may represent the social or family problems in the disease transmission.

Considering the distribution of occupation in patients in this study, we can clearly see that first of all, bitting activity of sand flies in this area is mostly sylvatic. Because most of infected patients have agricultural and unspecific activities, although the important number of students and housekeepers infected might suggest domiciliary bitting, at night, when doing respective works such as late night studies or breakfast preparation at early dawn. The poor distribution of other occupational activities in the rest of the patients strongly suggests that transmission occurs mostly in the forest. Because of many different places where patients came from, there is no doubt about the widespread of the disease in the entire Canton El Carmen and other areas of Manabi and neighbouring provinces.

Registered data on number, size and location of lesions could have been really useful to evaluate efficiency of treatment, if related to the different groups of the trial. Unfortunately these aspects were not specified for each group of patients. Most patients had a short duration time (between <1 and 4 months), but this important information was not related or specified for each group of treated patients neither. We must also recognize here that patients with a long duration time such as the one with four years, were probably not leishmanial cases, and diagnosis was wrong considering the lack of experience for diagnosis and the easy confusion occuring in some rural laboratories with other skin diseases, once in a while.

First two groups of the trial of treatment received an insufficient dose of Glucantime®, perhaps due to the fact that in that time there was no information about the strain of *Leishmania* causing the disease in the area, and low dose could have worked in case of benign or less virulent strains. Results clearly show the efficiency of treatment in the other groups, with the correct dosage. About the patient who did not heal

the lesion in group C, there is again the possibility of a diagnosis mistake. It is important to point here, that the time for follow-up has been too short in this trial; the minimun should be of one year. We must also add that during actual research we are doing in El Carmen, we have found several cases of false positive and negative diagnoses of leishmaniasis, confirmed by us (unpublished data). Probable side effects are the usual with the exception of fever which could have been caused by septic manipulation of lesions.

In conclusions, evidently, the incidence of leishmaniasis maintains an important rate in Manabi Province, specially in those remote areas where medical care is not available neither educational programs on public health are developed. Bitting activity and transmission occur mainly in the forest although there could be domiciliary transmission, specially in those areas where houses are surrounded by vegetation. For further investigation patients should be grouped by age, and conditions of lesions (number, size and location) for each treatment group. Good training and diagnostic conditions should be insured, to avoid misdiagnosis. Isolation of the parasite should be established as routinary activity, to know real distribution of *Leishmania* species in this province. Schemes of treatment should be kept as in groups D and E. Follow-up should be of one year as minimun. Alternative treatments with drugs similar to or more efficient than antimonials, but of easier administration should be taken into consideration, for field conditions.

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Chapter 6

Chemotherapy of Leishmaniasis Patients

1. Oral Treatment of New World Cutaneous Leishmaniasis with Antimalarial Drugs in Ecuador: a Preliminary Clinical Trial

ABSTRACT. The current study was designed to evaluate antileishmanial activity of mefloquine hydrochloride (Mephaquin®) and artesunate (Plasmotrim®) which are currently being used as antimalarial drugs. A totoal of 17 patients (volunteers) with cutaneous leishmaniasis were treated with these drugs in this study. Of these subjects, 16 were treated by the oral administration of a total dosage of 1500 mg (1 Lactab each for 6 days) mefloquine, *i.e.,* 4.2 mg/kg/day for 6 days, and if necessary the dosage was repeated with 3 weeks intervals. The majority of cutaneous lesions healed within 2 to 3 weeks after the commencement of mefloquine treatment, showing an average of 3.6 weeks of healing times with 100% cure rate. One slowly healing within 8 weeks after the commencement was observed; this case grew worse because of infection with *Tunga penetrans* at the late healing phase of leishmaniasis. The remaining one patient with an ulcer lesion was treated by the oral administration of a total dosage of 1200 mg (2 Lactab each for 3 days) artesunate, *i.e.,* 6.7 mg/kg/day for 3 days, and the same dosage was repeated 2 weeks later. The lesion healed within 6 weeks after the commencement of artesunate treatment. In the present study, all the patients received mefloquine or artesunate were treated without admission, performing their normal daily activities. No specified adverse reaction was noticed.

Introduction

The pentavalent antimonials sodium stibogluconate and meglumine antimonate remain as the first choice of drugs in the clinical treatment of different types of leishmaniasis, such as cutaneous, mucocutaneous and visceral forms (Bryceson, 1980; WHO, 1990). Since the introduction of these antimonials more than 50 years ago, many investigations have been done in order to find more efficient treatment without side effect for the disease. No satisfactory effective new drugs, however, have been developed, though several important advances have been made (Berman, 1988; Croft, 1988). There is, therefore, still a need to search for a new drug that is fully effective and orally applicable for most clinical forms of Old and New World leishmaniasis.

To date, trials of search for new drugs and treatment have been performed *in vitro* and *in vivo* using experimental animals and/or volunteer patients with leishmaniasis. In the present study we tried to treat cutaneous leishmaniasis patients with two types of antimalarial drugs, mefloquine and artesunate, which are clinically being used. The current paper describes the antileishmanial activities of these drugs, based on the data obtained from clinical trials at endemic areas of Ecuador.

Materials and Methods

In the present study, a total of 17 patients with

cutaneous leishmaniasis were examined. They came from different endemic areas, Zhucay and Manta Real (Province of Cañar), Zapotal (Province of Guayas), Muisne (Province of Esmeraldas) and Caluma (Province of Chimborazo). Of these subjects, 16 were treated orally with a total dosage of 1500 mg (1 Lactab® each for 6 days) mefloquine (Mephaquin®, Mepha Ltd., Aesch-Basle, Switzerland; each Lactab® contains mefloquine hydrochloride corresponding to 250 mg mefloquine base), *i.e.,* 4.2 mg/kg/day for 6 days, and if necessary the dosage was repeated with 3 weeks intervals after the end of initial treatment. In patients treated with mefloquine, 13 out of 16 were male and 3 were female, aged from 2 to 81 years. The remaining one patient (14 years old male) was treated orally with a total dosage of 1200 mg (2 Lactab® each for 3 days) artesunate (Plasmotrim®, Mepha Ltd; each Lactab® contains artesunate 200 mg), *i.e.,* 6.7 mg/kg/day for 3 days, and the same dosage was repeated 2 weeks later. All the subjects were informed of the purpose of the study and gave permission for drug administration and repeated physical examinations. The patients received treatment during their daily activities without admission. Almost all the volunteers (patients) lived in mountainous and dense forest areas in which no transportation systems are available (Fig. 6.1.1A). Their dwellings are located at very remote areas from our laboratory of health centers in each endemic area. All the volunteers were in a very poor economic condition. In such a field situation and an ethical consideration, no placebo treatment was performed in the current trial.

All the cutaneous leishmaniasis patients treated in this study were diagnosed by the demonstration of *Leishmania* amastigotes in smear specimens from the lesions. Treated patients received a follow-up physical examination every 2 or 3 weeks, and they were recorded photographically at the same time. When their dermal lesions were partially still active in the examination, an additional administration of the drug was made as mentioned above. During the treatment, the patients were asked for the presence of any complaint, such as vomiting, nausea, diarrhoea, fever and etc.

The evaluation of the results of treatment was made as described by El-On *et al*. (1986) but partially modified as follows: 1) rapidly effective (grade 1), no parasites detected in cutaneous lesions, followed by total healing within 1-3 weeks after the commencement of treatment; 2) less rapidly effective (grade 2), the same process (no parasites and total healing) occurring within 4-6 weeks after the commencement; 3) effective (grade 3), no parasites detected but healing within 7-8 weeks; 4) ineffective (grade 4), parasites still present in lesions and/or no clinical healing after 9 or 10 weeks of the commencement of treatment.

In the present subjects, species of *Leishmania* are not identified precisely, but our previous work indicates that *L. (Viannia) panamensis* is the most frequently identified organism by zymodeme and serodeme analyses, followed by *L. (V.) guyanensis* in and around the study area (unpublished data).

Results

The number of cutaneous lesions per person ranged from 1 to 4 with different size of lesions, ranging from 3 x 3 mm to 30 x 30 mm in diameter (Table 6.1.1). All the present patients had ulcerative lesions; the majority of these lesions were located on the exposed body surface, such as forearm, foot and face. The duration time of infection varied from one to 12 months (average: 3.6 weeks) at the time of the commencement of mefloquine treatment. Nine (56.3%) of the 16 patients healed within 3 weeks after the commencement of oral treatment of mefloquine, showing grade 1 category (see Fig. 6.1B-D), and other 6 cases took 4-6 weeks (grade 2) for healing. Only one case (patient No. 3) took 8 weeks for complete healing, because of *Tunga penetrans* infection in the cutaneous lesion which had been at the late phase of healing (4 weeks after the commencement of mefloquine treatment). No ineffective case was found in this study using mefloquine; the cure rate showed 100%. In the present trial of treatment with meflo-

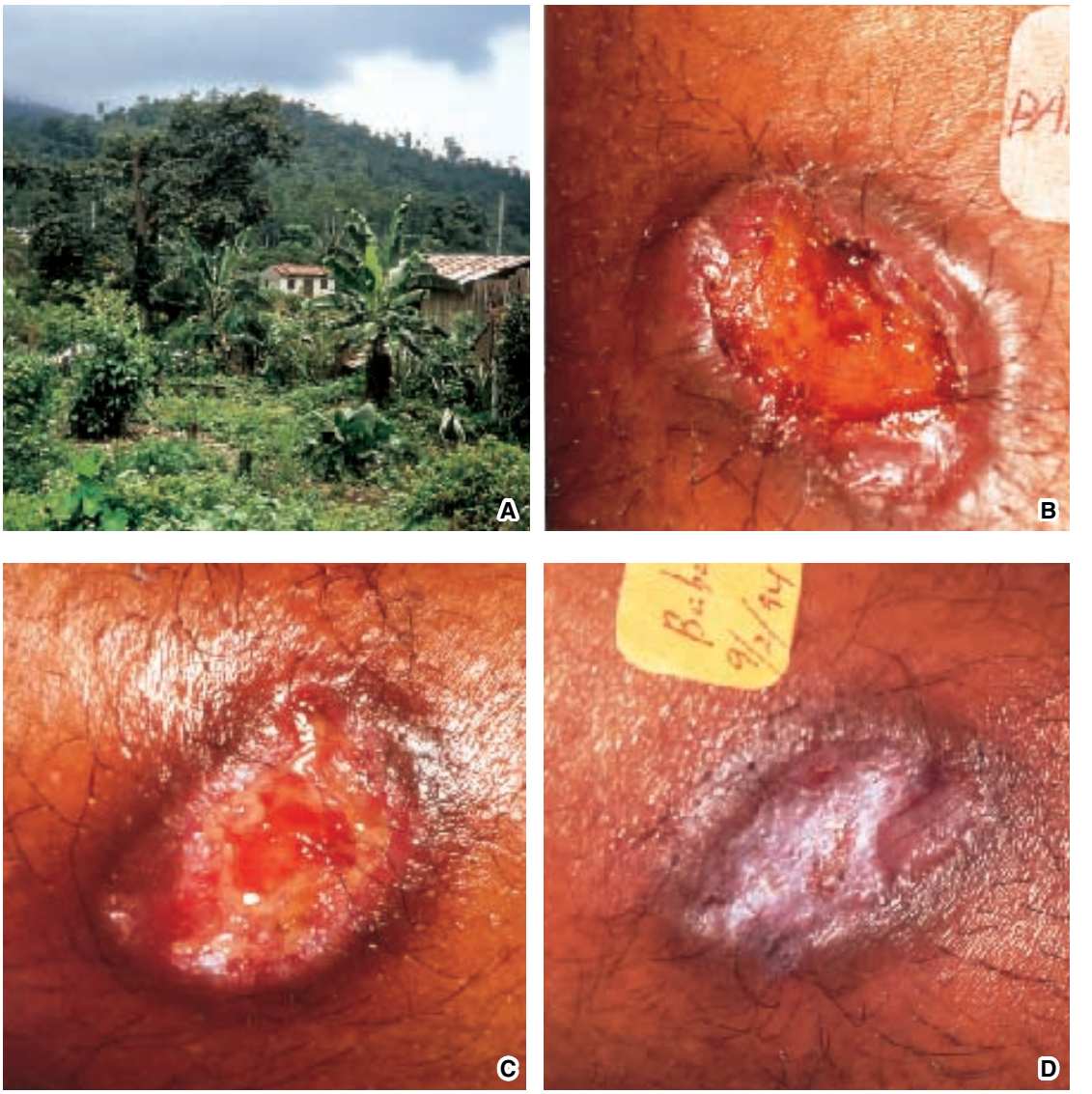

Figure 6.1.1. A, dwelling site of inhabitants in an endemic area (Manta Real) surrounded by a dense forest where the present patients (volunteers) came from. **B-D,** A cutaneous leishmaniasis lesion located on the lower extremity of a 22 years old female patient (No. 14). B, An ulcer (34 x 26 mm) with typically elevated border and marked induration around the lesion, before mefloquine treatment. C, After 1 week of the commencement of oral administration of mefloquine, the lesion reduced slightly in size and showed a gradual disappearance of the ulcer border and induration. A marked epidermidalisation was observed on the surface of the lesion. D, After 3 weeks of mefloquine treatment; the lesion was completely covered by epidermis without induration.

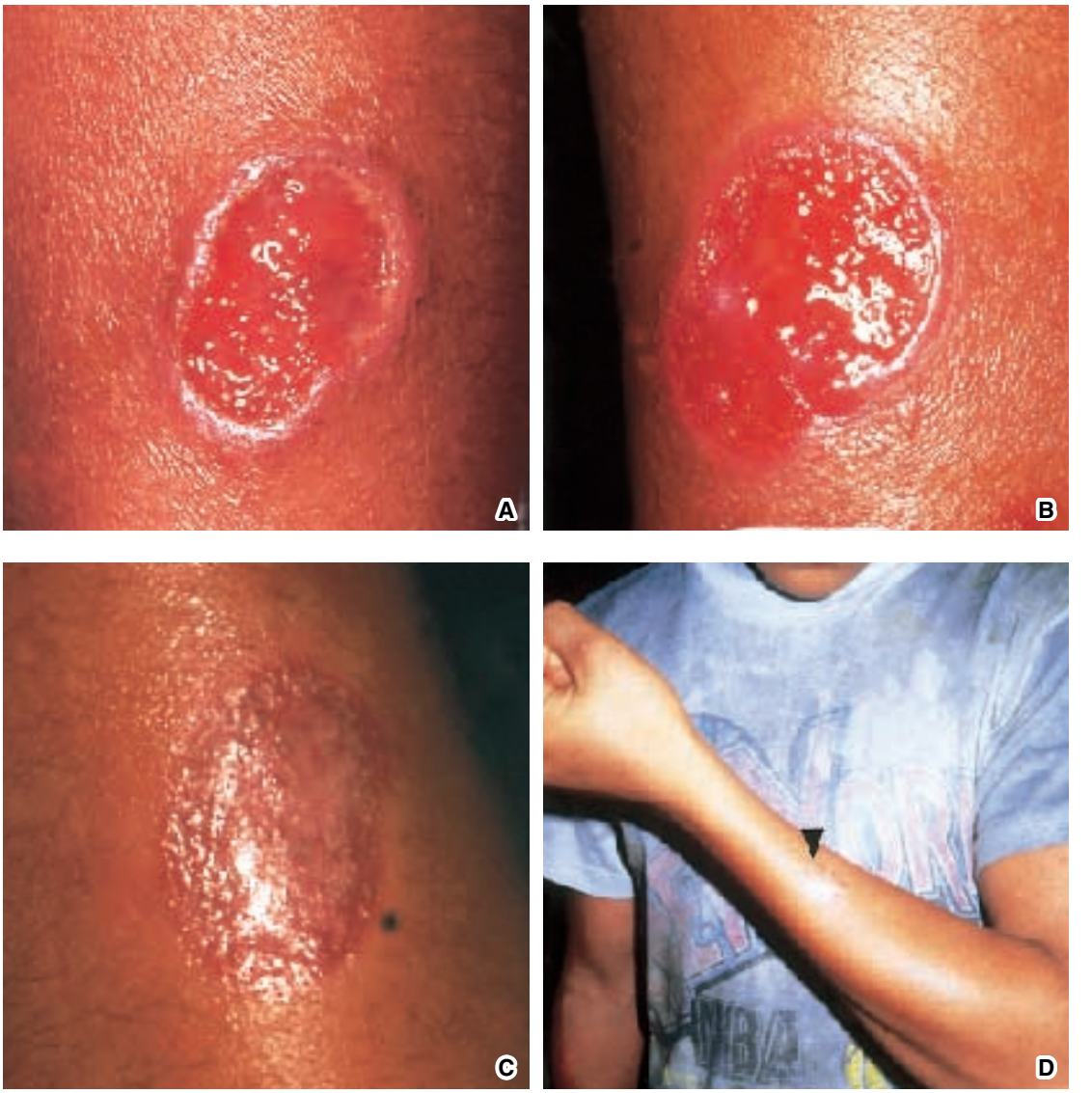

Figure 6.1.2 A-D. A cutaneous leishmaniasis lesion located on the upper extremity of a 14 years old male patient. A, A lesion (20 x 17 mm) showing a typical ulcer border, before artesunate treatment. B, After 2 weeks of the oral administration of artesunate, the lesion showed a marked granulation and reduced ulcer border. C, The cured lesion completely covered by epidermis without induration, after 6 weeks of artesunate treatment. D, The lesion (arrow) after 5 months of the treatment, showing a typical scar.

Table 6.1.1. Clinical data on the parasitologically-positive patients with cutaneous leishmaniasis who received Mephaquin® therapy

Patient No.	Age	Sex	No. of lesions	Size of lesions (mm)	Site of lesions	Type of lesions	Duration time of infection	Times (wks) for healing
1	41	♂	1	35×25	forearm	ulcer	2M*	6
2	16	♂	2	20×15	nose	ulcers	12M	3
				5×5	cheek			
3	8	♂	2	30*30	foot	ulcers	3M	8
				10*5				
4	12	♂	2	5×5	forearm	ulcers	3M	5
				5×5				
5	19	♂	1	20×10	forearm	ulcer	3M	5
6	81	♂	4	20×10	forearm	ulcers	2M	4
				15×15	shoulder			
				10×10				
				10×10				
7	26	♂	3	20×20	forearm	ulcers	1M	4
				10×10				
				10×10				
8	17	♂	1	15×10	neck	ulcer	1M	2
9	4	♂	4	10×10	face	ulcers	5M	2
				5×5				
				5×5				
				4×4				
10	22	♂	1	20×10	forearm	ulcer	1M	2
11	3	♂	2	4×4	face	ulcers	8M	2
				3×3				
12	13	♀	1	15×5	forearm	ulcer	2M	3
13	22	♂	1	4×3	forearm	ulcer	3M	3
14	22	♀	1	30×10	foot	ulcer	8M	3
15	31	♀	1	20×10	front	ulcer	2M	2
16	14	♂	1	34×26	foot	ulcer	3M	6

quine, age or sex of the patients, number and size of the lesions and duration time of the infection did not show any influence against the efficacy of drug. However, heavy bacterial infections and other infection such as *Tunga penetrans* were fully influential for the healing time, especially in lesions located on the lower extremities.

In a patient (14 years old male) treated with artesunate (Plasmotrim®), an ulcer (20 x 17 mm) on the forearm healed within 6 weeks after the commencement of treatment (Fig. 6.1.2A-C) and no recurrence was found after 5 months (Fig. 6.1.2D). In comparison with mefloquine, however, artesunate showed a slow effectiveness on the healing of the dermal lesion, especially in the early phase (1 week later) of oral treatment.

All the present patients treated with mefloquine or artesunate lived and worked in a rural and humid area

with hygienically bad conditions. There is no specified adverse reaction in the patients treated with the drugs, including skin eruptions or pruritus. Therefore, none of the subjects treated were withdrawn from the study because of adverse reactions.

Discussion

For the treatment of most forms of New and Old World leishmaniasis, pentavalent antimonial compounds are still remain as the drugs of choice. These drugs are given intramuscularly or intravenously, and generally cause serious adverse reactions, such as renal and liver disfunctions, nausea, headache and arthragia, in addition to pain at the injection site. For the treatment of Old World cutaneous leishmaniasis, El-On *et al.* (1986) reported topical application of paromomycin ointment, obtaining satisfactory cure rate as good as any currently used therapy. Recently, the therapy was also used for New World cutaneous leishmaniasis in Ecuador (Nonoka *et al.*, 1992; Krause *et al.*, 1994) and Belize (Weinrauch *et al.*, 1993), confirming the results reported by El-On *et al.* (1986). However, such a topical treatment using ointment has limitations in its usage even in cutaneous leishmaniasis cases. Application of ointment would be only useful for relatively mild and simple lesions which are caused by *L. (L.) mexicana* groups. It is however not feasible for cutaneous disease forms caused by *L. (V.) braziliensis* (Weinrauch *et al.*, 1993) and mucocutaneous or visceral forms caused by other *Leishmania* agents.

To date, various oral antileishmanial drugs, such as metronidazole, rifampicin, levamizole, ketokonazole, co-trimoxazole, dapsone and etc., have been used for different disease forms with variable results. In the present study, antimalarial drugs, mefloquine and artesunate were selected for clinical trials in the continuing search for oral antileishmanial drugs. These drugs were found to be effective against malarial parasites in the Old and New World. Mefloquine, a long-acting quinine analogue is a schizonticide and destroys the erythrocytic, asexual forms of the *Plasmodium* parasites in man, and the mean elimination half-life of the drug is culculated as 21.4 days ranging from 15 to 33 days (Desjardins *et al.*, 1979; Schwartz *et al.*, 1980, 1982). According to Schwartz *et al.* (1980, 1982), maximum plasma concentrations are reached 2-12 hours after a single oral dose and plasma concentrations approaching 1 μg/ml are present after a dose of 1,000 mg of mefloquine. They also showed that similar maximum concentrations are present in the steady state after administration of 250 mg (1 Lactab®) weekly; the concentration in the erythrocytes is almost twice as high.

In this study, using mefloquine, almost all cutaneous lesions healed within 6 weeks after the commencement of treatment showing 100% cure rate, in spite of a relatively low daily dosage (250 mg/day) of the drug compared with the dosage used in malarial cases. The precise mode of action of mefloquine against *Leishmania* has not been determined, although it has been shown that more than 98% of the active substance against *Plasmodium* schizonts is bound to plasma proteins (Schwartz *et al.*, 1982). To some extent, the mode of action of mefloquine against *Leishmania* parasites might be similar to that found in malarial cases, affecting amastigote-macrophage interactions.

In oral treatment using artesunate, only one case was experienced in this preliminary trial, suggesting that the drug might remain as a candidate for future study. Artesunate, a preparation for the killing of erythrocytic stage of *Plasmodium* asexual form, reacts with intraparasitic heme in its mechanism of anti-malarial action (Meshrick *et al.*, 1991), but its mode of action against *Leishmania* parasites is still unknown precisely. According to Jiang *et al.* (1982), an advantage of artesunate administration is the speed of onset of action and inhibitory effect on the maturation of malarial parasites. On the other hand, in the present leishmaniasis case, it is likely that the drug has a tendency to act slowly as compared with mefloquine, especially at the early phase of treatment.

The current treatment with mefloquine or artesunate was done in the subjects who made their normal daily activities without admission. When a similar treatment using these drugs was performed in well-controled subjects under admission, more rapidly healing would be found. Furthermore, a rapid healing might occur when antibiotics are used as complemental treatment to eliminate bacterial infections of cutaneous lesions. With regard to oral administration of the present drugs used, more suitable and effective dosage should be examined, in addition to their precise mechanism(s) of antileishmanial action.

In conclusion, the significant efficacy of antimalarial drugs, mefloquine and artesunate against *Leishmania* following the oral delivery, suggests that the novel antileishmanial activities of these drugs should be investigated further, and their potential as drugs for various clinical forms of leishmniasis including visceral forms needs more study.

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2. Further Studies on the Oral Treatment of Cutaneous Leishmaniasis with Antimalarial Drug Mefloquine (Mephaquin®) in Ecuador

ABSTRACT. Since 1994, because of the lack of antimonials in that year, we tried for the first time antimalarial drugs, mefloquine and artesunate, to treat cutaneous leishmaniasis, and got quite surprised by their probable efficiency. This paper follows the prior one, with the main purpose to study a larger number of patients with a new dosage of mefloquine first, and in a further work, of artesunate, to obtain more evidence on such cure capacity. A group of seventy-two selected patients (groups A, B and C) and a randomized group (D) of 16, were treated with mefloquine, 100 mg after breakfast and supper for ten days, for adults or children with more than 45 kg, and a total dose of 50 mg/kg given as one or two oral intakes of 50 mg after breakfast and supper during ten days, for children (or adults) with less than 45 kg. A group (E) was also treated with Glucantime® for comparison. Obtained results are quite interesting, with a high percentage of cure rate (clearance of parasites) and normally variable times for healing in the patients. Side effects were carefully evaluated, and confirmed to be mild, in most cases, when present.

Introduction

Antimonials have been for a long time the drug of choice for the treatment of leishmaniasis in Ecuador, like in many other countries. However, the difficult parenteral administration of antimonials, the toxicity, high cost and specially the usual lack of this drug in the country have required for investigators to search for alternative treatments, similar or better on terms of efficiency but specially, of easier administration. Several new treatments have been tried by us in recent years, specially some ointments prepared with different drugs (Nonaka *et al*., 1992; Hosokawa *et al*., 1994). In 1994, we started treatment of cutaneous leishmaniasis with antimalarial drugs, mainly with mefloquine hydrochloride (Mephaquin®) and secondarily with artesunate (Plasmotrim®). The idea of trying this drugs came from the information we had on the treatment of leishmaniasis with chloroquine, done by American doctors during World War Two for infected soldiers, with relative success. We thought that mefloquine, a much more effective antimalarial drug could also work in some manner. Interesting results showing probable antileishmanial action of these drugs (mefloquine and artesunate) obtained in the mentioned prior trial, encouraged us to do a larger study to obtain more detailed information about such interesting event.

Materials and Methods

Study areas

This study was carried out in three different endemic areas of Ecuador: Zhucay in Province of Cañar; Mamey in Province of Esmeraldas and El Carmen in Province of Manabi.

Selection of subjects

We know mefloquine (Mephaquin®) is a drug of low toxicity, but because of ethical procedure and security of patients as insured by our protocol, we were very careful to choose patients to be under treatment in this trial. Admission discernments to include a patient were: active cutaneous lesion(s); recent evolution; parasitological confirmation; no prior specific treatment; good general condition; consent by patient or relatives; no pregnancy; no breast feeding; no severe or chronic diseases. An additional selection was done

among excluded patients to conform 2 comparative study groups: one to be treated with Glucantime® and the other with mefloquine as a randomized group.

Management of subjects

Medical histories were elaborated; clinical examination was done. Cutaneous lesions were evaluated by type (ulcerous, nodular, verrucous etc) and size (small: <1cm; medium: >1- 2.5cm; big: > 2.5cm). The parasite was isolated when possible. Biopsies were taken before, during and after treatment. Density of parasites was evaluated before, during and after treatment (No. of parasites / 100 microscopic fields). Treatment was administered according to protocol. Contaminated lesions were treated with antibiotics (cefalexine) inocuous for *Leishmania* before starting administration of mefloquine. All patients were followed-up for a minimun of one year, after healing time of lesions.

Drug administration

Used therapeutical scheme was the following: adults or children with more than 45 kg: 100 mg (1 tablet of 100 mg) after breakfast and supper for 10 days; children with less than 45 kg: 50 mg/kg as a total dose, with oral intakes of 50 mg once or twice a day after meals to complete the total dose. This treatment could be repeated after 7 days in case of severe leishmaniasis (big lesions with high density of parasites).

Follow-up of treated subjects

Clinical and parasitological examination and photographic record were done on the first day and every 15 days until cure, and then, every 30 days to complete one year of follow-up.

Results

Under our selective procedure, 155 patients with cutaneous lesions from the three mentioned endemic areas were examined, of which, 118 were found infected with *Leishmania* amastigotes; 72 were selected for the study, 15 for comparative study group treated with Glucantime® (usual dosage) and 16 to comform a special randomized group treated with mefloquine (Mephaquin®) with same dosage than the principal group. This randomized group was not under selection protocol.

Only 16 (22%) patients had small lesions and conformed group A; 56 (78%) had medium and big lesions and were included in groups B and C. In group A, 18% of lesions were nodules, and 82% were ulcers; in groups B and C (56 subjects), 26.7% were nodules, 3.5%, verrucous and 69.8%, ulcers. Parasites species isolated in Zhucay are *Leishmania (Viannia) guyanensis* and *L. (V.) panamensis*; in Mamey, *L. (Leishmania) mexicana* and *L. (V.) panamensis*; in El Carmen, *L. (V.) panamensis*. Among selected patients (groups A, B and C), 41 (56%), cleared parasites in 15 days; 31 (44%) did it in 30 days. In randomized group (D: also treated with mefloquine), 5 persons (31.2%) cleared parasites in 15 days; 10 (62.5%) dit it within 30 days and 1 (6.3%) never did it. In group (E) treated with Glucantime®, all patients took 30 days for total parasite clearance. Healing time for 16 patients with small cutaneous lesions (group A) treated with mefloquine (Mephaquin®) is shown in Fig. 6.2.1. The minimun healing time was 15 days,

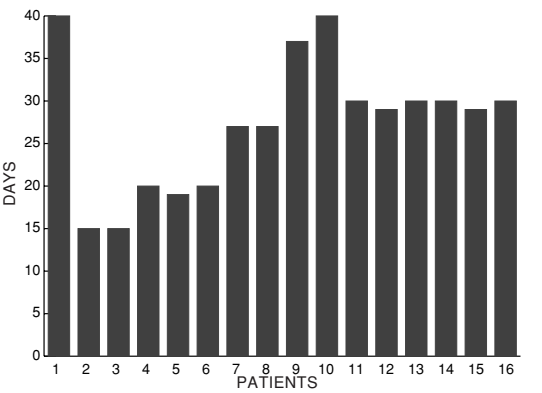

Figure 6.2.1. Healing time for subjects with small lesions (less than 1 cm in diameter) of cutaneous leishmaniasis (group A) treated with Mephaquin®.

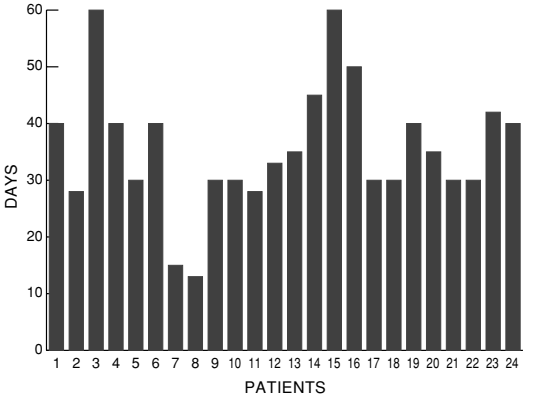

Figure 6.2.2. Healing time for subjects with medium and larger lesions (1.1 cm to 2.4 cm) of cutaneous leishmaniasis (group B) treated with Mephaquin®.

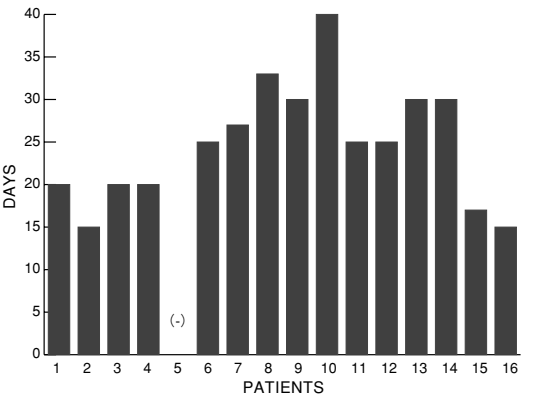

Figure 6.2.4. Healing time for subjects in randomized group (D) treated with Mephaquin®. A patient (No. 5) in this group showed no healing during the present trial.

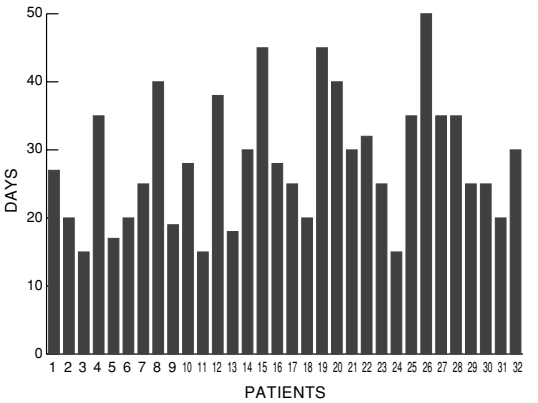

Figure 6.2.3. Healing time for subjects with larger lesions (2.5 cm or more) of cutaneous leishmaniasis (group C) treated with Mephaquin®.

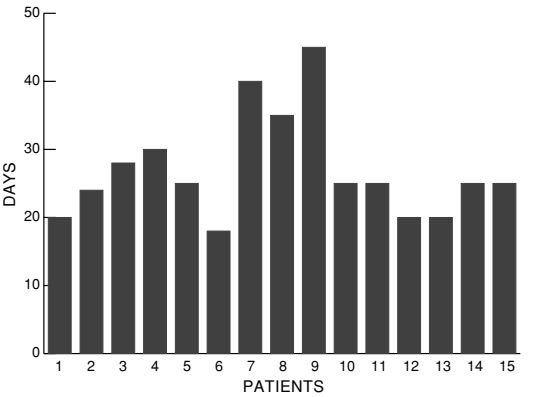

Figure 6.2.5. Healing time for subjects in randomized group (E) treated with Glucantime®.

maximun, 40 days, and average, 27.2 days. Results on healing time for 24 patients with medium and large cutaneous lesions (group B) treated with mefloquine is shown in Fig. 6.2.2. The minimum time was 13; maximum was 60; and average, 35.5 days. Results on healing time for last group of selected patients (32) with medium and large cutaneous lesions (group C), treated with mefloquine are shown in Fig. 6.2.3. The minimum time was 15; maximum, 50; and average, 28.2 days. In addition, the result on healing time for randomized group of 16 patients with cutaneous leishmanial lesions, treated with mefloquine is shown in Fig. 6.2.4. The minimum time was 15; maximum, 40; and average, 24.8 days. For comparison of drug efficacy, healing time for randomized group of cutaneous leishmaniasis patients treated with Glucantime® is shown in Fig. 6.2.5. The minimum healing time was 18; maximum, 45; and average, 27 days.

Forty two (58.3%) of patients in groups A-C presented side effects. Of these, only 2 (2.7%) had a clin-

Figure 6.2.6. A patient (8-year-old male) treated orally with mefloquine (Mephaquin®) and his dwellings. **A**: before treatment (14 Aug., 1996), **B**: 2 weeks after reatment (28 Aug., 1996), **C**: 4 weeks after treatment (11 Sept., 1996), **D**: dwellings of the patient.

ical symptom which may be considered important, because it was kind of moderate paresis; loss of sensibility in both legs for about 3 hours, after which it disappeared. Other side effects presented by the remaining subjects treated with Mephaquin® were: mild somnolence, 15 (21%); mild headache, 8 (11.1%); moderate headache, 7 (9.7%); severe headache, 2 (2.7%); mild somnolence and headache, 5 (6.9%); mild dizziness (on fast movements), 2 (2.7%); and moderate abdominal pain, 1 (1.3%). The rest of patients in groups A-C, 30 (41%) did not complain of any side effect. In randomized group D (Fig. 6.2.6), 9 (56.2%) complained of some symptoms as follows: moderate headache, 3 (18.7%); mild nausea, 3 (18.7%); mild dizziness, 2 (12.5%); and mild weakness, 1 (6.2%). The rest of patients in this group, 7 (43.8%) did not complain of anything.

In the group E treated with Glucantime®, side

effects were shown by 9 patients (60%) as follows: moderate headache, 3 (20%); moderate nausea, 4 (26.6%); and mild dizziness, 2 (13.3%). The rest of patients in this group, 6 (40%) did not have any problem.

Discussion

Different clinical features of leishmaniasis are not always severe. Many times, benign or small lesions cure spontaneously or with conservative treatments. Although antimonials are very effective to kill the parasite, parenteral administration of such drugs remains being a problem because of the multiple complications it may have, like painful muscles, inflamation, bacterial infections (*Chlostridia, Staphylococcus, Streptococcus* etc), viral infections (hepatitis, AIDS etc), several and serious adverse reactions (headache, nausea, arthralgia, liver and renal disfuctions etc) and so on, besides the necessity of experimented personal to apply the shots and high cost of the drug in market. Moreover, in Ecuador, we must add something else to all these problems, for example, the frequent lack of antimonials in this country, for many different reasons.

Like many other research groups, we have been looking for an alternative good treatment for several years, and recently in a previous paper we presented the first treatment done with antimalarial drugs, mefloquine (Mephaquin®) and artesunate (Plasmotrim®) in this country. Results obtained in the present work, with a larger number of patients, represent, in our opinion the confirmation of the real possibility of using this drugs for the treatment of cutaneous leishmaniasis caused by the mentioned species of parasites isolated in our study areas.

In carefully selected group (A-C), all of them (100%) cleared parasites. Cure rate was 100%; however it does not represent the real rate to be obtained in routinary treatment, because patients were carefully selected. That is why we also tried a randomized group (D) with the same treatment, where 93.7% cleared parasites, and cure rate was 93.7%. One patient of this group did not clear neither heal his lesion during 45 days he was under control but did not come again. So, we may suppose that cure rate for patients treated with mefloquine is between 90 and 93 %. Cure rate for patients (group E) treated with Glucantime® was 100%. Times for clearance of parasites were similar but did not show no relationship with times taken for healing of lesions in this study. Parasites are cleared by the drug (rate of efficiency), but healing is an individual biological process depending on many individual factors such as age, nutritional state, peripheral blood perfusion, chronic diseases (diabetes), contamination and etc. In the other hand, we must consider the fact that our patients were not hospitalized, kept doing usual activities and no complementary topical care was done, so if medical care is taken at a hospital (hygiene, topical care, rest and good nutrition), healing time should improve.

Detected side effects in patients treated with mefloquine (Mephaquin®) are in general mild, and kind of similar to those found with Glucantime®. Mefloquine should be tried against other types of leishmaniasis caused by different parasites. The possibility of combining these two drugs should be taken into consideration and tried in further studies to treat resistant species of *Leishmania*. Oral administration of mefloquine is quite convenient and combining drugs would decrease the dosage of antimonials to be used.

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3. Studies on Oral Treatment of Cutaneous Leishmaniasis with an Artemisinin Derivative, Artesunate (Plasmotrim®) in Ecuador

ABSTRACT. Our previous work revealed that antimalarial drugs, mefloquine (Mephaquin®) and artesunate (Plasmotrim®), had an efficacy when they were used as oral therapy of cutaneous leishmaniasis patients. In order to clarify if both antimalarial drugs have similar or different effects, it is necessary to carry out more detailed investigation based on additional cases of the treatment especially with artesunate. For this purpose, 15 patients with cutaneous leishmaniasis were treated with artesunate. The following dosage of the drug was used in the treatment: 1) adults with 45-59 kg body weight (b. w.) or children with more than 45 kg b.w. and/or older than 12 received 100 mg of Plasmotrim® (half tablet) after breakfast and supper for 10 days; and 2) adults with more than 60 kg b. w. received 200 mg of the drug (1 tablet) after breakfast and supper for 10 days. In both cases, the treatment could be repeated if necessary. Time for clearance of parasites was 15 days in 26.6% of patients and 30 days in 73.4%. The clearance times are quite similar to those obtained with Mephaquin®. Average time for clinical healing of cutaneous lesions was 34.5 days. Topical trial using 1% ointment of artesunate against cutaneous lesions was also done, and it showed a good result.

Introduction

In general terms, only two clinical forms of leishmaniasis have been confirmed to exist in Ecuador, so far. Studies on the epidemiological aspects of leishmaniasis and its mode of transmission have been continuously done in this country, and among a lot of information obtained since 1982, when the research work started, cutaneous and mucocutaneous forms are the only two clinical manifestations of leishmaniasis found in Ecuador (Hashiguchi and Gomez, 1991). Among cutaneous cases, there is one case, completely confirmed and described, of the diffuse type of cutaneous leishmaniasis, DCL (Reyna *et al*., 1994). Cutaneous leishmaniasis is the most important and frequent clinical form in the country, and it is found in the three principal natural geographic regions of Ecuador (Hashiguchi and Gomez, 1991). Mucocutaneous leishmaniasis is mostly found, not so often, in the Amazonian region (Amunarriz, 1982), but some cases have been described from other areas.

There is a great variety of types of cutaneous leishmaniasis, regarding to size, morphology, clinical evolution, and response to treatment, probably due to virulence of different *Leishmania* strains and/or immunological and physiological conditions of humans (hosts). Antimonials are still drugs of the first choice for the treatment of leishmaniasis in Ecuador, but, we have found, when visiting several endemic areas, that treatment with antimonials is not available for most patients living in remote, rural areas; that some people may get antimonials, after paying certain high amount of money, but do not find a qualified person to apply the injections and, that sometimes persons with small or benign lesions received an excessive dosage of antimonials. For these reasons, we have been looking for a good alternative treatment for cutaneous leishmaniasis, including ointments, lotions and lately oral treatment with antimalarial drugs mefloquine and artesunate (Gomez *et al*., 1995). In this paper we present the results of a clinical trial done with artesunate lactabs 200 mg (Plasmotrim®) to treat 15 patients with cutaneous leishmaniasis, and the experimental use of artesunate ointment in other one, which could not be given oral treatment because of breast feeding.

Materials and Methods

All patients came from the same endemic area, Canton El Carmen in Province of Manabi, Ecuador. Individuals were carefully selected, following our usual criteria for admission and requirements of our protocol. Those requirements were: active cutaneous lesion, recent evolution, parasitologically confirmed to be leishmaniasis, no prior antileishmanial treatment, good general condition, without severe or chronic diseases, no pregnancy or breast feeding, good nutritional condition and consent by the patient or relatives.

After selection of patients, clinical histories were taken. Cutaneous lesions were evaluated by number, type and size. When evident contamination was detected, patients received antibiotic treatment with a known inocuos for *Leishmania* drug, cefalexine. Parasite was isolated when possible, and isolates kept for further identification. Tissue smears and biopsies were taken before, during and after treatment to evaluate density of parasites and future electron microscopic studies. Density of parasites was determined by counting the number of parasites in 100 scope fields. Artesunate was given as follows: adults with 45-60 kg or children with more than 45 kg and older than twelve: 100 mg (half tablet) after breakfast and supper for ten days; adults with more than 60 kg: 200 mg (1 tablet) after breakfast and supper for ten days. This treatment could be repeated after 5 - 7 days if necessary. Clinical evaluation to detect side effects and photograph secuence was done before, during and after treatment (follow-up). Minimum time for following patients up was 1 year, with periodic evaluations every 30 days after cure.

Results

A total of 50 patients were examined, of which, 35 had cutaneous leishmaniasis. Among these 35, only 15 patients were admitted in the study, and treated with artesunate. A comparative group of patients treated with Glucantime® with usual dosage, was also structured.

Regarding to cutaneous clinical evaluation, 3 (20%) patients presented multiple (2-5) lesions, 12 (80%) presented only one; 4 (26.6%) had small lesions (<1cm), 11 (73.3%) had medium or big lesions (>1.1 - 2.4 cm; >2.5); 3 (20%) had nodules and 12 (80%) had ulcers.

Parasite species isolated in El Carmen is *Leishmania (Viannia) panamensis*. This species has a wide distribution in the north and central part of Manabi Province. About clearance of parasites, 4 patients (26.6%) cleared them within 15 days and 11 (73.3%) did it within 30 days. In comparative group (Glucantime®) all patients cleared parasites within 30 days. About healing time for patients treated with artesunate (Plasmotrim®) lactabs, the minimum was 15 days; maximum, 45 days; and average, 34.5 days as shown in Figures 6.3.1 and 6.3.2.

Average healing time for patients treated with Glucantime®, in the comparative group was 45 days.

The only patient (EC, No. 9) treated with 1 % ointment of artesunate in vaseline base was a breast feeding young female, who had a 2-month-old baby. She had two ulcerous lesions in her right thigh and two in her right arm which healed within 30 days and multiple nodules on her left elbow which healed within 60 days (Fig. 6.3.3).

Parasitological examinations after treatment per-

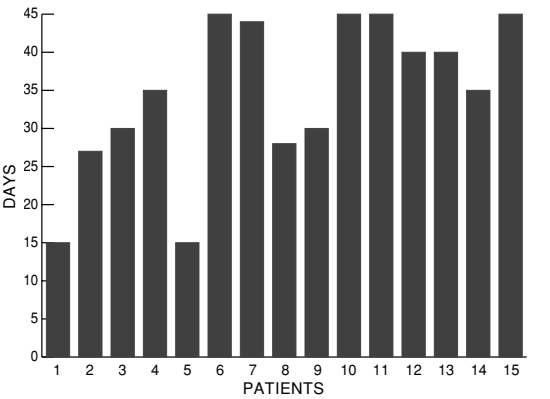

Figure 6.3.1. Healing time for patients with cutaneous leishmaniasis treted with artesunate (Plasmotrim®).

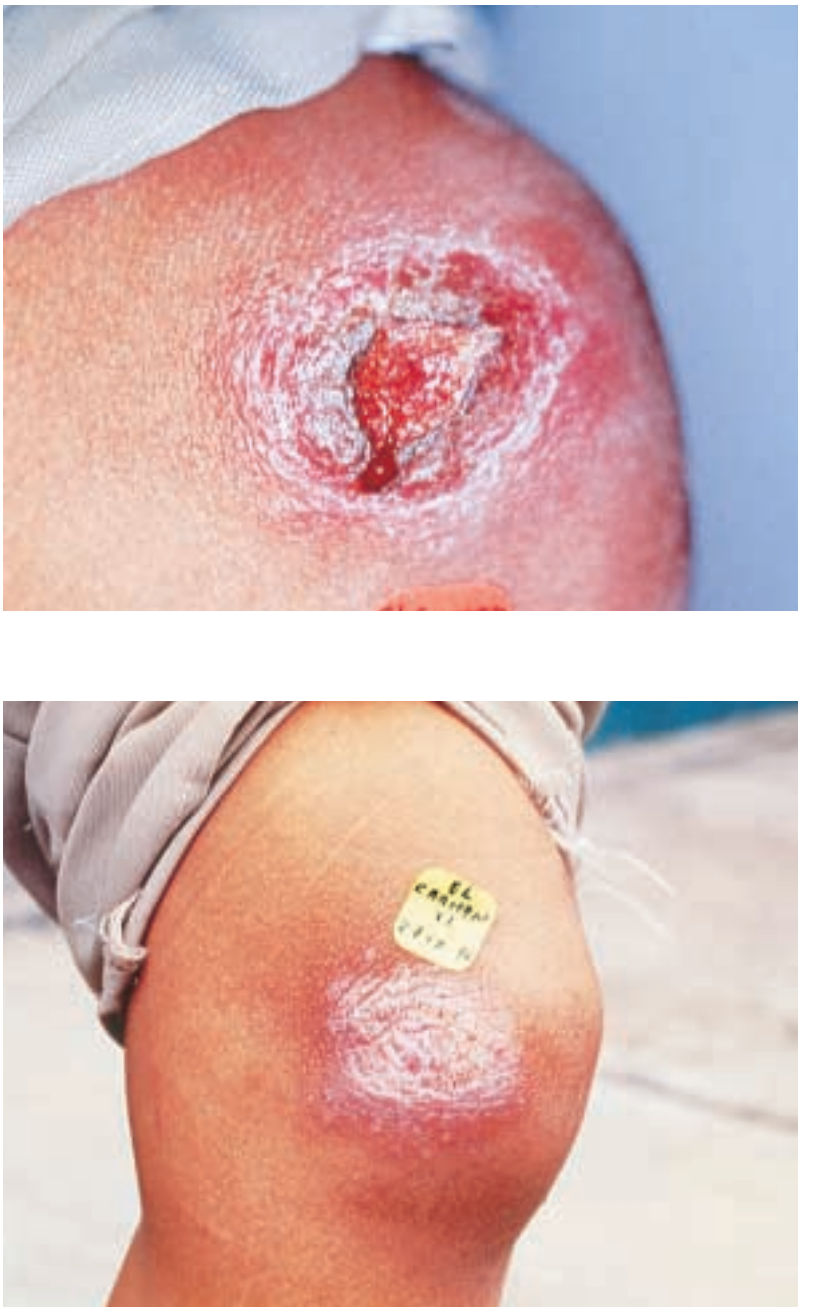

Figure 6.3.2. A male patient (EC-No. 12) with a large ulcer lesion on his knee. **Above**: before oral treatment with artesunate (Plasmotrim®) (13 Aug. 1996). **Below**: 45 days after the oral treatment (27 Sept. 1996).

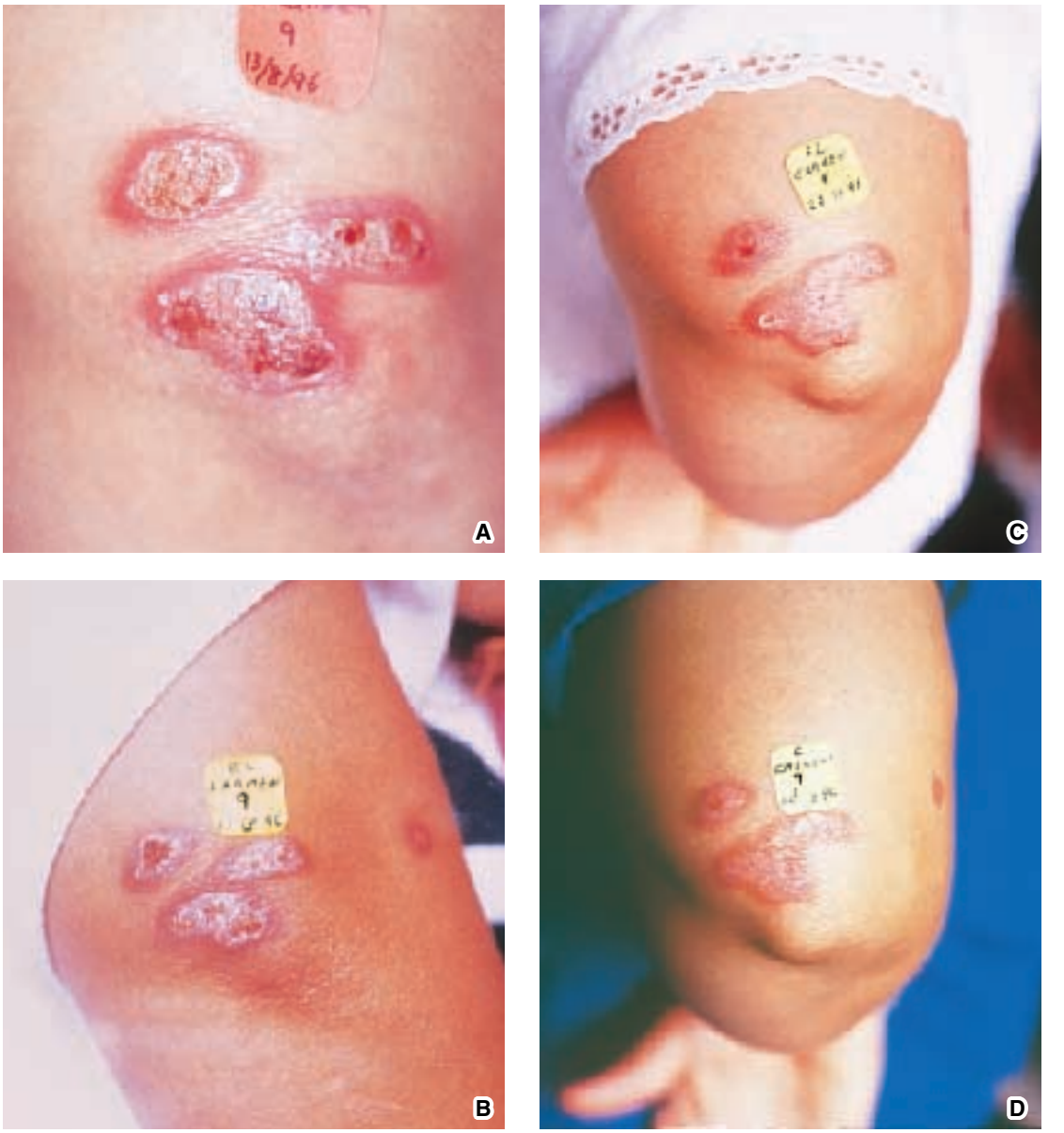

Figure 6.3.3. A female patient (EC-No.9; 22-year-old) with a 2-month-old breast feeding baby was treated with 1% ointment of artesunate (Plasmotrim®). She had 2 each of ulcerous lesions on her right thigh and right arm which healed within 30 days. However, multiple nodular type lesions on her left elbow took about 2 months for healing as shown in this figure. **A**: before the treatment (13 Aug. 1996), **B**: 27 days after the treatment (9 Sept. 1996), **C**: 42 days after the treatment (24 Sept. 1996), **D**: 60 days after the treatment (12 Oct. 1996).

mitted us to observe interesting changes in the structure of parasites after 15 days, which might be the result of the direct action of artesunate. Differences are evident when comparing the morphological characteristics of parasites on smear specimens taken before and after treatment. Cytoplasm of the few parasites is hardly seen, kinetoplast has disappeared in all of the parasites, nuclear granules are disorderly grouped and usual shape of the nucleus is gone. In smears taken before treatment, these figures are never seen, and parasites inside and outside of the macrophages were seen normaly and clearly. In smears taken after treatment, parasites are completely gone within 15 days in patients with low density of amastigote, but in those with high densities, more than 80% of parasites disappear but some may be still seen, showing the apparent morphological changes. Within 30 days every subject showed a complete clearance of amastigotes on smear specimens.

Discussion

Cure rate for patients treated with artesunate (Plasmotrim®) was 100%, but this rate may show any variation when a larger numbers of patients are studied. Clearance of parasites seems to have some direct relationship with the parasite density; the higher density of parasites, the longer time is required for clearance, but not with the size of the ulcer, which should be more related with healing time, in normal systemic health conditions.

Patients with short times for clearance were also those with low parasite densities; on the contrary, those with long clearance times had always high densities. If compared with antimonials (Glucantime®), artesunate (Plasmotrim®) seems to take a shorter time for clearing parasites, so far; however, this must be completely clarified when doing further research works.

Like in our prior work (Gomez *et al*., 1995; Chapters 6.1 and 6.2 in this text) done with mefloquine (Mephaquin®), times taken for healing lesions showed a variation difficult to analyze regarding to the effect of the drug which is really represented by the times taken for parasite clearance. Individual biological factors have an undoubtful participation in healing process, therefore healing must not be taken as indicator of drug efficiency. Many cases of reactivation of leishmaniasis are due to the fact that ones consider a patient as cured, based on healing without confirming if parasite clearance was completed. On the other side, patients who have cleared their lesions very fast, continue under treatment with antimonials because healing does not occur, due to other so many factors. As an example of the importance of this aspect, we insist the followings: during this work we selected a total of 16 patients for the treatment with artesunate. Few weeks after treatment we noticed that one of the patients who cleared parasites within 15 days, did not show any sign of healing. There was no contamination, and his clinical history did not show any helpful information. We asked the patient about the possibility of diabetes and he denied it. A blood sample was taken and diabetes was confirmed later on. Thus the patient confessed he did not tell us about his problem, to avoid to be excluded. He was cured of leishmaniasis, but his ulcer was never going to heal, without additional treatment for diabetes. The treatment was stopped and the patient was excluded.

About any relationship between the type of lesion and clearance of parasites, we think that if any, it will be determined for further studies with a larger number of patients. Perhaps the most important aspect of the treatment of cutaneous leishmaniasis with artesunate is the almost total abscence of undesired side effects. Only one patient complained of moderate headache, which easily responded to a weak analgesic.

The interesting results obtained with our female patient treated with artesunate ointment opens a new possibility to be investigated. If effective, the ointment could be combined with oral treatment with antimalarials mefloquine and artesunate, or, with parenteral antimonials, to decrease in both cases, the total dosage of those drugs, and to diminish risk of undesired side effects for the patients.

The way of action of artesunate against *Leishmania* parasites is the big question. Efficiency of topical ointment, if confirmed, would suggest the direct action of the drug against amastigotes. So far, we may suppose there is a direct action. We have observed very clear changes in smear taken after treatment. Besides the spectacular reduction of parasites density, lasting elements show alterations which might be considered the effect of the drug. However, more sophisticated techniques will have the good answer. In the meanwhile, careful observation of the smears taken before and after treatment should be done, to make sure, unless, that those alterations are not seen before treatment.

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4. Clinical Observation of Topical Treatment for Cutaneous Leishmaniasis for 5 Years in Ecuador

ABSTRACT. We tried to evaluate the topical treatment of several drugs for 5 years in Ecuador. The total number of patients treated with topical ointment and solution was 162, 82 males and 80 females. The mean age of patients was 17.60 (±1.19)-year-old in total, 17.11 (±1.87)-year-old in male and 18.09 (±1.47)-year-old in female. Young aged patients less than 20-year-old occupied more than 60 per cent of all the patients treated. In this study, healing process was divided into 4 grades according to the reaction against topical treatments employed as follows: 1) excellent, 2) good, 3) fair, and 4) no reactions. The treatment with meglumine antimonate solution produced excellent reaction in 13 patients, good reaction in 5 patients, fair reaction in 6 patients and no effect in one patient. The effective rate more than good reaction of this treatment was 75.0% in male, 69.2% in female and 72.0% in total. The treatment with paromomycin ointment showed the results of excellent reaction in 38 patients, good reaction in 19 patients, fair rection in 11 patients and no reaction in 4 patients. The effective rate more than good reaction of this treatment was 70.0% in male, 85.7% in female and 79.2% in total. The treatment with 10% paromomycin ointment showed excellent reaction in 10 patients, good reaction in 6 patients, fair reaction in 5 patients and no reaction in one patient. The effective rate more than good reaction of this treatment was 72.7% in total. The treatment with 2% paromomycin ointment showed the result of excellent reaction in 25 patients, good reaction in 13 patients, fair reaction in 6 patients and no reaction in 2 patients. The effective rate more than good reaction of this treatment was 66.7% in male, 92.9% in female and 82.6% in total. Topical treatment using low concentrations of paromomycin ointment and meglumine antimonate with mercury chrome solution was effective to 80% of cutaneous leishmaniasis patients. There was a low irritation to the wet ulcers of patients with cutaneous leishmaniasis. But, it was less effective to the non-ulcered lesions of the patients. Topical treatment to cutaneous leishmaniasis should be done carefully to the patients, who do not have any concern to mucocutaneous and visceral types of leishmaniasis.

Introduction

There are various forms of treatments for cutaneous leishmaniasis (Koff *et al*., 1994). Antimonial therapy is still well used in the world. But, this drug has side effects and it is very difficult to use this drug to the patients with pregnancy or small children. Previously, we undertook an evaluation of topical treatment for cutaneous leishmaniasis using paromomycin ointment and meglumine antimonate solution with mercury chrome (Nonaka *et al*., 1992). An evaluation of topical treatment for cutaneous leishmaniasis is not easy. The first problem is natural healing. Guderian *et al*. (1991) confirmed untreated cures on nine paitents during their therapeutic study for cutaneous leishmaniasis in Ecuador. The second is a reactivation of the lesions. The third is a possibility to transfer to mucocutaneous or visceral forms. In general, parasites of mucocutaneous and visceral leishmaniasis in New World are *Leishmania (Viannia) braziliensis* complex and *L. (Leishmania) chagasi*, respectively. We performed our therapeutic study in the areas infected by *L. (L.) mexicana* complex. Five years follow up study were performed in this study.

Materials and Methods

Subjects

A total of 162 patients with cutaneous leishmaniasis with informed concert were recruted in this study (Table 6.4.1). All the subjects continued their daily activity during the treatment without hospitalization. Follow-up of the treatment was performed from 1991-1995. Thirty-nine patients were treated with meglumine antimonate solution plus mercury chrome solution (Table 6.4.2). One hundred eleven patients were treated with paromomycin ointment (Table 6.4.3).

Preparation for topical ointment and solution

Paromomycin ointment was prepared in tow concentrations, 10% and 2%. Petrolatum was used for ointment base. Meglumine antimonate solution was prepared as follows: 250 ml of 30% meglumine antimonate, 750 ml of physiological saline and 1,000 ml of mercury chrome solution. This mixed solution was then divided into 25 ml each in small bottles.

Application procedure of topical ointment and solution

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

Efficacy criteria for the topical treatments

The effect of topical application was judged primarily by the clinical features. Photographs of the lesions were taken at each one week to two weeks, and the size and conditions of lesions were carefully observed and recorded. Especially, the ulcer sizes and indurations were measured at each clinical observation. The effects were graded into four criteria as follows: 1) no effect; no change of the eruption during treatment, 2) fair; slight improvement of the eruptions, 3) good; definite improvement of the eruptions; and 4) excellent; marked improvement or complete cure of the lesions.

Results

The results are summarized in Tables 6.4.1 to 6.4.5. The total number of patients treated with topical ointment and solution was 162, 82 males and 80 females (Table 6.4.1). The mean age of patients was 17.60 ($\pm$1.19)-year-old in total, 17.11 ($\pm$1.87)-year-old in male and 18.09 ($\pm$1.47)-year-old in female. Young aged patients less than 20-year-old occupied more than 60 % of all the patients treated. The treatment with meglumine antimonate solution produced excellent reaction in 13 patients, good reaction in 5 patients, fair reaction in 6 patients and no effect in one patient (Table 6.4.2). The effective rate more than good reaction of this treatment was 75.0% in male, 69.2% in female and 72.0% in total. The four patients who showed excellent reaction are described as follows. The case of No. 139 was a 4-year-old boy. The patient had many insect bites on the face and extremities. Four months earlier, his mother had noticed red papules and nodules on the same place, then these lesions increased gradually and some of them became ulcerated. When the patient visited our clinic, 7 lesions were observed on the right arm (Fig. 6.4.1A), right thigh and face. The smear from the elbow showed positive for the parasites, and the skin test also showed positive (18x18/22x23). The meglumine antimonate with mercury chrome solution was given to him. The nodules with ulcer changed to the lesion with crusts after 3 weeks of the treatment (Fig. 6.4.1B). Two month later, all the lesions healed completely. One year later, the lesions healed with slight scar and there was no recurrence (Fig. 6.4.1C). The case of No. 140 was a 6-year-old boy. Three lesions appeared on the elbow and face 4 months ago. The erythematous plaque with small ulcers on the left elbow was the largest lesion (Fig. 6.4.2A). The skin test showed positive (10x10/12x15). The meglumine antimonate solution was given to him. The ulcer disappeared after three weeks of the treatment (Fig.6.4.2B). The lesions

Table 6.4.1. Age of patients with cutaneous leishmaniasis

Age (year)	Male (%)	Female (%)	Total (%)
0－ 5	11 (13.41)	12 (15.00)	23 (14.20)
6－ 9	17 (20.73)	16 (20.00)	33 (20.37)
10－19	25 (30.49)	27 (33.75)	52 (32.10)
20－29	17 (20.73)	10 (12.50)	27 (16.67)
30－39	6 (7.32)	4 (5.00)	10 (6.17)
40－49	4 (4.88)	6 (7.50)	10 (6.17)
50－59	0 (0.00)	2 (2.50)	2 (1.23)
60－	2 (2.44)	3 (3.75)	5 (3.09)
total	82 (100.00)	80 (100.00)	162 (100.00)

Table 6.4.2. The effects of meglumine antimonate with mercury chrome solution

Effects	Male (%)	Female (%)	Total (%)
Excellent	6 (50.00)	7 (53.85)	13 (52.00)
Good	3 (25.00)	2 (15.38)	5 (20.00)
Fair	3 (25.00)	3 (23.08)	6 (24.00)
No effect	0 (0.00)	1 (7.69)	1 (4.00)
Subtotal	12 (100.00)	13 (100.00)	25 (100.00)
Unknown	9	5	14
Total	21	18	39

were completely healed after six months (Fig. 6.4.2C). The case of No. 145 was a 14-year-old girl, who complained of a nodule with ulcer on her right cheek for 8 months (Fig. 6.4.3A). The skin test was positive (12x13/18x22). The lesion became dry after two weeks of the topical treatment (Fig. 6.4.3B), and the lesion healed with a slightly elevated scar after 3 weeks (Fig. 6.4.3C). The scar became flatter after one year (Fig. 6.4.3D). The case of No. 146 was a 9-year-old girl, who complained of indurated scar with ulcers for 6 months. The lesion was a scar with indurated erythema and ulcers sized 50 mm by 30 mm on her right leg (Fig. 6.4.4A). The skin test was positive (25x22/35x40). The topical treatment was continued for 2 weeks, the lesion became dry (Fig.6.4.4B). One month later, the lesion was healed with slight crust (Fig. 6.4.4C), and that was completely healed after one year (Fig. 6.4.4D).

The treatment with paromomycin ointment showed the results of excellent reaction in 38 patients, good reaction in 19 patients, fair rection in 11 patients and no reaction in 4 patients (Table 6.4.3). The effective rate more than good reaction of this treatment was 70.0% in male, 85.7% in female and 79.2% in total. The treatment with 10% paromomycin ointment showed the results of excellent reaction in 10 patients, good reaction in 6 patients, fair reaction in 5 patients and no reaction in one patient (Table 6.4.4). The effective rate more than good reaction of this treatment was 72.7% in total. The treatment with 2% paromomycin ointment showed the result of excellent reaction in 25 patients, good reaction in 13 patients, fair reaction in 6 patients and no reaction in 2 patients (Table 6.4.5). The effective rate more than good reaction of this treatment was 66.7% in male, 92.9% in female and 82.6% in total. The four patients showed excellent reaction are described as follows. The case of No. 27 was a 40-year-old male, who complained of indurat-

Table 6.4.3. The effect of paromomycin ointment

Effect	Male (%)	Female (%)	Total (%)
Excellent	13 (43.33)	25 (59.52)	38 (52.78)
Good	8 (26.67)	11 (26.19)	19 (26.39)
Fair	7 (23.33)	4 (9.52)	11 (15.28)
No effect	2 (6.67)	2 (4.76)	4 (5.56)
Subtotal	30 (100.00)	42 (100.00)	72 (100.00)
Unknown	23	16	39
Total	53	58	111

Table 6.4.4. The effect of 10% paromomycin

Effect	Male (%)	Female (%)	Total (%)
Excellent	4 (36.36)	6 (54.55)	10 (45.45)
Good	4 (36.36)	2 (18.18)	6 (27.27)
Fair	3 (27.27)	2 (18.18)	5 (22.73)
No effect	0 (0.00)	1 (9.09)	1 (4.55)
Subtotal	11 (100.00)	11 (100.00)	22 (100.00)
Unknown	5	3	8
Total	16	14	30

Table 6.4.5. The effect of 2% paromomycin ointment

Effect	Male (%)	Female (%)	Total (%)
Excellent	8 (44.44)	17 (60.71)	25 (54.35)
Good	4 (22.22)	9 (32.14)	13 (28.26)
Fair	4 (22.22)	2 (7.14)	6 (13.04)
No effect	2 (11.11)	0 (0.00)	2 (4.35)
Subtotal	18 (100.00)	28 (100.00)	46 (100.00)
Unknown	10	7	17
Total	28	35	63

ed nodule with an ulcer for 5 months. The lesion was a indurated ulcer with dirty crust sized 25 mm by 15 mm on his right wrist (Fig. 6.4.5A). The skin test was positive (16x22/30x35). When the 10% paromomycin ointment was applied for 2 weeks, the lesion became dry and it's size was decreased (Fig. 6.4.5B). One month later, the lesion was healed with slight crust (Fig. 6.4.5C), and that was completely healed after one year (Fig. 6.4.5D). The case of No. 36 was a 14-year-old boy, who complained of an indurated ulcer on his right forearm since 2 months (Fig. 6.4.6A). The skin test was positive (20x20/38x32). The topical treatment with 2% paromomycin ointment was carried out. The lesion became dry after three weeks (Fig.6.4.6B), and it healed with a slightly elevated scar after 2 months. The scar became flatter after one year

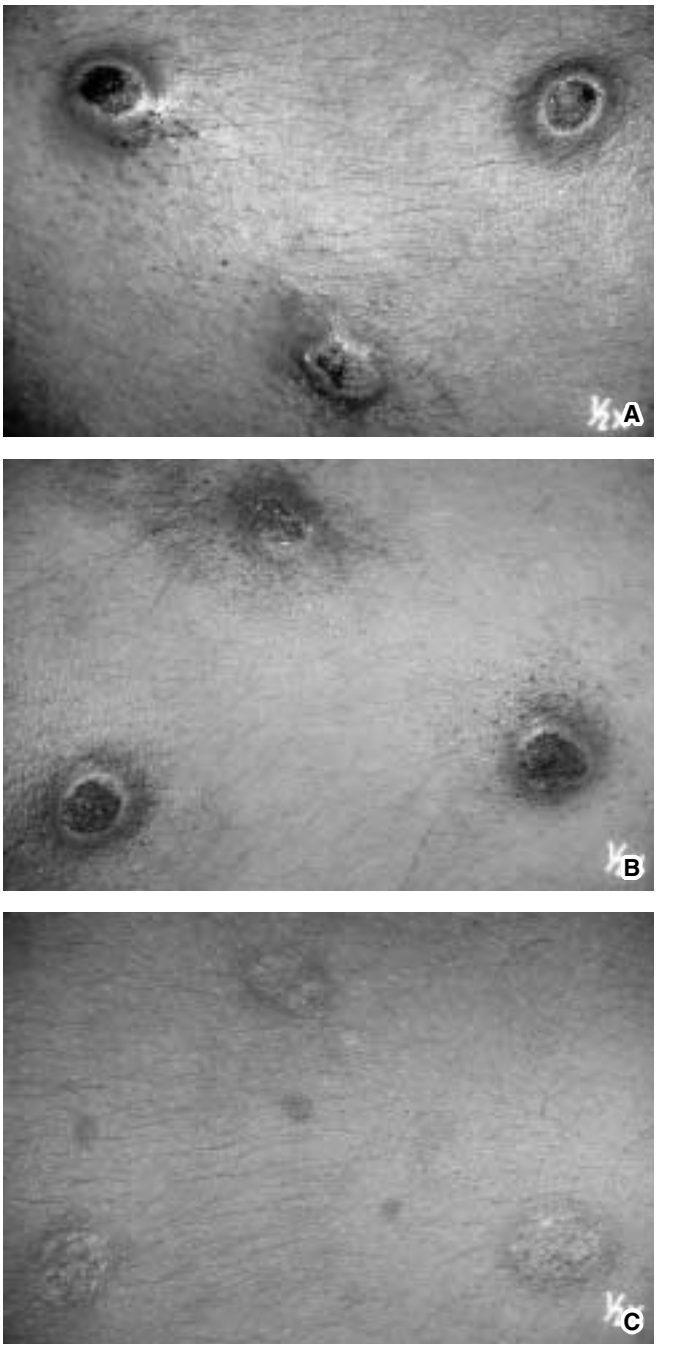

Figure 6.4.1. Case No. 139, a 4-year-old boy. **A**, before topical treatment with antimoniate lotion; **B**, 3 weeks later; **C**, 1 year later without recurrence.

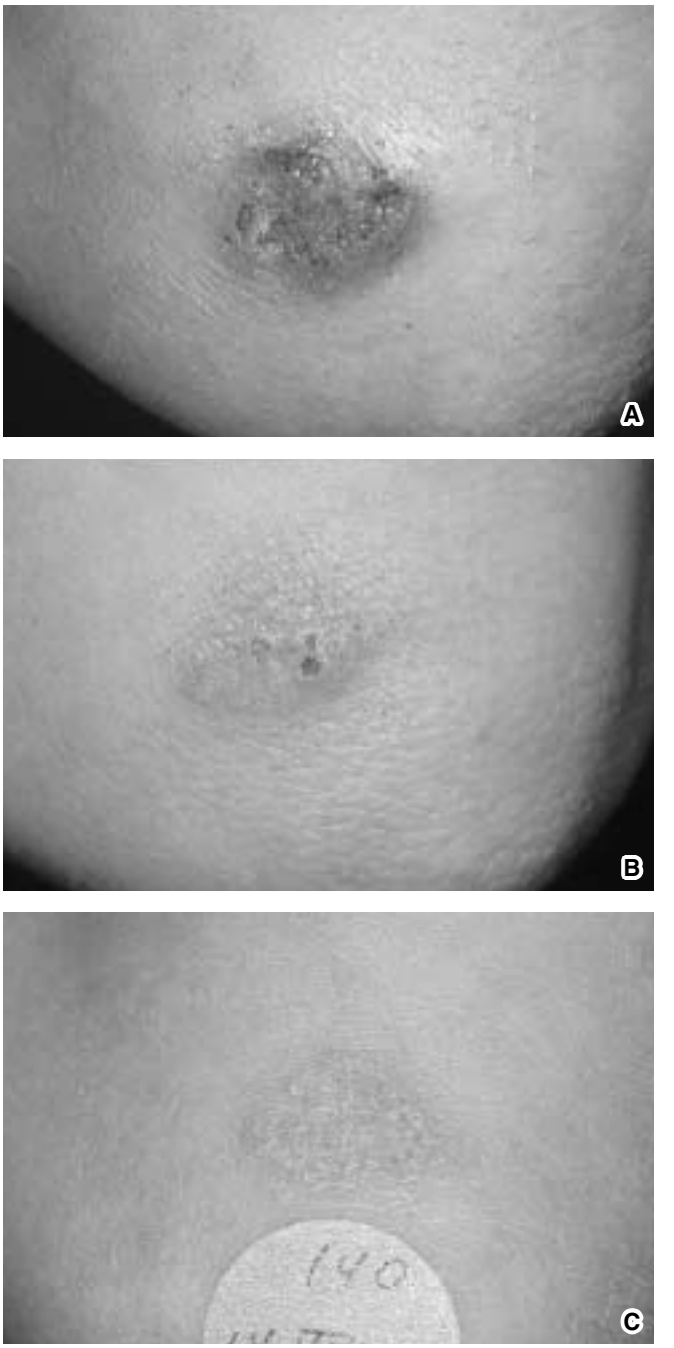

Figure 6.4.2. Case No. 140, a 6-year-old boy. **A**, before treatment with the lotion; **B**, 3 weeks later; **C**, 6 months later.

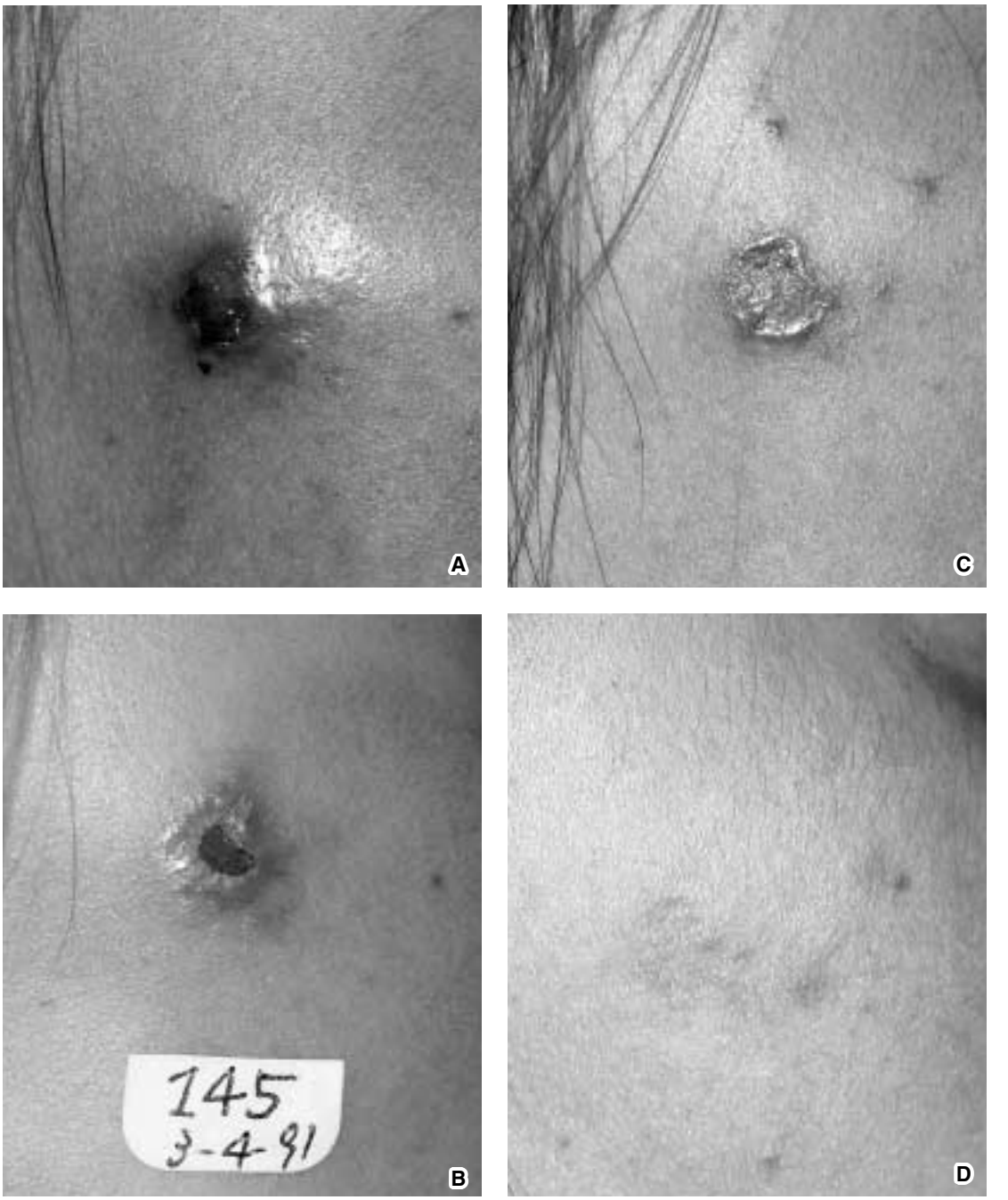

Figure 6.4.3. Case No. 145, a 14-year-old girl. **A**, before treatment with the lotion; **B**, 2 weeks later; **C**, 3 weeks later; **D**, 1 year later.

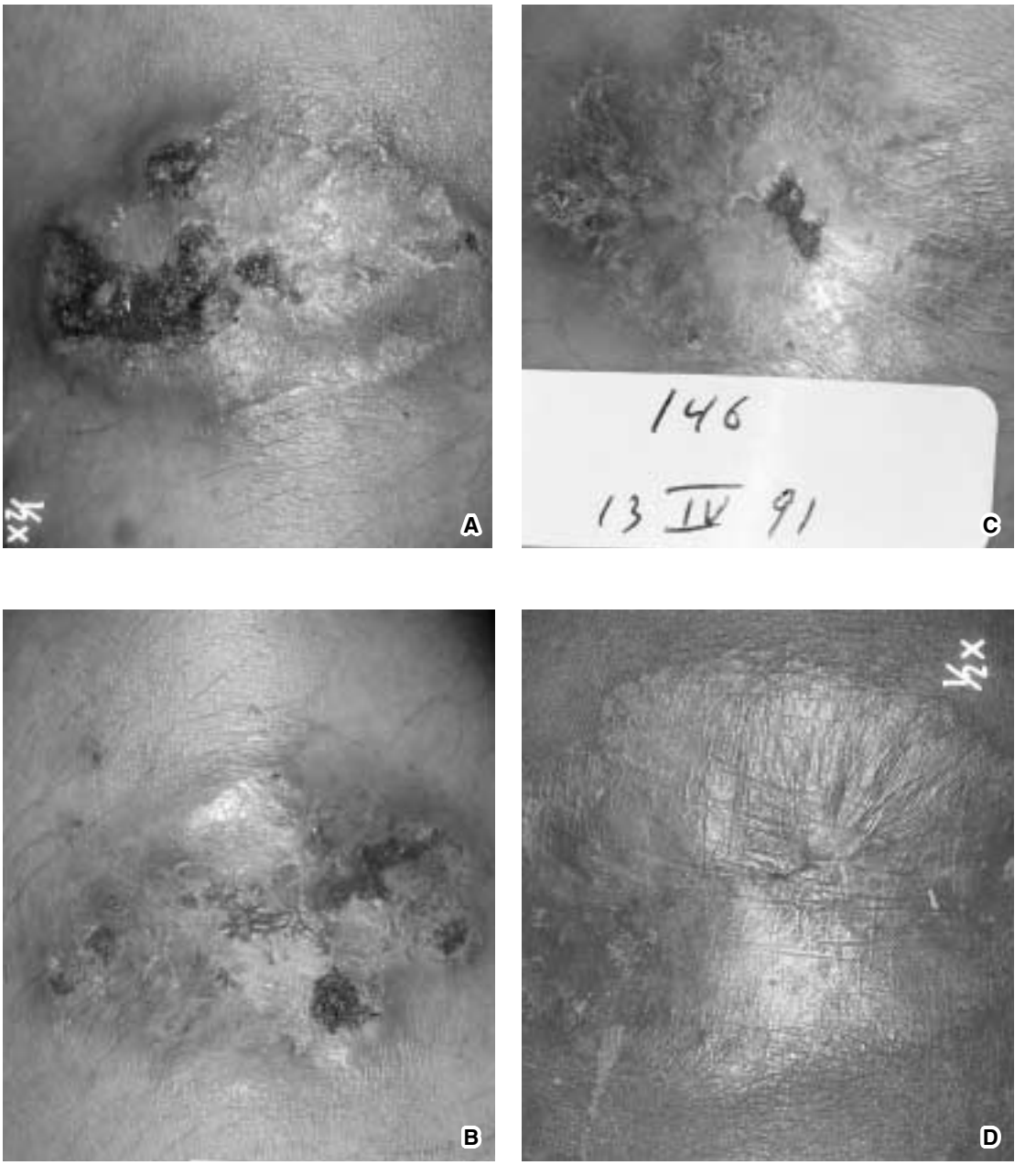

Figure 6.4.4. Case No. 146, a 9-year-old girl. **A**, before treatment with the lotion; **B**, 2 weeks later; **C**, 1 month later; **D**, 1 year later.

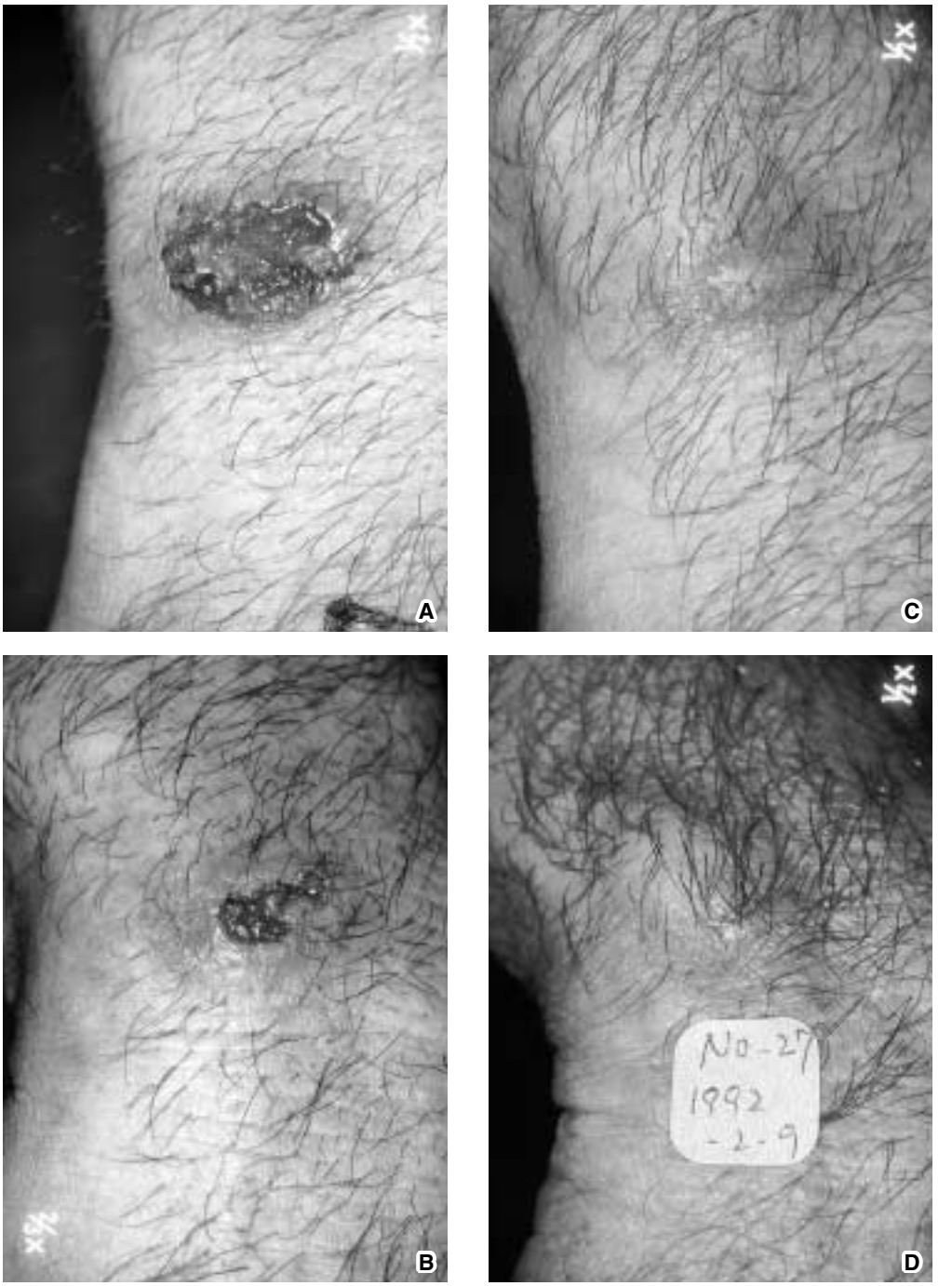

Figure 6.4.5. Case No. 27, a 40-year-old male. **A**, before topical treatment with paromomycin ointment; **B**, 2 weeks later; **C**, 1 month later; **D**, 1 year later.

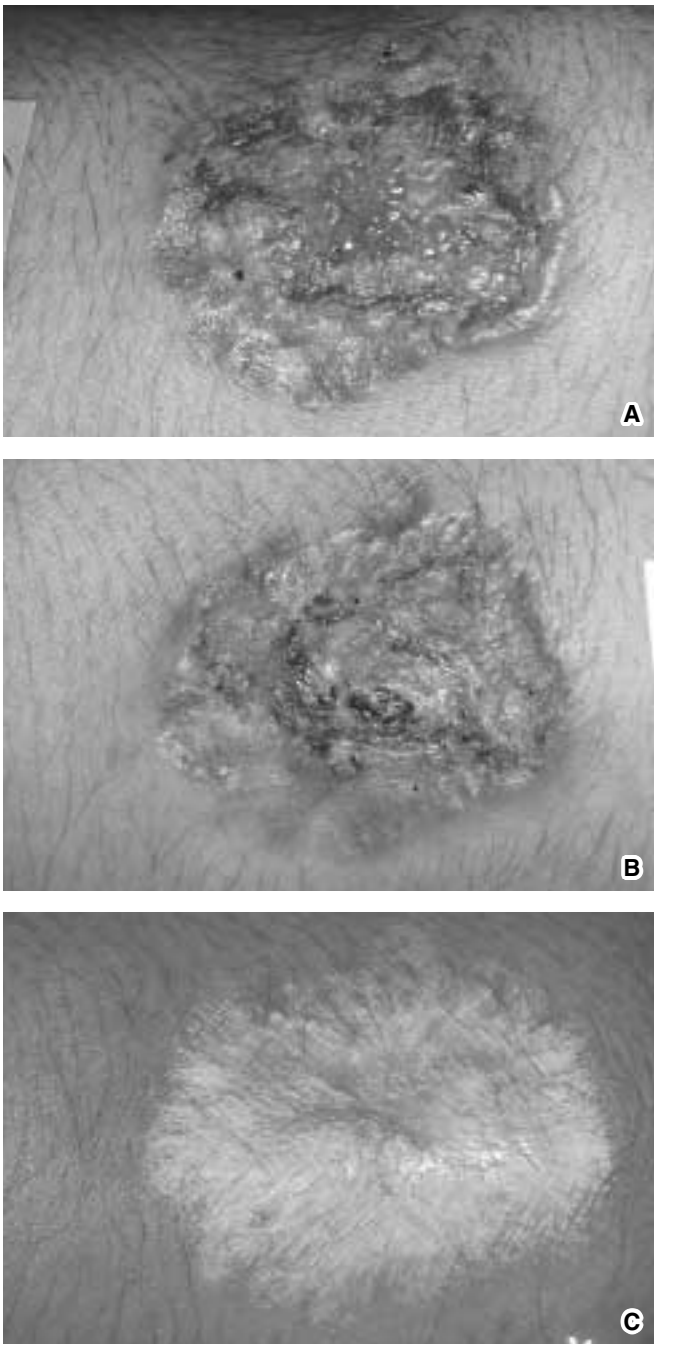

Figure 6.4.6. Case No. 36, a 14-year-old boy. **A**, before treatment with the ointment; **B**, 3 weeks later; **C**, 1 year later.

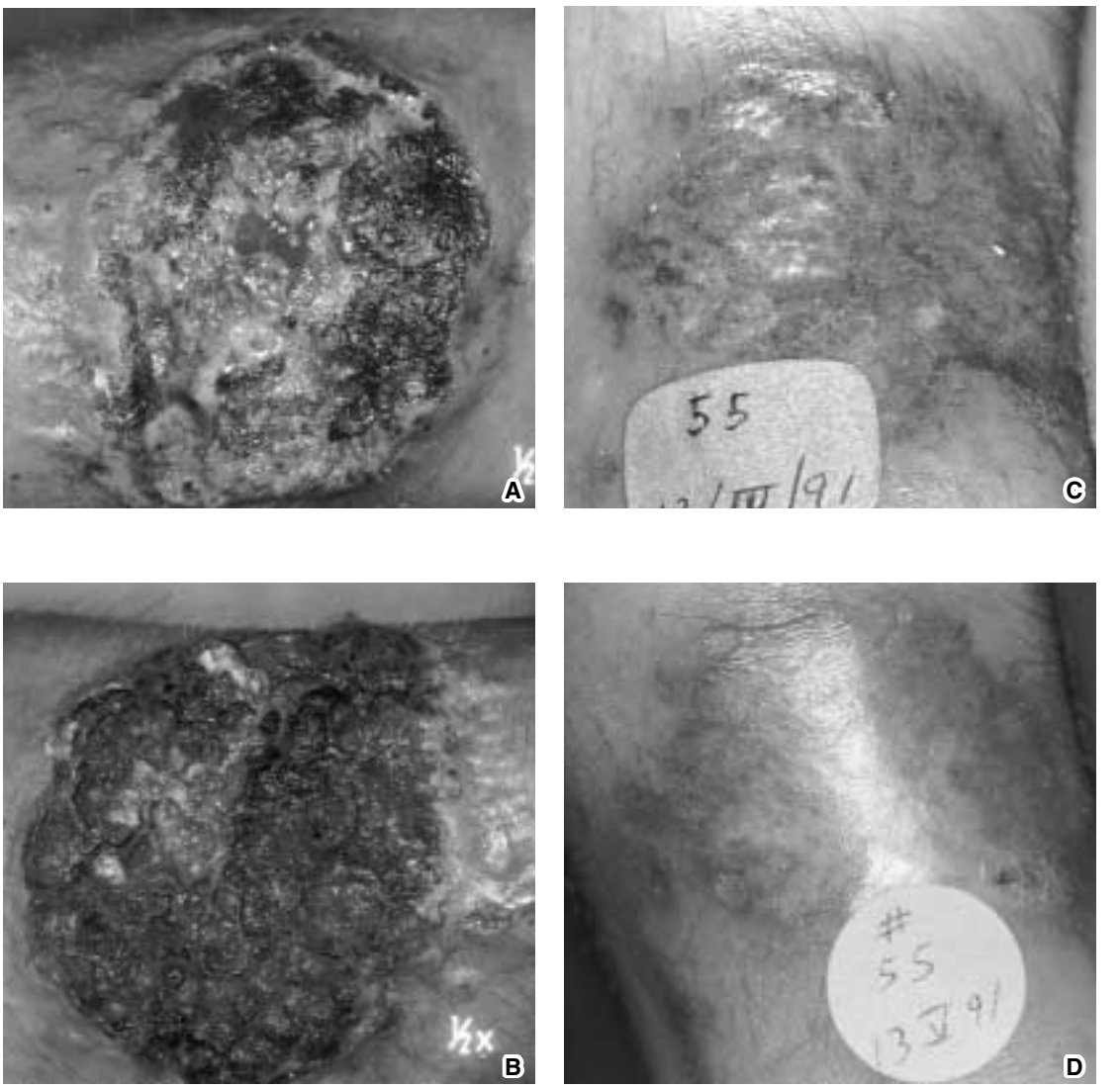

Figure 6.4.7. Case No. 55, a 13-year-old boy. **A**, before treatment with the ointment; **B**, 3 weeks later; **C**, 2.5 months later; **D**, 4 months later.

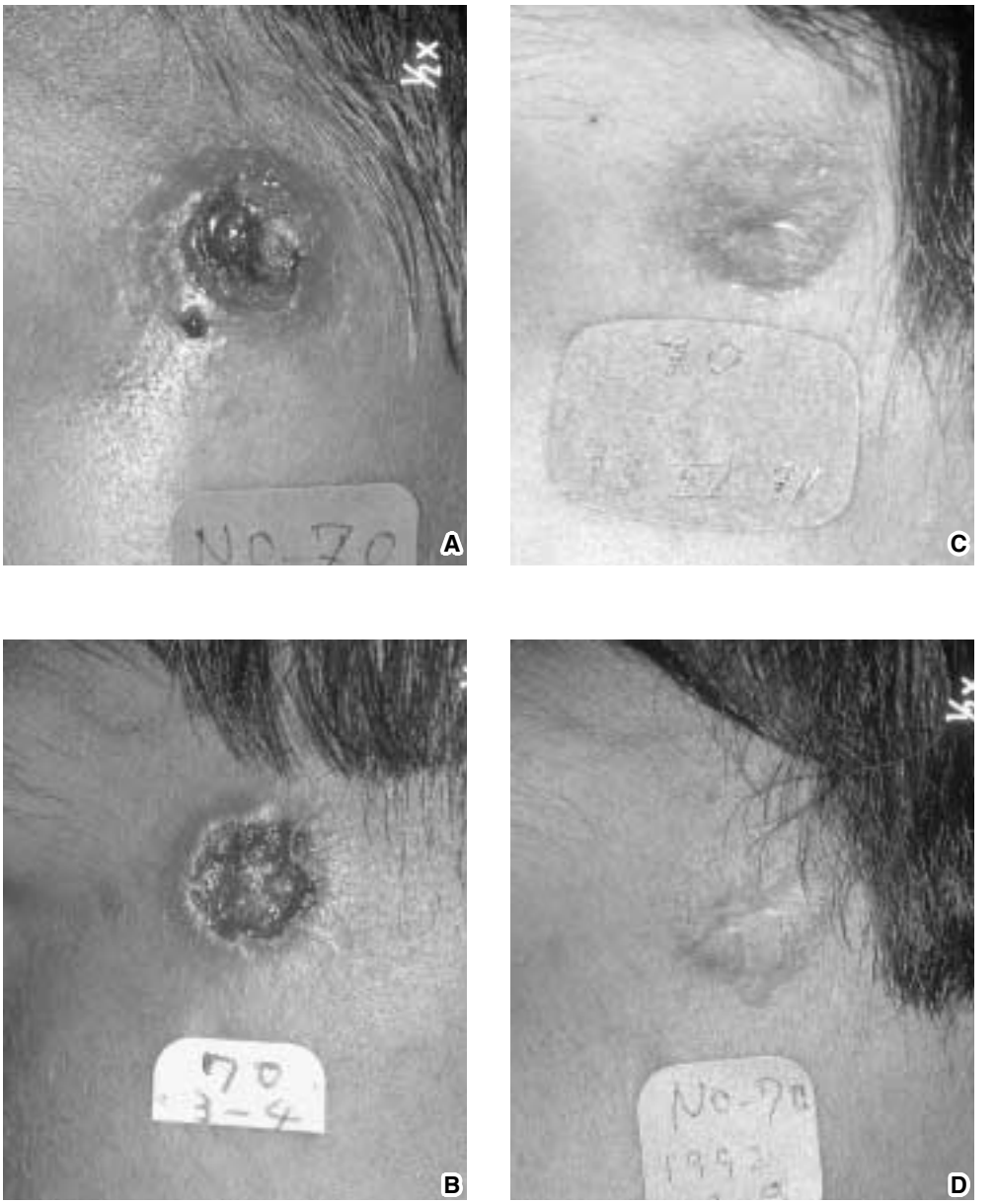

Figure 6.4.8. Case No. 70, a 9-year-old boy. **A**, before treatment with the ointment; **B**, 1 month later; **C**, 2 months later; **D**, 1 year later.

(Fig. 6.4.6C). The case of No. 55 was a 13-year-old boy, who complained of a large ulcer on his right lower leg for 2 months (Fig. 6.4.7A). There was a large ulcer with dirty crust sized 70 mm by 45 mm and small red papules were scattered surrounding the ulcer. The skin test was positive (25x21/28x25). The topical treatment with 2% paromomycin ointment for 3 weeks changed, the lesion dry (Fig. 6.4.7B), then meglumine antimonate solution was added to the lesions. The lesion was covered with epidermis after 2 and half months (Fig. 6.4.7C). Four months later, the lesion was completely healed (Fig. 6.4.7D). The case of No. 70 was a 9-year-old boy. The patient had complained of an ulcer on his face since 3 months. When the patient visited our clinic, there was an indurated ulcer sized 30 mm by 30 mm on the left cheek (Fig. 6.4.8A). The skin test showed positive (24x14/33x22). The 2% paromomycin ointment was given to the patient. The ulcer changed to the dry lesion with crusts after one month (Fig. 6.4.8B). Two months later, the lesion was healed completely with slight scar (Fig. 6.4.8C); the scar was diminished after one year (Fig. 6.4.8D).

Discussion

A 15% paromomycin sulfate and 12% methylbenzethonium ointment was effective to the patients with cutaneous leishmaniasis in Old World (El-On, 1986, 1992). The concentration of paromomycin was relatively high and the ointment was combined with paromomycin and methylbenzethonium. We used lower concentrations of paromomycin in Ecuador. Even a low concentration of 2% was effective in 80% of the patients in this study. There was no difference in effectiveness between the 10% and 2% of paromomycin ointments. Meglumine antimonate with mercury chrome solution was also effective. The effective rate of the solution showed similar tendency to paromomycin ointment. However, drug resistance of antimonial drugs to the cutaneous leishmaniasis is increasing (Grogl, 1992). Therefore, topical use of meglumine antimonate will have the same problem. We need a study how to avoid this drug resistance in the future. Chemotherapy for killing the parasites has always a similar contradiction. Immunotherapy may be necessary for the treatment of leishmaniasis.

Topical treatment using low concentrations of paromomycin ointment and meglumine antimonate with mercury chrome solution was effective to 80% of cutaneous leishmaniasis patients in this study. There was a low irritation to the wet ulcers of patients with cutaneous leishmaniasis. But, it was less effective to the non-ulcered lesions of the patients. Topical treatment against leishmaniasis should be done carefully to the patients, who do not have any concern to mucocutaneous and visceral types of the disease.

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Chapter 7

Experimental Studies on Antileishmanial Effect *in Vitro* and *in Vivo*

1. Effect of Plant Juices on the Growth of *Leishmania* Promastigotes in Culture

ABSTRACT. In this study, effects of crude components of plants which are distributed in *Leishmania* endemic area of Ecuador on the growth of *Leishmania* promastigotes in culture are investigated. Promastigotes cultured in medium 199 with the samples from mandarina and naranja showed a better development as compared with the control at 48 hours in culture. Although the samples from piñon and agave promoted the development of promastigotes until 24 hours in culture, the number of promastigotes in the medium with these samples were smaller than the controls at 72 hours in culture. In all experiments, flagellum length of promastigote extended with the lapse of time. At 72 hours in culture, flagellum length was about twice in length as compared with flagellum at the commencement of the culture. It was clear that naranja and mandarina contain some components which promote the growth of promastigotes in culture and agave and piñon enhance the growth of promastigotes during early cultivation period (~ 24 hours). On the other hand, mamei and the two *Leguminosae* plants used in this study inhibited the development of promastigotes. Those plants apparently contain some components which can adversely affect the growth of promastigotes in culutre.

Introduction

Leishmania parasites are transmitted by the bites of infected female sandflies to the vertebrate hosts. In sandflies the parasites as a promastigote form develop only in the gut and in vertebrate hosts they as an amastigote form develop in the macrophages. Although female sandflies feed on the blood meal from the vertebrates for oviposition, their common diets are plant-derived foods termed sugar meals. *Leishmania* infections are permanently in the gut of sandfly where they are exposed to these meals and are apparently affected by the components of these meals. Schlein and Jacobson (1994) reported some plant diets of *Phlebotomus papatasi*, which is the vector of *L. (Leishmania) major* in Israel, impair *L. (L.) major* infectons in the sandflies. Since the sandflies in the New World also feed on plants, the composition of plants may influence *Leishmania* infections in their gut. In this study, effects of the composition of plants which are distributed in *Leishmania* endemic area of Ecuador on growth of *Leishmania* promastigotes in culture were investigated.

Materials and Methods

Plants used in this study

Leaves of an agave (*Amaryllidace* sp.) (Agave) and two bean-plants (*Leguminosae*) (Mame 1 and Mame 2) were collected from Huigra in the Province of Chimborazo. Leaves of piñon, fruits of naranja (*Citrus* sp.) (Piñon and Naranja) and mandarina (*Citrus* sp.) (Mandarina) were collected from San Sebastian in the Province of Los Rios which is an endemic area of *L. (Viannia) panamensis*. In addition, nuts of mamei (Mamei) were collected from La Esmeralda

in the Province of Los Rios.

Leishmania *parasite*

The WHO reference strain, *L. (V.) panamensis* (MHOM/PA/71/LS94), which is a major species in the endemic area from where plant samples were obtained, was maintained in the culture medium, tissue culture medium 199, with 10% heat inactivated fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 24℃.

Preparation of plant samples

The plant materials were kept at -80℃ until use. The plant samples were squeezed and then at temperature of 4℃, centrifuged at 12000 rpm for 15 minutes. The supernatant (plant juice) was used for the experiment after filtration through a 0.22 μm pore membrane filter (MILLEX-GS, MILLIPORE Co., Ltd., U.S.A.).

Growth curves

Ten to the fifth of promastigotes (10^5)/ ml in logarithmic-phase were seeded in 96-well cell culture plate (Cluster Dishes, Coster, U.S.A.) with the tissue culture medium 199. The plant samples of one hundredth volume of the medium were added to the wells individually and then incubated at 24℃. At 6, 12, 24, 48, and 72 hours after starting the culture, 50 μl of the culture was collected and the number of parasites was counted using a cell count chamber. For observations of morphological shapes of cultured promastigotes, the remaining culture medium was used.

Morphological mesurements of cultured promastigotes

Promastigotes from 6, 12, 24, 48, and 72 hours cultures were put on a slide glass, air-dried, fixed with methanol, and stained with Giemsa solution. The lengths of the body and the flagellum were determined by measuring 30 randomly selected culture forms.

Results

Growth patterns

Promastigotes cultured without any plant samples (the control) increased in number with the lapse of time until 72 hours from 1 x 10^5 to 5.69 x 10^5 /ml (Table 7.1.1). Promastigotes cultured with the samples from mandarina and naranja showed about two-fold development (6.77 x 10^5 and 7.52 x 10^5 /ml, respectively) as compared with the control (4.01 x 10^5 /ml) at 48 hours in culture. Although the samples from piñon and agave promoted the development of promastigotes (3.37 x 10^5 and 2.34 x 10^5 /ml, respectively) until 24 hours in culture, the number of promastigotes in medium 199 with these samples were smaller than the control's at 72 hours in culture. The two Mame and Mamei controlled the development of promastigotes in culture.

Morphological changes during growth in medi-

Table 7.1.1. Growth of promastigotes in culture

	Control	Agave	Mame 1	Mame 2	Piñon	Naranja	Mandarina	Mamei
0 time	1.0							
6 hours	1.44	1.07	1.01	1.93	2.04	2.98	2.78	1.64
12 hours	1.62	1.93	1.96	1.62	2.38	3.23	2.34	1.58
24 hours	2.49	2.34	1.35	2.30	3.37	3.82	3.18	1.98
48 hours	4.01	5.07	2.29	2.02	5.34	7.52	6.77	2.35
72 hours	5.69	4.24	1.38	1.52	4.83	6.56	7.55	1.79

* No. is shown as x 10^5 promastigotes/ml.

** Agave; Leaves of an agave (*Amaryllidace* sp.), Mame 1 and Mame 2; two bean-plants (*Leguminosae*), Piñon; Leaves of piñon, Naranja; fruits of naranja (*Citrus* sp.), Mandarina; mandarina (*Citrus* sp.).

um 199 with/without plant samples

In all experiments, flagellum of promastigote increased in length with the lapse of time (Table 7.1. 2). At 72 hours in culture, flagellum length was about twice as long (from 11.1 μm to 23.2 μm) as flagellum at the commencement of the culture. Some little growth of the body length was recognized in culture with the plant samples from Agave, Naranja and Mandarina (20.1 μm, 22.8 μm and 19.3 μm, respectively). In contrast, the body length of promastigotes reduced from 18.1 μm to 15.3 μm, with those from Mame 1.

Discussion

Sandfly vectors do not only feed on blood meals from vertebrates but also feed on sugar meals from

Table 7.1.2. Morphology of parasites in culture

Plant juice* and culture medium		Full length	Flagellum length (μm)	Body length (μm)	F/B**
0 time control		29.2	11.1	18.1	0.37±0.067
Control,	6 hours	35.6	15.2	20.4	0.42±0.064
	12 hours	35.8	15.3	20.5	0.42±0.066
	24 hours	40.1	20.4	19.7	0.50±0.079
	48 hours	44.1	22.5	21.6	0.51±0.047
	72 hours	43.8	23.2	20.6	0.53±0.072
Agave	6 hours	29.8	11.4	18.4	0.37±0.094
	12 hours	29.3	12.4	16.9	0.42±0.071
	24 hours	31.2	13.7	17.5	0.43±0.064
	48 hours	47.7	24.0	23.7	0.50±0.044
	72 hours	43.6	23.5	20.1	0.53±0.063
Mame 1	6 hours	37.6	16.2	21.4	0.42±0.074
	12 hours	34.1	14.3	19.8	0.42±0.040
	24 hours	33.6	14.7	18.9	0.43±0.075
	48 hours	39.0	20.7	18.3	0.55±0.076
	72 hours	39.8	24.5	15.3	0.62±0.123
Naranja	6 hours	33.0	13.6	19.4	0.41±0.071
	12 hours	33.1	14.5	18.6	0.43±0.081
	24 hours	32.1	14.9	17.2	0.46±0.054
	48 hours	47.4	24.0	23.4	0.50±0.060
	72 hours	49.3	26.5	22.8	0.53±0.044
Mandarina	6 hours	31.6	12.5	19.1	0.39±0.076
	12 hours	29.8	12.6	17.2	0.42±0.058
	24 hours	35.0	16.6	18.4	0.47±0.058
	48 hours	51.7	26.0	25.7	0.50±0.041
	72 hours	41.3	22.0	19.3	0.53±0.046

* Agave, Leaves of an agave (*Amaryllidace* sp.); Mame 1, bean-plants (*Leguminosae*); Naranja, fruits of naranja (*Citrus* sp.); Mandarina, mandarina (*Citrus* sp.). ** Flagellum/body length.

juices of plants in the field. Honeydews consist mainly of various sugars and amino acids of plant saps (Brown 1975) and these compositions might be useful for the development of promastigotes in the gut of the sandfly. Honeydews lack many of the components of plant tissues. Although in the wild, feeding of sandflies only on juices has been confirmed, in the laboratory, they can feed by biting into plant tissues (Schlein and Warburg, 1986). Therefore, plant food may give a wide range of influence on the *Leishmania* parasites in the sandflies.

In this study, we investigated the effects of components of plants collected from endemic areas of leishmaniasis in Ecuador on *Leishmania* promastigotes in culture. It was clear that naranja and mandarina contain some components which promote the growth of promastigotes in culture and agave and piñon enhance the growth of promastigotes during early cultivation period (~ 24 hours). On the other hand, mamei and the two *Leguminosae* plants used in this study inhibited the development of promastigotes. Those plants apparently contain some components which can adversely affect the growth of promastigotes in culture. Although it is not confirmed that sandflies feed on those plants in the field, it may be considered that if sandflies infected with *Leishmania* feed above mentioned plants, the development of the parasites in the sandflies are either accelerated or hindered.

Sugar meals from juices of plants affect infectivity of *Leishmania* promastigotes in the gut of the sandflies (Schlein, 1993, Young *et al*., 1980). However, plant feeding of the sandflies is not only beneficial but also impaire the development of the parasites in the gut. Feeding on *Ricinus communis*, *Capparis spinosa* and *Solanum luteum* cause more than 50% mortality and deformation of promastigotes (Schlein and Jacobson, 1994). In the present study, measurements of para ites showed a marked tendency to diminish in flagellum/body (F/B) length ratios due to an increase in the length of the flagellum on successive days in culture. The round-shaped cell body of promastigotes in the early culture period became spindle-shaped in all of experiments except in cultures with the plant samples from two *Leguminosae*. In those cases, the development of promastigotes was hindered and about 40% of the promastigotes showed rounding of the cell body, which died by 72 hours in culture.

Since the plants used in this study affected the development of promastigotes in culture, they may also affect activity of promastigotes in the sand flies (Rey *et al*., 1990).

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2. Effect of Mefloquine on Visceral Leishmaniasis in Mice

ABSTRACT. We evaluated an effect of oral administration of mefloquine on experimental visceral leishmaniasis in mice inoculated with *Leishmania (Leishmania) donovani*. Mice orally received mefloquine at a dose of 75 mg/kg for 2 days before infection showed a 50% reduction in number of parasites in the liver. However, administration of drug at the same dose for 2 days after infection had no therapeutic effect on elimination of parasites in the liver of infected mice.

Introduction

Recently we found that antimalarial drugs, mefloquine hydrochloride (Mephaquin®) and artesunate (Plasmotrim®), had an effect when they were used as oral therapy of cutaneous leishmaniasis patients at endemic areas of Ecuador (Gomez *et al*., 1995). The patients treated with the oral administration of a total dosage of 1500 mg mefloquine or 4.2 mg/kg/day for 6 days showed healing of almost all skin lesions within 6 weeks. These clinical trials raised a question whether the same drugs were also effective against visceral leishmaniasis. Here we report results of oral administration of Mephaquin® to mice experimentally infected with *Leishmania (Leishmania) donovani*.

Materials and Methods

Parasites

A virulent clone of *L. (L.) donovani* 2S-25M-C2 (Katakura and Kobayashi, 1985, 1988) was used in this study. Promastigotes of the clone have been maintained in Medium 199 containing 20% heat-inactivated fetal calf serum (HIFCS) and 100 μg/ml gentamycin at 25-26℃.

Infection of mice and determination of parasite burden in the liver

As described previously (Katakura and Kobayashi, 1985, 1988), female BALB/c mice of 8 weeks of age were inoculated intravenously *via* the tail vein with 1 x 10^8 promastigotes harvested from the stationary phase cultures. Two weeks after the infection, the number of amastigotes was counted per 500 liver nuclei in Giemsa-stained liver stamp preparations and multiplied by liver weight in mg to express the parasite load as Leishman-Donovan Units (LDU) (Bradley and Kirkley, 1977).

Treatment of infected mice with mefloquine

For *in vivo* experimental chemotherapy, we used Mephaquin® Lactab® (Mepha Ltd., Aesch-Basle, Switzerland); each tablet contains mefloquine hydrochloride corresponding to 250 mg mefloquine base. The mefloquine tablet was ground in a pestle and mortar with sterile saline and resultant drug suspension was inoculated into the mouse stomach by stomach tube. A group of four infected mice were given the drug at a dose of 75 mg/kg for 2 days before infection (days -1 and 0) and the other group of six infected mice were given at the same dose at 7 and 8 days after infection (days +7 and 8). Six infected mice remained untreated as a control group.

In vitro *drug susceptibility test*

For *in vitro* drug susceptibility test, we used mefloquine hydrochloride powder which was supplied by M. Andrial, Mepha Ltd. Promastigotes of *L. (L.) donovani* were harvested from the stationary phase cultures and suspended in Medium 199 with 20% HIFCS at a concentration of 4 x 10^5 per ml. The organisms (4 x 10^4) in a volume of 100 μl were added to each well of 96-well flat-bottomed microplates (Nunc, Denmark) as described before (Katakura *et al*., 1992, 1994). Two-fold dilutions of mefloquine hydrochloride in a volume of 100 μl were added to duplicate rows of the plate. The plate was incubated at 26℃ for 4 days until

control cultures with no drug reach to the maximum growth. The number of parasites in each well was counted on a hemocytometer. 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

Effect of Mephaquin® on experimental visceral leishmaniasis in mice

We assessed LDU in the liver of infected mice at 2 weeks of infection because a peak of number of parasites in the liver was seen at 2 weeks of infection in our murine model using BALB/c mice and *L. (L.) donovani* 2S-25M lines (Katakura, 1986). Infected BALB/c mice received 75 mg/kg mefloquine for two days before infection had an average of 575 LDU in the liver, showing about a 50% reduction in number of amastigotes compared to that in untreated control infected mice (Table 7.2.1). However, infected mice received the drug at 7 and 8 days after infection exhibited no substantial reduction in number of parasites in the liver.

Antipromastigote activity of mefloquine hydrochloride in vitro

The susceptibility profile of *L. (L.) donovani* promastigotes to mefloquine hydrochloride was monophasic. Few proliferating parasites were seen in the presence of 2.5 μg/ml mefloquine and the ED_{50} value was about 1.35 μg/ml (Fig.7.2.1).

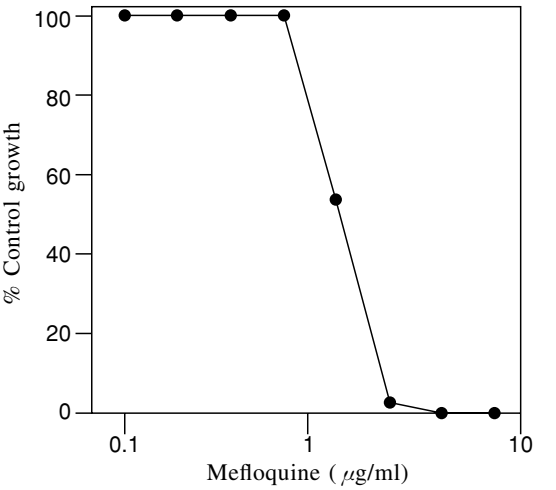

Figure 7.2.1. Susceptibility of *L. (L.) donovani* promastigotes to mefloquine hydrochloride *in vitro*. Promastigotes were cultured in the presence of various concentrations of mefloquine hydrochloride and inhibitory effect of the drug on parasite growth was expressed as a percentage of the control growth.

Discussion

Chemotherapeutic drugs for leishmaniasis have been extensively examined by *in vitro* drug susceptibility test against promastigotes in cell-free cultures and amastigotes in cultured mammalian cells as well as by *in vivo* animal models for cutaneous and visceral leishmaniasis (reviewed by Neal, 1987). We still need, however, to make an effort to search for rational drugs which are orally applicable for different types of leishmaniasis.

In Ecuador, we tried oral treatment of cutaneous leishmaniasis patients with mefloquine, an antimalarial drug. Surprisingly we found that almost all cuta-

Table 7.2.1. Effect of mefloquine on *L. (L.) donovani* infection in mice

Mefloquine	LDU ± SD in the liver on day 14 (No. of mice examined)
Non-treated	1170 ± 284 (6)
75 mg/kg/day (days -1 and 0)	575 ± 211 (4)
75 mg/kg/day (days +7 and +8)	1120 ± 279 (6)

neous lesions of the patients healed within 6 weeks (Gomez *et al*., 1995). Mefloquine, a quinine analogue, destroys asexual erythrocytic forms of *Plasmodium* species and has been used for chemotherapy of malaria patients. Possible mechanisms of action of mefloquine against malarial parasites are explained as follows. Mefloquine binds to lipid membranes of erythrocytes with high affinity and may inhibit the invasion of erythrocytes by merozoites (Mu *et al*., 1975). The action of mefloquine appears to be linked to hemoglobin digestion by the parasites which produces ferriprotoporphyrin IX, a membrane lytic compound. Mefloquine strongly binds to ferriprotoporphyrin IX to form a complex which is toxic to the parasites while *Plasmodium* hem polymerase detoxifies this metabolite by catalyzing formation of an inert malarial pigment, hemozoin (Goldberg and Slater, 1992; Wellems, 1992). In addition, ionisable nitrogens of the side chains of mefloquine may be important to increase *P. falciparum* vesicle pH and inhibit parasite growth (Krogstad *et al*., 1985). Although the precise mode of action of mefloquine against *Leishmania* parasites is unknown, it is possible that mefloquine also acts for increasing lysosome pH in *Leishmania* and inhibit metabolic pathways of the parasites.

Peters *et al*.(1980a, b) reported that mefloquine was more effective against experimental cutaneous leishmaniasis than visceral leishmaniasis. Against mice infected with *L. (L.) major* or *L. (L.) amazonensis*, they subcutaneously injected a total dose of 350 mg mefloquine by giving 70 mg/kg/day for five consecutive days from the day of infection. The drug activity was recorded as a moderate level for both *Leishmania* species because the mean size of the skin lesions developed at the base of the tail was reduced by 1/3 to 2/3 of that in untreated control infected mice. In addition, they also examined the activity of mefloquine against visceral leishmaniasis in mice. The same amounts of mefloquine were subcutaneously administered into the *L. (L.) infantum*-infected mice from 5th or 6th day after infection for five consecutive days. As a result, they concluded that the drug activity was scored as a low level because the number of parasites in the liver of mice treated with mefloquine remained 50-90% of that in untreated control infected mice. The reasons why dermotropic *Leishmania* spp. are more sensitive to mefloquine than viscerotropic species remain to be elucidated

In this study we also detected that mefloquine was not effective against visceral leishmaniasis in mice when the drug was given after infection. However, when the drug was administrated before infection, a moderate level of prophylactic effect of mefloquine against visceral leishmaniasis was evident. This effect may be due to the direct antipromastigote activity of the drug. We speculated that mefloquine concentrations in the plasma of mice infected with *L. (L.) donovani* were probably higher than 1.35 μg/ml, the ED_{50} against promastigotes of this species *in vitro*, because the mice were given a total dose of 150 mg/kg mefloquine and it has been reported that mice received 8 mg/kg mefloquine showed the maximum drug concentration of 1.55 μg/ml in the plasma at 24 hr after the drug administraion (Rozman *et al*., 1978). Furthermore, administration of a total dose of 1000 mg mefloquine for healthy volunteers resulted in the maximum drug concentration of about 1 μg/ml in the plasma and the mean half life of the drug is calculated as 21.4 days ranging from 15 to 33 days. (Schwarts *et al*., 1980, 1982).

In conclusion, therapeutic effect of mefloquine on visceral leishmaniasis is unlikely, whereas some prophylactic effect of the drug against various types of leishmaniasis may be expected when malaria patients residing in endemic areas of both malaria and leishmaniasis are treated with mefloquine.

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Chapter 8

Leishmaniasis and its Related Skin Diseases

1. Clinical Comparison of Cutaneous Changes of Patients with Diffuse Cutaneous Leishmaniasis and Leprosy in Ecuador

ABSTRACT. The cutaneous changes of a patient with diffuse cutaneous leishmaniasis who had been mistreated for lepromatous leprosy were reported. The patient was a 24-year-old male, born in Province of Esmeraldas in Ecuador. Diffuse type of cutaneous leishmaniasis was not detected by the screenings of family members, the relatives and the neighbouring people. The eruptions observed in this case were similar to those of leprosy, lepromatous leprosy and borderline group leprosy. However there were some differences in dermatological findings, such as types of eruptions and their distribution, between these two infectious diseases.

Introduction

Clinically, patients with cutaneous leishmaniasis have various cutaneous manifestations such as insect bite-like papules, nodules, ulcers and erythematous plaques. Therefore, differential diagnosis between cutaneous leishmaniasis and other skin diseases including leprosy is very important, especially in Ecuador where those infectious diseases are relatively common. It is reported that early nodular lesions of cutaneous leishmanisis are similar to those of lepromatous leprosy (LL) (Jopling, 1984).

The purpose of the assignment given to us in our medical team from 1992 until 1995 was to compare the lesions between cutaneous leishmaniasis and leprosy. We, therefore, have been investigated and summarized cutaneous changes observed on the skin lesions of patients with cutaneous leishmaniasis (Nonaka *et al*., 1990; Hosokawa *et al*., 1994). In this report, the cutaneous changes of a patient with diffuse cutaneous leishmaniasis who had been previously treated for LL are reported. Though the eruptions of this case were similar to those of leprosy, there were some differences in clinical and dermatological findings between these two diseases. Furthermore, based on the observation of the cutaneous manifestations of this case, we will add a brief comment on a possibility of ulcer formation on the lesion and non-specific immunodeficiency against leishmaniasis.

Case report

A 24-year-old male, born in Province of Esmeraldas, Ecuador, visited our clinic in August of 1994. He grow up in San Ignacio (Muisne), a rural and mountainous area far from Esmeraldas city, where his family actually lived. There were no patients with a diffuse cutaneous leishmaniasis in his family members and neighbouring people. When the patient was 16-year-old, papules appeared on his left knee and right cheek. The eruption gradually increased in size and in number. For about two years, he received various medications without confirmed diagnosis. About half a month prior to his visit to an outpatient clinic in Guayaquil city, he was diagnosed clinically as leprosy and started receiving medication for it. In 1989, after clinical and laboratory examinations, he was diag-

nosed as a diffuse cutaneous leishmaniasis at the clinic. Physical examinations revealed pea to rice-grain-sized reddish papules on both the ear lobes and the auricles. Induration of the lesions was palpable (Fig. 8.1.1). Miliary to pea-sized reddish papules were observed on the face (Fig. 8.1.2). Miliary to pea-sized reddish papules and nodules were also observed on the left shoulder. Many miliary to hen-egg-sized papule, nodule, erythema, infiltrated erythema and brownish colored fleckles were observed on the upper extremities (Fig. 8.1.3). The surfaces of some of the nodules were scaly and crusted (Fig. 8.1.4). Rice-grain to thumb-sized reddish papules grouped on the brownish colored fleckle lesion on the lower back were observed. Miliary-sized papules were scattered showing satellite lesions around the plaque-like lesions (Fig. 8.1.5). Thumb to palm-sized erythemas were observed on the thigh showing no loss of sensation (Fig. 8.1.6). Various kinds of eruptions, such as papules, nodules, infiltrated erythemas, brownish colored fleckles, were observed also on the lower extremities (Figs. 8.1.7, 8.1.8 and 8.1.9). There were no eruptions on the hands, the feet, the scalp, the axillary, epigastric, inguinal, perineal and anal regions, except a few verruca vulgaris lesions on the dorsal aspect of the left hand (Fig. 8.1.3). There was no sensory loss on all over the body surface and no hypertrophy of peripheral nerve. Leishmanin skin test showed negative. Numerous *Leishmania* amastigotes were observed in the stained smears from the skin lesion though no acid fast bacilli were observed in the skin slit smear materials with Ziehl-Neelsen staining method. The parasites were isolated in culture both *in vivo* and *in vitro*. Zymodeme and karyodeme analysis revealed that they were *L. (Leishmania) mexicana* (Katakura *et al*., 1994). Histological findings of the specimen taken from nodule on the thigh showed hyperkeratosis and mild acanthosis of the dermis. There was dense cellular infiltration in the dermis (Fig. 8.1.10). Proliferations of blood vessels were also visible. In the foamy cells, numerous *Leishmania* amastigotes were observed (Fig.8.1.11). There was no such epithelioid cell granuloma as seen in leprosy. No acid fast bacilli were observed in the dermis. From these result of various examinations, the patient was diagnosed as a diffuse cutaneous leishmaniasis (Reyna *et al*., 1994).

Comments

Lepromatous leprosy is similar to diffuse cutaneous leishmaniasis in that the disease is generalized; the skin tests negative for their specific and pathogenic organisms, Lepromin test and Leishmanin test; there are many organisms in the lesions; it progresses chronically during several years; and it is resistant to specific treatment (Convit *et al*., 1972). In the present case of a diffuse cutaneous leishmaniasis, the similar cutaneous manifestations to those of leprosy, especially multi-bacillary leprosy, were observed. As the patient had various kinds of eruption similar to various type of leprosy (Jopling, 1984.; Hidano, 1968; Walter, 1983), it might be convenient to summarize some points for making differential diagnosis between these two diseases as follows. Examination of sensory function is the most important test for diagnosis of leprosy. In LL, especially in its early stage, as sensory loss sometimes is not clear or unfold, this examination should be done carefully at the wide region on all over the body surface including normal colored skin regions. Palpation of peripheral nerves is also important. But in multi-bacillary leprosy, especially in LL, sometimes hypertrophy of peripheral nerves is not observed or they show only mild changes without pressure pain and irradiation pain. Bacteriological examinations such as acid fast staining for diagnosis and classification should be performed in all patients suspected with leprosy. Ziehl-Neelsen staining method is easy and simple. In some prefectures in Ecuador, we saw the indeterminated leprosy case which had been misdiagnosed and medicated as LL. The distribution of the eruptions in this case was relatively bilaterally symmetric and similar to borderline lepromatous leprosy (BL). The distribution of the skin lesions of this case was similar to that of leprosy. That is, the eruptions were

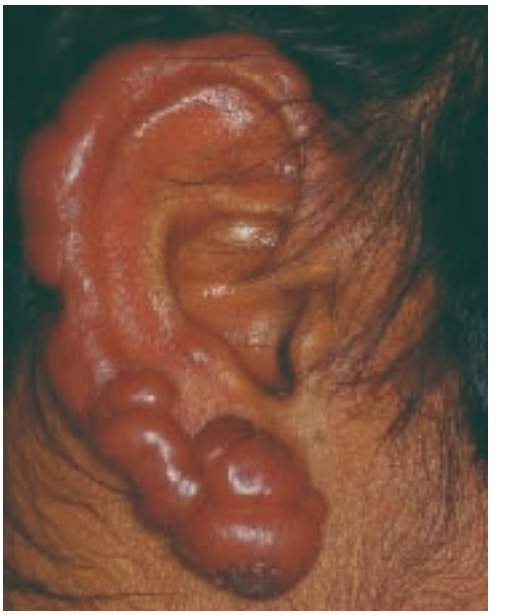

Figure 8.1.1. Rice to pea sized papules on the right ear.

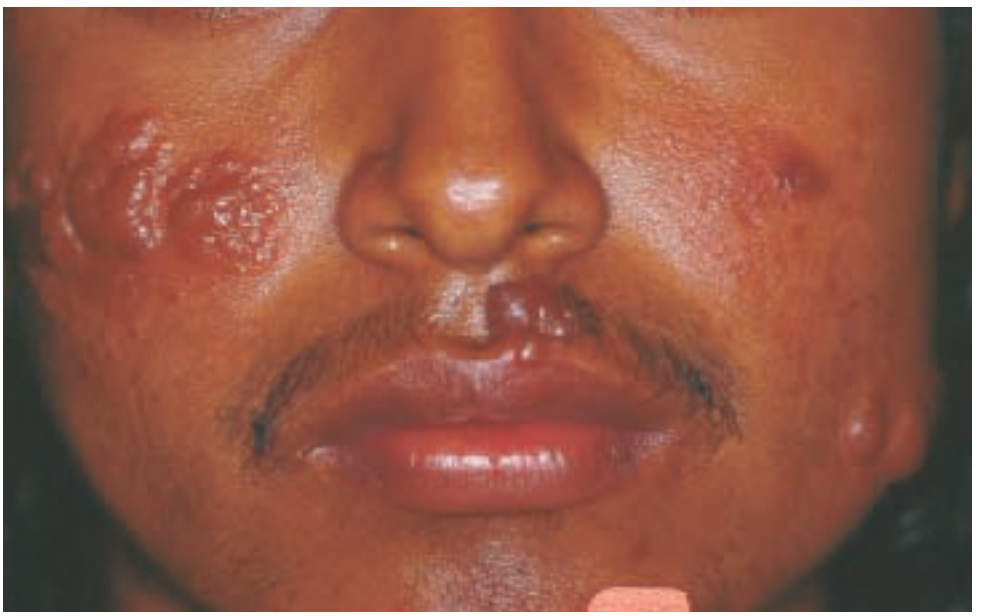

Figure 8.1.2. Various sized papules on the face. On the right cheek, rice grain sized papules grouped and make plaque-like lesion.

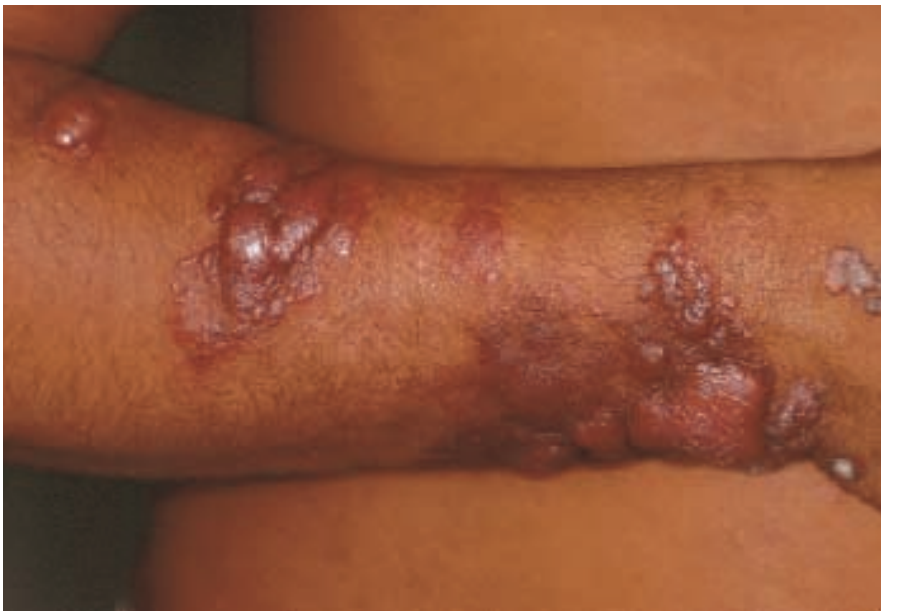

Figure 8.1.3. Various kinds of eruption on the right upper extremity. Verruca vulgaris scattered on the dosal aspect ot the hand.

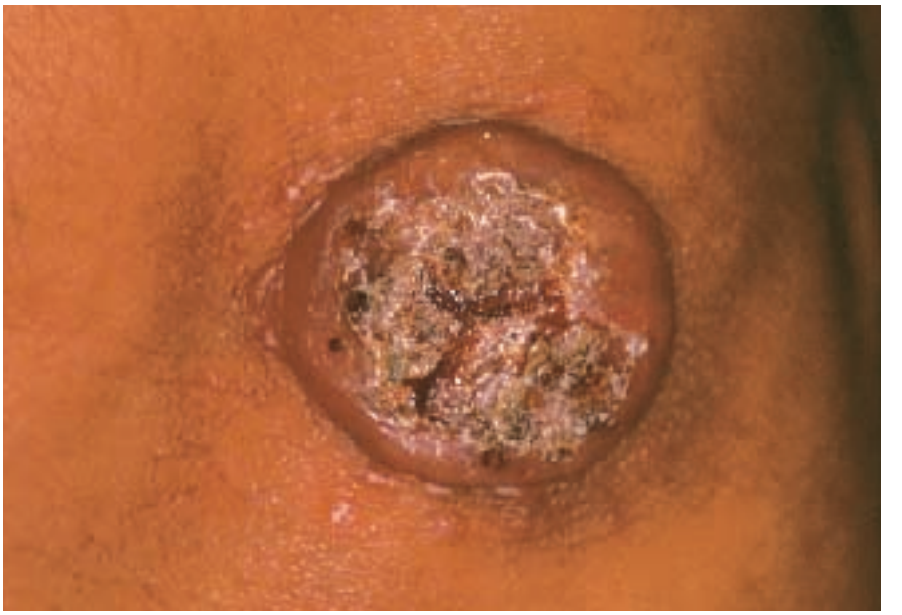

Figure 8.1.4. Keratoachantoma-like nodule on the elbow. Scaly and crusted shallow ulcer is observed.

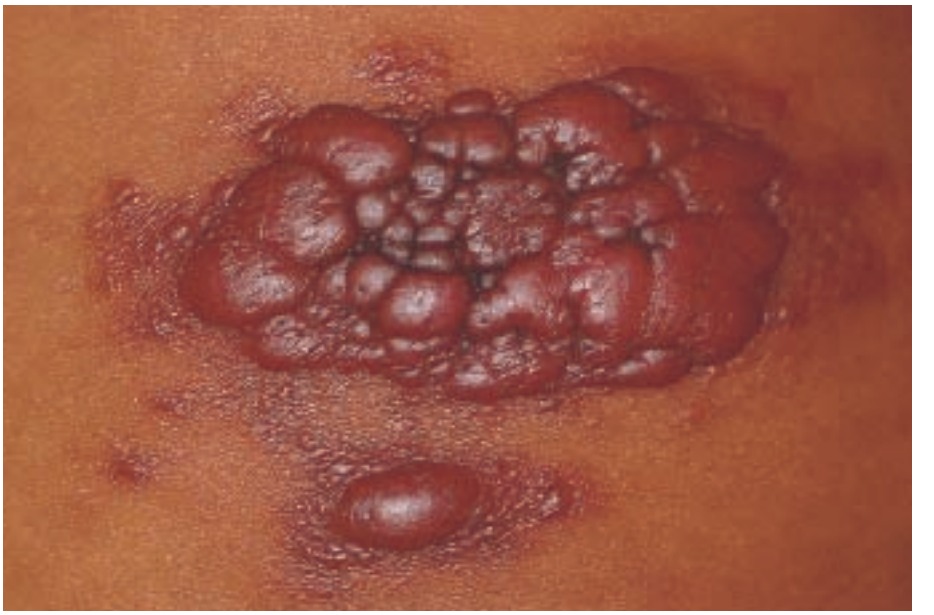

Figure 8.1.5. Various sized papules and nodules grouped on the lower back.

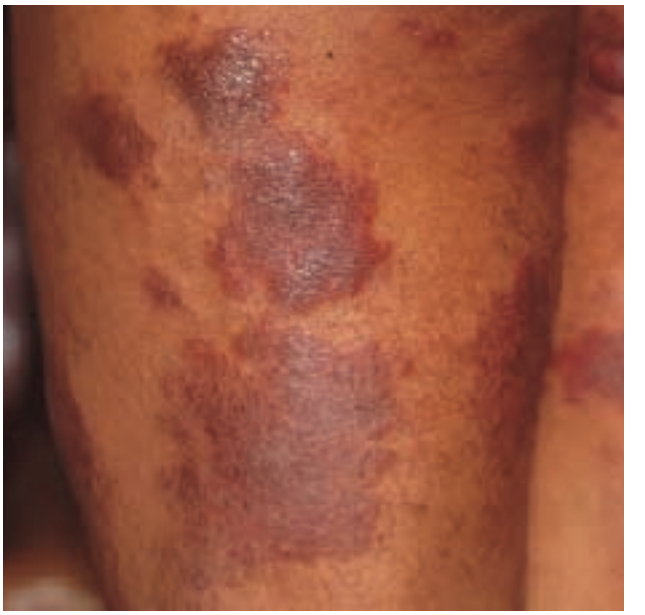

Figure 8.1.6. Thumb to palm sized infiltrated erythemas, partially annular, and brownish colored fleckles on the right thigh.

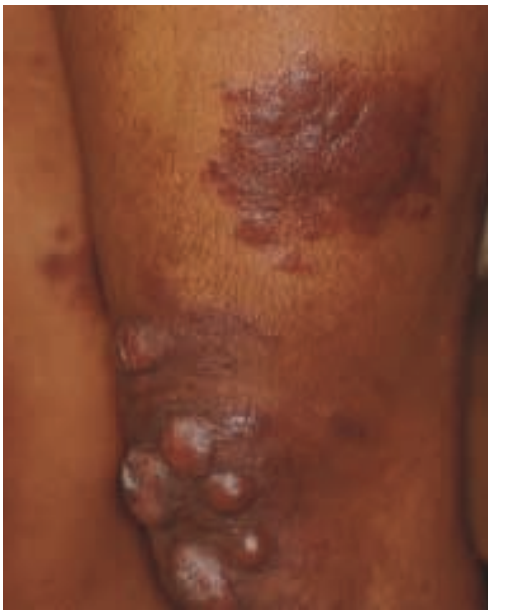

Figure 8.1.7. Various kinds of eruptions on the left thigh.

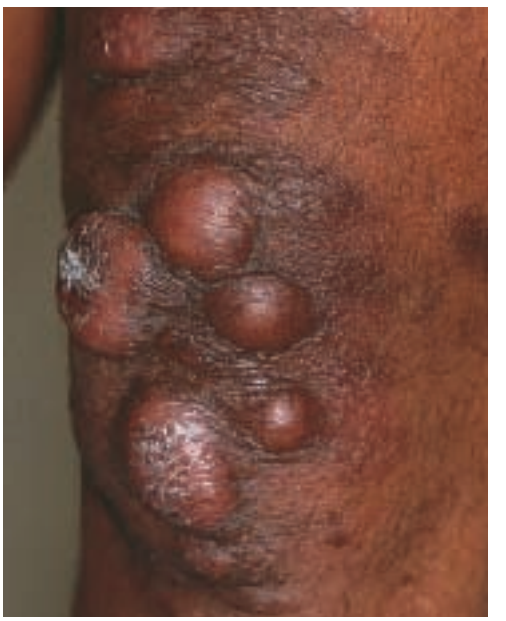

Figure 8.1.8. Nodules grouped on the left knee.

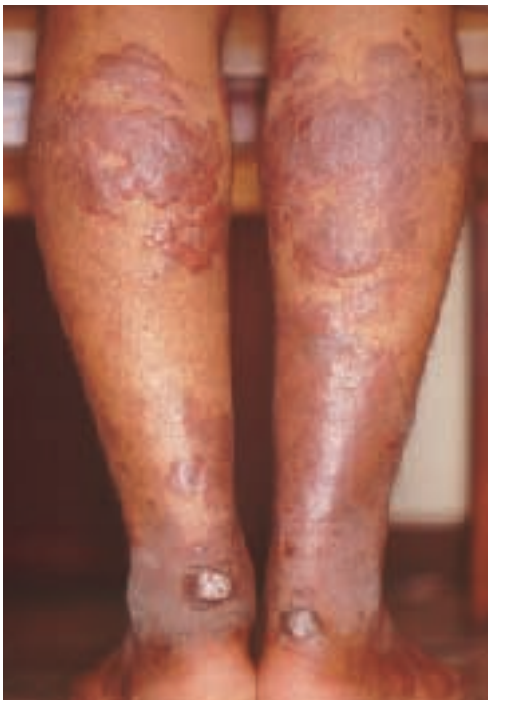

Figure 8.1.9. Brownish colored fleckles and infiltrated erythema on the extensor aspect of the both legs.

observed on the ear lobes, the auricles, the face and the all extremities except on the hands and feet. Furthermore, no eruptions were observed on the scalp, the axillary, epigastric, inguinal and perineal regions. As ulceration is not always observed on the lesions of leprosy, it can not be a ground for differential diagnosis between the two. In LL, papules are multiple and their size is monotone, distributed bilaterally and symmetrically. In this case, although papules were multiple, their distribution was not bilaterally symmetric over all the affected region and their size varied from miliary-sized to pea-sized. Although the thumb-sized nodules were observed on the extremities in this case, in LL and BL the size of nodules is not so big. The plaques in this case resembled those of tuberculoid leprosy (TT) and borderline group leprosy in appearance, but could be differentiated by the fact that sensory loss exists in the lesion of leprosy. Annular erythema usually observed in TT and borderline group leprosy was not observed in this case. The brownish pigmentation probably caused by the inflammation or caused by taking clofazimin, resembled to that of leprosy in appearance, but could be also differentiated by examination of sensory functions. Although Leon *et al*. (1990) reported that diffuse cutaneous leishmaniasis form non-ulcerated nodules, crusted lesions were observed on the nodules in our case. The crusted lesion on the relatively large nodules might have been caused by ulceration, exudate and bleeding. Therefore, it is suspected that ulceration occured in the lesions of the disease. From this point of view, the case of Ecuadorian diffuse cutaneous leishmaniasis reported by Zerega (1961), in which immunlogical and parasitological examinations had not been performed, should be reexamined for the true diagnosis. In leprosy, clinical and histological findings show various features caused by some factors, such as site of biopsy, choice and stage of eruptions (new, old, lepra reaction, medicated, cured or spontanously healed eruption etc.), and especially

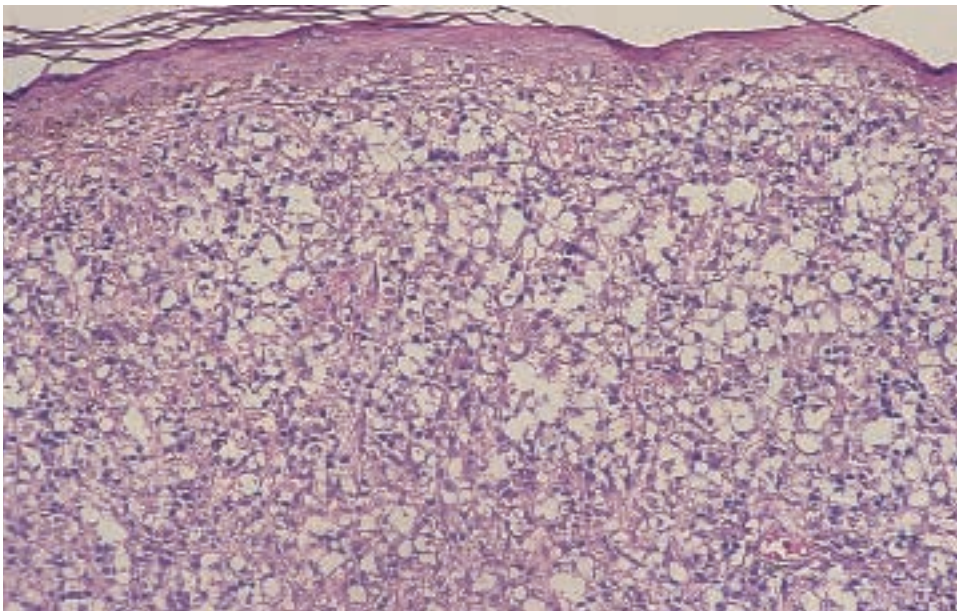

Figure 8.1.10. Numerous foamy cells are observed in the dermis stained with H&E (x100).

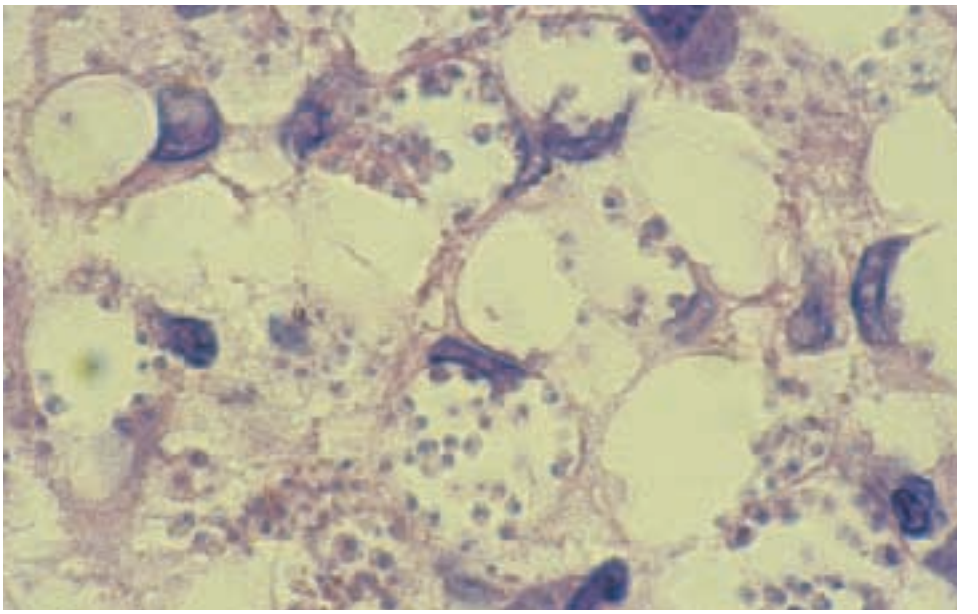

Figure 8.1.11. *Leishmania* amastigotes are observed in the foamy cells stained with H&E (x1000).

immunological relation between hosts and parasites.

It has been speculated that host-parasite immunological relationships may modify cutaneous manifestations of such diseases as tuberculosis, candidiasis, dermatophytosis, chromomycosis, sporotrichosis and other infectious diseases (Hosokawa *et al*., 1996). If the similar features to those in leprosy are observed in diffuse cutaneous leishmaniasis, they might be caused by some of the above mentioned immunological factors. In other respects of the dermatological findings of this case, no eruptions were observed on the scalp, the axillary, epigastric, inguinal, perineal and anal regions, where the temperature of the body surface was relatively high. This fact is usually observed in the patients with leprosy (Hosokawa *et al*., 1996; Hosokawa *et al*., 1994). It was speculated from this fact that the causative agents might clinically prefer low temperature of the body surface (Sheperd, 1965). In this case, leishmanin (Montenegro) skin test showed negative reaction and tuberculin, candidine and trichophytine tests showed positive reactions, demonstrating the existence of specific anergy against *Leishmania* antigen (Reyna *et al*., 1994). On the other hand, the existence of verruca vulgaris on the forearm of the 24-year-old patient suggests that he might suffer from some immunodeficiency besides the above mentioned specific immunodeficiency against *Leishmania*. In consideration of the differential diagnosis of diffuse cutaneous leishmaniasis, it may be difficult to diagnose it only by cutaneous manifestations. Therefore, during the investigation of this case, we re-recognized that sensory test and biological examinations by simple skin slit smear for discovering the organisms were very important for correct diagnosis and prevention of misdiagnosis of the diseases.

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2. A Case Report of Chromomycosis from an Endemic Area for Cutaneous Leishmaniasis in Ecuador: a Differential Diagnosis between Leishmaniasis and Chromomycosis

ABSTRACT. A patient with chromomycosis was seen in an area endemic for cutaneous leishmaniasis, Babahoyo city, Province of Los Rios, Ecuador. The patient was a 60-year-old male who daily worked outdoors as a farmer. The cutaneous manifestation on the dorsal aspect of the right hand in this case was similar to that of verrucous type of cutaneous leishmaniasis. Mitochondrial DNA analysis for *Fonsecaea pedrosoi* cultured from this case proved to be type 4 of *F. pedrosoi*, which is usually detected in South, Central and North America. It is well known that chromomycosis, cutaneous leishmaniasis and leprosy have a number of common clinical characteristics. Therefore it is important to perform mycological, parasitological and bacteriological examinations using epidermal scrapings or skin slit smears, and to perform sensory tests on the wide area of the body surface when the differential diagnosis of the three diseases needed.

Introduction

Fungal infections still remain a frequent health care problem in Latin American countries including Ecuador (Rios-Fabra *et al*., 1994). In the current study, one of the purposes is to make a differential diagnosis about the skin lesions found in the patients with cutaneous leishmaniasis, leprosy and various skin disoders including fungal infections. We found a case of chromomycosis in an endemic area for cutaneous leishmaniasis in Ecuador. Cutaneous manifestations in chromomycosis sometimes showed a similarity to those of cutaneous leishmaniasis. Both diseases have a chronic infectious, granulomatous findings histopathologically. The clinical feature of our case was thought to be similar to that of verrucous type of cutaneous leishmaniasis. Initially, as the scrclotic cells were found in the epidermal scrapings from the surface of the nodule of the patient, the case was considered to be rare with concominant cutaneous leishmaniasis and chromomycosis. Several examinations proved that the lesion had been caused only by chromomycosis. This paper deals in detail with the case of chromomycosis caused by *Fonsecaea pedrosoi* and we consider a differential diagnosis between cutaneous leishmaniasis and chromomycosis. The patient gave an informed consent to participate to every examination.

Case report

The patient (File No. Baba 6) was a 60-year-old male, farmer, living in rural area of Babahoyo city, Province of Los Rios, Ecuador. None of his family members had the similar cutaneous symptoms. He denied any history of local traumatic injury prior to the development of skin lesion. He did not receive any topical treatment of corticosteroid. About a year earlier, the patient noticed a rice-grain-sized, scaly papule on the dorsal aspect of his right hand. The scaly papule gradually increased in size to form a superficial nodule. He visited our clinic, the Multin y Casa Hospital in Babahoyo city, for diagnosis and treatment.

Physical examination revealed an irregular, scaly, crusted, verrucous, firm, nontender, brownish, hen-egg-sized nodule on the dorsal aspect of his right hand (Fig. 8.2.1). The nodule was non-pedunculated, whose top was nearly flat, and raised about 1 cm. The surface was rough and irregular with cauliflower-like apprearance. There was no associated regional lym-

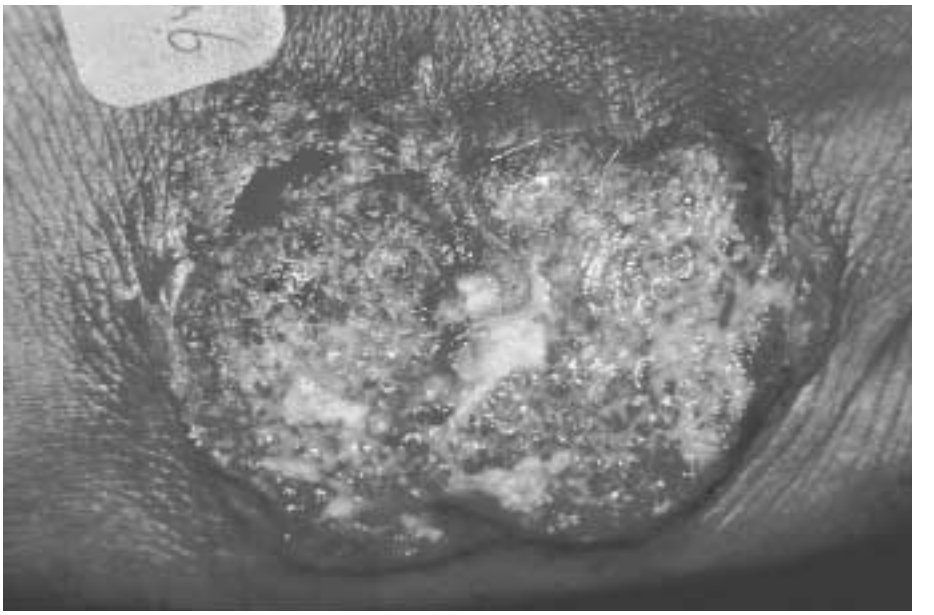

Figure 8.2.1. Verrucous lesions of the patient caused by *F. pedrosoi*.

phadenopathy. The differential diagnoses included chromomycosis, cutaneous leishmaniasis, paracoccidiomycosis, skin cancer and tuberculosis verrucosa cutis.

Laboratory examinations

Smear specimens of the lesion revealed neither acid-fast bacilli nor *Leishmania* amastigotes. Epidermal scrapings from the lesion were collected for mycological evaluation. Under direct microscopic examination of the specimens using 15% potassium hydroxide (KOH) solution, the fungal elements found in the scales were dark brown in color and were in the forms of sclerotic cells and hyphae (Fig. 8.2.2). Epidermal scrapings from the lesion were cultured on Sabouraud-dextrose agar at room temperature. Tissue sample taken from the lesion was also cultured on blood agar medium for detection of *Leishmania* promastigotes. About two weeks later, a black colored, heaped colonies were observed on the both culture media (Figs. 8.2.3 and 8.2.4). The colony showed three kinds of conidation: *Cladosporium*-like (Figs. 8.2.5 and 8.2.6), *Rhinocladiella* and *Phialophora* conidation (Figs. 8.2.7 and 8.2.8). They were identified to be *F. pedrosoi*. Mitochondrial DNA analysis for *F. perosoi* showed that this strain was included in group 4 (Fig. 8.2.9 and Table 8.2.1). Mitochondrial DNA analysis was perfomed by the method of Kawasaki *et al*. (1993).

Histopathological study of the skin biopsy specimen from the border of the lesion revealed features of chromomycosis. Biopsy of the nodule revealed marked irregular hyperkeratosis and acanthosis and pseudocarcinomatous hyperplasia (Fig. 8.2.10) of the epidermis with papillomatosis. The dermis and epidermis showed a granulomatous infiltration consisting admixture of epitheloid cells, giant cells of both Langhans and foreign body types, plasma cells, lymphocytes, eosinophils and polymorphonuclear leucocytes forming microabscesses. A small number of dark-brown, rounded, thick walled, and septate fungal cells, that is sclerotic cells, were seen both within the giant cells (Fig. 8.2.11) and in the microabscesses. Culture of biopsy specimen taken from the surface

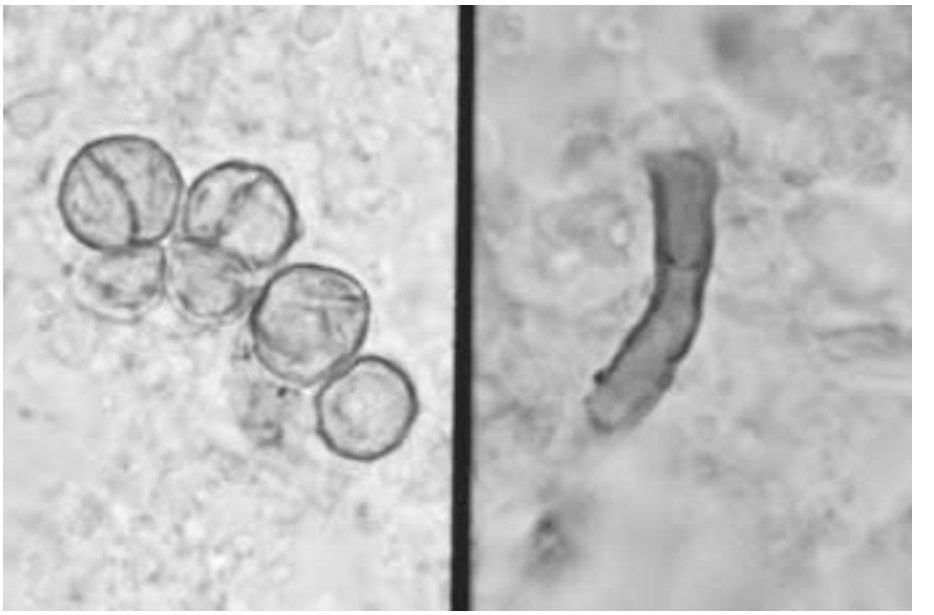

Figure 8.2.2. Brownish colored sclerotic cells (left) and hypha (right) observed by microscopic direct examination of a tissue fragment in KOH (original magnification, x1000).

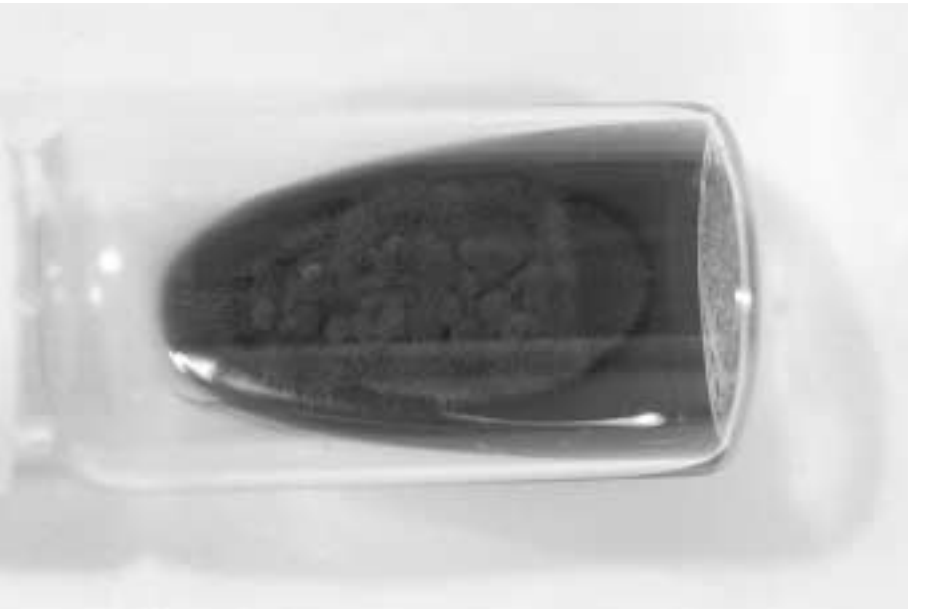

Figure 8.2.3. Culture of the scales taken from the surface of the nodule, in Sabouraud-dextrose agar revealed growth in about 2 weeks. Heaped up dark colonies with a brown velvety surface of *F. pedrosoi* is observed.

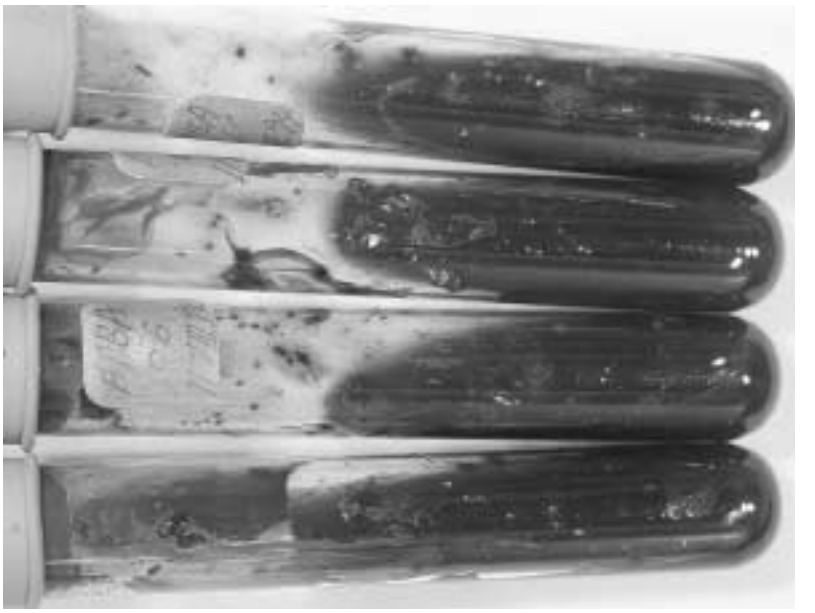

Figure 8.2.4. Culture of the tissue taken from the border of the nodule. Morphology of *F. pedrosoi* colony, heaped up dark colonies with a brown velvety surface, on blood agar after about a month incubation.

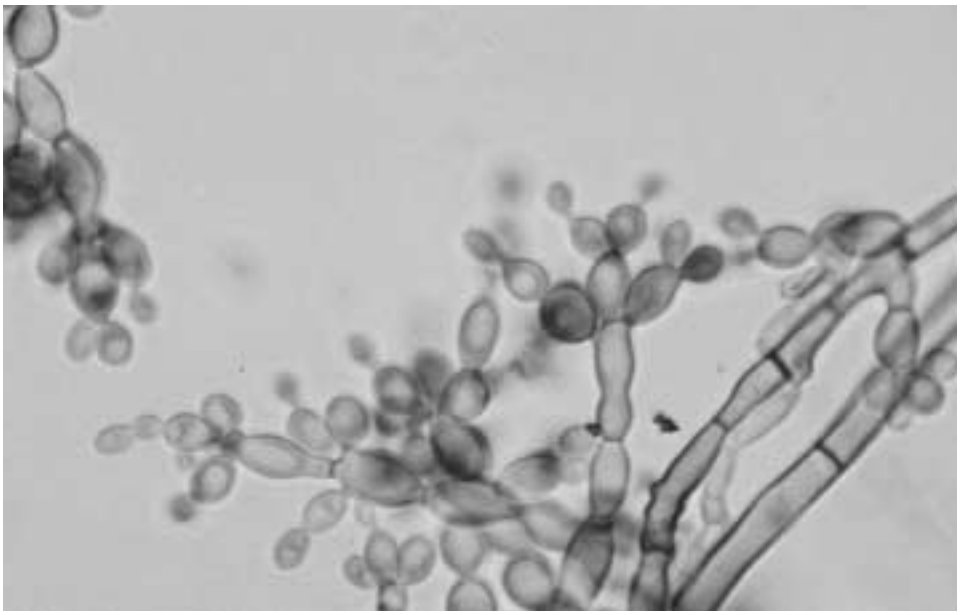

Figure 8.2.5. Slide culture of *F. pedrosoi* detected from this case. Forms of *Cladosporium* conidation (original magnification, x1000).

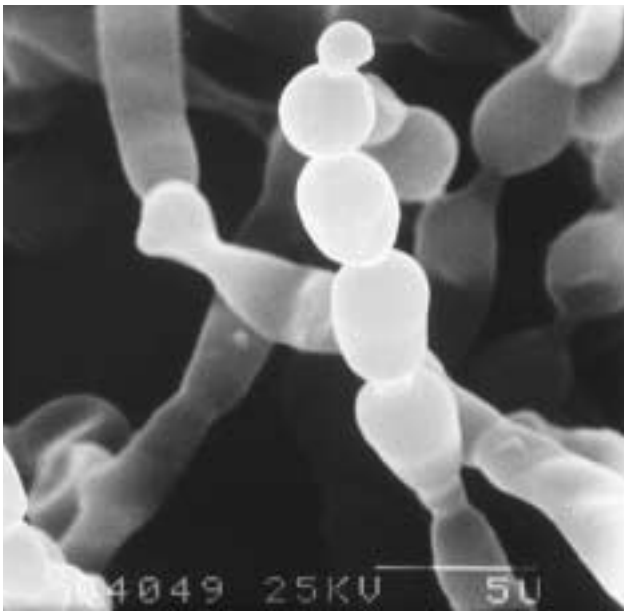

Figure 8.2.6. *Cladosporium*-like conidation of *F. pedrosoi* detected from this case is demonstrated cleary by the scanning electron microscope.

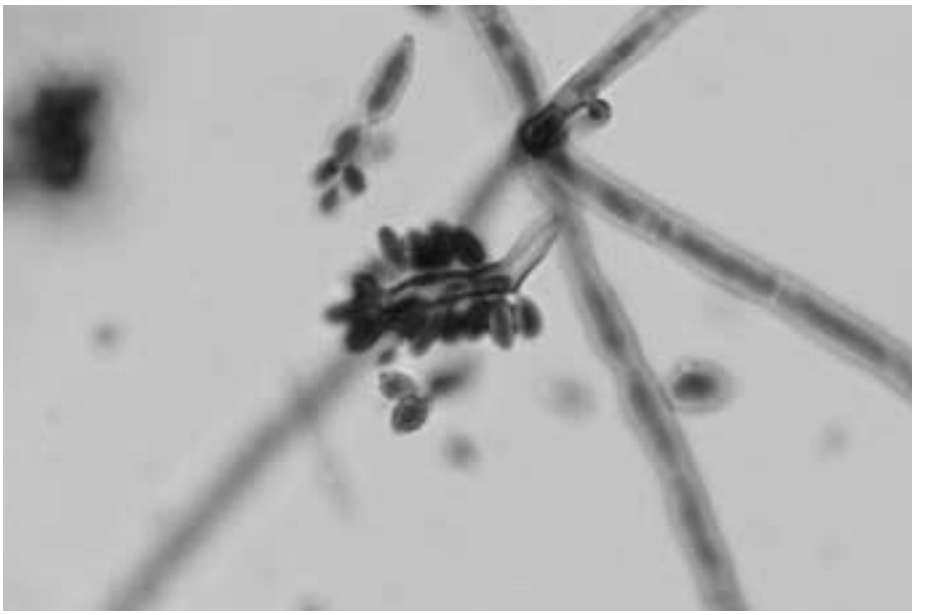

Figure 8.2.7. Slide culture of *F. pedrosoi* detected from this case. *Rhinocladiella*-type structure is observed (original magnification, x1000).

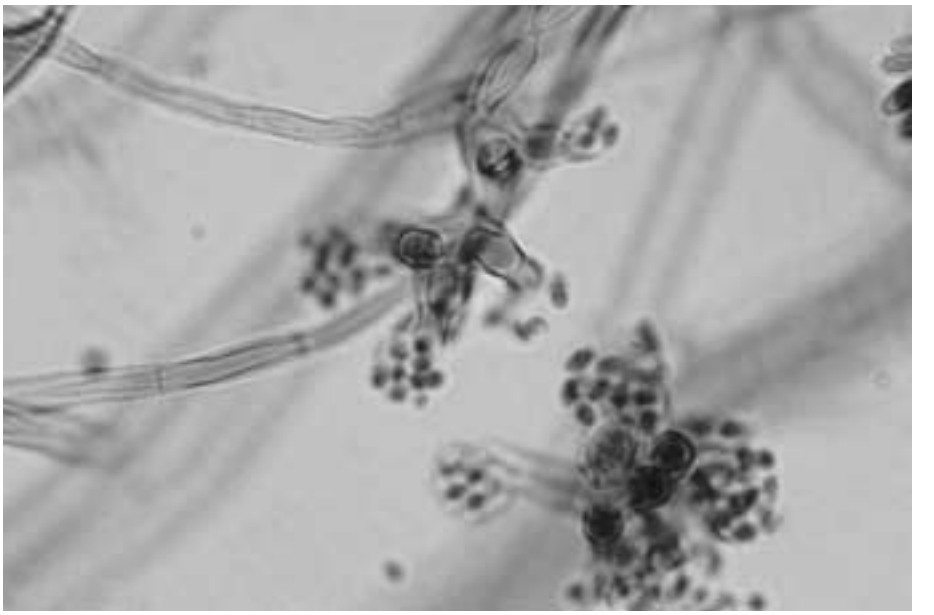

Figure 8.2.8. *Phialophora* fructification of *F. pedrosoi* is observed by slide culture of the fungus taken from the lesion of this case (original magnification, x1000).

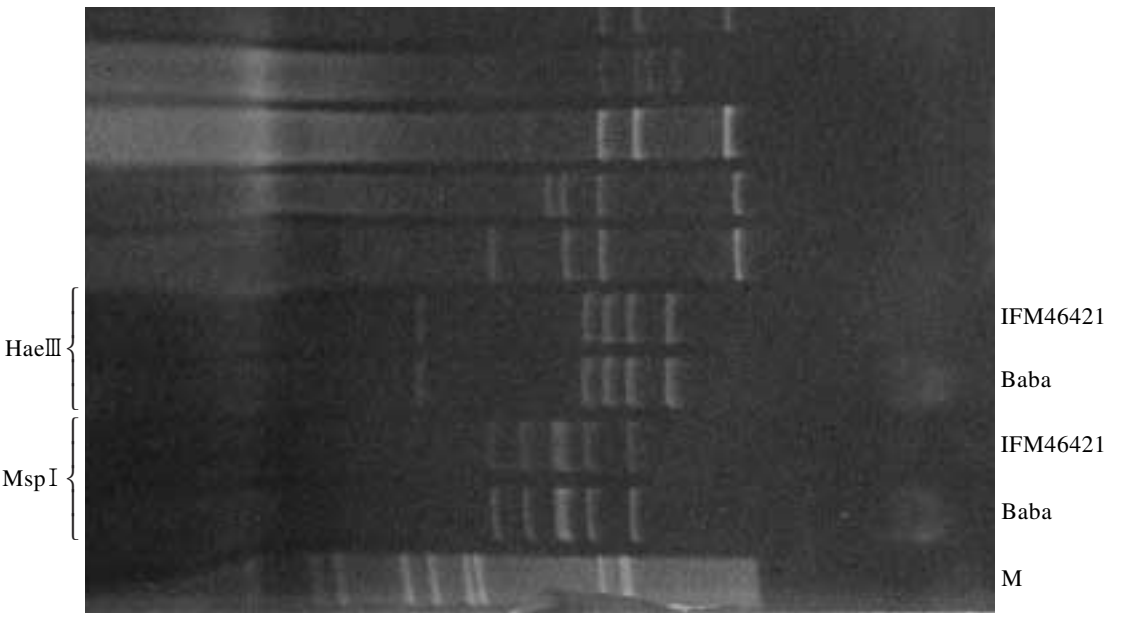

Figure 8.2.9. Mitochondrial DNA analysis for the present strain showing *F. pedrosoi* group 4. Restriction fragment length polymorphism of the mtDNA digested with HaeⅢ and MspI. M, size marker; Baba, the present case; IFM46421, control.

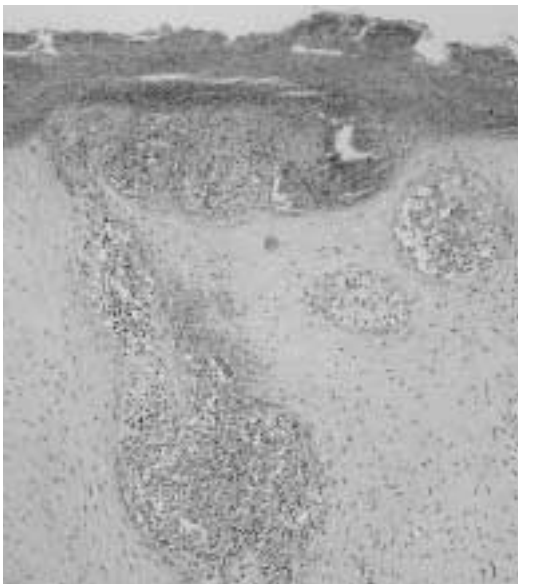

Figure 8.2.10. Biopy of the nodule revealed marked irregular hyperkeratosis and acanthosis and pseudocarcinomatous hyperplasia of epidermis with papillomatosis. Dermis and epidermis showed a granulomatous infiltration consisting admixture of epitheloid cells, giant cells of both Langhans and foreign body types, plasma cells, lymphocytes, eosinophils and collections of polymorphonuclear leucocytes forming microabscesses (original magnification, x100).

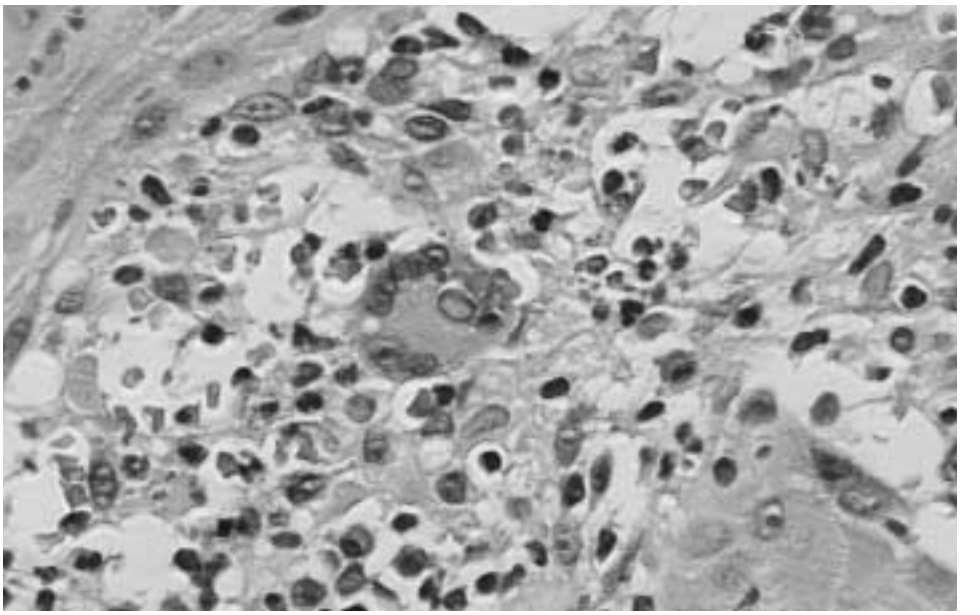

Figure 8.2.11. A small number of dark brown rounded thick-walled and septate fungal cells 'sclerotic cells' were seen within the giant cells and in the microabscesses (original magnification, x400).

Table 8.2.1. Mitochondrial DNA analysis for *F. pedrosoi* (Kawasaki *et al*., 1993)

Strain	Origin	Type	Strain	Origin	Type	Strain	Origin	Type
TIMM 530	Japan	①	TIMM 677	Japan	①	IFM 41517	Venezuela	③
TIMM 533	Japan	①	TIMM 683	Japan	①	IFM 41518	Venezuela	③
TIMM 606	Japan	①	TIMM 684	Japan	①	IFM 41519	Venezuela	③
TIMM 622	Japan	①	TIMM 685	Japan	①	IFM 41520	Venezuela	④
TIMM 634	Japan	①	TIMM 686	Japan	①	IFM 1415A	Colombia	④
TIMM 637	Japan	①	TIMM 687	Japan	①	IFM 41867	Colombia	⑤
TIMM 639	Japan	①	TIMM 688	Japan	①	IFM 41868	Colombia	⑥
TIMM 642	Japan	①	TIMM 689	Japan	①	IFM 41705	China	①
TIMM 656	Japan	①	TIMM 690	Japan	①	IFM 41706	China	①
TIMM 661	Japan	①	Yakou	Japan	①	MMC 42	Thailand	②
TIMM 664	Japan	①	TIMM 532	USA	④	IFM 4886	USA	④
TIMM 666	Japan	①	IFM 4915	Mexico	④	IFM 41704	China	①
TIMM 669	Japan	①	IFM 4916	Mexico	④	IFM 41934	?	④
TIMM 671	Japan	①	IFM 4917	Venezuela	④	*(F. compacta)*		
TIMM 676	Japan	①	IFM 4918	Venezuela	④			

TIMM: Research Center for Medical Mycology, Teikyo University, Tokyo, Japan.
IFM: Research Center for Pathogenic Fungi Microbial Toxicoses, Chiba University, Chiba, Japan.
MMC: School of Medicine, Chiang Mai University, Chiang Mai, Thailand.

of the nodule, in Sabouraud-dextrose agar (Slant-N) also revealed a growth of colonies in about two weeks at room temperature. The fungus in the colony was also identified to be *F. pedrosoi*.

Slit-skin smear from the lesion, as above mentioned, did not show acid fast bacilli with Ziehl-Neelsen staining method, though screlotic cells and hyphae were stained clearly dark blue with methyleneblue (Fig. 8.2.12).

Comments

Chromomycosis is a localized chronic mycosis of skin and subcutaneous tissues. It is characterized by verrucous, ulcerated and crusted lesions which may be nearly flat and or may be raised 1 to 3 centimeters. Many of the raised lesions are pedunculated and have surfaces so rough and irregular that they looks like a cauliflower (Chester, 1970). In general, early lesions of chromomycosis must be differentiated from those of blastomycosis. They lack sharply raised borders containing minute abscesses, the fairly rapid lateral spread and central healing with flat and thin scar, all of which are present in the former. In older lesions of chromomycosis with widespread or confluent flat verrucous lesions and concurrent lymph stasis, the diagnosis of "mossy foot" must be excluded by demonstrating the fungi in crusts or tissues. Only in the classic type of raised or pedunculated lesion, the clinical diagnosis can be made with considerable confidence (Chester, 1970). It is well known that clinical findings of cutaneous leishmaniasis sometimes resemble to those of sporotrichosis and other infectious diseases (Nonaka, 1990). In the current survey in Ecuador, one of the purpose was to make a differential diagnosis about the lesions found in the patients with cutaneous leishmaniasis, leprosy and other skin diseases. The lesions of chromomycosis must be also differentiated from those of cutaneous leishmaniasis, cutaneous tuberculosis, tertiary syphilis, yaws and leprosy, especially from tuberculoid leprosy, borderline tubercu-

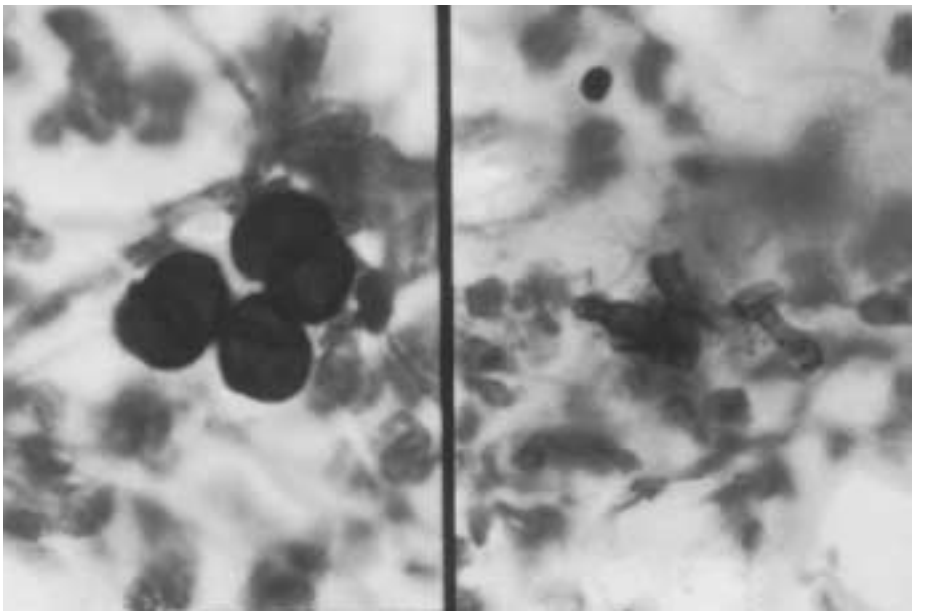

Figure 8.2.12. Slit-skin smear from the lesion did not show acid fast bacilli with Ziehl-Neelsen staining method, though screlotic cells and hyphae were stained dark blue with methyleneblue.

loid leprosy and chronic discoid lupus erythematosus (Torrealba *et al*., 1995). Though it is stated that leprosy may be rarely associated with other systemic granulomatous diseases of infectious origin (Pavithran, 1988) including chromomycosis and cutaneous leishmaniasis, one case of chromomycosis has been misdiagnosed as tuberculoid leprosy (Pavithran, 1992; Torrealba, 1995) and eight patients with concomitant leprosy and cutaneous leishmaniasis in Ethiopia (Barnetson, 1978) had been reported. As chromomycosis, cutaneous leishmaniasis and leprosy have a number of similar clinical characteristics, it is important that skin slit smears should be taken from several lesions and that sensory test should be performed on the wide area of the body surface when the coexistence of the three infections is suspected.

At first, when we observed the eruption of our patient, we suspected that of verrucous type of cutaneous leishmaniasis. Even after the demonstration of the sclerotic cells in the crusts from the surface of the nodule, the lesion was suspected to be induced by the two infectious microorganisms and the medication for cutaneous leishmaniasis was started. Though this tentative diagnosis was considered initially, the lesion was found to be caused only by chromomycosis on repeated examinations finally. Though its association with cutaneous leishmaniasis has not been reported, the examination, especially detection of the microorganism, should be performed for confirming the diagnosis and adequate treatment. Histopathological study of the nodule revealed all the features characteristic of chromomycosis. No *Leishmania* amastigotes were seen in the smear materials and the biopsy specimen. These were further confirmed by the culture of biopsy specimen in Sabouraud dextrose agar. Culture of the tissue of the dermis taken from the lesion's border by a syringe in blood agar medium for *Leishmaia* promastigotes further strengthened this diagnosis.

Lesions of chromomycosis are usually localized to the lower leg but may be occur on the skin surfaces at the sites of cutaneous injuries (Zaror *et al.*, 1987). Satellite lesions arise by autoinoculation, by lymphatic spread to adjacent areas and rarely by hematogenous

spread to the brain (Lever, 1983). There is a predominance of infection in males, probably because they have greater opportunity for soil contact and predisposition to injury while working in the field. The majority of the patient are between the age of 0 and 50 years. As above mentioned, the feet and the legs are the most frequented sites of infection, except Venezuela (Rios-Fabra *et al*., 1994) where the more frequently involved sites are the shoulders, chest and trunk, and in Cuba, the upper extremities. The most common agent is *F. pedrosoi*, which is found in humid tropical zones or wet areas within torrid zones (Rios-Fabra *et al*., 1994; Londero *et al*., 1976). In Ecuador, 95% or more of the cases were caused by *F. pedrosoi* (Londero *et al*., 1976).

Recently mitochondrial DNA (mtDNA) analysis for various fungi was considered as one of the useful methods for identification of the species (Kozlowski, 1982; Taylor, 1986; Corby, 1987; Helga, 1988; Suzuki, 1988; Takeda, 1991; Nishio, 1992; Kawasaki, 1993). As shown in Table 8.2.1, mtDNA analysis for *F. pedrosoi* shows that all strains discovered in North, Central and South America are group 4 (Yamagishi *et al.*, 1994). Our analysis proved that the agent of this case is also included in the group 4 of *F. pedrosoi*.

Our patient was a 60-year-old male farmer living in a rural area in Babahoyo city. The site of lesion was dorsal aspect of the right hand. Though he denied a history of traumatic injury locally prior to the development of the lesion, we suspected that he had had many opportunities to be exposed to the causal agent of chromomycosis. Because he worked daily in the field. The agent of his disease was *F. pedrosoi* (type 4). Nishimoto *et al*. (1984) stated that more than 300 cases of chromomycosis had been repored in the Japanese literature and they were noted to have several characteristics different from those found in the cases in tropical or subtropical areas of Central and South America. The cutaneous manifestations of chromomycosis observed in our case were not common in Japan. Although the lesion did not show typical pedunculated form in our case, we consider that this case is relatively typical of chromomycosis in Ecuador. We consider that direct microscopic examination in potassium hydroxide (KOH) solution is very important for diagnosis of fungal diseases.

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3. Case Reports of Cutaneous Myiasis due to *Dermatobia hominis* from an Area Endemic for Cutaneous Leishmaniasis in Ecuador

ABSTRACT. Two cases of patients with cutaneous myiasis, due to *Dermatobia hominis* (myiasis-producing fly), were seen in an area endemic for cutaneous leishmaniasis, Zhucay, Province of Cañar, Ecuador. These cases were diagnosed during a survey for cutaneous leishmaniasis in the area. Clinical manifestations of cutaneous myiasis showed a similar appearance to those of furuncle and other skin diseases. One case of 70-year-old male had a furunculous lesion with mild pain on the right elbow and another case of 7-year-old male had the folliculitis-like lesion with itching on the scalp. Cutaneous myiasis is a rare disease in Japan, and therefore it is difficult for Japanese doctors to diagnose it correctly. The incidence of myiasis will augment following increased air travels in recent years.

Introduction

During our survey for cutaneous leishmaniasis in an endemic area, Zhucay, Province of Cañar, two patients with cutaneous myiasis visited our clinic for group examination. When the eruption of cutaneous myiasis was observed, it was first suspected that the lesion might be the furuncle, folliculitis, bacterially caused traumatic lesion or some rare type of cutaneous leishmaniasis similar to Andean type. Clinically, patients with cutaneous leishmaniasis have various types of cutaneous changes such as insect bite-like papules, nodules, ulcers, sporotrichosis-like lesion, chromomycosis-like verrucous lesion and erythematous plaques. It is therefore important to differentiate leishmaniasis from these dermal changes. On the other hand, myiasis is the invasion of intact tissues by larvae of certain species of flies. The flies are found in coastal areas as well as on the inland plains and in the jungles (Zumpt, 1973). Two main forms are recognized, (a) external or dermal, and (b) internal myiasis. Cutaneous myiasis in man usually results from the invasion of skin, mucous membrane, nose, mouth and anus, or conjunctiva by the fly larvae (Edington, 1969). The current paper deals with the two cases with cutaneous myiasis in detail. The patients gave an informed consent to participate to the examination and treatment.

Case report

Case 1 (File No. Zhucay 24): A 70-year-old male visited our rural clinic in Zhucay, Province of Cañar, Ecuador on August 26, 1995, with a complaint of nodule of a few months duration on the extensor aspect of left elbow. The patient was a farmer and worked usually outdoors. He had not noticed any insect bite on the lesion site. A few months earlier, however, he noticed a reddish papule. The lesion gradually incresed in size. He regarded the furunculous lesion as cutaneous leishmaniasis and came to the clinic to get a specific treatment. The reddish nodule was 1.5 x 1.0 cm in diameter and a small fistula, 2-3 mm in diameter, was open at the center of the nodule (Fig. 8.3.1). In the fistula, the spiracle (mouth) of the fly larva moving for respitration was observed. Swellings of the axillary and inguinal lymph node were not palpable. The patient complained of a mild pressure pain. A public health nurse of the clinic diagnosed clinically the lesion as cutaneous myiasis on the basis of the cutaneous appearance. At first, we tried to extract larva by a forceps with application of gentle pressure to both sides of the lesion (Fig. 8.3.2). As it was relatively large, it was extracted by a surgical procedure using a small incision under local anaesthesia (Figs. 8.3.3 and 8.3.4). After removal of the larva (maggot), oral antibiotics and antiseptic solution were

prescribed for the lesion. It was identified as the third instar larva of *Dermatobia hominis*. The larva was 21 mm in length and 5 mm in width (Fig. 8.3.5). There were many small spines lined around the body. Nine rows of spines and a few oblique rows behind them were counted on the surface from the mouth to the end of the body (Figs. 8.3.6 and 8.3.7). In the mouse, two small pharyngeal skeletons were observed and around the spiracle, numerous small tiny spines were observed (Fig. 8.3.8).

Case 2 (File No. Zhucay 22): A 7-year-old male schoolboy visited the same clinic in Zhucay on August 26, 1995 with a complaint of papule of a few weeks duration on the scalp. In addition, the patient suffered from a nodule and an ulcer of a few months duration on the dorsal aspect of right forearm. The crusted reddish nodule was 1.5 x 1.3 cm in diameter (Fig. 8.3.9) and the ulcer with induration at the margin was 1.3 x 0.5 cm in diameter (Fig. 8.3.10). These eruptions were diagnosed as cutaneous leishmaniasis clinically and parasitologically. Besides these eruptions, a few weeks earlier, an eruption appeared on his scalp and gradually increased in size (Fig. 8.3.11). He regarded the eruption as cutaneous leishmaniasis as he had done with the eruptions on the forearm. He came to the clinic to receive a specific treatment. He had not noticed any insect bite on the scalp. On physical examination, the indurated erythema of 1 cm in diameter without tenderness was crusted slightly. A small sized fistula, about 1 mm in diameter, was opened at the center of the eruption (Fig. 8.3.10). In the fistula, the mouth of the fly larva moving for respiration was observed. Axillary and inguinal lymphnode swellings were not palpable. Again, the public health nurse diagnosed clinically the lesion as cutaneous myiasis. After she applied an insecticide on the lesion, surgical extraction was performed by tweezers. After the removal of the larva, ointment of antibiotics was prescribed on the lesion. It was identified as second instar larva of *D. hominis*. The length of the larva was 16 mm and width, 2mm. There were many small spines lined around the body. Four rows of spines and a few oblique rows behind them were counted on the surface from the mouth to the end of the body. In the mouth, two small pharyngeal skeletons were observed. Around the spiracle, many small tiny spines were observed. These two cases were summarized in Table 8.3.1.

Comments

Though cutaneous myiasis is not a relatively rare disease in developing countries, the incidence of myiasis will increase especially in tourist following increased air travel in recent years. We should add cutaneous myiasis to the list of differential diagnosis for leishmaniasis when we enter into the area endemic for the disease and when we examine a patient who has an experience to have visited an endemic area. In Japan, for example, 13 cases of cutaneous myiasis due

Table 8.3.1. Two cases of myiasis from the endenic area of cutaneous leishmaniasis, Zhucay, Province of Cañar, Ecuador.

	Case 1 (File No. 24)	Case 2 (File No. 22)
Age	70	7
Sex	male	male
Site of lesion	extensor aspect of left elbow	scalp (vertex)
Size of lesion	10×15 mm	13×15 mm
Symptom of lesion	reddish furunculous subcutaneous nodule with mild pain	crusted slightly elevated erythema with mild itching
Treatment	surgical extraction under local anaesthesia	application of insecticides on the lesion and of gentle pressure to both sides of the lesion

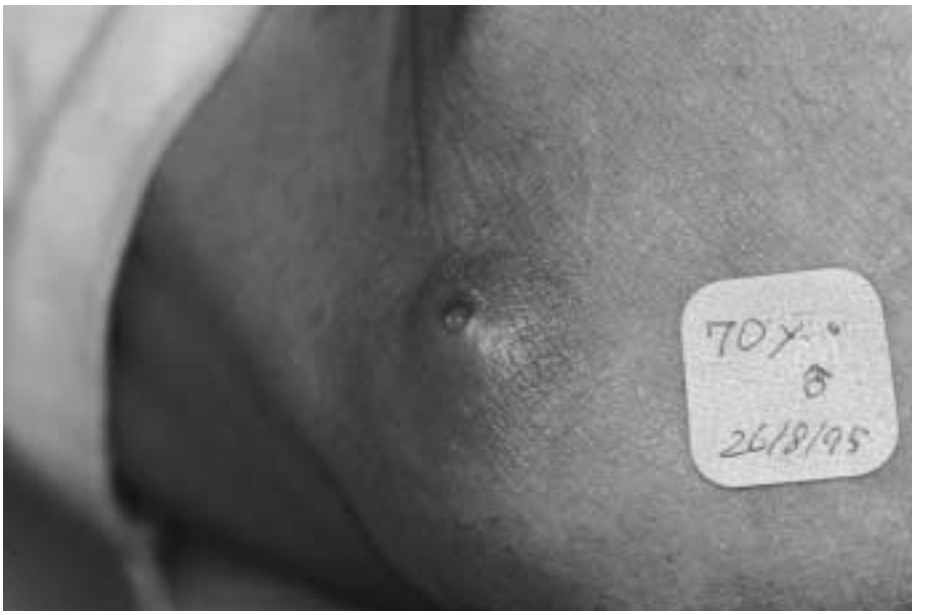

Figure 8.3.1. Thumb sized reddish furunculous nodule on the extensor aspect of left elbow. Small size fistula was opened at the center of the nodule. In the fistula, the spiracle (mouth) of the fiy larva moving for respiration was observed.

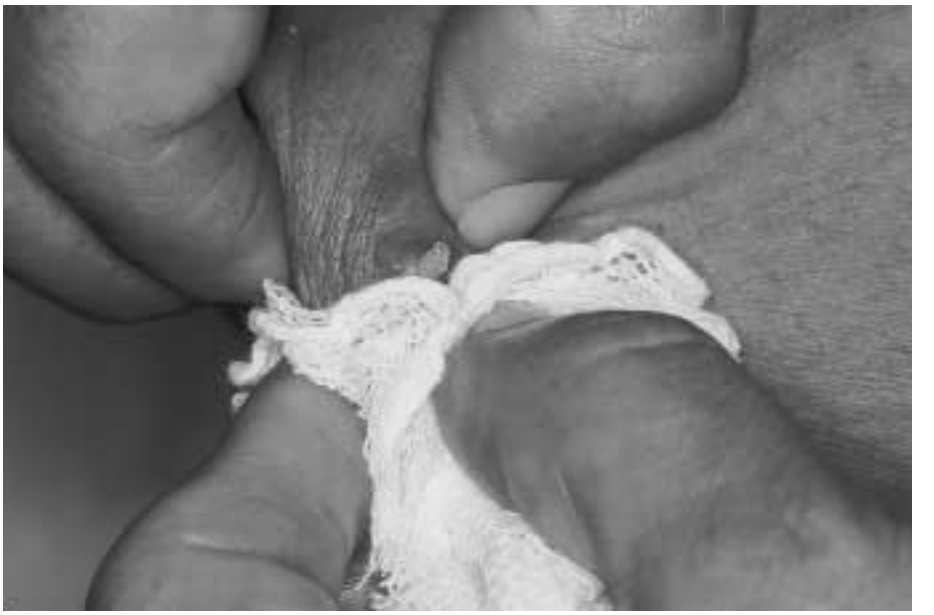

Figure 8.3.2. The larva was tried to extract by a forceps under application of gentle pressure to both sides of the lesion.

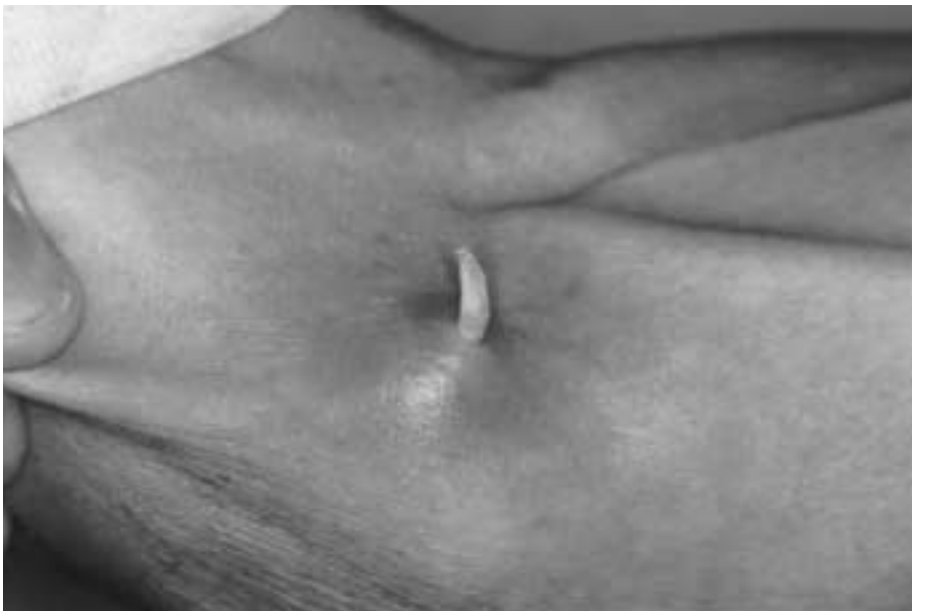

Figure 8.3.3. As the larva was relatively large, it was not extracted by the treatment mentioned in the explanation for Figure 8.3.2.

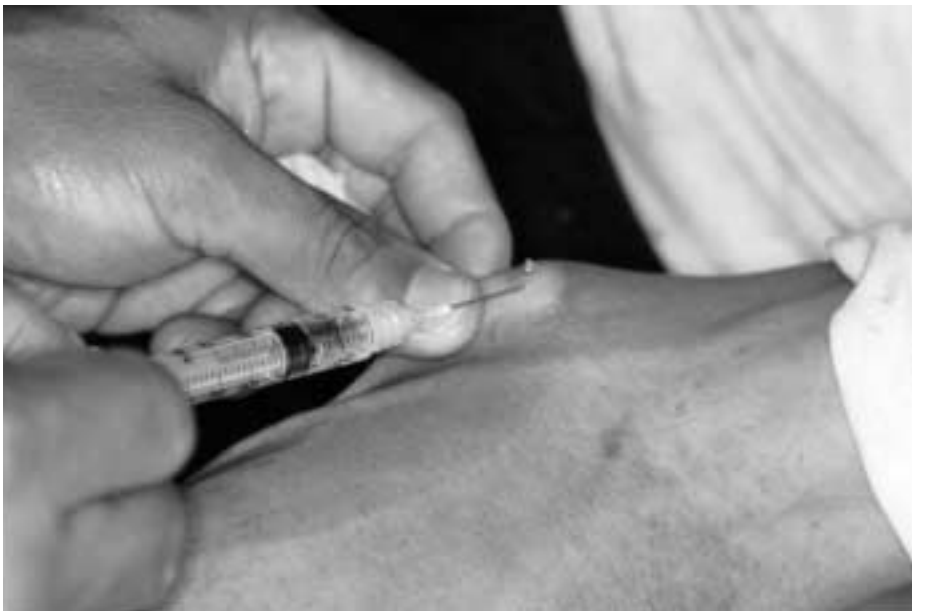

Figure 8.3.4. The larva was extracted by a surgical procedure using a small incision under local anaesthesia.

Figure 8.3.5. The larva extruded from the elbow of Case 1. There are many small spines lined around the body. Nine rows of spines and a few oblique rows behind the mare counted on the surface from the mouth to the end of the body.

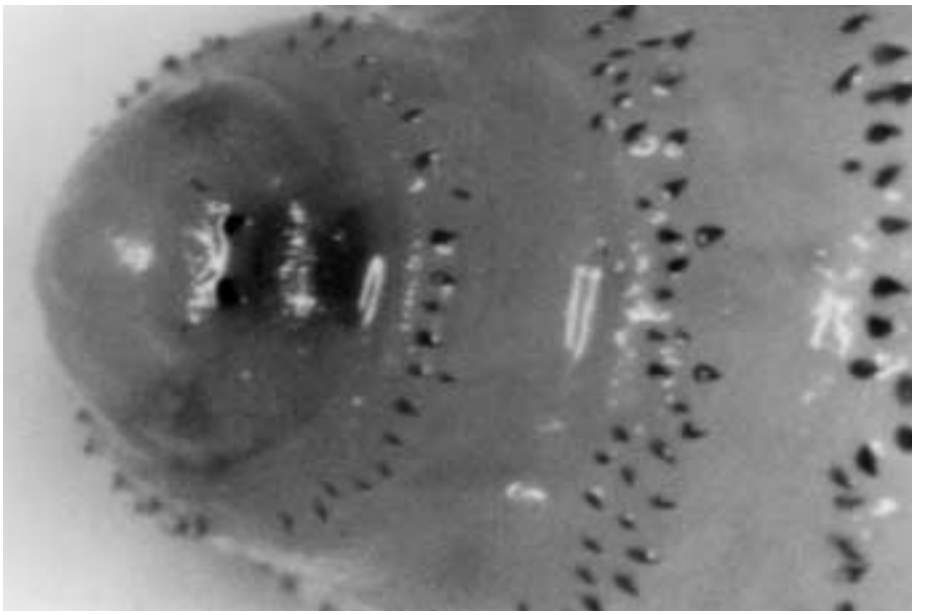

Figure 8.3.6. The anterior end of the larva. Rows of spines are seen.

Figure 8.3.7. A scanning electron microscopic feature, showing the structure of the anterior end of the larva.

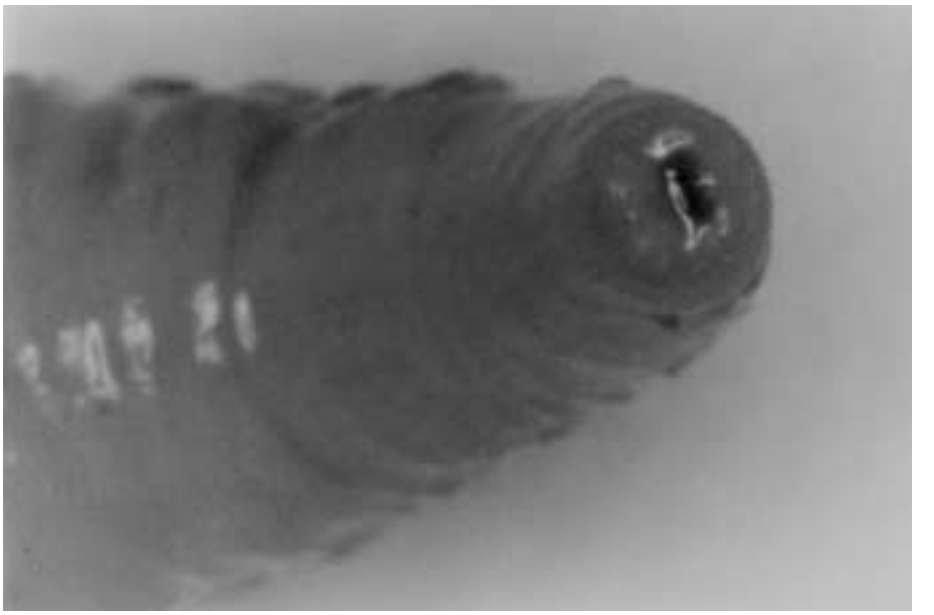

Figure 8.3.8. The posterior end of the larva. Two small pharyngeal skeletons were observed in the openning. Around the spiracle, numerous small tiny spines existed.

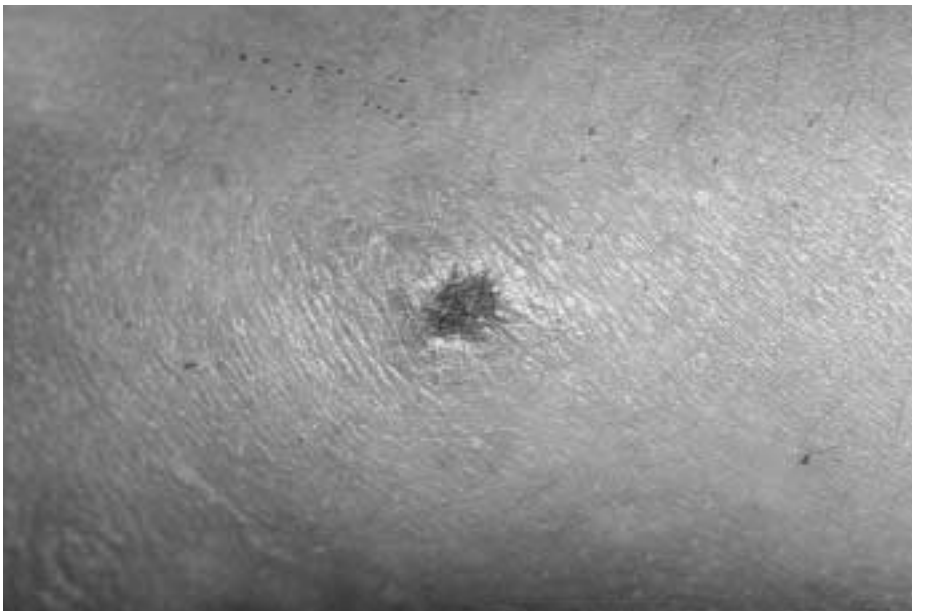

Figure 8.3.9. A cutaneous leishmaniasis lesion located at proximal site of the right forearm of Case 2.

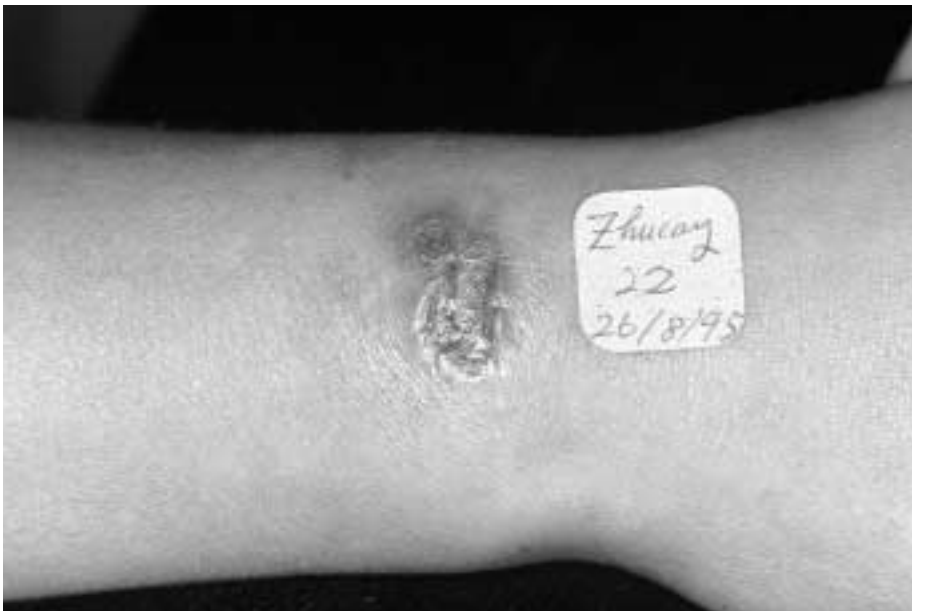

Figure 8.3.10. An ulcer, with induration at the margin, of cutaneous leishmaniasis located at distal site of the right forearm of Case 2.

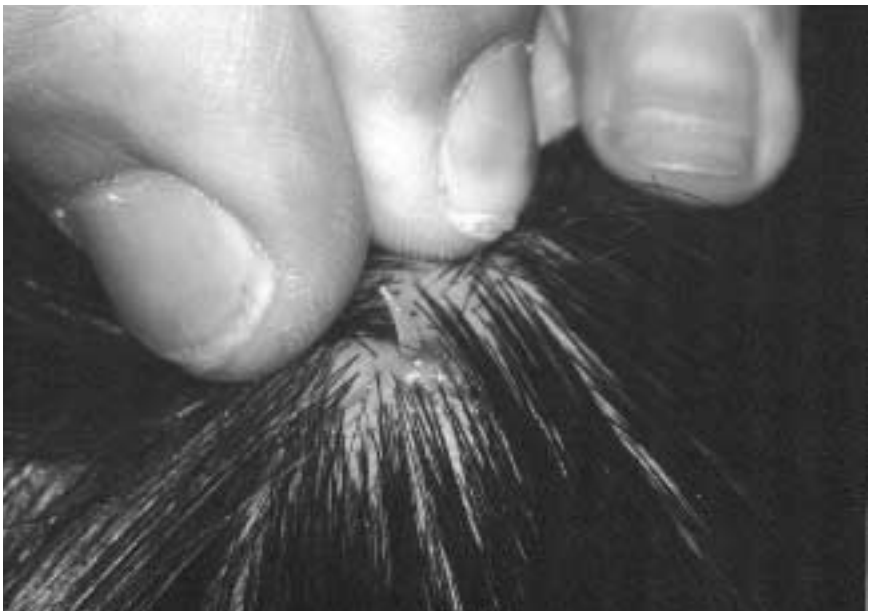

Figure 8.3.11. On the scalp of Case 2, crusted slightly indurated pea sized erythema without tenderness. A small size fistula was opened at the center of the eruption. In the fistula, the mouth of the fly larva moving for respiration was observed.

to *D. hominis* have been reported from 1974 to 1996 by Taniguchi (1996). They reported the number of patients and countries of infestations. Out of 13, 7 (53.8%) cases were from Brazil, 2 (15.4%) cases were from Mexico, one case from Paraguay, one case from Belize (Central America), one case from Honduras (Taniguchi *et al*., 1996).

Dermatological findings of cutaneous myiasis

1) Papular or furunculoid lesions: The opening at the summits of the lesions, through which the larva breathes and which may be diagnostic, is characteristic of the human or tropical bot, *i.e., D. hominis*, in the American tropics (Miller, 1978). The lesions, produced after the larva has penetrated, are usually, but not necessarily, on exposed parts of the body, and are single-grouped, or widely separated. They commonly contain one larva, but sometimes several. Symptoms progress from minor itching and pain to local tenderness and severe pain as the lesions increase in size (to 20 mm or more) over a period of usually several weeks. Open lesions may produce a serious discharge, but there is little pus unless secondary bacterial infection and abscess formation occur after death or escape of the larva (Miller, 1978).

2) Migratory lesions: These lesions induce itching and serpentine, superficial, red tunnels caused by the young larvae of botflies. *D. hominis* does not produce such lesions, because it does not migrate into the skin.

3) Traumatic cutaneous lesion: The lesion originates in the open wounds where the flies deposit eggs or larvae, they may be caused by over 50 different species of flies including *D. hominis*.

Thomas *et al*. (1995) reported that five out of six patients with cutaneous myiasis due to *D. hominis* had complained of pain, sometimes severe, at the lesions. A furunculous, often painful lesion was formed, in which the larvae had remained for up to 90 days (Arellano *et al*., 1985). The duration of the symptoms

before referral was markedly long in this group, compared with the patients infected with *Cordylobia anthropophaga* of whom only two out of six had complained of pain. In Case 1, the lesion had had no pus and patient complained of only mild pressure pain. In Case 2, the lesion showed minor itching on the scalp without pus, and was relatively clean. The patient did not complain of pain at all. From these clinical findings and the reports in their references, it was considered that the severity of pain at the lesions might be induced by the secondary bacterial infection.

Myiasis producing fly (D. hominis) *and its vectors*

Most common infestation of cutaneous myiasis is due to *D. hominis* (Fig.8.3.12) and *C. anthropophaga* (Thomas *et al*., 1995). *D. hominis* is endemic in the American tropics. *D. hominis* fly, which shows a neotropical distribution from the Gulf coast of Central Mexico to Argentina, is a member of warble or botfly group, which includes the sheep nasal bot (*Oesrus ovis*), the cattle bots (*Hypoderma* spp.), the horse bots (*Gasterophilus* spp.), the Russian gadfly (*Rhinoestrus purpureus*), and other species which generally less parasitize humans. The flies are found in the coastal areas as well as on the inland plains and in the jungles of tropical America, frequently near forests (Zumpt, 1973).

It is considered that both *Dermatobia* and its vectors seem to be forest insects, though *Dermatobia* will occasionally leave the forests accompanying a host (Bates, 1942). The adult fly does not seek its host directly, but almost always utilizes some other species of insects for the transmission of its eggs. The adult female *D. hominis* acts in the daytime and seeks insects of moderate size and moderately active habits on which it deposits its eggs. The *Dermatobia* female usually captures the mosquitoes or other blood-sucking arthropods in flight and, while hovering, manages to fasten some 14 to 25 whitish elongated-ovoid eggs to its abdomen without causing any injury. Their common vectors are several species of *Psorophora* (subgenus *Janthinosoma*) in Central America and northern South America, *Culex* among the mosquitoes, *Stomoxys calcitrans* of the blood-sucking muscoids *Siphona* sp. (biting flies) in Brazil, *Fannia* spp. of the

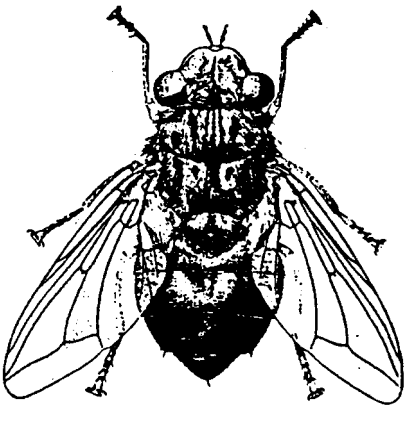

Figure 8.3.12. *Dermatobia hominis*, female (myiasis-producing fly) (from James, 1947).

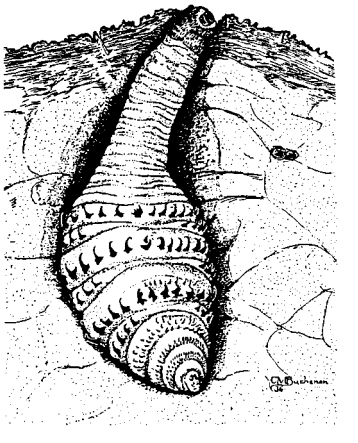

Figure 8.3.13. *Dermatobia hominis*, maturing larva in human skin. The posterior end of the larva is at the outer end of the tunnel, so that breathing may occur through the posterior spiracles (from Faust *et al*., 1970)

nonblood-sucking muscoids, and the tick, *Amblyomma cajennense* (Paul, 1984; Taniguchi, 1996). When the favorite vectors of the *Dermatobia*-eggs alights on a warm-blooded host, the young larvae, stimulated by the warmth of the host's body and ready to hatch, emerge from the egg-membranes through a small opercular opening, and in less than an hour invade the skin frequently through the puncture wounds made by the vector and/or by their own mouth parts. Within the host's skin layers, the larva feeds, grows, and moults twice. Each larva penetrates independently in the skin and forms a boil-like pouch in the subdermis. It communicates with the exterior through a small fistula (Fig. 8.3.13). Near the anterior of the second and third thoracic segments, are short, stout, posteriorly directed spines or hooklets that anchor the larva in the skin and the larva does not migrate within the skin. The duration for larvae development requires from about 46-50 days (Dunn, 1930; Taniguchi *et al*., 1996) to as long as 3 months, after which the larva works its way out of the skin of the hosts, falls to the ground, and pupates in the soil, and then develops into adult fly.

Hosts of the myiasis

Domestic and wild mammals, humans, cattle, dogs and birds are most commonly parasitized by the larvae. The *Dermatobia* population may differ from forests to forests with no obvious relation to the population of cattle, usually the chief host, in the vicinity (Bates, 1942). Humans are usually infested when associated with domestic animals (Paul, 1981).

Medical treatment

It is stated that larvae should be removed from the skin, because they can lead to abscess formation. Several methods of removal have been known. One of them is to remove them by forceps or a needle under application of gentle pressure to both sides of the lesion. Application of mineral oil onto the opening of the lesion, injection of lidocaine into the larvae, use of insecticide spray, placement of vaseline, bacon fat, butter onto the lesion, and various other methods have been proposed to ease the larvae out (Rawlsins, 1988; Omar, 1992; Loong, 1992; Brewer, 1993; Nunn, 1994; Taniguchi, 1996; Thomas, 1995). These materials cut off the larval air supply by blocking the spiracle and stimulate extrusion. Other method is to extract them by a surgical procedure using a small incision under local anaesthesia. That is, larvae may be removed by slightly widening the already present opening under local anaesthesia, and then gently squeezing out the larva. Cleansing and use of antiseptics and antibiotics are indicated to combat secondary infection. Generally, surgical excision is unnecessary unless larvae are alive, but it has been used for removal of dead or decayed larvae (Arosemana, 1993). In Case 1, the larva was removed by the surgical treatment using a small incision under local anaesthesia and under application of gentle pressure to both sides of the lesion. In Case 2, after the application of the insecticides onto the opening of the lesion, the larva was removed by forceps under application of gentle pressure to both sides of the lesion. On the other hand, it was reported that spontaneous extraction was seen in four patients out of 13 Japanese patients with cutaneous myiasis due to *D. hominis* in Japan, and it was speculated that application of ointment containing vaselin and antibiotics onto the treated lesions might have caused the spontaneous extrusion of lavae. In 1995, when we visited Guayaquil city in Ecuador, we encountered a woman, a city dweller who had travelled in the rural areas, suffering from cutaneous myiasis on the leg. In this case, the larva moved out from the furunculous lesion spontaneously (unpublished data). In general, oral medicines are not applied to the patients with cutaneous myiasis due to *D. hominis*. It is reported that the oral application of ivermectin is not a standard procedure in the treatment of infection with *Hypoderma lineatum* but might be of value in individual cases (Thomas,1995). We have the same opinion as theirs and generally the oral application of ivermectin may not be needed.

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Summary

In this text, the results obtained from field surveys in different endemic areas of cutaneous leishmaniasis in Ecuador, and those obtained from laboratory works based on the materials collected during 1994 and 1996 were mainly compiled, from the parasitological, molecular biological, dermatological and pharmacological points of view. During the present investigation, special emphasis was given on the evaluation of different types of drugs which would be suitable for oral or topical treatment of the disease. In addition, currently available molecular biological techniques are also evaluated briefly, in order to have good diagnostic tools which are especially applicable for field surveys at endemic areas of developing countries in tropical and subtropical regions. The results obtained are summarized as follows.

A note on Leishmania-*HIV co-infection*

Recently, leishmaniases, especially its visceral forms are noticed as one of the important opportunistic infections of acquired immunodeficiency symdrome (AIDS) in several areas of the world where both diseases distribute sympatrically. Since the mid-1980s there has been a dramatic increase in the number of *Leishmania* infections in human immunodeficiency virus (HIV) positive patients concurrent with the spread of the viral epidemic to areas traditionally endemic for leishmaniasis in the world. In southern Europe, for example, especially in Spain, Italy and France, leishmaniasis is a growing problem with several hundreds of HIV co-infection cases. Similar problems are also reported from Asian countries. Therefore, in the present text, such cases of *Leishmania*-HIV co-infections were briefly reviewed, in order to give an attention to inhabitants living in areas endemic for both diseases, leishmaniasis and AIDS.

Molecular parasitological findings

DNA karyotype of *Leishmania* isolates from cutaneous leishmaniasis patients at endemic areas of Ecuador was analyzed by pulsed field gel electrophoresis. From the results obtained, it is worth to note that DNA karyotyoe variation was evident among *Leishmania (Viannia) panamensis* isolates in the Pacific coastal regions while karyotype homogeneity was detected previously in *L. (Leishmania) mexicana* isolates in the Andes mountain regions. *Leishmania* isolates collected during the period from 1990 to date at 13 provinces of leishmaniasis-endemic areas of Ecuador were also analyzed by ELISA, based on more than 100 isolates, and their geographical distribution was shown.

Ultrastructural studies on leishmaniasis

A comparative electron microscopic observation was made between the skin biopsy materials taken from diffuse cutaneous (DCL) and localized cutaneous leishmaniasis (LCL) patients in Ecuador. Large parasitophorous vacuoles and disconnected cell membranes of *Leishmania* amastigotes were observed only in DCL. From the results obtained, it was suggested that proteo-high molecular weight phosphoglycan (proteo-HMWPG) was released from the disconnected site of the membranes of the amastigotes, and that production of proteo-HMWPG was accelerated in DCL more than in LCL. Pathological difference between DCL and LCL was also investigated immunohistochemically by using anti-T cell, CD45RO antibody and anti-lysozome antibody. The results obtained showed that the macrophages may not play their role as antigen presenting cells in the DCL case. Microscopic studies on patients treated orally with an antimalarial drug, Mephaquin®, was carried out by examining skin biopsy materials from the patients. After the oral treatment, inflammatory cell infiltration was remarkably decreased in H-E staining specimens, and the activity and cytotoxicity of macrophages were remarkably diminished in anti-asialo GM1 antibody staining specimens.

Diagnostic trials using molecular techniques

A trial to detect *Leishmania* parasites in paraffin-embedded skin biopsies of Ecuadorian cutaneous leishmaniasis patients, using polymerase chain reaction (PCR). In the study, a specifically amplified DNA by PCR using genomic DNA extract from *Leishmania* organisms was confirmed, and specific DNA was detected in some of the formalin-fixed and paraffin-embedded skin specimens. A comparative study of conventional and PCR-based diagnosis of cutaneous leishmaniasis in Ecuador was made. The results showed that PCR was consistently more sensitive than any of the 3 conventional diagnostic methods, microscopic examinations of 1) smear specimens, 2) *in vitro* culture materials and 3) histological specimens. In the other trial, template DNAs were prepared by boiling for 10 min in 5% Chelex solution, and *Leishmania* amastigotes in skin biopsy materials were detected by PCR using primers designed from minicircle (13A and 13B) and mini-exon gene (S-1629 and S-1630). The latter primer never amplified non-specific products even in human template, and enabled the subgenus level identification of the genus *Leishmania.*

Clinical and epidemiological studies

During 5 years from 1991 to 1995, a total od 348 cutaneous leishmaniasis patients were examined clinically and parasitologically in epidemiological surveys at different endemic areas of cutaneous leishmaniasis; the study sites distributed into 4 provinces, Manabi, Los Rios, Azuay and Esmeraldas, Ecuador. In this retrospective study, clinical and epidemilogical features of cutaneous leishmaniasis, such as age-composition of patients and clinical forms of the disease, and number, size, location and duration time of lesions, were thoroughly analyzed. From the clinical and epidemiological analysis of data, it was recommended that control and/or treatment of the disease in Ecuador should be done based on not only clinical knowledges but also entomological, ecological, environmental and anthropological knowledges. A retrospective study was also made in an endemic area of the Pacific coastal region, Province of Manabi, Ecuador. Clinical cases registered during 1985 and 1996 in a public hospital were briefly evaluated, in order to get an information on the global situation of the disease. In this study area, the following preliminary trials of chemotherapy of patients with antimalarial drugs were done.

Oral and topical treatment using antimalarial drugs and others

Antimalarial drugs such as mefloquine hydrochloride (Mephaquin®) and artesunate (Plasmotrim®) which are currently being used for malaria cases were evaluated for their antileishmanial activities. Almost all of cutaneous leishmaniasis patients were highly sensitive for both drugs, showing a high cure rate. The healing time of lesions tended to depend on the size and/or secondary infections of lesions, including other health conditions of each patient. Topical treatment using 2 preparations, a low concentration of paromomycin ointment and a meglumine antimonate lotion with mercury chrome was also effective against 80% cutaneous leishmaniasis patients. These topical treatment used showed a low irritation agaist patients with ulcer lesions, and less effective against those with non-ulcered lesions. Naturally, such a topical application of drugs should be tried in the areas where no risk of mucocutaneous or visceral types of the disease exists.

A laboratory assay of plant juices and mefloquine for antileishmanial activity in vitro *and* in vivo

Crude components of plants which are distributed in leishmaniasis-endemic areas of Ecuador are evaluated on their antileishmanial effects of promastigotes *in vitro*. From the results obtained it was suggested that naranja and mandarina contain some components which promote the growth of promastigotes in culture, and agave and piñon enhance the growth of the parasites during early cultivation time within 24 hours. On the other hand, mamei and the two *Leguminosae* plants used in this study inhibited the development of promastigotes; these plants apparently contain some components which can adversely

affect the parasite growth in culture. An antileishmanial effect of mefloquine (Mephaquin®) was evaluated using *Leishmania donovani*-infected visceral leishmaniasis model mice. The animals were treated orally with the drug at a dose of 75 mg/kg for 2 days before infection showed a 50% parasite reduction in the live, while no parasite reduction was found when the same dose was given after infection.

Related diseases

A clinicl comparison of cutaneous chages between patients with diffuse cutaneous leishmaniasis and leprosy was done based on dermatological findings, such as types of eruptions and their distribution. Furthermore, case reports of chromomycosis and myiasis due to Dermatobia hominis were reported from areas endemic for cutaneous leishmaniasis, and a differential diagnosis between these diseases and cutaneous leishmaniasis was briefly mentioned in the text.

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. Sinembargo no se han hecho intentos para determinar definitivamente al vector o vectores principales de la enfermedad, mediante el hallazgo de la infección natural en los insectos incriminados potencialmente. Cuando la investi-

gación 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 flebotominós 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 ubicacion en el tubo digestivo del huesped invertebrado.

Al examinar los ejemplares recolectados a diferentes alturas sobre el nivel del mar, 350 m, 600 m, 950 m, 1,200 m y 1,500 m, *Lu. trapidoi* 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 1,200 m 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 600 m, 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 Generacion de Phlebotomus de Laboratorio en el Ecuador: el Metodo de Crianza, Mantenimiento y su Contribucion al Futuro de la Investigacion 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 Medicne and Hygiene, 15(1), 1987, 7-15

8. Leishmaniasis in Different Altitudes on Andean Slope of Ecuador

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

ABSTRACT. An epidemiological survey was performed in a leishmaniasis-endemic area along highway which was established about 15 years ago on the Andean slope of Ecuador; the area ranged from 300 m to 1,500 m above sea level. In general survey, 64 (14.3%) of the 446 subjects examined were positive for leishmanial signs. In order to know leishmanial infections in relation to the altitudes of dwelling sites of subjects, analysis was made on 224 children with 5 to 15 years of age. At 4 different sites with 500 m, 1,000 m, 1,300 m and 1,500 m above sea level, the infection rates of the subjects from the individual sites were 17.4, 18.8, 5.6 and 8.8%, respectively. A statistically significant difference was recognized between the altitudes, 500-1,000 m and 1,300-1,500 m ($0.01<p<0.05$, $\chi^2 = 5.314$), but not between 500 m and 1,000 m and between 1,300 m and 1,500 m. Leishmanial infec tions 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 ($\chi^2 = 7.06$, 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 regions. The majority of leishmaniasis cases reported were 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 mammalian 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 lowland areas, including *Lu. trapidoi, Lu. hartmanni*, and *Lu. gomezi*, three proven vectors of *Leishmania*, since glanular secretions were found in both parous and nulliparous females.

Nettai, 22, 1989, 68-82

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

Yoshihisa Hashiguchi

ABSTRACT. A brief review is given of recent developments in leishmaniasis research worldwide, including details of the transmission of the three clinical forms of the disease, *viz*., cutaneous, muco-

cutaneous, 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 erradicated through the application of efficient control measures and sanitary improvements. This has resulted in a tendency for Japanese researchers to have little interest in parasitology and the control of parasitic diseases, at a time when research on these topics is urgently required in the third world countries. (In Japanese)

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

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

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

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

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

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

Yoshihisa Hashiguchi y Eduardo A. Gomez L.

ABSTRACT. Se examina brevemente el estado actual de los conocimientos sobre la leishmaniasis en el Ecuador, 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 of 97 (2%) *Lutzomyia ayacuchensis* Caceres and Bianchi were found to be naturally infected with *Leishmania* promastigotes. The parasites were confined to the midgut of the sand fly, indicating they did not belong to the subgenus *Leishmania (Viannia).*

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

19. Phlebotomes of Paraguay: Species Identification in Three Endemic Areas (Diptera, Psychodidae, Phlebotominae)

Alba Inchausti, Yoshihisa Hashiguchi and Antonieta De Arias

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

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

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

Yoshihisa Hashiguchi (ed.)

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

Leishmaniasis and its endemic area of Ecuador

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

Leishmania isolates from humans and animals and their characterization

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

Sand fly fauna and human leishmaniasis vectors in Ecuador

In eight Departments of Ecuador where human leishmaniases are endemic, the phlebotomine sand fly was sampled. A total of 40 species was collected, of which at least 11 represented new records for Ecuador. This record increased the number of sandfly 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 activ-

ity, of the flies in the area suggesting a high transmission intensity during the rainy season. The validity of the ovarian accessory glands of seven sandfly 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.

Comments on combating leishmaniasis in Ecuador

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

Strategies for future molecular epidemiology in Ecuador

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

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

21. A Review of Leishmaniasis in Ecuador

Yoshihisa Hashiguchi and Eduardo A. Gomez L.

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

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

22. Evaluation and Characterization of Partially Purified Skin Test Antigens Prepared from *Leishmania panamensis* Promastigotes

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

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

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

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

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

ABSTRACT. Between 1986 and 1988, epidemilogic studies were carried out in a small rural community in an Andean region of Ecuador, where cutaneous leishmaniasis is highly endemic. A total of 25 human cases, positive for *Leishmania* parasites by culture and/or smear, were examined. Fourteen

of the cases were in infants less than one year of age, suggesting intradomiciliary transmission of the disease. Clinically, many of these cases were similar to descriptions of "uta," a form of cutaneous leishmaniasis which occurs in Andean regions of Peru and is reportedly caused by *L. peruviana*. Of the 11 positive cultures obtained from human cases in the present study, eight were identified by molecular characterization as *L. mexicana* and three were identified as *L. major*-like. Two additional isolates of *L. mexicana* were also made from an infected dog and from a sand fly, *Lutzomyia ayacuchensis*, living in the region, thus implicating the latter species as possible reservoir and vector, respectively, of *L. mexicana* in this highland community. The significance and validity of recent isolates of *L. major*-like parasites from the New World are also discussed.

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

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

iasis in the Ecuadorian Andes which has a completely different species of causative agents and vectors from those of Peruvian uta. In this text, we briefly reviewed Andean leishmaniasis including uta and revised an ecological model of the disease in the Andean plateau. Furthermore, in this text autochthonous Andean leishmaniasis cases were reported from two regions of Ecuador, Huigra (1,300 m above sea level) and Alausi (2,300 - 2,500 m a.s.l.), Department of Chimborazo. In the areas 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 hyperendemic 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 hyperendemic area.

Japanese Journal of Tropical Medicine and Hygiene, 20(3), 1992, 203-215,

31. Histopathological Observations of Golden Hamsters Infected with an Ecuadorian Isolate of *Leishmania mexicana*

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

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

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

32. The Successful Treatment of Intralesional Injection of Meglumine Antimonate for Cutaneous Leishmaniasis

Motoi Takenaka, Taro Ohgami, Hikotaro Yoshida, 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)

American Journal of Tropical Medicine and Hygiene, 48(5), 1993, 707-715

33. 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.

Journal of Dermatology, 21 (3), 1994, 178-184

34. 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 endothilial 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.

Journal of Pakistan Association of Dermatologists, 3, 1994, 17-32

35. 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.

Japanese Jaunal of Parasitology, 43(3), 1994, 173-186

36. New World Leishmaniasis and its Transmission, with Particular Reference to Andean Type of the Disease, Uta

Yoshihisa Hashiguchi

ABSTRACT. In the text, New World leishmaniases were geographically divided into lowland and Andean highland forms, and were briefly reviewed. As to Peruvian uta, its short research history and more recent information on the taxonomic problem of the causative agent, *Leishmania (Viannia) peruviana*, were briefly discussed. From 1982 to 1993, the author and his co-workers worked with leishmanisis in Ecuador, in order to disclose the transmission mechanism(s). During the study, a new type of leishmaniasis was found in three endemic areas of Andean highlands, Paute (2,300 m -1,500 m above sea level), Alausi (2,300 m -2,500 m a.s.l.) and Huigra (1,200 m -1,500 m a.s.l.). Clinically, the disease forms in Ecuador were found to be very similar to those in Peru. However, the parasites and vectors were completely different between the two countries. In Ecuador, the organisms isolated from humans, sandflies *(Lutzomyia ayacuchensis)* and dogs *(Canis familiaris)* were identified as *L. (Leishmania) mexicana* by zymodeme, serodeme, schizodeme and karyodeme analysis. In addtion, another species of the genus *Leishmania*, was also isolated from humans living in Paute, Ecuador, and characterized as *L. (L.) major*-like, by molecular techniques mentioned above. Thus, the current review pointed out that Andean leishmaniases would have more complicated features of the epidemiology and ecology in different endemic areas than were previously considered. Besides, a model to show how local conditions affect transmission of the disease in the Andes was also shown.

Japanese Journal of Tropical Medicine and Hygiene, 22(4), 1994, 219-223

37. Case Report of Leprosy and a Trial of Screenings for the Family Members in Ecuador

Atsushi Hosokawa, Shigeo Nonaka, Juan J. Alava P., Eduardo A. Gomez L., Hugo M. Jurado S. and Yoshihisa Hashiguchi

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 them (borderline lepromatous leprosy and indeterminate one) in a single family and result of screenings for the family members were reported. It was suggested that family examination of leprosy patient might be useful for early detection of leprosy in a low endemic areas for leprosy, such as Department of Manabi. A nine banded armadillo kept by the family was examined, but no acid-fast bacilli was observed in the liver materials.

Japanese Journal of Tropical Medicine and Hygiene, 22(4), 1994, 179-184

38. Seroepidemiological Surveys for Leprosy in Ecuador

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and
Yoshihisa Hashiguchi

ABSTRACT. Serological examination of leprosy in endemic areas of cutaneous leishmaniasis were carried out using the 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 (42.4%, 154/365) of the subjects from other areas of Ecuador. It was suggested that serological survey of families of leprosy patients might be useful for screening of household contacts in a low endemic area, such as Department of Manabi, Ecuador.

Kochi, Japan: Kyowa Printing, Research Report Series, No. 4, 1994, 1-193

39. Studies on New World leishmaniasis and its Transmission, with Particular Reference to Ecuador

Yoshihisa Hashiguchi (ed.)

ABSTRACT. 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 (IFA) 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 snadflies 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 into 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 (1200-1500 m above sea level), Department of Chimborazo. The results obtained were compared to those in Alausi (2,300-2,500 m), Department of Chimborazo and Paute (2,300-2,500 m), 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 karyotype 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 a 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 sero-positive 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.

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40. Oral Treatment of New World Cutaneous Leishmaniasis with Anti-malarial Drugs in Ecuador: a Preliminary Clinical Trial

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and
Yoshihisa Hashiguchi

ABSTACT. The current study was designed to evaluate anti-leishmanial activity of mefloquine hydrochloride (Mephaquin®) and artesunate (Plasmotrim®) which are currently being used as malaria drugs. A total of 17 patitents (volunteers) with cutaneous leishmaniasis were treated with these durgs in this study. Of these subjects, 16 were treated by the oral administration of a total dosage of 1,500 mg (1 Lactab® each for 6 days) mefloquine, 4.2 mg/kg/day for 6 days, and if necessary the dosage was repeated with 3 weeks intervals. The majority of cutaneous lesions healed within 2 to 3 weeks after the commencement of mefloquine treatment, showing an average of 3.6 weeks of healing times with 100% cure rate. One slowly healing within 8 weeks after the commencement was observed; this case grew worse because of infection with *Tunga penetrans* at the late healing phase of leishmaniasis. The remaining one patient with an ulcer lesion was treated by the oral administration of a total dosage of 1,200 mg (2 Lactab® each for 3 days) artesunate, *i, e*., 6.7 mg/kg/day for 3 days, and the same dosage was repeated 2 weeks later. The lesion healed within 6 weeks after the commencement of artesunate treatment. In the present study, all the patients received mefloquine or artesunate were treated without admission, performing their normal daily activities. No specified adverse reaction was noticed.

Okinawa Medical Journal, 33, 1995, 44-47

41. A Trial of Topical Treatment using 2% Fluorouracil (5FU) Ointment for Cutaneous Leishmaniasis at the Pacific Coastal Lowland of Ecuador

Atsushi Hosokawa, Shigeo Nonaka and Yoshihisa Hashiguchi

ABSTRACT. In this paper, a topical treatment of an anticancer drug ointment (Fluorourail: 5FU) was evaluated against cutaenous leishmaniasis. 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 77 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 useful for the relatively small sized shallow ulcerative lesions, but not so effective against non-ulcerative lesions. (In Japanese)

Hihu-Rinsho Derma, 38, 1996, 547-556

42. Cutaneous Leishmaniasis

Yoshihisa Hashiguchi

ABSTRACT. Leishmaniases distribute widely in tropical and subtropical countries of the New and Old World; and 350 million people are at risk. The causative agents of the disease belong to the genus *Leishmania* parasitic to reticuloendotherial cells, especially macrophages, of mammals including man. The genus *Leishmania* which are divided into 2 subgenus, *Leishmania* and *Viannia*, includes about 20 species parasitic to human in the New and Old World. In human case the clinical forms are very variable, depending on the causative species of *Leishmania*, host immunological and physiological conditions and characteristics of each endemic area. Clinically, the disease forms are largely classified into 3 categories, cutaneous (CL), mucocutaneous (MCL) and visceral (VL) types in general. In this text, however, they are devided into 6 categories in order to compare their clinical features in detail as follows: 1) CL including simple and self-healing type, leishmaniasis recidivans type and sporotricoid type; 2) DCL (diffuse cutaneous leishmaniasis); 3) DICL (disseminated cutaneous leishmaniasis); 4) MCL; 5) VL; 6) PKDL (post-kala-azar dermal leishmaniasis). Furthermore, a brief review was also done on the infection mechanism(s) and the clinical classification of the disease based on histopathological findings reported. In addition, clinical and immunological features of leishmaniasis are compared among DCL, DICL and CL. Finally, the vector sand flies, reservoir hosts, diagnosis, treatments and control measures of the disease are discussed briefly. (In Japanese)

Japanese Journal of Dermatology, 106(12), 1996, 1471-1481

43. Leishmaniases

Yoshihisa Hashiguchi

ABSTRACT. Leishmaniases are caused by a unicellular organism of the genus *Leishmania*, and transmitted by phlebotomine sandflies, the genus *Phlebotomus* in the Old World and the genus *Lutzomyia* in the New World. There are at least 21 different species *Leishmania* that cause human infections. The protozoan parasites, *Leishmania* spp., produce a wide range of clinical infections in both humans and vertebrate aminals as zoonosis. In humans, clinical leishmaniasis ranges from a simple, often self healing cutaneous form to those producing destructive mucocutaneous ulcers of the nasopharynges, uncurable diffuse cutaneous lesions, and a visceral form known as kala-azar, a severe chronic infection of the reticuloendothelial system which is often fatal if left untreated. The disease is endemic in many tropical and subtropical regions and is classified as one of the six tropical diseases targeted by the World Health Organization (WHO) for study by the Tropical Disease Research Program (TDR). It is estimated that there may be some 12 million infected people in the world and 370 mil-

lion at risk, of whom some 0.4 to 1 million will be infected each year in the 67 countries affected. Some 90% of the visceral leishmaniasis cases are reported from two regions, a wide zone from northeast India and Bangladesh to southern Nepal and Sudan; and 90% of cutaneous cases including mucocutaneous and diffuse ones are found in Afganistan, Iran, Saudi Arabia and Syria in the Old World, and Brazil and Peru in the New World. (In Japanese with English summary)

Internal Medicine, 35(6), 1996, 434-435

44. Leishmaniasis: its Changing Pattern and Importance as an Imported Disease

Yoshihisa Hashiguchi

ABSTRACT. During the past few decades, the parasitic diseases such as leishmaniasis, malaria and trypanosomiasis have not been considered priority public health problems or to be of medical importance in Japan and in other developed countries. Therefore, such diseases were sometimes relegated to the status of simply an academic curiosity in these countries, and few physicians or parasitologists felt the need to understand the details of the diagnostic procedures and treatment regimens associated with these parasitic infections. Recently, however, increasing worldwide travel has raised the numbers and a variety of parasitic diseases have been imported into non-endemic areas of the diseases. In such a circumstance, imported cases of a variety of parasitic diseases should be adequately diagnosed and treated by knowledgeable medical personnel. Here, the changing pattern of leishmaniasis and its importance as an imported disease are briefly discussed in order to stimulate the interest of medical personnel in the field of parasitic diseases.