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

2001

Research Report Series No. 6

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

edited by

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Scientific Research Team
funded by the Ministry
of Education, Science
& Culture, Japan

Printed by
Kyowa Printing & Co. Ltd.
Kochi City, Kochi, Japan
2001

This study was supported by a grant under the International Scientific Research Programme, the Ministry of Education, Science and Culture, Japan (Research Grant Nos. 08041177 and 10041199), with the cooperation of the Departamento de Medicina Tropical, and Instituto de Biologia Molecular, Facultad de Medicina, Universidad Catolica de Santiago de Guayaquil, Guayaquil, Ecuador, Unidad de Inmunologia, Medicina Tropical, Centro de Biomedicina, Universidad Central del Ecuador, Quito, Ecuador, and Departamento de Investigaciónes Clinicas, Medicina Tropical, Hospital Vozandes, Quito, Ecuador, South America.

The present text reports on the data and materials
mainly collected during the period from 1998
to 2000 in Ecuador, South America

Emerging and re-emerging diseases: its changing pattern and importance. 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 other medical personnel felt need to understand the details of the diagnostic procedures and treatment regimens associated with these parasitic infections. Recently, however, increasing world-wide travelers, global warming, environmental changes and etc., have raised the numbers and a variety of parasitic diseases have been imported into non-endemic areas (countries) of the diseases. (Hashiguchi, 1996: Int. Med., 35, 434)

Regarding leishmaniasis, the disease is found in five continents and is endemic in the tropical and subtropical regions of 88 countries: 16 are developed countries, 72 are developing countries and 13 of them are among the least developed. There is an overall prevalence of 12 million cases world-wide. Globally the yearly incidence is believed to be 1.5 to 2 million new cases of cutaneous leishmaniasis and 500,000 new cases of visceral leishmaniasis. (WHO/LEISH/2000.42,1)

Is leishmaniasis extending its range ? The recent report of leishmaniasis in people in East Timor raises a number of important issues (Cheavalier *et al.,* 2000: Clin. Inf. Dis., 30, 840). In November 1999, a medicosurgical group from Europe, forming part of the International Force for East Timor (INTERFET), identified 46 patients who had cutaneous lesions characteristic of leishmaniasis; diagnosis was made on the basis of clinical presentation and direct microscopical examination of lesion specimens. This finding represents the emergence of a serious vector-borne parasitic disease in a region distant from known endemic foci. It not only poses a "new" threat to the area, but also has the potential to spread. (Thompson and Reid, 2000: Parasitol. Today, 16, 370)

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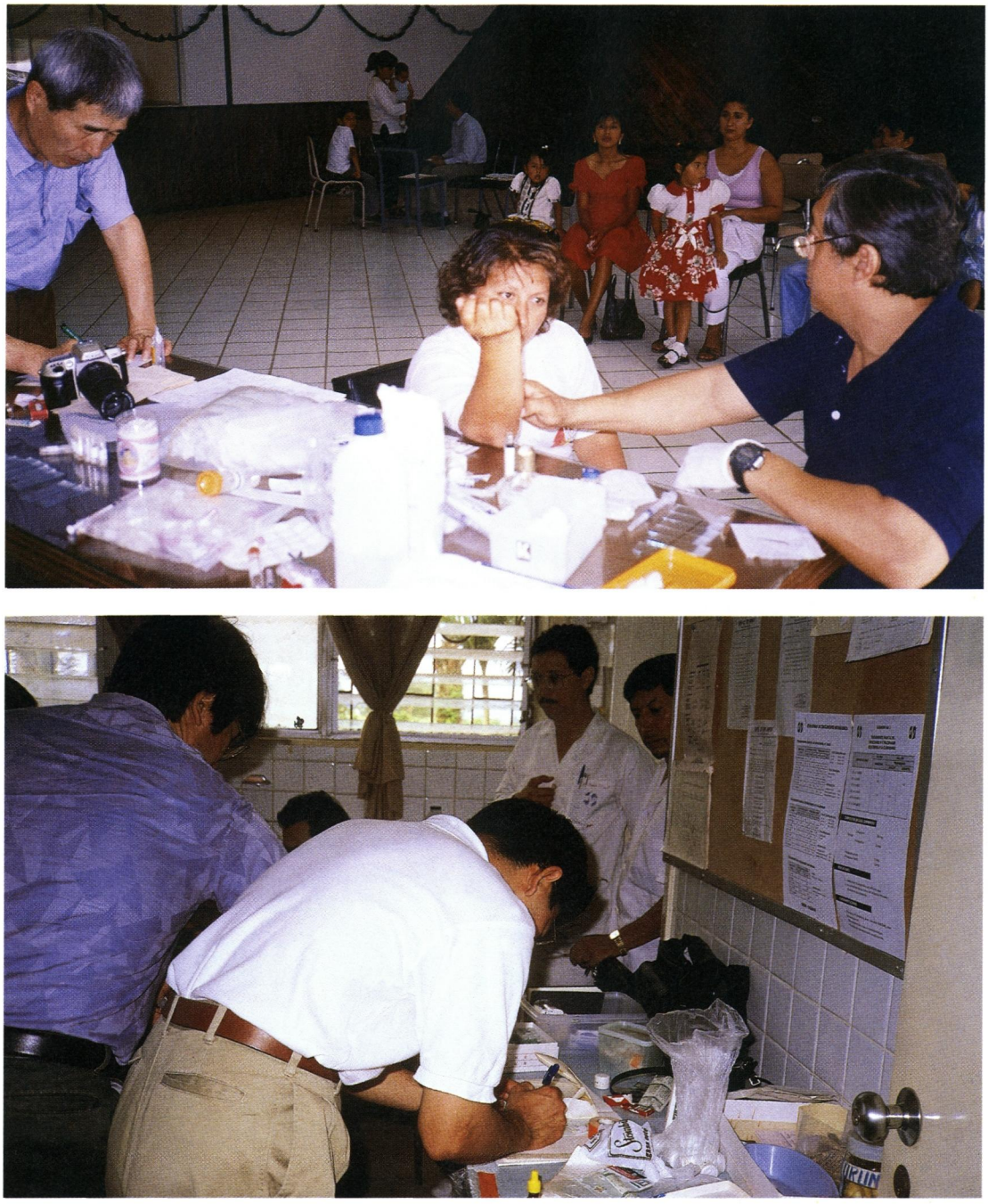

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Preface

Investigations on leishmaniasis and its transmission in Ecuador by our research group were first commenced in 1982 and continued until 1984, under the financial support of the Japan International Cooperation Agency (JICA). During this preparatory phase, we strongly felt that more intensive and countrywide investigation of the disease by multidisciplinary experts should be done thoroughly, in order to disclose the epidemiological features of the disease in each endemic area of Ecuador. Fortunately, we were able to perform continued studies of leishmaniasis in that country after 1986, receiving the financial support of the Ministry of Education, Science and Culture, Japan. In our field phase of investigations, we have principally aimed at obtaining data on human cases, vector sandflies and reservoir mammals for a better understanding of epidemiological features including the transmission mode of the disease in different endemic areas of Ecuador. On the other hand, in the laboratory phase, we analysed the materials collected during field studies from leishmaniasis patients, vectors and reservoirs, and if necessary animal experiments using the materials were also made for further analysis.

The data obtained were summarized in the Research Report Series Nos. 1-5, entitled "Studies on leishmaniasis and its transmission, with particular reference to Ecuador", published in 1987-1997. The reports mainly include the information and description of the causative agents, *Leishmania* spp., vector sandflies, *Lutzomyia* spp., and clinical features of the disease in different endemic areas of Ecuador. The current report, Series No. 6, mainly deals with the results obtained from field surveys and laboratory works in our leishmaniasis research project during 1998 and 2000. Much of the materials and data collected have yet to be examined and analyzed; the results will be published in detail elsewhere in future, under the authorship of all research workers participated in the study. A further study of leishmaniasis and its transmission in Ecuador will be continued from 2001 onwards, with the main intention of employing molecular techniques to elucidate epidemiological and clinical features of the disease.

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Acknowledgements

The current study could not have been performed without the thoughtful support of many persons and colleagues in Japan, Ecuador, and other countries. Our special thanks are due to Ecuadorian colleagues who have given us the use of every available facility at the Instituto de Biologia Molecular Tropical, Facultad de Medicina, Universidad de Catolica de Santiago de Guayaquil, Guayaquil, Ecuador, the Instituto de Inmunologia, Facultad de Medicina, Universidad Central del Ecuador, Quito, Ecuador, Departamento de Investigaciones Clinicas, Medicina Tropical, Hospital Vozandes, Quito, Ecuador, Hospital de La Mana, Pichincha, Ecuador, Hospital Cantonal El Carmen, Manabi, Ecuador, and Subcentro de Salud de San Sebastian, Manabi, Ecuador. We are grateful to Dr. Layes, Director, Instituto de Biologia Molecular Tropical, Dr. Rodorigo Armijo, Instituto de Inmunologia, Universidad Central del Ecuador, Dr. Juan J. Alava, Instituto de Higiene y Medicina Tropical, Portoviejo, Manabi, Dr. Milton Ayala, Hospital de La Mana, Pichincha, Dr. Moran and Mr. Victor Hugo Cañarte, Hospital Cantonal El Carmen, Manabi, Ms. Gloria Esthela Losa, Subcentro de Salud de Zhucay, Cañar, Ecuador. Thanks go to Drs. Hisao Ikeda (President), Yusuke Sagara, Syouhei Ogoshi (Vice Presidents), and Prof. Tetsuo Sugiura, Kochi Medical School, Nankoku-shi, Kochi, Japan, and also go to Dr. Michael Dumont (Vice President), Universidad de Catolica de Santiago de Guayaquil, for their encouragement and support at different phases of the present study. Finally, valuable assistance by Dr. Masataka Korenaga, Ms. Kyoko Imamura, and Mr. Jorge Diego Marco, Department of Parasitology, Kochi Medical School is gratefully acknowledged.

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

Introduction

In the New World, cutaneous (CL), mucocutaneous (MCL) and visceral (VL) leishmaniases are endemic at a wide range of Central and South American countries, and it constitutes a significant public health problem in each country. Control of the disease in these regions is complicated by the variety of factors, such as different species of the genus *Leishmania,* diverse clinical forms and epidemiological patterns in each leishmaniasis-endemic area. In many regions of the New World, two or more *Leishmania* species are sympatric. The species belonging to the subgenus *Viannia,* are known as the causative agents of CL and MCL. Among the *Viannia* group, *L. (V.) braziliensis* shows the most wide range of distribution, affecting many populations and causing CL and MCL. In the New World, almost all of the disease forms of leishmaniasis are zoonoses, and man is usually an incidental host of the parasite, *Leishmania.* In the subgenus *Leishmania* group, *L.(L.) mexicana* is mainly reported from the Central American countries and from a part of the northern regions of the South America including Ecuador. The species of the subgenus *Leishmania, mexicana, amazonensis* and *pifanoi* are important not only as causative agents of CL but also as those of diffuse cutaneous (DCL) leishmaniasis. *L.(L.) chagasi* (*infantum* ?) is main or only species which causes visceral leishmaniasis (VL) and/or CL in the New World with a wide range of distribution from Mexico to Brazil. In Ecuador, however, no such a parasitologically confirmed VL case has not been reported, though only clinically diagnosed one case was reported about 50 years ago (Leon, 1949); during about 20 years from 1982 to date of our epidemiological survey, no VL-suspected human case and the vector sandfly species, *Lutzomyia longipalpis* and *Lu. evansi* were found.

Ecuador is one of the small countries in South American continent, and each geographic region has specific features relating to the transmission factors of leishmaniasis such as terrain, environment and life style of the inhabitants. In that country, leishmaniasis was first reported in 1920 by Valenzuela, but it has remained one of the least studied of Ecuadorian tropical diseases. From 1982 to date, we have studied almost all of the suspectable endemic foci of leishmaniasis, in order to disclose the transmission mechanisms, clinical forms and etc. It was found that leishmaniasis is widespread in most provinces of Ecuador, showing a considerable health problem. The disease occurs in many populations living in rural and mountainous areas on both sides of the Andes, including those living in the Andean valleys. Of the 22 provinces of Ecuador, 18 are endemic; 6 are located in the Pacific Coastal region, 6 are situtuated in the Andes and 6 in the Amazonean regions. As to the causative agents of leishmaniasis in Ecuador, seven species of the genus *Leishmania, viz.*, three of the subgenus *Leishmania, mexicana, amazonensis,* and *major*-like, four of the subgenus *Viannia, braziliensis, guyanensis, panamensis* and *equatorensis* were specified based on zymodeme, serodeme, schizodeme and karyodeme analysis. Four sandfly species of the genus *Lutzomyia, trapidoi, hartmanni, gomezi* and *ayacuchensis,* and 8 species of mammals, anteaters, sloths (2 spp.), squirrels (2 spp.), kinkajous, rats and dogs were incriminated as possible vectors and reservoirs, respectively, in the endemic areas of Ecuadorian lowland and highland areas. Regarding clinical forms of Ecuadorian leishmaniasis, ulcer and nodular types of CL including Uta type in the Andes, generalized types of ulcer CL, DCL, and MCL were found, but any case of VL was not observed as mentioned before; Uta type was first described by our reseach group in 1986 from Paute, Azuay province.

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Chapter 1

A Retrospective Review of the Present Leishmaniasis Research Project in Ecuador

ABSTRACT. The present leishmaniasis research project entitled "Studies on New World leishmaniasis and its transmission" was first commenced in 1982 and then continued to date. In the current text, therefore, a brief retrospective review was made focusing on the main topics obtained during about 18 years. As the causative agents of leishmaniasis in Ecuador, seven species of the genus *Leishmania, viz., L. (L.) mexicana, amazonensis* and *major*-like, and *L.(V.) braziliensis, panamensis, guyanensis* and *equatorensis*, were characterized by molecular analyses such as zymodemes, serodemes, schizodemes and karyodemes. Four species of the genus *Lutzomyia, trapidoi, hartmanni, gomezi* and *ayacuchensis*, were determined as probable vectors of the disease at different endemic areas. *Leishmania* parasites were detected from eight species of the mammals, sloths (2 spp.), squirrels (2 spp.), kinkajous, anteaters, rats and dogs examined, and these were recorded as reservoir hosts of leishmaniasis in Ecuador. Epidemiological and clinical features of the disease were thoroughly investigated and discussed performing a wide range of field studies. In our project, moreover, intensive studies for more effective and convenient treatment, including a search for more simple and field applicable diagnostic tools, were made continuously. Topically applicable lotions and ointments, and oral drugs, antimalarials such as Mephaquin and Artesunate were investigated. With regard to the diagnosis of cutaneous leishmaniasis, it was recommended that scrape/exudate sampling method for PCR is a better alternative to skin biopsy samples. For this purpose, in our project, subgenus- and species-specific primers were designed, specially turgeting on the main *Leishmania* species in the New World, *L. (L.) mexicana, amazonensis,* and *L. (V.) braziliensis, panamensis* and *guyanensis*. We also performed a preliminary study on leishmaniasis in Paraguay, and the results obtained were mentioned briefly.

The current leishmaniasis research project was first commenced in 1982 under the financial support of the Japan International Cooperation Agency (JICA). After that, the project has been continued to date under the support of the Japanese Ministry of Education, Science and Culture during about 15 years from 1986 to 2000. In this session, therefore, a brief retrospective review was made on the results obtained in our project, focusing on the main topics.

1. Causative agents of leishmaniasis in Ecuador

In Ecuador, the first case of leishmaniasis was reported in 1920 by Varenzuela, based on typical ulcer lesions found on the forearm and thorax of a female patient from the province of Esmeraldas, very close to the Colombian borders. In the country, however, the precise characterization or identification of the causative agents was not done, and the *Leishmania* species has been mentioned as *L. braziliensis* or *L. mexicana* complexes based on the clinical features of patients. The first precise characterizations of *Leishmania* parasites isolated from humans, reservoir animals and vector sandflies were made by Mimori *et al.* (1989), analysing zymodeme and serodeme; they reported two species of the genus *Leishmania, amazonensis* and *panamensis,* belonging to the subgenera *Leishmania* and *Viannia,* respectively. Such efforts for the characterization of the parasites isolated during our field research were made continuously, then further five species, *L. (L.) mexicana, L.(L.) major*-like, *L.(V.) braziliensis, L.(V.) guya-*

nensis and *L.(V.) equatorensis* were listed until now. As is known, it is very difficult to identify *Leishmania* parasites, using generally recognized taxanomic criteria. In our research project, therefore, molecular techniques such as PCR (polymerase chain reaction) were also employed to characterize these parasites at the subgenus or species levels. Katakura *et al.* (1993) tried to perform karyodeme analysis among three species of the genus *Leishmania, L.(L.) mexicana, L.(L.) major*-like and *L.(V.) panamensis,* isolated from Ecuadorian patients, using pulsed-field gel electrophoresis (PFGE). From the results obtained, it was suggested that the molecular karyotyping employed would be useful for the species and/or strain characterization. In their trials, chromosomal locations of dihydrofolate reductase-thymidylate synthetase and P-glycoprotein genes revealed further differences in chromosomal organizations among the *Leishmania* species examined in Ecuador. In this project, a DNA fingerprint study of the major *Leishmania* species was performed by arbitrarily primed polymerase chain reaction (AP-PCR) and the subgenus-specific amplified DNA bands were identified (Mimori *et al.,* 1998). The sequences of these subgenus-specific DNA arrangements were determined, and primers that recognize the subgenus-specific genes were designed. Some sets of these primers amplified the subgenus-specific genes, which contained several polymorphic base changes depending on their species. Polymorphism-specific primers were therefore designed based on a comparison of the DNA sequences of the subgenus-specific amplified genes and a polymorphism-specific PCR (PS-PCR) for yielding species specific amplification was performed. Consequently, a PCR panel was established for distinguishing all five major *Leishmania* species, *L. (V.) braziliensis, panamensis* and *guyanensis,* and *L. (L.) mexicana* and *amazonensis,* causing New World cutaneous and mucocutaneous leishmaniasis.

2. Sandfly vectors of leishmaniasis in Ecuador

In Ecuador, for a long time, the vector sandfly species of leishmaniasis had not been known. In our project, sandfly collections and dissections were made intensively during the early phase of project, in order to decide the vector, detecting the natural infections with *Leishmania* promastigotes. To date, a total of four species of the genus *Lutzomyia, viz., Lu. trapidoi, hartmanni* and *gomezi* from lowland, and *Lu. ayacuchensis* from Andean highland, were recorded as probable vectors of leishmaniasis in Ecuador (Hashiguchi *et al.*, 1985, 1991; Takaoka *et al.*, 1990).

In that country, more than 60 sandfly species were recorded, among them we listed 46 species performing fly collections in 12 of the 21 provinces (Alexander *et al.*, 1992). In addition to these data, other factors relating to leishmaniasis transmission, such as biting activities, seasonal variations or fluctuations of natural infections with *Leishmania,* and parous and nuliparous rates of the sandflies were also investigated (Hashiguchi *et al.,* 1985; Takaoka *et al.*, 1989; Gomez and Hashiguchi, 1991).

3. Reservoir hosts (mammals) of leishmaniasis and their roles in Ecuador

In the New Word, leishmaniases are typical zoonoses, mainly distributing in the dense forested and/or mountainous areas of Central and South Americas. In such an epidemiological characteristics of the disease in the regions, man is usually a dead-point-host for the parasites, *Leishmania* spp., and therefore no man to man or man to sandfly to man transmission cycles are hardly available. In the future control of the disease, therefore, it is important for medical personnel to know details of the roles of reservoir hosts (mammals) in each leishmaniasis-endemic area. For these purpose, we tried to determine probable reservoirs examining *Leishmania* parasites in cultured or smeared materials from wild or domestic mammals at different endemic areas (Hashiguchi *et al.,* 1985). In our trials, the following eight species of the mammals from Pacific lowland, Andean slope, and highland leishmaniasis-endemic areas of Ecuador were recorded: *Bradypus variegatus ephippiger* (three-toed sloth), *Choloepus hoffmani didactylus* (two-toed sloth), *Sciurus granatensis* (red squirrel), *S. vulgaris* (brown squirrel), *Potos flavus* (kinkajou), *Tamandua tetradactyla* (anteater),

Rattus rattus (brown rat), and *Canis familialis* (dogs). Among the parasites isolated from the animals, those from sloths, kinkajous and anteaters were characterizad as *L. (L.) amazonensis,* and those from dogs were *L.(L.) mexicana* based on zymodeme and serodeme analysis (Mimori *et al.*, 1989; Hashiguchi *et al.*, 1991)

4. Epidemiological and clinical features of leishmaniasis in Ecuador

1) Epidemiology and transmission of the disease

Ecuador is, in all aspects, a highly varied country, and geographical, climatic, ecological, ethnical, socio-economical and pathological differences are found between each natural region of the country. The Andes, a range of mountains which traverses Ecuador from north to south, divides the country into three natural regions, the littoral or Pacific coast region, the Andean region, and the Amazonian region. The relationship between the distribution of leishmaniasis and the ecological aspects mentioned above should be thoroughly analyzed. The ecological features influenced on the epidemiology and clinical manifestations of leishmaniasis at different endemic areas. After the first description of a clinical case of the disease in Ecuador in 1920 by Varenzuela, it has been known that leishmaniasis is prevalent countrywide, distributing from lowlands to Andean highlands including Andean slopes and Andean valleys (Hashiguchi and Gomez. 1991); however, no detailed information on the transmission at each endemic area has not been available. In this project, from the beginning phase, we tried to perform intensive examinations on the patients, the natural infections of sandflies and mammals with *Leishmania,* and other transmission relating factors (Hashiguchi *et al.*, 1984, 1985). Among the epidemiological data obtained in this project, the most important topic is the description of Andean type of leishmaniasis, which is very similar to Peruvian Uta. However, the Ecuadorian cases are caused by *L. (L.) mexicana* or *L .(L.) major*-like, but not caused by *L. (V.) peruviana,* and transmitted by the sandfly, *Lu. ayacuchensis* (Hashiguchi *et al.*, 1991; Takaoka *et al.*, 1990). Dogs and rats *(Rattus rattus)* are recorded as the reservoir hosts in the endemic areas. To date, it was found that the Andean forms of leishmaniasis in the country are prevalent at four foci, Paute (2200-2500m above sea level), Azuay province, and Alausi (2300m a.s,.l.), Chanchan (1500m a.s.l.) and Huigra (1200-1300m a.s.l.), Chimborazo province, affecting mainly less than 5 years of age groups.

2) Clinical features of the disease

In Ecuador, only one case of visceral leishmaniasis was reported in 1949 by Leon, based on the clinical finding of a 3 years old boy without demonstrating *Leishmania* parasites. During our 18 years countrywide surveys, we could not observe any suspectable such a case in spite of our efforts and interest. In this text, therefore, cutaneous (CL) cases and mucocutaneous (MCL) cases examined thoroughly by dermatologists were mentioned in the present project. The clinical forms in Ecuador are mainly divided into following froms: CL in lowland and highland, MCL, DCL, generalized CL (GCL) and sporotricoid type CL. In the lowland, larger and multiple lesions with a heavy secondary infections were frequently observed, caused by species of the subgenus *Viannia, panamensis* and *guyanensis* at the Pacific regions, and *braziliensis* at the Amazonian regions, and those of the subgenus *Leishmania, mexicana* and *amazonensis* at the Pacific coast. On the other hand, lesions found in patients from the Andean highlands, Paute, Alausi and Huigra, were very small and superficial, caused by two species of the subgenus *Leishmania, mexicana* and *major*-like, as mentioned before. Biopsy materials from patients were electron-microscopically studied, and a lot of new information was accumulated hitherto (Bhutto *et al.,* 1994a). In the country, MCL forms were found in around 7% of the total cases (Hashiguchi and Gomez, 1991), and only one parasitologically confirmed DCL case was reported by our research group; the latter is highly resistant for presently available drugs including antimonials (Glucantime and Pentostam). The parasites *(L. (L.) mexicana)* isolated from DCL and those from simple CL were experimentally inoculated into hamsters, and then the animal-derived parasites were electron-microscopically observed but no difference was recognized morphologically (Bhutto *et al.*, 1996).

Differential diagnosis of CL was also made, performing dermatological examinations at different leishmaniasis-endemic areas; special attention was paid to the difference between CL and Hansen's disease.

5. Treatment and diagnosis of leishmaniasis in Ecuador

1) Treatment

In leishmaniasis, at the moment, antimonial drugs, Glucantime and Pentostum, are the first choice for the treatment in Ecuador. However, these drugs have several drawbacks such as side effects, administration difficulties and etc., at rural areas endemic for the disease. There is, therefore, still necessity to develop or to find more effective drugs easy for administration. In our project, topically applicable treatment was searched for CL cases. For this purpose, we prepared a lotion composed of Glucantime and mercury chrome, and an ointment composed of paromomycin and vaseline; both preparations were highly effective for lowland CL (Nonaka *et al.*, 1992). On the other hand, antimalarial drugs such as Mephaquin and Artesunate were orally given to CL patients. From the results obtained, it was found that these two drugs are highly effective for Ecuadorian CL caused by *L. (V.) guyanensis* and *panamensis* at the Pacific coast lowlands (Gomez *et al.*, 1995); the biopsy materials from treated patients with Mephaquin were examined electron-microscopically and the killing process of the parasites by the drug was observed.

2) Diagnosis

For CL diagnosis, the parasitological observations of *Leishmania* parasites in culture or smear specimens are the most reliable. These trials are, however, time consuming and need well experienced personnels; moreover, the detection rates of such examinations are very low, demonstrating a considerably high false negative, depending on the disease forms and/or the causative agents, *Leishmania* spp. At the beginning phase of our project, we prepared skin test antigens from *Leishmania* promastigotes. Using this tool a countrywide epidemiological survey was carried out, in order to know the prevalence of CL at different endemicities. ELISA was also performed, and the results obtained were compared with skin test results. In the comparison, it was found that both tools are useful for screening of the disease in the endemic areas, showing a relatively high specificity and sensitivity. With regard to the reactivity of these tools, skin test had a tendency to demonstrate stronger reaction in cured subjects than in the active ones. By the analysis of the data from both skin test and ELISA, it was suggested that leishmanial ulcer lesions seemed to be deteriorated depending on the immunological factors such as cellular and humoral immunity, especially at the beginning phase of the evolution (Mimori *et al.*, 1987). Recently, polymerase chain reaction (PCR) methods have been used widely to diagnose leishmaniasis. In our project, we also designed *Lishmania* species- and subspecies-specific primers, and *Leishmania* species were identified using formalin-fixed biopsy materials (Mimori *et al.*, 1998; Uezato *et al.*, 1998). It was our great favor to have more simple and convenient diagnostic methods including suitable procedures for taking sample materials at field conditions. We, therefore, tried to compare the results of PCR diagnosis based on the materials from three different procedures, *viz.*, scrape/exudate, syring-sucked fluid and biopsy samples from ulcer lesions (Matsumoto *et al.*, 1999). From the results obtained, it was recommended that scrape/exudate sampling method is a better alternative to skin biopsy for CL diagnosis. The scrape/exudate samples had the same sensitivity as the biopsy samples, and its collection from skin lesions was easy and painless for the patients compared with the syringe-sucked or biopsy methods; the biopsy method of sample collection sometimes yields undesirable results causing various secondary infection, especially in field conditions at leishmaniasis-endemic areas.

6. Leishmaniasis in Paraguay

In our project, a preliminary study was done in three localities, Alto Parana, Caaguzu, and Caazapa provinces situated at the eastern regions of Paraguay, close to the Brazilian and Argentine borders. In the country, clinical examinations of inhabitants, and survey for vector sandflies and reservoir mammals were made at leishmaniasis-endemic areas of the provinces mentioned

above. At Limoy, Alto Parana, the skin testing reaction using antigens prepared from *L. (V.) panamensis* promastigotes was observed in 149 subjects; 74 (50%) of them revealed positive reactions and 88 (59%) had typical active or cured CL and MCL lesions (Hashiguchi *et al.,* 1991). From these results and information collected at different endemic areas, it was suggested that the disease is prevalent countrywide and a further detailed epidemiological study in that country was needed in the future. With regard to sandfly vectors, many fly samples were collected and dissected at different areas; the following nine anthropophilic species were recorded: *Lutzomyia whitmani, intermedia, shannoni, migonei, fischeri, pessoai, cortelezzii, walkeri* and *longispinus*. Among these, only one specimen of *Lu. whitmani* showed positive for *Leishmania* promastigotes parasitic at the hind-gut of the fly, suggesting subgenus *Viannia* group of the genus *Leishmania;* no precise characterization of the parasites isolated was possible (Hashiguchi *et al.,* 1992). In our study, species compositions of sandflies and their possibilities as probable vectors were also examined, based on the materials collected at different areas. In that country, however, no detailed information on the epidemiology of leishmaniasis, including transmission relating factors such as vectors and reservoirs, are available, although many clinical cases have been reported.

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Chapter 2

A Global Situation of Leishmaniases

ABSTRACT. A global situation of leishmaniasis in the world was brieflly reviewed, especially focusing on the changing patterns of the transmission, clinical forms, prevalence and etc. at different endemic foci. *Leishmania*/HIV co-infection cases are increasing annually due to different factors, such as human behavioral, environmental and epidemiological changes, especially in the south-western Europe, Spain, Italy, France and Portugal. Such co-infection cases have also been reported from other countries of different continents, Asia, Africa, and Central and South Americas. In the case of *Leishmania*/HIV co-infection cases, serological diagnosis is of little use. To overcome this problem in HIV-infected patients, an indirect xenodiagnosis of visceral leishmaniasis using laboratory colonized sandflies were recently developed by Spanish workers; the usefulness was shortly discussed in the present text as a topic.

1. Changing patterns of transmission and clinical forms

Leishmaniases are caused by different species belonging to the genus *Leishmania,* and the genus includes two subgenera, *Leishmania* and *Viannia*; more than 20 species of them are described as the causative agents of human leishmaniasis in the world. The parasites are haemoflagellate protozoans which are exclusively transmitted by the bite of a tiny 2 to 3 millimeters long female sandflies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World. Of the 500 phlebotomine species recorded to date, only about 30 of them have been incriminated as vectors of human leishmaniasis in the world. The diseases show a wide range of clinical manifestations depending on *Leishmania* species and/or immunological or physiological conditions of the host patients. Clinically, the diseases are mainly classified into the following forms: visceral (VL), cutaneous (CL), mucocutaneous (MCL), diffuse cutaneous (DCL), and post-kala-azar dermal (PKDL) leishmaniasis, although there are a variety of clinical manifestations in each category of the clinical forms. Among these clinical cases, VL is the most serious forms characterized by irregular bouts of fever, substantial weight loss, swelling of the spleen and liver, and anaemia (Bryceson, 1996). If left untreated, the fatality rate of VL cases can be as high as 100% (WHO, 1993). CL forms produce self-healing or non-healing skin ulcers or nodules leaving permanent scars on the exposed skin surface of the body such as the face and the upper and lower extremities, conferring a life-long immunity on the host against the challenge with the same species or subspecies of the genus *Leishmania.* Furthermore, MCL, DCL and PKDL forms, especially the second form (DCL), are usually resistant for the drugs available commercially, and MCL leads to partial or total destruction of the mucous membranes of the nose, mouth and throat cavities and surrounding tissues including the cartilage accompanying with serious secondary infections. The scars and disfigurings caused by leishmaniasis can cause serious social prejudice, and cast out the patient from society (WHO, 1996).

2. Changing patterns of the prevalence and magnitude of the disease

The diseases are endemic in 88 countries on the five continents, with a total of 350 million people at risk, and the diseases afflict at least 12 million peo-

ple worldwide (WHO, 1996). These numbers are however probably underestimated; figures of 2 million new cases per year, including 1.5 million cases of CL and 500,000 of VL are likely (Desjeux, 1996). Over the last ten years, endemic regions of leishmaniasis have been spreading further afield and there has been a sharp increase in the number of recorded cases of the disease (Hashiguchi, 1996). In Tunisia, for example, 1,300 cases of CL were reported in 1983, then the figure had reached 6,000 in 1991; in the northern states of Brazil, 2,000 cases were reported in 1980, and 9,000 in 1990. Furthermore, in the Stare of Bihar, India, where 38 out of 42 districts are affected, between 250,000 and 300,000 cases were estimated in 1992 - five times the official figure (WHO, 1993); the number of VL cases reported officially from Bihar during 1985 and 1993 increased from 13,030 to 75,523 and the number of deaths, from 35 to 1,417 (Dhanda *et al.,* 1996). Of all VL cases in the world, 90% cases occur in Bangladesh, India, Sudan, and Brazil; 90% of CL occur in Afghanistan, Iran, Saudi Arabia, Syria, Brazil and Peru; and 90% of MCL are reported from Brazil, Bolivia and Peru (WHO, 1993). Thus, leishmaniasis endemic regions and clinical cases are increasing annually without specific measures effective for the mass control after the first report from Turkey in 1756, about 245 years ago. Since 1993, geographic spread of the disease is also found due to the factors related mostly to development, including massive rural-urban migration, agro-industrial projects that being non-immune urban dwellers into endemic rural areas and man-made environmental changes like dams, irrigation systems and wells (WHO, 1996).

3. Leishmania/*HIV co-infection and the diagnosis and treatment of CL*

During the past few years, AIDS and other immunodepressed conditions have increased the risk of *Leishmania*-infected people developing visceral forms. *Leishmania*/HIV co-infection has emerged as a result of the increasing overlap between VL and AIDS, which is due to the spread of the AIDS pandemic in rural areas and that of VL in suburban areas, although some CL cases have been reported (WHO, 2000). Cases of co-infection have so far been reported in 33 countries worldwide. Most of the cases have been notified in south-western Europe. Of the total 1,440 *Leishmania*/HIV co-infection cases which have been reported to WHO since 1990, 49.8% cases were reported from south-western Europe (Spain, Italy, France and Portugal) during January 1996 and June 1998. Total number of reported cases in these countries increased greatly during 1990-1998 and 1996-1998 as follows: 835(1990-1998)/412(1996-1998) in Spain, 259/132 in France, 229/85 in Italy and 117/88 in Portugal (WHO, 2000). According to WHO (1996), of the cases reported, 90% were male, 86% were young adults (20-40 years of age), and 71% were intravenous drug users. In these Mediterranean basin *L. (L.) infantum* is the causative agent of VL, one of the vectors being *Phlebotomus perniciosus,* and dogs being a principal reservoir host. In these areas, however, direct infections from man to man by contaminated syringe, and anthroponotic infections (man-sandfly-man) are also frequent because of a high suitability of co-infected patients with abundant parasites as reservoir hosts (WHO, 1996). In eastern African countries (Ethiopia, Kenya, Malawi and Sudan) and the Indian subcontinent (India, Bangladesh and Pakistan), *Leishmania*/HIV co-infection is also increasing, owing to the simultaneous spread and geographical overlap of both diseases as well as periodic epidemics of VL (WHO, 1996, 2000). In the Mediterranian endemic areas, approximately 50% of all VL cases in adults are associated with HIV infection (Alvar *et al.,* 1992). Thus, in many areas of the world, the risk of overlap of leishmaniasis and HIV is increasing due to a number of factors such as mass migration, civil unrest war, resettlement programs, and promiscuity and prostitution in refugees camps.

Serologic diagnosis is of little use in *Leishmania*/HIV co-infected patients, because of absence of anti-*Leishmania* antibodies in 30%-43% of such cases (Alvar *et al.,* 1989; WHO, 2000). Therefore, immunologic diagnosis of VL in co-infected patients is difficult, requiring the use of combination of several

techniques for confirmation of the disease. Bone marrow aspirate remains the safest and first selective techniques, but spleen aspirate and liver biopsy are also used; in some cases search for the parasites can be done in peripheral blood samples (WHO, 1996). In the blood, the frequency of *Leishmania* is particularly noticeable; buffy-coat staining and culture increase diagnostic sensitivity; and leukocyte concentration is considered as an easy, fast and inexpensive technique (WHO, 2000). Molina *et al.* (1994) performed an indirect xenodiagnosis of VL in HIV-infected patients using laboratory colonized sandflies, *Phlebotomus perniciosus* females were fed on a membrane-feeding apparatus containing 2 ml blood from each patient, with heparin, EDTA, or sodium citrate as anticoagulants; blood samples were kept at 4 C for 6-96 hrs until use. Sandfly guts were dissected and examined for parasites 2-7 days after membrane feeding. Among their diagnostic methods used, indirect xenodiagnosis was only method that proved 100% effective in the co-infected patients, confirming it as an alternative in *Leishmania*/HIV co-infected patients. However, its use should be restricted to those cases where the more usual diagnostic techniques have failed and there is a strong suspicion of leishmaniasis in HIV-infected patients (Molina *et al.,* 1994). With regard to treatment for co-infected patients, pentavalent antimonials showed a positive response in 83% of cases, with 52% relapsing rate of co-infected patients within a period of one month to three years; the main alternative drugs include pentamidine, amphotericin B and liposomal amphotericin B (WHO, 1996). Recently, an oral drug, miltefosine (hexadecyl phosphocholine), a phosphocholine analogue which interferes with cell signaling pathways in tumours has been shown to be highly effective for VL (Sunder *et al.,* 1998, Jha *et al.,* 1999). The same drug was used in a 29-year-old male VL/HIV co-infected patient from Bihar, India, and a good and satisfactory result was obtained; the patient was an antimonial resistant case (Thakur *et al.,* 2000). Therefore, in the future, a trial of this drug should be undertaken in a larger number of such co-infection cases, especially in the Mediterranean region where *Leishmania*/HIV co-infection is estimated to be found in 3-7% of total HIV patients (Alvar, 1994; Gorgolas and Miles, 1994).

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Chapter 3

Further Comments on the Andean Leishmaniasis

ABSTRACT. Andean highland leishmaniasis was reviewed briefly, especially emphasizing on the causative agents of the disease forms in Peru and Ecuador. Ecuadorian form was clinically very similar to Peruvian uta, but the causative agents were completely different between the two countries. In the Andes regions, several *Leishmania* species were reported from different countries as follows: *L.(V.) peruviana* in Peru, *L.(L.) mexicana* and *L. (L.) major*-like in Ecuador, *L. (L.) mexicana* in Colombia, *L. (L.) garnhami* in Venezuela and *L. (V.) braziliensis (braziliensis* complex) in Argentina, but the last species from Argentina is doubtful because of the old report more than 70 years ago. A brief review was made on the problem of species status of *L. (V.) peruviana,* focusing on the discussions done in the literatures for a long time widely.

Andean leishmaniasis was principally named to the disease forms found in the Andes of Peru, and it was called uta. In this text, a brief review was given to the Andean highland leishmaniases including uta found in the New World, focusing on the form in Ecuador. Cutaneous leishmaniases (CL) from the Andean highland of 800-2,900m above sea level in the New World were reported from five countries, *viz.,* Peru, Ecuador, Colombia, Venezuela and Argentina, hitherto. As the causative agents of the disease from these countries, the following five species of the genus *Leishmania* were reported: *L. (Viannia) peruviana* from Peru, *L. (Leishmania) mexicana* and *L. (L.) major*-like from Ecuador, *L. (L.) garnhami* from Venezuela, and *L. (V.) braziliensis (L. braziliensis* complex) from Argentina. With regard to the identification or distribution of the species from these areas, there is a different opinion; *L. (L.) garnhami* might be synonymous with *L. (L.) amazonensis* (Grimaldi and Tesh, 1993), and *braziliensis* complex from the Andes of Argentina is doubtful because of its old publication reported more than 70 years ago (Mazza, 1926). A search for vector sandfly is not thoroughly done in the Andean regions; to date four species of the genus *Lutzomyia, viz., peruensis, verrucarum, ayacuchensis* and *youngi* were incriminated as vectors of CL at different endemic countries (Table 3.1). Investigations on the reservoir hosts of Andean highland leishmaniasis have not also been done intensively. To date, only three species of wild or domestic mammals were reported from the following countries: *Canis familialis* (dogs) from Peru and Ecuador, *Rattus rattus* (brown rat) from Ecuador, and *Didelphis marsupialis* (opossums) from Venezuela (Hashiguchi *et al.,* 1991; Herrer, 1982; Scorza *et al.,* 1979).

1. Peruvian uta and its causative agents, vectors and reservoirs

Uta was first described from Peru in 1913 by Strong *et al.* who recognized different types of skin lesions from those found in other Central and South American countries. As to the scientific name, the specific name, *peruviana* was already used by Velez in the same year (1913), before Strong *et al.* (Gardener, 1977). After that, anyway, the Andean form of leishmaniasis from 800-2,900m above sea level has been mentioned as a unique type of the disease clinically totally different from the lowland disease forms (Lumbreras and Guerra, 1985). It was considered for a long time that the reservoir host of Peruvian uta would be only domestic dogs, without giving much attention to other mammals; recently, it was found that wild mammals in the area also have an

Table 3.1. Andean highland leishmaniasis in the New World: its causative agents, *Leishmania* spp., vector sandflies, *Lutzomyia* spp. and reservoir mammals in different countries

Country	Altitude (a.s.l.)	*Leishmania* spp.	*Lutzomyia* spp.	Mammalian reservoirs
PERU	900-2,900m	*L. (V.) peruviana*	*Lu. peruensis* *Lu. verrucarum* *Lu. ayacuchensis*	*C. familialis*
ECUADOR				
Paute	2,300-2,500	*L. (L.) mexicana* *L. (L.) major*-like	*Lu. ayacuchensis*	*C. familialis* *R. rattus*
Huigra	1,200-1,500	*L. (L.) mexicana*	*Lu. ayacuchensis*	
Chanchan	1,500	*L. (L.) mexicana*	*Lu. ayacuchensis*	
Alausi	2,300-2,500	*L. (L.) mexicana*	*Lu. ayacuchensis*	
COLOMBIA	1,500	*L. (L.) mexicana*	not determined	not determined
VENEZUELA	800-1,600	*L. (L.) garnhami*	*Lu. youngi*	*D. marsupialis*
ARGENTINA	1,200	*L. (V.) braziliensis*	not determined	not determined

(Modified from Hashiguchi, 1994)

important role in the transmission of the disease (Perez *et al.,* 1991).

Clinically, in uta one or several ulcer or papular lesions are characteristic, and the lesions are usually spontaneous healing without any specific treatment; no mucocutaneous cases are found in uta (Lumbreras and Guerra, 1985). Such a lesion is clinically very similar to the Oriental sore caused by *L. (L.) tropica;* therefore, in the past few decades it was considered that the causative agent of uta would have been imported (or transported) into the Andes region, by some unknown routes. However, it was suggested that the agent of uta should belong to *braziliensis* complex, but not to *L. (L.) tropica,* based on the results *in vitro* cultures and hamster infections, in addition to the clinical features (Lainson and Shaw, 1972). Moreover, the agent, *L. (V.) peruviana* has several characteristics such as ecology and/or epidemiology showing restricted endemicity in the Andean valleys, hind-gut localization of the parasites in sandflies, and zymodeme patterns. From these features, *L. (V.) peruviana* has been mentioned as distinct species from *L. (V.) braziliensis* (Lainson, 1983; Lainson and Shaw, 1987; Walton, 1987). Thus, the problem of species status of *L. (V.) peruviana* has been discussed widely. Some researchers suggested that the species may simply be a variant of *L. (V.) braziliensis* and not valid species (Grimaldi, Tesh and McMahon-Pratt, 1989). But others had different opinions on this point. During these discussions, many workers made a wide range of investigations on the species status of *L. (V.) peruviana,* isolating the parasites mainly from uta patients, and performing different molecular analyses based on zymodemes, serodemes, schizodemes and karyodemes.

Consiquently, it was suggested that the two species, *L. (V.) peruviana* and *L. (V.) braziliensis* would be closely related species and/or might belong to the same species (Grimaldi *et al.,* 1987; Romero *et al.,* 1987; Lopez *et al.,* 1988). On the other hand, Arana *et al.* (1990) reported that *L. (V.) peruviana* could be clearly differentiated from *L. (V.) braziliensis,* by observing the two isozymes, MPI (mannose phosphate isomerase) and MDH (malate dehydrogenase). Recently, Dujardin *et al.* (1993a,b) made an intensive studies on karyotype polymorphism and conserved characters in the *L. (V.) braziliensis* complex explored with chromosome-derived probes, and also made studies on karyotype variation in *L. (V.) peruviana,* indicating geographical populations in Peru distributed along a north-south cline. Furthermore, Banuls *et al.* (2000) performed a characterization of a set of 38 *Leishmania* stocks from the Andean valleys of Peru, by employing multilocus enzyme electrophoresis (MLEE) and random amplified polymorphic DNA (RAP). Their data were analyzed in

terms of taxonomy and evolutionary genetics, and supported the view that the two species of the genus *Leishmania* correspond to two closely related, but distinct monophyletic lines (clades) and can therefore be considered as "discrete typing units". As Banuls *et al.* (2000) mentioned, confirmation of the distinctiveness of these two species is all the more desirable since they are associated with very distinct clinical forms, *L. (V.) peruviana* causing simple uta but *L. (V.) braziliensis,* espundia, a serious mucocutaneous lesion.

With regard to sandfly vectors in the Andes, a relatively little investigation has been done in comparison with other regions. It may be due to the application of control measures for bartonellosis which exists at the same region in the Andes and transmitted by sandflies; as a result, the measure has been causing difficulties for sandfly surveys, especially for their specimen collections. In the Andean foci of uta, *Lutzomuyia peruensis* and *Lu. verrucarum* were incriminated as probable vectors and recently *Lu. ayacuchensis* was also incriminated (Herrer, 1982; Perez *et al.,* 1991; Dujardin *et al.,* 1993b).

2. Andean leishmaniasis in Ecuador: its agents, vectors and reservoirs

An intensive leishmaniasis research has been done from 1982 to date, by our project. During the period, Andean highland leishmaniasis was first reported in1986 at a small town Paute, Province of Azuay, located at 2,300-2,500m above sea level (a.s.l.) of the Andes valley. By reviewing clinical cases from different areas of the country, we found several cases from Andean highland, and visited the place (Paute) from where the patients came; the small and superficial lesions observed were completely different from the lesions observed in lowlands of Ecuador. And then a similar type of cutaneous leishmaniasis was also found in other three areas, Alausi (2,300-2,500m), Chanchan (1,500m) and Huigra (1,200-1,500m). The lesions observed in the patients from these areas in Ecuador were very similar to Peruvian uta reported by Lumbreras and Guerra (1985), in addition to ecological similarities of the endemic areas. However, the causative agents are completely different between the two countries, Peru and Ecuador; *L. (V.) peruviana* in Peru and *L. (L.) mexicana* and *L. (L.) major*-like in Ecuador. The parasites, *L. (L.) mexicana* were isolated from *Lu. ayacuchensis,* rats *(Rattus rattus)* and dogs (Takaoka *et al.,* 1990; Gomez and Hashiguchi, 1991; Hashiguchi *et al.,* 1991). From the results obtained, it was suggested that Andean leishmaniasis would have more complicated epidemiological and ecological features of the disease at different endemic areas of Ecuador.

In order to disclose transmission mechanism(s) or ecology of Andean leishmaniasis in Ecuador, we made a longitudinal study on the natural infection of sandfly *Lu. ayacuchensis* with *L. (L.) mexicana,* in addition to epidemiological surveys and a search for reservoirs (Gomez and Hashiguchi, 1991; Hashiguchi *et al.,* 1991). Based on these data, we proposed a model for *Leishmania* transmission in the regions endemic for Andean leishmaniasis. From the model, it was suggested that transmission cycle of leishmaniasis in the Andes involved variable overlapping of two sets of the biological entities with a degree of overlap governed by micro- and macro-climatic conditions, depending on the seasonal climatic conditions in the area. The first set of the entities consisted of three categories of habitats (open field, periurban area and urban area). Each of these habitats was occupied at the same time by humans and domestic and wild mammals such as dogs and others. The second set consisted of the relationship between the sandfly vectors and the principal reservoir hosts presumed to rats *(Rattus rattus)* and other rodents. A high intensity of transmission in Andean leishmaniasis-endemic area (Paute) occurred from October to December in open field, largely between the wild mammals living in rock crevices and the vector sandflies. During the rainy season from January to February, the sandfly population increased gradually. The new sandfly generation expanded their biting activity towards periurban area, and transmission of *Leishmania* parasites to humans by sandfly bites intensified in this season. During March to April, there was a high density of infected sandflies and an increased population of wild rodents living peridomiciliary was also found in the periurban area (unpublished data); sandfly biting activity

extended into urban area with maximum overlap of the domestic and wild hosts and the vector sandflies. Therefore, many leishmaniasis cases were observed in this season at urban area (Hashiguchi *et al.,* 1991). During May to June, the end of the rainy season caused a rapid decline of sandfly density; flies disappeared from the urban area of Paute town, although they were still observed in periurban areas and open fields. From July to September, sandfly density fell to a minimum, because of extremely dry conditions in the Andes plateau. Nevertheless, rock crevices and animal burrows in the open field allowed the breeding of sandflies even in this dry seasons. From these results, it was suggested that *Leishmania* transmission in the open field mainly occurred between wild mammals (rats) and sandflies throughout the year, keeping *Leishmania* life cycle in the area. In periurban area of the Andes, transmission time continued to May or June lasting about five or six months. On the other hand, it was very short in urban areas, lasting only two months from March to April with high incidence. Changes in the incidence were considered to be the result of both sandfly and reservoir migrations in three habitats (urban, periurban and open field). In conclusion, we recommended that in the future, control measure should be applied during dry seasons when the transmission was extremely restricted to open field, including rock crevices and animal burrows.

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Chapter 4

Parasitology and Vector Entomology

1. Detection of *Endotrypanum* Using Polymerase Chain Reaction (PCR) and Southern Blotting

ABSTRACT. *Endotrypanum* and *Leishmania,* which infect sloths (reservoirs) and sandflies (vectors), are morphologically, biochemically and molecular-biologically similar in many aspects making it difficult to differentiate them. *Endotrypanum* and some of the pathological *Leishmania* species infect the same mammalian reservoirs and sandfly vectors in the New World. It is, therefore, epidemiologically important to differentiate the *Leishmania* spp. from the *Endotrypanum* spp. using polymerase chain reaction (PCR) and Southern blotting. Many vacuoles of similar morphology were found under electron microscope in the cytoplasms of both the *Endotrypanum* and *L. (Viannia) equatorensis* examined. It was difficult to distinguish *Endotrypanum* from *L. (Leishmania) major* morphologically. A specific band for *Endotrypanum* was observed in PCR using primer we designed. A positive signal on the same band was obtained by Southern blotting. The base sequences of the PCR products of *Endotrypanum* were almost same. However, there were bands of identical base sequences for *Endotrypanum* and *L. (V.) equatorensis,* which was used as a negative control. From the results obtained in this study, we concluded that it is possible to detect *Endotrypanum* using the present primers. It can also be suggested to have a strong posibility that *L.(V.) equatorensis* belongs to the genus *Endotrypanum.*

Introduction

Endotrypanum and *Leishmania* species infect the same mammalian reservoirs (sloths) and insect vectors (sandflies). It, therefore, is epidemiologically important to differentiate the promastigotes isolated from the sandflies and sloths. Genus *Endotrypanum* parasites exist in the red blood cells of sloths in Central and South-America. The vector is the sandfly belonging to the genus *Lutzomyia* (Shaw, 1964). It is difficult to discern *Endotrypanum* and *Leishmania* promastigotes morphologically in the gut of the sandflies (Christensen and Herrer, 1976, 1979). In leishmaniasis-endemic areas, however, the pathological *Leishmania (Viannia)* species share the same mammalian intermediate host reservoirs and insect vectors (Arias *et al.,* 1985). Therefore, it is very important to determine whether parasite isolated from the sandflies or mammalian reservoirs are disease-causing *Leishmania* species or harmless *Endotrypanum* species.

Polymerase chain reaction (PCR), Southern blotting and election microscopy were performed to differentiate the *Leishmania* spp. from the *Endotrypanum* spp. *(E. monterogeii, E. schaudinni).*

This report states that it is possible to distinguish between *Endotrypanum* and *Leishmania* by PCR, Southern blotting and sequence analysis.

Materials and Methods

Sources of parasite stocks

Sources of parasite stocks used in this study are listed in Table 4.1.1.

DNA purification and PCR assay

The parasites were cultured in RPMI1640 medium (Nakaraitesuku, Japan) with inactivated 10% FBS (Bio Whittaker, USA). The parasites were then centrifuged and collected. They were suspended in 500μl lysis buffer (2% SDS, 10mM Tris-HCl (pH8.0), 150mM NaCl, 10mM EDTA) and left overnight with proteinase K 500 μg/ml at 56℃. Phenol-chloroform and chloroform-isoamylalcohol were added and then centrifuged again. A precipitate was produced by adding 5N NaCl and Ethanol.

DNA was collected from the precipitate. The DNA was dissolved in dH_2O and 250ng/ml was used as a template. The primer we designed and used was 5'-CGACCGACCTACCGCCAGCAG-3', 5'-CAAGGT-GCATCACTCGCGTG-3'. The PCR conditions were, an initial denaturation for 3 min at 94℃, followed by 35 cycles each of denaturation for 1 min at 94℃, annealing for 1 min at 60℃ and extension for 1 min at 72℃. Final extension at 72℃ for 5 min was performed at MJ Research Minicycler, USA. The Taq polymerase and PCR solutions used were from Takara Co. LTD., Japan.

Cloning of PCR products and sequence analysis

The amplified PCR products were electrophoresed on a 1% SeaKam GTG agarose gel(FMC, USA). The gel with the amplified band was cut and purified on a SUPREC I and SUPREC II column (Takara, Japan). The purified DNA was ligated to pT7 Blue T vector (Novagen, USA), and transformed DH5 α *E.Coli.* After blue-white selection and subcloning the *E. Coli* was cultured in a LB culture medium for 16 hrs at 37℃. The plasmids were collected with a QIAprep Spin Miniprep Kit (Qiagen, USA) and the base sequence was determined by the Hitachi SQ5500 sequencer.

Hybridization assays

The non-RI kit used for southern blotting was the ECL 3'-oligolabelling and detection system (Amersham Life Science, USA). The probe used had a common base sequence with each stock, 30bp, 5'-ACGAGGT-GTTGTTCAAGCTGGCGGATG GT-3'. In the Southern blotting method, PCR was performed and after electrophoresis the products were alkalynized with 0.2N NaOH-0.6M NaCl solution. This was transferred to a nylon membrane Hybond™N+ (Life Science, USA). The nylon membrane was washed with 0.1XSSC (Na_3 citrate, NaCl), at 50℃.

Electron microscopy

Parasites in cultures were harvested by centrifu-

Table 4.1.1. *Leishmania* and *Endotrypanum* strains used in this study

International codes	Species
MCHO/EC/82/LSP-1	*L. (V.) equatorensis*
MSCI/EC/82/LSP-2	*L. (V.) equatorensis*
MHOM/EC/88/INH-03	*L. (V.) braziliensis*
MHOM/BR/75/M4147	*L. (V.) guyanensis*
MHOM/PA/71/LS94	*L. (V.) panamensis*
MHOM/VE/76/JAP78	*L. (L.) garnhami*
MHOM/BR/73/M2269	*L. (L.) amazonensis*
25-25M-C2-2M	*L. (L.) donovani*
MHOM/VE/57/LL1	*L. (L.) pifanoi*
MHYC/BZ/62/M379	*L. (L.) mexicana*
MHOM/BR/74/M2682	*L. (L.) chagasi*
MHOM/SU/58/Strain OD	*L. (L.) tropica*
MHOM/EC/88/PT-115	*L. (L.) major-like*
MHOM/SU/73/5ASKH	*L. (L.) major*
MCHO/CR/62/A9	*E. monterogeii*
MCHO/BR/80/M6159	*E. schaudinni*

gation, washed with cacodylate buffer (0.1M sodium cacodylate), and fixed with 1% osmium tetroxide for 1 hour at 4℃, and then dehydrated with an ethanol series and propylene oxide. The samples were 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 (JEOL, Tokyo, Japan).

Results

Analysis of PCR and Southern blotting using the PCR products

Positive bands were found at the same size in *L. (V.) equatorensis* (LSP-1=MCHO/EC/82/LSP-1, LSP-2=MSCI/EC/82/LSP-2), *E. monterogii* = MCHO/ CR/62/A9, *E. schaudinni* = MCHO/ BR/80/ M6159 and at the different size in 25-25M-C2-2M=*L. (L.) donovani* with our primer. No positive bands were found in other parasites, such as MHOM/ EC/88/INH-03 *(L. (V.) braziliensis),* MHOM/BR/75/M4147 *(L. (V.) guyanensis),* MHOM/PA/71/LS94 *(L. (V.) panamensis),* MHOM/ VE/ 76/ JAP78 *(L. (L.) garnhami),* MHOM/ BR/73/M2269 *(L. (L.) amazonensis),* MHOM/VE/57/ LL1 *(L. (L.) pifanoi),* MHYC/ BZ/62/ M379 *(L. (L.) mexicana),* MHOM/BR/74/M2682 *(L. (L.) chagasi),* MHOM/SU/58/Strain OD *(L. (L.) tropica),* MHOM/EC/ 88/PT-115 *(L. (L.) major*-like), MHOM/SU/73/5ASKH *(L. (L.) major)* (Fig. 4.1.1a). We used base sequence of 30bp common to four species of protozoans as probe, and performed Southern blotting. Positive signal was found in *L. (V.) equatorensis* (LSP-1, LSP-2), *E. monterogii* and *E. shaudinni,* but the result was negative in *donovani* (Fig. 4.1.1b).

Determination of the base sequence of the amplified DNA

The size of the base sequence obtained by the above methods was 164bp in three kinds of protozoa such as *L. (V.) equatorensis* (LSP-1, LSP-2), *E. monterogii* and *E. shaudinni.* But the size was different from 155bp in *L. (L.) donovani,* and the homology of the base sequence of *L. (V.) equatorensis* (LSP-1, LSP-2) and *E. monterogii* was exactly same except 1 base. On the other hand, the base sequence of *E. shaudinni* had a difference of 13 bases (Fig. 4.1.2).

The results of Electron microscopy

Eectron microscopic findings revealed that all four parasites showed slender to pear-shaped forms with flagellum, indicating promastigote forms (Fig. 4.1.3A-D). There were more vacuoles and droplets in the cytoplasms of three parasitess such as *L. (L.) equatorensis* LSP-1 and 2, *E. schaudinni* than *L. (L.) major.* Some vocuoles contain electron-dense materials in the same density as lipid droplets, which suggests that the vacuoles were the lysosomes which were losing their lipid contents. These findings were seen in the samples of *L. (V.) equatorensis* LSP-1 and *E. schaudinni.* In contrast, the cytoplasmic vacuoles were not so many in the cytoplasm of *L. (L.) major.* Virus-like particles (VLPs) were observed only in *L. (L.) major* in this study (Fig. 4.1.3E.)

Discussion

Until recently, the classification of Trypanosomatidae was done using morphological criteria of the parasites. However, it was difficult to classify clearly and differentiate each species morphologically. In recent years, there have been attempts to classify the parasites by their biochemical, and molecular biological characteristics (Beverley *et al.,* 1987; Ashall and Miles, 1988; Lopes and McMahon-Pratt, 1989; Lopes *et al.,* 1990; Fernandes *et al.,* 1993; Branquinha *et al.,* 1994, 1995; Medina-Acosta *et al.,* 1994a,b; Franco *et al.,* 1996; Croan *et al.,* 1997). However, search is still going on for a suitable method to clearly differentiate between the *Leishmania* spp. and the *Endotrypanum* spp. In the *Endotrypanum* spp. life-cycle, the organism takes the shape of an epimastigote or a trypomastigote in blood of its vertebrate host (sloths). In the sandfly vector, and in the culture medium they take the form of promastigotes (Shaw, 1969, 1992). Currently, there are two known species of the *Endotrypanum* spp., the *E.*

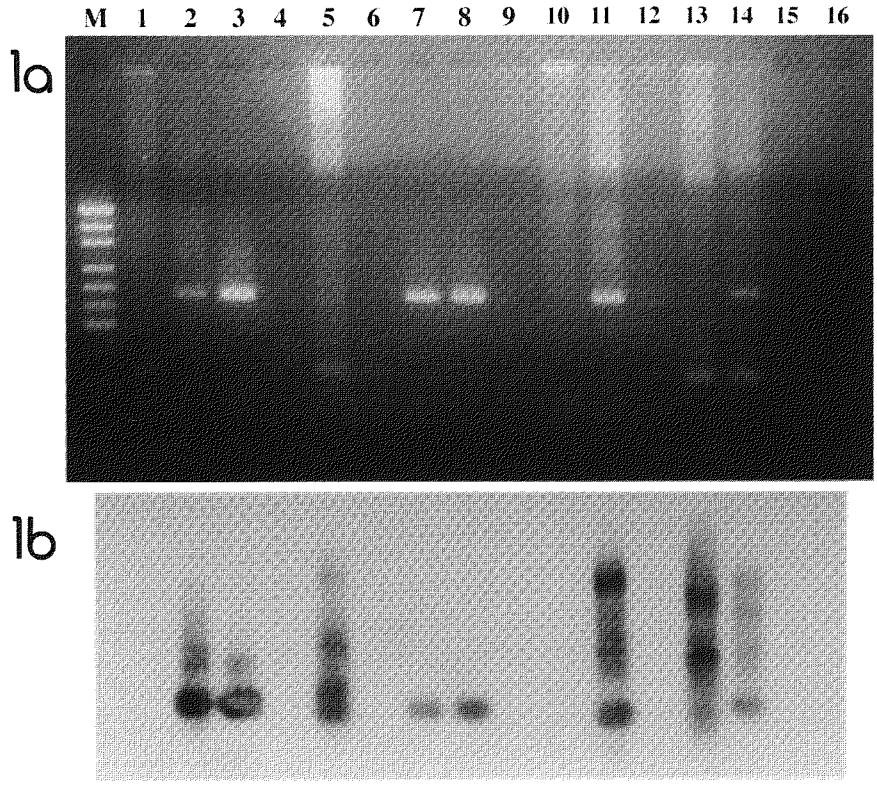


Figure 4.1.1. 4.1.1a Agarose gel electrophoresis of PCR products from *E. monterogeii* and *E. schaudinni* and *Leishmania* strains. DNA was electrophoresed through a 2.5% gel and visualized by staining with ethidium bromide. Lane 1, *L. (V.) equatorensis* (LSP-1); Lane 2, *L. (V.) equatorensis* (LSP-2); Lane 3, *L. (V.) braziliensis;* Lane 4, *L. (V.) guyanensis;* Lane 5, *L. (V.) panamensis;* Lane 6, *L. (L.) garnhami;* Lane 7, *L. (L.) amazonensis;* Lane 8, *L. (L.) donovani;* Lane 9, *L. (L.) pifanoi;* Lane 10, *L. (L.) mexicana;* Lane 11, *L. (L.) chagasi;* Lane 12, *L. (L.) tropica;* Lane 13, *L. (L.) major-like;* Lane 14, *L. (L.) major;* Lane 15, *E. monterogeii;* Lane 16, *E. schaudinni.* M, Molecular size marker is pUC19 digested with Hap II. **4.1.1b.** Southern blotting of PCR products hybridized with the probe in common with the inner portion of the amplified DNA.

schaudinni and the *E. monterogeii* (Croft *et al.,* 1980). The vertebrate host sloths and the vector sandflies are infected not only by the *Endotrypanum* spp. but also by a subgenus *Viannia* (Christensen and Herrer, 1979; Arias *et al.,* 1985; Shaw, 1969). It is important to differentiate between the *Leishmania* and the *Endotrypanum* spp. as it is difficult to do so morphologically in their cultured state (Christensen and Herrer, 1976, 1979).

On the other hand, there are some reports of attempts to detect *Endotrypanum* by the use of molecular-biological methods. For example, Greig *et al.* (1989) reported that it was possible to detect *Leishmania* and *Endotrypanum* by dot-blot analysis using *L. donovani* total parasite DNA as a probe. They reported that it was difficult to differentiate the two genera because both gave positive signals. Pacheco *et al.* (1990) performed a kDNA cross-hybridization on *Endotrypanum* and *Leishmania.* The probe they used was *E. schaudinni* kDNA. Signals were seen in both *E. schaudinni* and *L. (V.) braziliensis.* From these results, they concluded

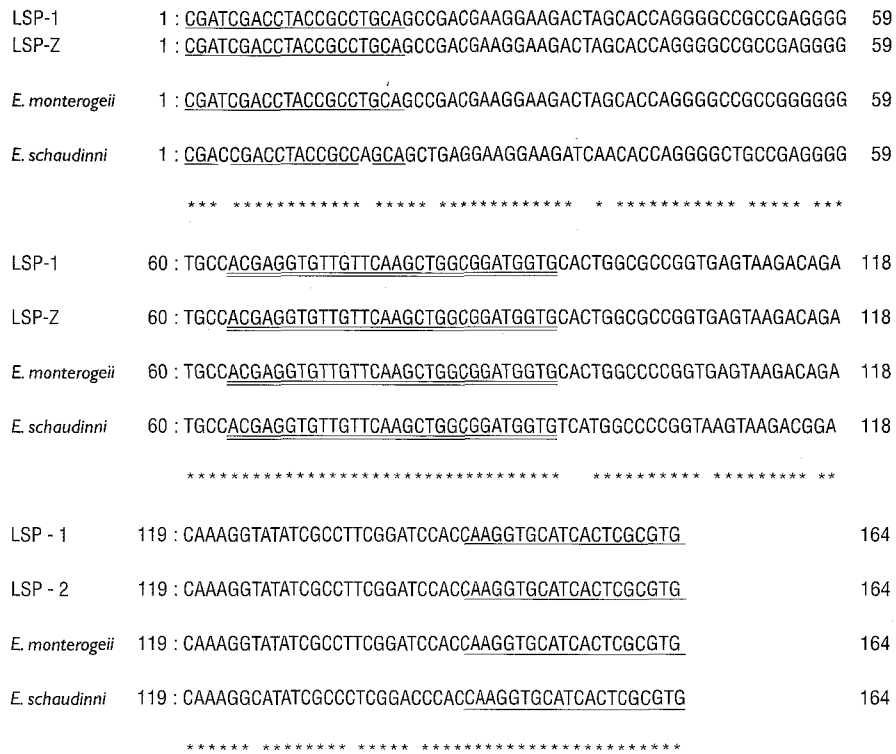


Figure 4.1.2a. Nucleotide sequence alignments of the *L. (V.) equatorensis,* LSP-1. LSP-2; *E. monterogeii* and *E. schaudinni.* Identical bases are indicated by an asterisk. The primers are underlined and the probe is double underlined.

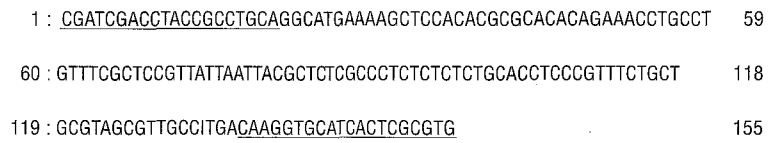
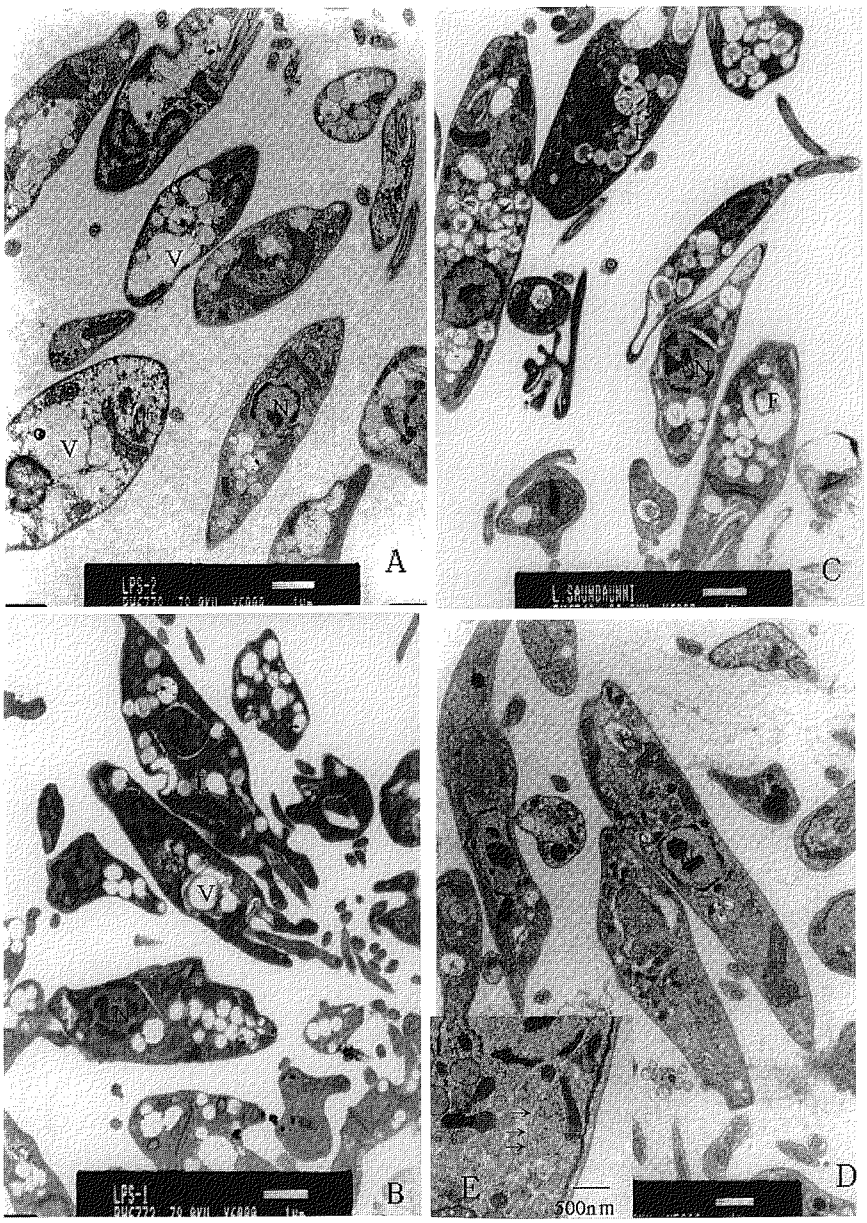


Figure 4.1.2b. Nucleotide sequence of the *L. (L.) donovani.*

that there was a close relationship between *Endotrypanum* and *L. (V.) braziliensis.* However, in our PCR results we found that there were no amplified DNA bands for the *Leishmania* spp. including *L. (V.) braziliensis* but there were identical positive bands for *L. (V.) equatorensis* LSP-1, LSP-2, *E. monterogeii* and *E. schaudinni.* Furthermore, in lane 8 *(L. (L.) donovani),* a thin band of amplified DNA consisting of several different sizes was obtained (Fig. 4.1.1a). This finding does not support the results of Pacheco *et al.* (1990). The *Endotrypanum* and *Leishmania* are not closely related but rather different and separate species. Fernandes, *et al.* (1993) detected *E. schaudinni* by PCR and Southern blotting using the *Endotrypanum* mini-exon gene as a primer. This primer provides the means to detect *E. schaudinni* and since the intergenic probe of *E. schaudinni* used in Southern blotting did not cross-hybridize with the New World *Leishmania* species, it is possible to dif-



Bar =1 μm, N=nucleus, V=vacuole, L=lipid droplet, F=flagellum, arrow= virus-like particles

Figure 4.1.3. Electron microscopic findings revealed many vacuoles in the cytoplasm of each parasite. **A.** *L. (V.) equatorensis* (LSP-2), **B.** *L. (V.) equatorensis* (LSP-1), **C.** *E. schaudinnii,* **D.** *L. (L.) major.* **E.** Virus like particles (arrows). Bar = 1nm, N = nucleus, V = vacuole, L = lipid droplet, F = flagellum.

ferentiate between the genus *Leishmania* and *Endotrypanum schaudenni.* It can be further concluded that this method can be used in epidemiological studies for the vectors and the mammalian reservoirs. Using our primers, a positive band was noted for *Endotrypanum, L. (V.) equatorensis* and *L.(L.) donovani.* However, *Endotrypanum* and *L. (V.) equatorensis* gave band size of 164bp while *L.(L.) donovani* showed band size of 155bp and in a different position making distinction easy. With Southern blotting, signals were obtained with *Endotrypanum (E. monterogeii, E. schaudinni)* and *L. (V.) equatorensis* (LSP-1, LSP-2) but not for *L. (L.) donovani.* The amplified DNA base sequences of *Endotrypanum (E. monterogeii, E.schaudinni)* and *L. (V.) equatorensis* (LSP-1, LSP-2) were almost identical. However, they were totally different in base sequence in comparison to *L. (L.) donovani.* It can therefore be concluded that it is possible to detect and differentiate *Endotrypanum* with the primer we used (Fig.4.1.2a, b).

Cupolillo *et al.* (1998) reported that *L. (V.) colombiensis* and *L.(V.) equatorensis* possibly belonged to the *Endotrypanum* spp. They concluded this by stating the results of the studies by Grimaldi *et al.* (1992), and also performing the minicircle kDNA heterogeneity analysis and the measurement of sialidase activity of *L. (V.) equatorensis.* Our results showed that *L. (V.) equatorensis* and *Endotrypanum* spp. were almost same and supported their conclusions. This strongly suggests the possibility that *L. (V.) equatorensis* belongs to the *Endotrypanum* spp. of trypanosomatids, requiring further investigations.

It was difficult to classify and differentiate clearly *Endotrypanum* spp. from *Leishmania* spp. morphologically. We inspected the *E. schaudinni, L. (V.) equatorensis* (LSP-1, LSP-2) and *L. (L.) major* by electron microscope. Megasomes of amastigote-like form of *L. (L.) mexicana,* containing lysosomal enzymes, may vary the homogeneity of their contents depending on nutritional or stress condition (Pral *et al.,* 1993). In the cytoplasm of promastigote-form of *L. (L.) mexicana,* vacuoles were observed under the short-time incubation with dermaseptin, an antifungal peptide (Hernandez *et al.,* 1992). *L. (L.) mexicana* promastigote as well as the amastigote-form may show the morphological appearance of the cytoplasmic vacuoles if the promastigotes are cultivated in the nutritionally poor conditions. In this study, *L. (V.) equatorensis* (LPS-1, LPS-2) and *E. schaudinni* showed cytoplasmic vacuoles which suggests that these three kinds of promastigote-form parasites might have been exposed to the nutritionally poor condition as compared to *L. (L.) major.* More appropriate conditions than the culture conditions in this study may be needed when these three kinds of promastigotes are cultivated *in vitro.* On electron microscopic examination, VLPs were frequently observed in the cytoplasm of some *Endotrypanum* spp. and *Leishmania spp.*(Croft *et al.,* 1980; Soares *et al.,* 1991; Molyneux, 1974). VLPs in this study were observed only in *L. (L.) major* as mentioned before.

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2. Studies on the Detection of Subgenus *Leishmania* Parasites Using Polymerase Chain Reaction (PCR) and Southern Blotting

ABSTRACT. In this study, an attempt was made to identify different *Leishmania* species by polymerase chain reaction (PCR) and Southern blotting. Fourteen *Leishmania* strains from stock were tested by PCR and southern blotting. A pair of primers were employed that anneal to the kinetoplast DNA sequence conserved among subgenus *Leishmania*. Of the 14 *Leishmania* strains used in this study, 6 strains showed strong bands of approximately 170 bp, and all the positive strains belonged to the species of the subgenus *Leishmania*, viz., *L. (Leishmania) garnhami, L.(L.) amazonensis, L. (L.) pifanoi, L. (L.) mexicana, L. (L.) chagasi* and *L. (L.) major*. All the species belonging to the subgenus *Viannia* used in this study were negative by PCR. Southern blotting of the PCR products showed identical results. These results suggest that the primer pair may be useful for identification of the species belonging to the subgenus *Leishmania* of the New World as well as to distinguish subgenus *Leishmania* from subgenus *Viannia*.

Introduction

Leishmaniasis is caused by a protozoan parasite of the genus *Leishmania* causing cutaneous leishmaniasis (CL), diffuse cutaneous leishmaniasis (DCL), mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL). There exist 21 different *Leishmania* species belonging to either subgenus *Leishmania* (includes the *Leishmania* species which develops in the foregut and midgut of the sand fly) or *Viannia* (develops in the midgut and hindgut of sand fly), affecting an estimated 12 million people of the World and producing 2 million new cases each year with an annual toll of 150,000 lives; 50% of the deaths are due to MCL and the rest due to VL (Lainson and Shaw, 1987; Desjeux, 1993; Desjeux, 1998; Klaus and Frankenburg, 1999).

For epidemiologic and therapeutic purposes, identification of the causative *Leishmania* species is very important. For the last several years developments have been achieved in identifying *Leishmania* species using different techniques such as isoenzyme analysis, polymerase chain reaction (PCR) and using monoclonal antibodies. But to carry out these procedures isolation of the parasite in culture is necessary which is very difficult in case of some parasites such as *L. (Viannia) brasiliensis* and *L. (L.) infantum* from some cutaneous lesions (Ben-Ismail *et al.*, 1992). Again the isolation rate is rarely more than about 70% efficient even in case of easily cultured parasites (Noyes *et al.*, 1998). In the recent years, PCR has been proved to be a sensitive, specific as well as rapid method for detection of a variety of parasites in different clinical samples (Smits and Hartskeerl, 1995). A number of PCR based methods for diagnosis of leishmaniases by detecting parasite genomic DNA have been reported with varying specificity (Rodgers *et al.*, 1990; De Bruijn and Barker, 1992; Piarroux *et al.*, 1993); but a few could identify the parasite precisely.

In our previous studies, we developed a method for species identification of subgenus *Viannia* from clinical samples using PCR and Southern blotting analysis (Uezato *et al.*, 1998a,b). In the present report, we have extended these previous studies and attempted to identify the *Leishmania* species precisely of the subgenus *Leishmania* applying PCR and Southern blotting.

Materials and Methods

1.Parasites and in vitro culture conditions

Fourteen strains of *Leishmania* from stock were used in this study including some strains isolated from leishmaniasis patients and reservoir hosts from Ecuador (Table 4.2.1). They were cultured *in vitro* in RPMI 1640 (Nakaraitesuku, Japan) medium containing 10% heat inactivated fetal bovine serum (FBS) (Bio Whittaker, USA) supplemented with antibiotics (30 μg/ml Ampicillin + 100 μg/ml Gentamycine) at 24℃. Genomic DNA was extracted from cultured promastigotes as described previously (Uezato *et al.*, 1998 a, b).

2. PCR conditions and visualization of PCR products

A sense primer (US2 outer) 5'-TGGTGGAAATTG GTGGGAAA-3', and an anti- sense primer (13B) 5'-ATTTTACACCAACCCCCAGTT-3' were employed. The PCR reaction mixture (50 μl) contained various amounts of genomic DNA, 100 pmol each of forward and reverse primers, 2.5 nmol of dNTP, 1 U Taq DNA polymerase in the PCR buffer (Takara Co., Ltd., Japan). The reaction was carried out in MinicyclerTM (MJ Reseach, USA) amplification cycle consists of denaturation at 94℃ for 3 min, followed by 35 cycles of denaturation at 94℃ for 1 min, annealing at 54℃ for 1 min, extension at 74℃ for 2 min. The PCR products were separated by electrophoresis in 2.5% agarose gel with 0.5 μg/ml ethidium bromide.

3. Sequence determination of the PCR products

Following the agarose gel electrophoresis, major bands of approximately 170 bp were excised. The DNA in the bands were extracted and inserted into pT7 blue T-vector (Novargen, USA). After transformation into DH5a *E. coli* and plasmid purification, nucleotide sequences of cloned inserts were determined.

4. Sensitivity and specificity of the primers

In order to examine the sensitivity and specificity of the primers, a dilution cascade series was prepared using 20 ng/μl, 10 ng/μl l, 1 ng/μl, 0.5 ng/μl, 0.1 ng/μl, 10 pg/μl, 1 pg/μl, 0.5 pg/μl, and 0.1 pg/μl of the final total DNA concentration of the respective parasite DNA as a template. PCR was performed under the above mentioned conditions by using a final volume of 50 ul l of PCR reaction solution prepared by mixing 1 μg/μl of human DNA to 1 μl of each parasite total DNA with the respective concentration. Each of the PCR solution was electrophoresed in 2.5 μlg/ml agarose gel. Southern blotting was performed to examine the band specificity of the primers. To degenerate the DNA obtained by the above procedure, 0.2N NaOH and 0.6M NaCl were used. The degenerated DNA was transferred to a positively charged nylon membrane (HybondTM-N+, Amersham Life Science, USA). Southern blotting was performed using a non-RIECL 3'-oligolabelling and detection systems kit (Amersham Life Science, USA) by following its protocol. The probe was examined to amplify six stocks of base sequence and was decided 32 bp (5'-GRAAAATGRGTGCAGAAAMCCCGTTCATW WTT-3' (R=A/G: M=C/T: W=A/T)) common to the base sequence as probe. The blot was prehybridized at 42 C for 30 min in 5 x SSC (1 x SSC = 0.15M sodium chloride +0.015M trisodium citrate), 0.1% hybridization buffer component, 0.02% SDS (sodium dodecyl sulphate), and 20 fold dilution of the liquid block in the kit. Hybridization was performed at 42 C for 2 hrs after adding 10 ng/ml of the labeled probe. Washing was done two times under the conditions: 5 min in 1 x SSC, 0.1% SDS at room temperature and 15 min in 0.2 x SSC, 0.1% SDS at 60 C, respectively. The blot was exposed for 2 hrs to X-ray film.

Results

Six out of 14 *Leishmania* strains examined in this study, showed strong bands of approximately 170 bp when specificity of the primer set was examined by PCR and Southern blotting using relatively large amount (1μg) of genomic DNA of these 14 strains (Fig.4.2.1). Nucleotide sequence determination of amplified DNA confirmed that primers were precisely targeted to intended regions of kinetoplast DNAs (Fig.4.2.2). All the positive strains belonged to the subgenus *Leishmania* and no strain of the subgenus *Viannia* showed positive results in this study (Table

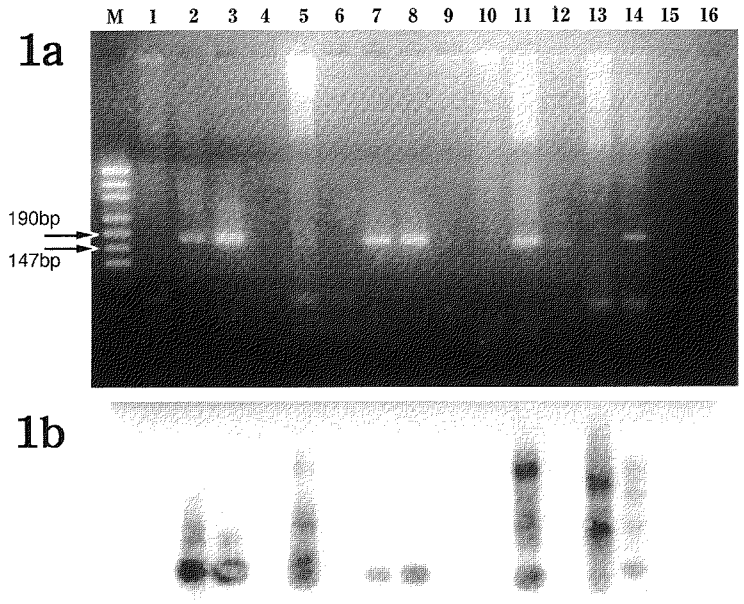

Figure 4.2.1. 4.2.1a The results of PCR with 14 *Leishmania* strains. M: size marker; pUC19/HapII; lane 1 = *L. (V.) braziliensis;* lane 2= *L. (L.) garnhami;* lane 3 = *L. (L.) amazonensis;* lane 4 = *L. (V.) braziliensis;* lane 5 = *L. (L.) donovani;* lane 6 = *L. (V.) guyanensis;* lane 7 = *L. (L.) pifanoi;* lane 8 = *L. (L.) mexicana;* lane 9 = *L. (V) panamensis;* lane 10 = *L. (V) equatorensis;* lane 11 = *L. (L.) chagasi;* lane 12 = *L. (L.) tropica;* lane 13 = *L. (L.) major-like;* lane 14 = *L. (L.) major;* lane 15 = normal human skin DNA; lane 16 = PCR solution. Lanes 2, 3, 7, 8, 11 and 14 showed positive bands. **4.2.1b.** The results of Southern blotting using 14 *Leishmania* strains. Lanes 2, 3, 7, 8, 11 and 14 showed positive signals.

```
No. 1  1:TGGTGGAAATTGGTGGGAAAAATGGGCCGAAAATCCAAACTTTTCTGCCCCGTGGGGG-AGGGGCGTTCTGCGATTTTGGGAAAAATGAGTGCAG
No. 2  1:TGGTGGAAATTGGTGGGAAAAATGGGTCCCGGC-CCAAACTTTTCTGCCCCGTGGGGG-AGGGGCGTTCTGCGGATTGGCGAAAAATGAGTGCAG
No. 3  1:TGGTGGAAATTGGTGGGAAAA-TGCCGAAAATC-CCAAACTTTTCTGGTCC-TCCACCGAGGGGCGTTCTGCGGGGACCTGGAAAATGAGTGCAG
No. 4  1:TGGTGGGAAATGGCCTGAAAA-TGGGGGAATTC-CCAAACTTTTCTGCCCCGTGGGGG-AGGGGCGTTCTGCGAAAATGGGAAAAATGAGTGCAG
No. 5  1:TGGTGGAAATTGGTGGGAAAAGTGGGTCCCGGT-CCAAACTTTTCTGCCCCGTGGGGG-AGGGGCGTTCTGCGATTTTGGGAAAAATGAGTGCAG
No. 6  1:TGGTGGGAAATGGTCAAAAA--TAGCTCATTTTTCCAAACTTTTCTGGTCCC-GCGGGTAGGGGCGTTCTGCGAAATTC-GAAAAATGGGTGCAG

No. 1  95:AAACCCCGTTCATAATTTAGGGGAATTTCTCGGAATCCGGCTCCGGGCGTGGAAACTGGGGGTTGGTGTAAAATA  169
No. 2  94:AAACCCCGTTCATAATTTGGGGGATTTTGGAGAATTCCGGCTCCGAGGCTCGAAACTGGGGGTTGGTGTAAAATA  168
No. 3  93:AAACCCCGTTCATAATTTCCCCAAAAATGCCCAAAAATGCCTCGGGGCGTCGAAACTGGGGGTTGGTGTAAAATA  167
No. 4  95:AAACCCCGTTCATAATTCCGGGGAAATTCCCGAAAATCGGCTCGGGGCGTCGAAACTGGGGGTTGGTGTAAAATA  169
No. 5  94:AAACCCCGTTCATATTTTGGTCGGAAATCCCGAAATTCGGCTCGGACGGTCACAACTGGGGGTTGGTGTAAAATA  168
No. 6  92:AAATCCCGTTCATTTTTGGCCAGAAAATGTCAATTTTGGGCTCGGAGGCGGGAAACTGGGGGTTGGTGTAAAATA  166
```

Figure 4.2.2. Alignment of base sequence homology of six species of the subgenus *Leishmania.* No. 1, *L. (L.) garnhami;* No. 2, *L. (L.) amazonensis;* No. 3, *L. (L.) pifanoi;* No. 4, *L. (L.) mexicana;* No. 5, *L. (L.) chagasi;* No. 6, *L. (L.) major.* A dash is gap. The primers are underlined and the probe is double underlined.

Table 4.2.1. *Leishmania* strains used in this study and results of PCR

No.	Strain designation	Species	PCR
1	MHOM/EC/88/INH-03	*L. (V.) braziliensis*	-
2	MHOM/VE/76/JAP78	*L. (L.) garnhami*	+
3	MHOM/BR/73/M2269	*L. (L.) amazonensis*	+
4	MHOM/BR/75/M2904	*L. (V.) braziliensis*	-
5	25-25M-C2-2M	*L. (L.) donovani*	-
6	MHOM/BR/75/M4147	*L. (V.) guyanensis*	-
7	MHOM/VE/57/ LL1	*L. (L.) pifanoi*	+
8	MHYC/BZ/62/M379	*L. (L.) mexicana*	+
9	MHOM/PA/71/LS94	*L. (V) panamensis*	-
10	MSCI/EC/82/Lsp-2	*L. (V) equatorensis*	-
11	MHOM/BR/74/M2682	*L. (L.) chagasi*	+
12	MHOM/SU/58/StrainOD	*L. (L.) tropica*	-
13	MHOM/EC/88/PT-115	*L. (L.) major-like*	-
14	MHOM/SU/73/5ASKH	*L. (L.) major*	+

4.2.1).

The genomic DNA from parasites cultured *in vitro* contained no additional DNA from any other species. The sensitivity of this PCR method and possible influence by human genomic DNA were examined by using diluted parasite genomic DNA (20 ng to 0.1 pg) to PCR in the presence of 1 μg of human genomic DNA. It was shown that, even 0.5 pg of genomic DNA from *L. (L.) amazonensis* gave a single clear band of approximately 170 bp, and the normal human DNA did not show any band (Fig.4.2.3). These observations indicate that the PCR method employed in this study is quite sensitive and that human DNA does not influence the results at all. Even 0.1 pg of the parasite genomic DNA, that gave no visible band with ethidium bromide staining, gave a very strong signal, when a degenerate internal probe was designed that anneals to the 32 bp sequence conserved among these PCR products (Fig.4.2.4) and used to probe the same PCR products as above after Southern transfer onto a nylon membrane, and the result was not influenced by human DNA.

Discussion

Leishmaniases caused by different *Leishmania* species are distinct in their clinical manifestations, prognosis, and responses to medication (Lainson and Shaw, 1987; Navin *et al.*, 1992). Precise identification of causative parasite species gives an important information for the choice of drug (Navin et al., 1992). But it is impossible to distinguish the species of *Leishmania* morphologically. To identify the species high resolution techniques such as, isoenzyme electrophoresis or PCR is necessary. Till few years back, culture of the parasite was necessary to carry out these procedures, which again is a cumbersome process and time consuming. Again the isolation rate of some parasites by culture is not up to the expected level. Therefore, the researchers have began to apply PCR directly on clinical samples to identify the parasite (Rodgers *et al.*, 1990; De Bruijn and Barker, 1992). The dermatologists have recently begun to employ molecular biological techniques for detection of these parasites from skin lesions. However, a definite method has yet to be established. For instance, a method based

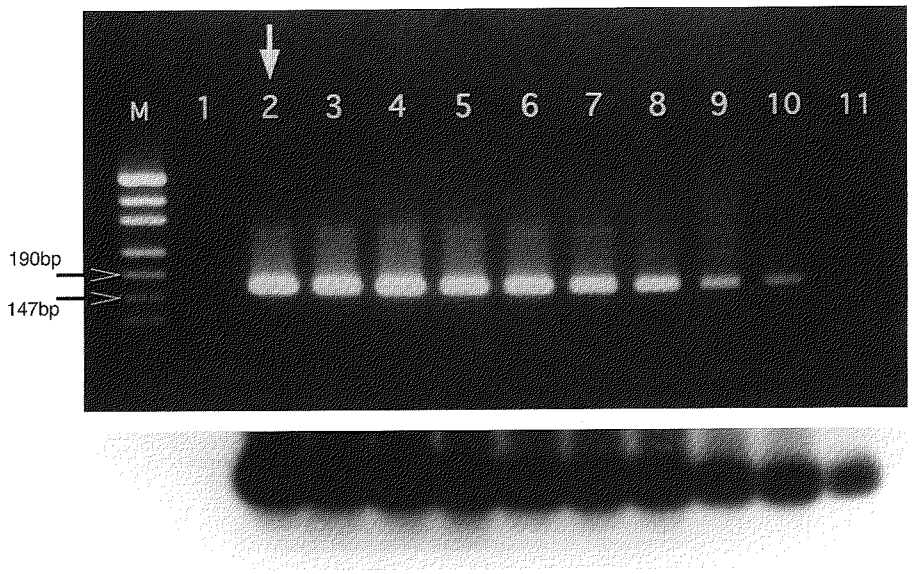

Figure 4.2.3. Results of PCR and Southern blotting using the PCR products from the dilution cascade series of the total positive DNA of *L. (L.) amazonensis;* human DNA (concentration: 20 ng/µl) was added in each lane except lane 2. Positive bands were found from lane 2 to lane 10 using PCR. Positive signals were found from lane 2 to lane 11 using Southern blotting.

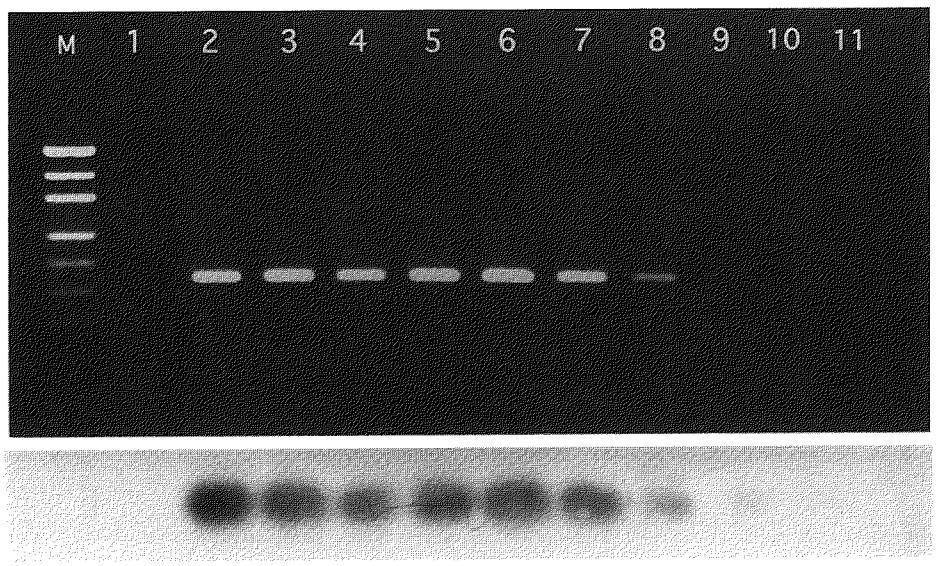

Figure 4.2.4. Results of PCR and Southern blotting using the PCR products from the dilution cascade series of the total positive DNA of *L. (L.) mexicana;* human DNA (concentration: 20 ng/µl) was added in each lane except lane 2. Positive bands were found from lane 2 to lane 9 using PCR. Positive signals were found from lane 2 to lane 9 using Southern blotting.

on PCR and Southern blotting reported by Rodriguez et al. (1994), could detect two species belonging to the genus *Leishmania, L. (L.) mexicana* and *L. (V.) braziliensis,* but data regarding other species have not yet been reported.

We have previously reported specific detection of subgenus *Viannia* parasites, by using PCR and Southern blotting, from skin lesions of Ecuadorian patients and Japanese patients immigrated from South America (Uezato *et al.,* 1998a, b). The aim of the present study was to apply the similar method in detecting the species of the subgenus *Leishmania.* A pair of primers were employed that anneal to the kinetoplast DNA sequence conserved among subgenus *Leishmania.* Of these, the reverse primer in our PCR (13 B) was same as that reported by Rodriguez et al. (1994), but the other (US2 outer) was designed by ourselves. The results (summarized in Table 4.2.1) indicate that this pair of primers could distinguish subgenus *Leishmania* from subgenus *Viannia.* All but 3 species of the parasites examined in this study belonging to the subgenus *Leishmania,* gave positive signals, but those belonging to the subgenus *Viannia* gave no signal. Among the three *Leishmania* species negative to PCR in this study, two were from the Old Wold and only the *L.(L.) major*-like was from the New World which is not a frequent agent of leishmaniasis. This specificity apparently comes from our new primer, since the primers used by Rodriguez *et al.* (1994) gave a positive signal with *L. (V.) braziliensis,* which belongs to subgenus Viannia. Since the sensitivity of detection was enhanced by combining PCR with Southern blotting in our previous studies (Uezato et al., 1998a, b), we examined again the effectiveness of this combination in the present study.

The primers used in this study are not only capable of detecting a number of species belonging to the subgenus *Leishmania* but also can exclude species belonging to the subgenus *Viannia.* Since all the positive strains belonging to the subgenus *Leishmania* used in this study were from the New World and most of the negative strains were from the Old World, and though the present study is yet to be extended in other settings with more number of strains and on clinical samples, it may be concluded from these results that the primer pair used in this study may be useful to detect species of the subgenus *Leishmania* of the New World and to differentiate subgenus *Leishmania* from the subgenus Viannia. Therefore, it may be a useful technique for screening of leishmaniases in the endemic areas of the New World.

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3. Detection of Natural Infections of Individual Sandfly with Leishmania Parasites in the Andean Areas of Ecuador Using Polymerase Chain Reaction (PCR)

ABSTRACT. Aims of the study were to find out the infection rate of the sandflies by *Leishmania* parasite and to know the usefulness of polymerase chain reaction (PCR) to detect *Leishmania* species from sandfly. Prevalence of *Leishmania* infections of the sandflies was studied in two endemic areas of leishmaniasis; Andean slope site, Chanchan (1,500 m above sea level) and Andean highland site, Alausi (2,300 m above sea level), Chimborazo, Ecuador. Sandflies were examined individually under microscope for *Leishmania* promastigotes and by subsequent PCR test. Eighty two sandflies from Chanchan and 141 from Alausi were identified as *Lutzomyia ayacuchensis* and subsequently dissected under microscope. *Leishmania* promastigotes were found in midguts of 5 (6.1 %) of the 82 sandflies caught from Chanchan; one of them was captured from inside and 4 from outside of houses. Thirteen (9.2 %) of the 141 sandflies captured from outside of houses of Alausi, were positive for flagellates under microscope. PCR results showed the band of *L. (Leishmania) mexicana* in all the samples prepared from promastigote positive sandflies. However, 3 (7,3 %) of the 41 promastigote negative sandfly samples were positive for *L. (L.) mexicana* DNA. So PCR may be a useful tool to detect *Leishmania* species from infected sandflies and thus may be used for future epidemiological surveys in leishmaniasis endemic areas.

Introduction

Leishmaniases have a worldwide distribution in the tropical and subtropical zones, approximately 12 million people in the world are already affected by this disease, where they present a considerable health problem (Desjeux, 2000). The precise identification of *Leishmania* species is important for the appropriate treatment regimen and public health surveillance since different species cause different clinical features of the disease.

Zymodeme (Kreutzer *et al.*, 1992), serodeme (Grimaldi *et al.*, 1987; Mimori *et al.*, 1989) and schizodeme (Baker, 1987) analyses have been performed for identification of *Leishmania* species. These techniques need a large quantity of materials. Polymease chain reaction (PCR) methods are also being used for identification and diagnosis of *Leishmania* parasites (Lopez *et al.*,1993; Bhattacharyya *et al.*, 1993). Recently, we designed *Leishmania* subgenus and species specific primers, and the five principal species; *L. (Viannia) panamensis, L. (V.) braziliensis, L. (V.) guyanensis, L. (Leishmania) mexicana* and *L. (L.) amazonensis* were identified using formalin fixed biopsy samples (Mimori *et al.*, 1998), and exudate/scrape samples (Matsumoto *et al.*, 1999).

Sandflies (*Lutzomyia* spp.) are the vectors of *Leishmania* parasites in the New World. However, it is very difficult to get *Leishmania* infected sandflies because of low rate of infections among these flies. Also search for the promastigotes under microscope in dissected sandflies is difficult in the field and it needs expertise.

In this study, we examined the usefulness of PCR technique to assess the infection rate among sandflies in leishmaniasis-endemic areas of Ecuador by comparing with parasite detection under microscope.

Materials and Methods

The study sites

Sandflies were collected from Chanchan, Province of Chimborazo, located at slope of the Andes, 1,500 m above sea level, and Alausi, Province of Chimborazo, located at plateau of the Andes, 2,300 m above sea level; both are endemic for Andean type of cutaneous leishmaniasis caused by *L. (L.) mexicana*. Sandflies were captured by using aspirator from protected human baits or resting sites inside and/or outside the houses, during 18:00 and 20:00. The collected sandflies were dissected, and then identified mainly based on the morphology of their spermathecae. These flies were examined for *Leishmania* promastigotes under the microscope. After these procedures, all the parasite-negative and positive flies were individually fixed in 0.5 ml absolute ethanol for future analysis by PCR.

DNA preparation

After centrifuging the specimen of sandflies, by washing distilled water, they were resuspended with 200 µl InstaGene™ Matrix (Bio Rad). The solution was incubated for 2 hr at 56℃ for separating genomic DNA, extracted with phenol and phenol/chloroform and precipitated with ethanol. The DNA was subjected to the PCR analysis.

PCR procedure for identification of Leishmania *at subgenus level*

PCR reaction was done in a 15 µl volume containing 10 mM dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 75 ng each primer, 0.3 Units r*Tth* DNA polymerase KL (Perkin Elmer) and 1 ml DNA template in a GeneAmp PCR System 2400 (Perkin Elmer). The primers, V; V1 (5'-GCTTCTCG-TTTCGCTTTGAAC-3') and V2 (5'-CAAGACAAGAAAAAAG GCGGC-3') for the detection of subgenus *Viannia* group, L; L1 (5'-GGTCACTCGGCATTTTTGC-3') and L2 (5'-GTGC-CCTGACTTGCATGTC TA-3') for the identification of subgenus *Leishmania* group (Mimori *et al.,* 1998), after at 95℃ initial denaturation for 5 min, 35 cycles (each comprises 30 sec at 95℃, 30 sec at 60℃, 30 sec at 72℃) followed by final extension at 72℃ for 10 min. Seven µl of each PCR product was analyzed by electrophoresis through a 2 % agarose gel containing ethidium bromide at 170 V for 0.5 hr.

PCR procedure for identification of Leishmania *at species level*

Polymorphism specific (PS)-PCR (Mimori *et al.,* 1998) was performed for identification of *Leishmania* at species level by using the following condition; in a 15 µl reaction volume using 0.2 Units of r*Tth* DNA polymerase for 35 cycles (30 sec at 95℃, annealing for 90 sec at 63℃, 30 sec at 72℃). Species specific primers were used as primer m; m1 (5'-TGCGAG-GATAAAGGGAAAGAG-3') and m2 (5'-GTGCC-CTGACTTGCATGTCTA -3') for *L. (L.) mexicana* and a; a1 (5'-TGCGAGGATAAAGGGAAAGA A-3'), and a2 (5'-GTGCCCTGACTTGCATGTCTA-3') for *L.(L.) amazonensis.*

Results

Sandfly species were identified and *Leishmania* parasites were detected in individual sandfly by microscopical examination and PCR analysis.

Microscopical examination

In this study, all the 223 sandflies examined were identified as *Lutzomyia ayacuchensis.* Eighty two sandflies from Chanchan and 141 from Alausi were dissected under microscope (Table 4.3.1). *Leishmania* parasites were found in the midgut of 5 (6.1 %) of the total 82 sandflies caught from Chanchan, one of them was captured from inside and 4 from outside of the houses in that area. On the other hand, *Leishmania* promastigotes were found in 13 (9.2 %) of the 141 sandflies trapped from outside of the houses in Alausi area.

PCR tests

The 78 bp products of DNA were observed in all the 18 samples prepared from the promastigote positive sandflies by PCR with primer 'L' and were identified as *L. (Leishmania)* subgenus. Subsequently, 62 bp PCR products were also detected with primer 'm' in all these 18 samples, and were identified as the

Table 4.3.1. Results of natural infections of individual sandfly *(Lutzomyia ayacuchensis)* by microscope and by PCR

Collectiong site	Microscope examination	No. of PCR examined	Result of PCR	Species
Chanchan, inside of house (1500 m)	Positive 1 (2.8 %)	1	Positive 1 (100 %)	*L. (L.) mexicana*
			Negative 0	
	Negative 3 5	2 9	Positive 3 (10.3%)	*L. (L.) mexicana*
			Negative 2 6	
Chanchan, outside of house (1500 m)	Positive 4 (8.7%)	4	Positive 4 (100 %)	*L. (L.) mexicana*
			Negative 0	
	Negative 4 2	1 2	Positive 0 (0 %)	
			Negative 1 2	
Chanchan, Total (1500 m)	Positive 5 (6.1 %)	5	Positive 5 (100 %)	*L. (L.) mexicana*
			Negative 0	
	Negative 7 7	4 1	Positive 3 (7.3 %)	*L. (L.) mexicana*
			Negative 3 8	
Alausi, outside of house (2300 m)	Positive 1 3 (9.2 %)	3	Positive 3 (100 %)	*L. (L.) mexicana*
			Negative 0	
	Negative 1 2 8	Not done		

species *L. (L.) mexicana* (Fig.4.3.1 A, A'; C, C'). However, 3 (7.3 %) of the 41 promastigote negative sandfly samples were also positive for *L. (L.) mexicana* DNA by PCR (Fig. 4.3.1B, B').

Discussion

Epidemiological survey of leishmaniasis was performed on the diagnosis and treatment for human patients, the reservoir hosts and the identification of vectors in Ecuador (reported by Hashiguchi *et al.*, 1987, 1990, 1992, 1994, 1997). In these studies, much information could not be provided regarding the identification of *Leishmania* species in sandflies since it was very difficult to isolate the parasite in culture medium without bacterial and/or fungal contaminations from the infected sandflies.

Recently, there were many reports that PCR was the useful tool for the diagnosis of the leishmaniasis compared with microscopic detection of the parasite in stained smear (De Bujin *et al.*, 1993; Laskay *et al.*, 1995: Mathis and Deplazes, 1995; Andresen *et al.*, 1996). A small quantity of parasite DNA in a sample can show positive results by PCR and thus this technique could directly be applied to diagnose the clinical cases using biopsy materials without parasite cultivation. In our previous reports, we showed that the identification of *Leishmania* subgenus and/or species could be detected by PCR using subgenus and/or species specific primers (Mimori *et al.*, 1998; Matsumoto *et al.*, 1999). There were some reports of identification of *Leishmania* from pooled sandflies samples by PCR (Rodriguez *et al.*, 1999; Calvopiña, 1999). In the present study, individual sandfly caught from two endemic areas of the Andes of Ecuador, was examined for *Leishmania* infection by microscopy and

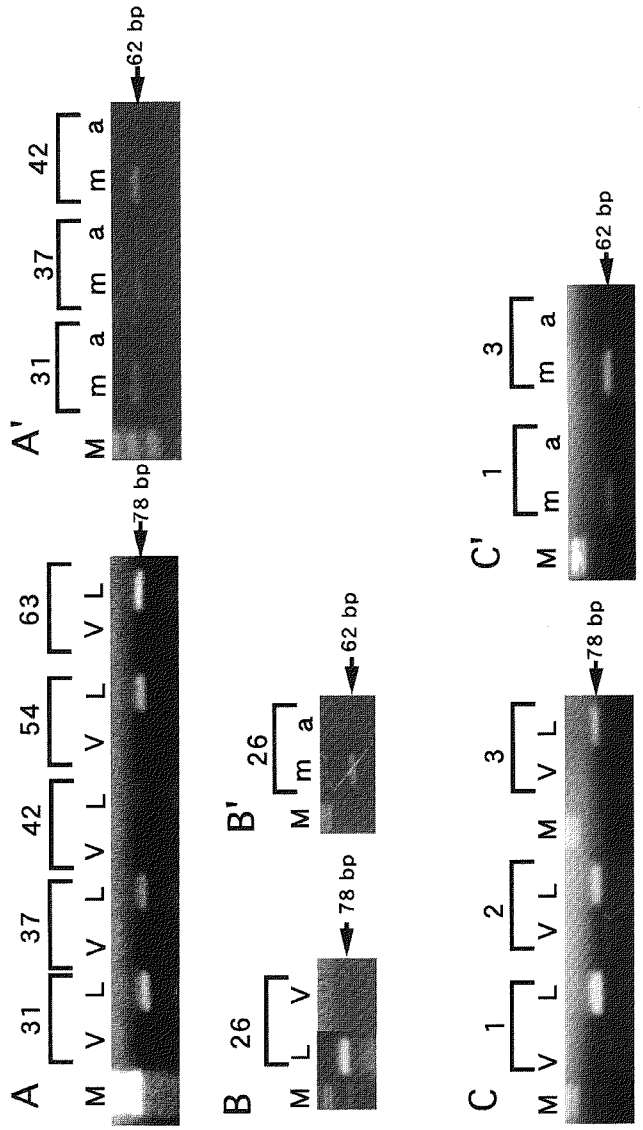

Figure 4.3.1. PCR products amplified from *Leishmania* parasites, separated on 2 % agarose gels containing ethidium bromide. M; 1 kb ladder. A and A': Samples of individual snadfly *(Lutzomyia ayacuchensis)* from Chanchan, promastigotes were observed under microscope. Nos. are individual sandfly number. B and B': Sample of individual snadfly *(Lu. ayacuchensis)* from Chanchan (1,500 m), promastigotes were not observed under microscope. No. is individual sandfly number. C and C': Samples of individual snadfly *(Lu. ayacuchensis)* from Alausi (2,300 m), promastigotes were observed under microscope. Nos. are individual sandfly number. A , B and C: PCR using primer V (V1-V2) for identification of the subgenus *L. (Viannia)* and primer L (L1-L2) for identification of the subgenus *L. (Leishmania).* A', B' and C': PCR using primer m (m1, m2) for identification of the species *L. (L.) mexicana* and primer a (a1, a2) for identification of the species *L. (L.) amazonensis.*

by PCR using our previously published PCR technique (Mimori *et al.,* 1998). It was known that the study sites, Chanchan and Alausi, were highly endemic areas for cutaneous leishmaniasis caused by *L. (L.) mexicana* (Gomez and Hashiguchi, 1991; Katakura *et al.,* 1997). In our study, all the PCR samples of the 18 promastigote positive sandflies showed bands identical to *L. (L.) mexicana* which was in accordance with the report of Katakura *et al.* (1997). There was no false negative result in PCR in samples which were positive for *Leishmania* promastigote under microscope. Moreover, PCR could identify parasites at the species level in this study. However, 3 (7.3 %) of the 41 microscopy negative sandfly samples were PCR positive for *L. (L.) mexicana.* It was, however, a question whether these 3 PCR positive cases were false positive or real positive. It was interesting that these sandflies, negative on microscopy but positive in PCR, were captured from inside of houses of the endemic area. As the PCR method has been proved to be more sensitive than microscopic detection of the parasite, so it may be presumed that those 3 sandflies might have infections with low number of parasites, or those were trapped in very early stages of parasites development in the gut of the sandfly. So further studies with more samples should be carried out urgently by this method. Therefore PCR may be a useful tool in the field survey of epidemiology for leishmaniasis.

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4. Isolation and Characterisation of the *Leishmania* Strains of Ecuador and Argentina by Using Molecular Tools and Monoclonal Antibody Based ELISA

ABSTRACT. To isolate and identify the *Leishmania* species of the New World, a total of 17 clinical samples were taken from the leishmaniasis lesions; 15 of them were from Ecuador and 2 from Argentina. All were smear positive, and *Leishmania* parasites were isolated in culture medium from all the samples. Nuclear DNA (internal transcribed spacer) and kinetoplast DNA were amplified, and sequencing of the PCR product was done along with characterisation of *Leishmania* species by serodeme analysis. Among the Ecuadorian strains, all the 8 *Leishmania* isolates from Huigra were identified as *L. (L.) mexicana,* all the 6 isolates from Puerto Quito and one from La Mana were identified as *L. (V.) panamensis.* Two isolates from Salta province of Argentina were characterised as *L. (V.) brasiliensis.*

Introduction

In the New World, leishmaniasis is endemic in many areas of Central and South Americas including Ecuador and Argentina (WHO, 1998). At least 18 *Leishmania* species cause diverse clinical forms of leishmaniasis with unique epidemiological pattern caused by each species. *L. (Viannia)* and *L. (Leishmania)* groups of parasites are the causative agents of cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and diffuse cutaneous leishmaniasis (DCL), and *L. (L.) chagasi* causes relatively less common visceral leishmaniasis (VL) in this area of the globe (Hashiguchi, 1992; 1997). Although some *Leishmania* species cause specific type of lesions, it is also evident that similar lesions are produced by different *Leishmania* species.

Accurate identification of the *Leishmania* species is necessary for the diagnosis of the disease especially in the cutaneous forms, whose prognosis can not be assumed by clinical features. Identification at species level can not be possible by conventional methods, such as smear, culture and histological observations. Many cases show variable clinical features and drug resistance depending on the parasite species. For example, sodium stibogluconate could cure lesions caused by *L. (V.) brasiliensis* but not the lesion caused by *L. (L.) mexicana,* and that ketokonazole cured the lesions caused by *L. (L.) mexicana* but could not cure the lesion by *L. (V.) brasiliensis* (Navin *et al.,* 1992). It was also reported that metastatic lesions caused by *L. (V.) brasiliensis* should be treated by systemic chemotherapy avoiding local administration of drugs. Moreover, with the spread of HIV/AIDS unusual presentation often occurs both in the Old and New Worlds. *Leishmania* species that normally cause only cutaneous disease may present with visceral leishmaniasis depending on the immune status of the host (Hernandez *et al.,* 1993; Roberts *et al.,* 2000). Identification of the species is also important in each endemic area (country) to understand the epidemiology of the disease and to take appropriate measures to control the diseases and to develop vaccine for endemic areas.

Zymodeme analysis is the standard and accurate method for the identification and classification of the parasite (Kreutzer *et al.,* 1980). This method, however, needs culture of the parasite which is a cumbersome and time consuming procedure. The other way to identify *Leishmania* species is by serodeme analysis by monoclonal antibody based ELISA (Mimori *et al.,* 1989; Furuya *et al.,* 1998). Recent efforts are going on to identify the *Leishmania* species by polymerase chain reaction (PCR) and many more

researchers are now concentrating on this technique to identify *Leishmania* parasite from nuclear and mitochondrial (kinetoplast) DNA (Rogers *et al.,* 1990; De Bruijn and Barker, 1992; Rodriguez *et al.,* 1994; Mimori *et al.,* 1998). In this study, we tried to characterize *Leishmania* isolates from Ecuador and Argentina by PCR, DNA sequencing, and monoclonal antibody based ELISA.

Materials and Methods

Leishmania *isolates and WHO reference strains used*

Fifteen samples were collected from Ecuador. Eight of them were from Huigra, Chimborazo, six were from Puerto Quito, Pichincha, and one was from La Mana, Bolivar. Two samples were collected from Oran, Salta province of northwestern Argentina. These 17 isolates and 5 WHO reference strains used in this study are shown in Table 4.4.1.

Ethical aspects

All the procedures were carried out following the medical ethics of the respective countries. Informed consent was obtained from each patient or in case of children, from their guardians before taking samples.

Collection of samples from skin lesions

Following the procedure of Matsumoto *et al.* (1999), some exudate was collected by syringe from the skin lesions suspected to have CL, MCL or DCL. A part of the syringe sucked aspirate was then inoculated in to modified NNN medium for culture of the *Leishmania* parasite and incubated at 25℃. One or two drops of the aspirated exudate was taken on to slide glass for smear preparation. The *Leishmania* strains grown in the culture medium was then transported to the Parasitology Department of Kochi Medical School, Japan for further study. All the *Leishmania* strains were maintained in USMARU medium (Evans, 1987) at Kochi Medical School.

Staining and light microscopy

All the smears were stained with Giemsa stain and examined under microscope for *Leishmania* amastigotes.

Extraction of DNA

Extraction procedure of DNA was performed according to the classical protocol using phenol:chloroform:isoamyle alcohol as well as DNA extraction kit (Qiagen, Tokyo, Japan) following the instructions of the company. The extracted DNA was then preserved at 4℃ after determining the DNA concentration by spectrophotometer (ULTROSPEC 3000, UV/visible spectrophotpmeter, Phammacia Biotech).

Primers used in this study

a) The following primers were used for identifying nuclear DNA (internal transcribed spacer)

U1 5' CAA CTC GGG GAG ACC TAT G 3'
U2 5' AAT ATG CGC ACA ACA CAA AC 3'

b) The following primers were used for identifying the kinetoplast DNA (Mimori *et al.,* 1998)

V1 (5'-G'CTTCTCGTTTCGCTTTGAAC -3')
V2 (5'-CAAGACAAGAAAAAAGGC GGC-3')

L1 (5'-GGTCACTCGGCATTTTTGC-3')
L2 (5'-GTGCCCTGACTTGCATGTCTA-3')

p1 (5'-GGTCGGATCTGCATG CATCAC-3'),
p2 (5'-CAAAAAGCGAGGGACTGCGGG-3')

b1 (5'-GTGGGCGTATCTGCTGATGAC-3'),
b2 (5'CAAAAAGCGAGGGACTGC GGA-3')

g1 (5'-GGTCGGATCTGCATGCATCAT-3')
g2 (5'-CAAAAAGCGAGGGACT GCGGG-3')

m1 (5'-TGCGAGGATAAAGGGAAAGAG -3')
m2 (5'-GTGCCCTGACTTGCATGTC TA -3')

a1 (5'-TGCGAGGATAAAGGGAAAGAA-3'),
a2 (5'-GTGCCCTGACTTGCATGTCTA-3')

PCR reactions

PCR amplification was carried out in a 25 μl final

volume containing 2 µl DNA, 2.5 µl 1xPCR buffer, 1.5 M $MgCl_2$, 25 µM of each dNTP, 10 pmoles of each primer, and 1.25 units of Taq DNA polymerase enzyme. Samples were subjected to initial denaturation at 94℃ for 10 min followed by 35 cycles of 94℃ for 45 sec, 55℃ for 30 sec and 72℃ for 1 min and 30 sec, followed by final extension at 72℃ for 10 min. For the detection of kinetoplast DNA by primers set 'b', the annealing temperature was set at 60℃ keeping the other conditions unchanged. After amplification, PCR products were separated through 1.5% agarose gel with ethidium bromide and the bands amplified were compared to those obtained with positive *Leishmania* DNA control. All the chemicals without DNA sample were also run in each reaction as negative control.

Table 4.4.1. WHO reference strains and *Leishmania* isolates from Ecuadorian and Argentine leishmaniasis patients used in this study

Stock	Disease forms	Species
WHO reference strains of the New World:		
MHOM/BR/75/M2904	cutaneous	*L. (V.) braziliensis*
MHOM/PA/71/LS94	cutaneous	*L. (V.) panamensis*
MHOM/BR/75/M4147	cutaneous	*L. (V.) guyanensis*
MHYC/BZ/62/M379	cutaneous	*L. (L.) mexicana*
MHOM/BR/73/M2269	cutaneous	*L. (L.) amazonensis*
[a]Clinical isolates from Ecuador:		
Strain Huigra 1	cutaneous	*L. (L.) mexicana*
Strain Huigra 2	cutaneous	*L. (L.) mexicana*
Strain Huigra 3	cutaneous	*L. (L.) mexicana*
Strain Huigra 4	cutaneous	*L. (L.) mexicana*
Strain Huigra 5	cutaneous	*L. (L.) mexicana*
Strain Huigra 6	cutaneous	*L. (L.) mexicana*
Strain Huigra 7	cutaneous	*L. (L.) mexicana*
Strain Huigra 8	cutaneous	*L. (L.) mexicana*
Strain PQ 4	cutaneous	*L. (L.) panamensis*
Strain PQ 13	cutaneous	*L. (L.) panamensis*
Atrain PQ 14	cutaneous	*L. (L.) panamensis*
Strain PQ 17	cutaneous	*L. (L.) panamensis*
Strain PQ 18	cutaneous	*L. (L.) panamensis*
Strain PQ V2	cutaneous	*L. (L.) panamensis*
LM 001	cutaneous	*L. (L.) panamensis*
[a]Clinical isolates from Argentina:		
AZ-1	cutaneous	*L. (L.) brasiliensis*
AZ-3	cutaneous	*L. (L.) brasiliensis*

[a], all the samples were amastigote positive in stained smears.

Band analysis

After running the PCR products in 1.5% agarose gel the bands were analysed by KODAK 1D image analysis software (Eastern Kodak Company, Scientific Imaging systems, Rochester, NY, USA) using the device Kodak 1 DAS 290.

DNA sequencing

After separating the PCR products in 1.5% agarose gel the band of DNA was cut and purified in filter column (Quantum Prep™ Freeze N Squeeze DNA Gel Extraction Spin Columns, Bio-Rad Laboratories, California, USA). The purified DNA was then precipitated with ethanol and resuspended in 8 µl 1/10 TE buffer and aliquotes were sequenced using the ABI PRISM kit. PCR primers were used as sequencing primers. The mixtures were then purified according to the manufacturer's instructions (ABI) using spin columns (CENTRI SEP, Applied Biosystems, Foster City, CA) and applied to an ABI PRISM Genetic Analizer. Alignment analyses were done using the programme Gentyx-Mac (ver. 11.0).

Sample preparation for ELISA

The harvested promastigotes were suspended in lysis buffer (10 mM Tris-HCL, pH 7.5, 2 mM EDTA, 1.6 mM phenylmethylsulfonyl fluoride, 2 mM iodoacetamide, 100 µg/ml leupeptin). After 3 bouts of sonication for 2 min each with an interval of 1 min the homogenate was centrifuged at 20000 g for 15 min. The protein concentration of the supernatant was determined and kept at -30℃ until use.

Species identification of Leishmania *by monoclonal antibodies*

We used different monoclonal antibodies (7 in total) for the New World *Leishmania* strains by enzyme-linked immunosorbent assay (ELISA) (Furuya *et al.,* 1998). 96 well micro-ELISA plate was coated with *Leishmania* antigen (10 µg/well), incubated at 4℃ overnight, and washed, and then diluted monoclonal antibodies were added and incubated. Peroxidase conjugated second antibody was added, and washed, and enzyme substrate (all from Kirkegaard and Perry Lab., MD, USA) was used and absorbance was measured at 405 nm.

Results

PCR and DNA sequencing

Using the primers U1 and U2, all the 5 New World *Leishmania* reference strains used in this study (Table 4.4.1) could be identified by observing bands of 350 bp to 510 bp. Results of band analysis using KODAK 1D image analysis software showed that the members of the *Viannia* subgenus gave the bands between 350 to 410 bp and those of Leishmania subgenus showed bands of around 500 bp (Fig. 4.4.1). Species of *Leishmania* could be presumed by observing these bands. *L. (L.) mexicana* gave a band of about 500 bp. Bands of about 350 bp, 380 bp, 410 bp and 510 bp are shown by *L. (V.) brasiliensis, L. (V.) panamensis L. (V.) guyanensis* and *L. (L.) amazonensis* respectively (Fig.4.4.2). DNA sequencing confirmed the diagnosis. All the strains of Huigra, Puerto Quito and La Mana area of Ecuador were identified as *L. (L.) mexicana, L. (V.) panamensis* and *L. (V.) panamensis* respectively using the primers U1 and U2. Two strains of Argentina were identified as *L. (V.) brasiliensis.* (Fig. 4.4.2C-E). Using other sets of primers (b), all the strains isolated from the patients of Huigra, Ecuador were characterised as *L. (L.) mexicana* by PCR, and confirmed by DNA sequencing. Using the primers m1 and m2, all the *Leishmania* strains isolated from patients of Huigra showed a band of 62 bp which corresponded to the 62 bp band of *L. (L.) mexicana* reference strain (Fig.4.4.4A, B). Similarly, all the strains isolated from patients of Puerto Quito, Ecuador showed positive bands of 79 bp with primers p1 and p2 identical to the 79 bp band of *L. (V.) panamensis* reference strain, and were identified as *L. (V.) panamensis* (Fig. 4.4.4C). Two isolates from Argentina were characterised as *L. (V.) brasiliensis* using primers b1 and b2.

Serodeme analysis

All the isolates from patients of Huigra, Ecuador were identified as *L. (L.) mexicana,* and all those of Puerto Quito and the isolate from La Mana were identified as *L. (V.) panamensis.* Two isolates from Argentina were characterised as *L. (V.) brasiliensis* by

using species specific monoclonal antibodies.

Discussion

Accurate diagnosis of leishmaniasis is important to ensure the early treatment to reduce the morbidity and mortality, and to assess the prognosis. The conventional diagnostic methods are to demonstrate *Leishmania* amastigote in the stained films of aspirated fluid and/or biopsy materials obtained from the CL, MCL, and DCL lesions and to observe the promastigotes in a suitable culture medium. But the sensitivity of these methods is low. So determination of *Leishmania* DNA by PCR may be the alternative method to diagnose the cases as it is highly sensitive and specific.

The objective of this study was to identify the *Leishmania* species of the New World by PCR and sequencing. A series of recent studies have focused on the use of PCR amplification of kinetoplast DNA and nuclear DNA from different clinical samples (Rogers *et al.,* 1990; De Brujin and Barker 1992; De Brujin *et al.,* 1993; Rodriguez *et al.,* 1994; Katakura *et al.,* 1997). Using the primers (U1, U2) constructed from the internal transcribed spacer, all the 5 New World WHO reference strains used in this study such as *L. (L.) mexicana, L. (V.) brasiliensis, L. (V.) panamensis L. (V.) guyanensis* and *L. (L.) amazonensis,* could be identified by observing bands of 350 bp to 510 bp. By observing these bands the species of *Leishmania* could be assumed. DNA sequencing confirmed the diagnosis. From the results obtained it may be suggested that the primers (U1, U2) employed may be useful for the screening of leishmaniasis as well as *Leishmania* species identification in the New World. In this study, all the *Leishmania* strains isolated from Huigra showed a band of about 500 bp when the internal transcribed spacer was amplified which was identical to the band shown by *L. (L.) mexicana* reference strain. All the strains of Puerto Quito and La Mana showed bands of approximately 380 bp similar to the band of *L. (V.) panamensis* reference strain. Two Argetine strains gave 350 bp bands similar to that of *L. (V.) brasiliensis* reference strain. All these were confirmed after sequencing the amplified PCR products. So sequencing of the amplified DNA may be a powerful tool to identify the *Leishmania* species. It

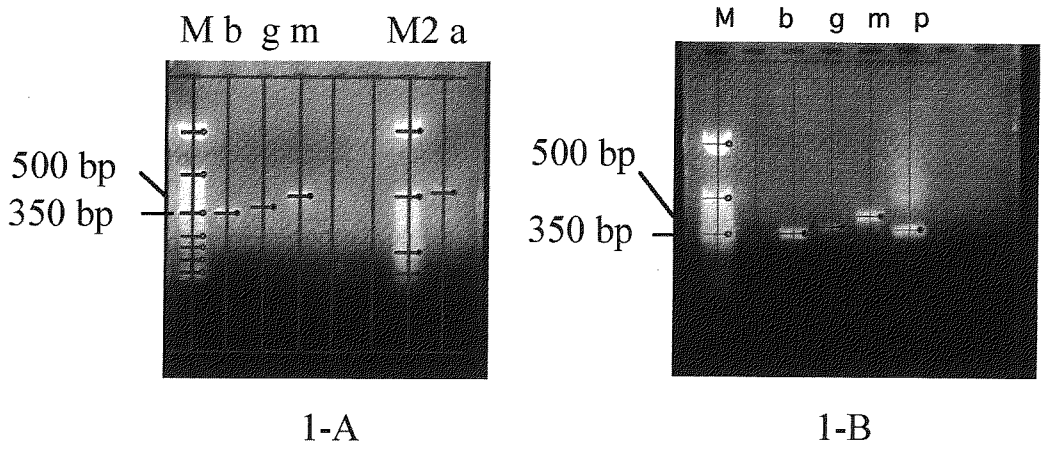

Figure 4.4.1. 1-A and **1-B.** Analysis of bands of PCR products amplified by primers U1 and U2 using KODAK 1D image analysis software by Kodak 1 DAS 290 device. M, 50 bp DNA ladder; M2, 25 bp DNA ladder; b, *L. (V.) braziliensis* (MHOM/BR/75/M2904); g, *L. (V.) guyanensis* (MHOM/BR/75/M4147); m, *L. (L.) mexicana* (MHYC/BZ/62/M379); p, *L. (V.) panamensis* (MHOM/PA/71/LS94) and a, *L.(L.) amazonensis* (MHOM/BR/73/M2269).

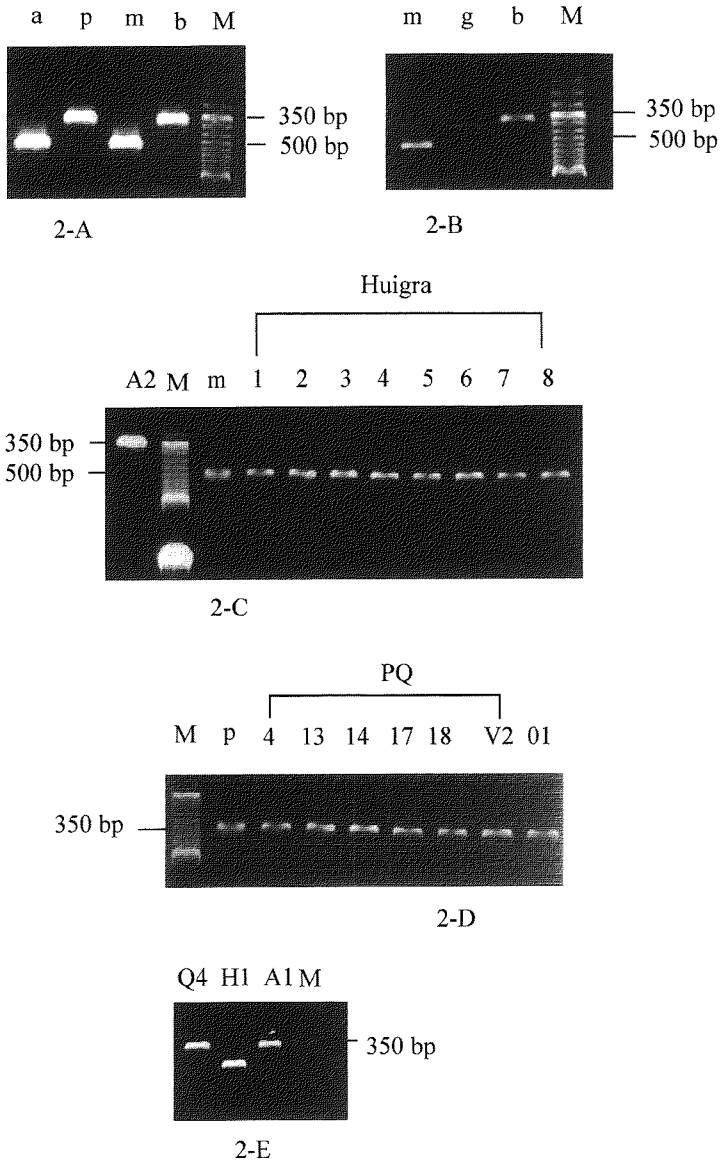

Figure 4.4.2. Amplified internal transcribed spacer of nuclear DNA from different *Leishmania* strains using primers U1 and U2. M, 50 bp DNA ladder. **2-A.** Amplified PCR products from WHO reference strains. b, *L. (V.) braziliensis;* m, *L. (L.) mexicana;* p, *L. (V.) panamensis;* a, *L. (L.) amazonensis.* **2-B.** Amplified PCR products from WHO reference strains. b, *L. (V.) braziliensis;* g, *L. (V.) guyanensis* and m, *L. (L.) mexicana.* **2-C.** A2, AZ-2; m, *L. (L.) mexicana* WHO reference strain; Huigra 1-8, strains isolated from patients of Huigra. **2-D.** p, *L. (V.) panamensis* WHO reference strain; PQ 4-V2, strains isolated from Puerto Quito, Ecuador and 01, LM-001, a strain isolated from La Mana, Ecuador. **2-E.** Q4, PQ4; H1, Huigra-1 and A1, AZ1.

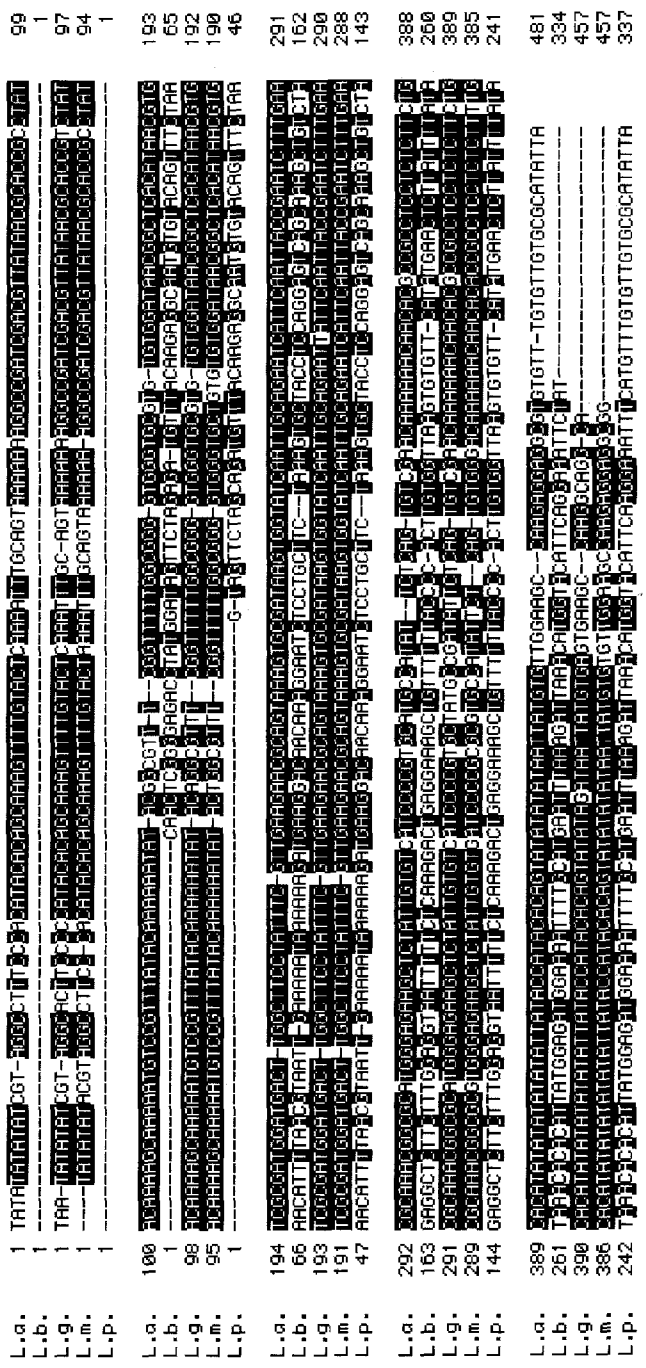

Figure 4.4.3. Multiple alignment of sequences of the amplified region of internal transcribed spacer of different *Leishmania* WHO reference strains. L.a., *L. (L.) amazonensis;* L.b., *L. (V.) braziliensis;* L.g., *L. (V.) guyanensis;* L.m., *L. (L.) mexicana;* L.p., *L. (V.) panamensis*

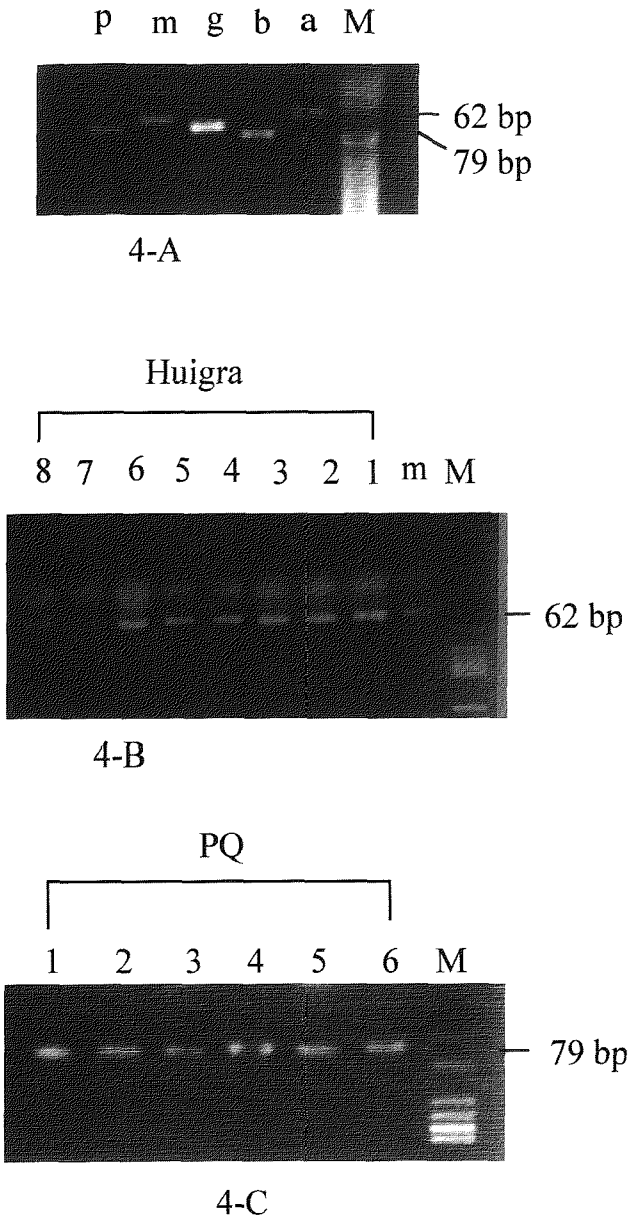

Figure 4.4.4. PCR products amplified from kDNA molecules from cultured *Leishmania* parasites using species specific primers separated on 1.5% agarose gels containing ethidium bromide. M, 50 bp DNA ladder. **4-A.** PCR products of the following WHO reference strains with specific primers of their own: p, *L. (V.) panamensis* (primers p1 and p2); m, *L. (L.) mexicana* (primers m1 and m2); g, *L. (V.) guyanensis* (primers g1 and g2); b, *L. (V.) braziliensis* (primers b1 and b2); a, *L. (L.) amazonensis* (primers a1 and a2). **4-B**. 62 bp DNA products of isolates from Huigra with primers m1 and m2; m, *L. (L.) mexicana* WHO reference strain. **4-C.** PCR products amplified from strains isolated from Puerto Quito: 1, PQ4; 2, PQ13; 3, PQ14; 4, PQ17; 5, PQ18 and 6, PQV2.

may be invaluable for epidemiological studies in different leishmaniasis-endemic areas (countries). Several reports have been published regarding successful identification of *Leishmania* species from clinical samples using same sets of primers constructed from kDNA (primers set 'b') used in this study (Mimori *et al.,* 1998; Matsumoto *et al.,* 1999). So more detailed studies using these primers are necessary on more *Leishmania* isolates from different parts of the New World.

Till to date, the different *Leishmania* species isolated from Ecuador are *L. (L.) mexicana, L. (L.) amazonensis, L. (L.) major*-like belong to the *L. (Leishmania)* group, and *L. (V.) brasiliensis, L. (V.) guyanensis L. (V.) panamensis* and *L. (V.) equatorensis* belong to the *L. (Viannia)* group. The results obtained in the present study were in accordance with previous studies reported in this project (Hashiguchi, 1987; 1990; 1992; 1994; 1997). The two isolates from Salta province of Argentina were characterised as *L. (V.) brasiliensis* by PCR and serodeme analysis. This finding coincides with a few published reports about the prevalence of *Leishmania* species in that country (Cuba *et al.,* 1996; Segura *et al.,* 2000). Considering the geographical location, the Salta province is very close to the endemic areas of Paraguey where *L. (L.) amazonensis, L. (V.) panamensis* and *L. (V.) brasiliensis* infections are prevalent (Mimory *et al.,* personal communication). There is, therefore, a possibility of presence of *Leishmania* species, other than *L. (V.) brasiliensis* in that area of Argentina. A further study should be carried out urgently on large number of isolates to realise or to exclude this possibility.

In conclusion it may be said that the primers (U1, U2) constructed from ITS region of DNA used in this study may be extremely useful to indentify the New World *Leishmania* species.

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Chapter 5

Diagnosis

1. Diagnosis of Cutaneous Leishmaniasis with Polymerase Chain Reaction (PCR) Technique in Comparison with Conventional Methods

ABSTRACT. In this study the diagnosis of cutaneous leishmaniasis was evaluated comparing the polymerase chain reaction (PCR) method with other currently recommended procedures: the smear technique, the cultivation on specific medium and the histopathology of biopsies. This study was realized on 72 residents of the canton El Carmen (province of Manabi, Ecuador) with cutaneous lesions presumably caused by *Leishmania* spp. infection. The PCR with specific oligonucleotids for the variant *braziliensis* was more sensitive (90.9%) than any other of the three methods used. The cultivation in the Navy-MacNeal-Nicolle (NNN) medium reached a sensitivity of 45.5% although 19% of the samples had to be excluded because of contamination with fungi or bacteria. The smear merely achieved a sensitivity of 40.9% and the histopathological evaluation, 36.4%. According to these results, the PCR proved to be more sensitive, specific and faster in diagnosing cutaneous leishmaniasis in endemic areas of Ecuador. These benefits could help reducing the mortality, sequels, costs and risks associated with unappropriated treatment.

Introduction

Leishmaniasis is one of the seven diseases of highest priority to the Tropical Disease Research, World Health Organization in matter of research and control. It is estimated that 12 million people are infected worldwide and that further 350 million live in areas of high risk of infection. Every year about 400,000 new cases are reported (Ahford *et al.,* 1992).

The leishmaniasis tegumentaria americana (LTA) includes a group of zoonotic disease with different clinical features depending on the *Leishmania* species, the immune response of the host and variable conditions regarding the vector's saliva (Grimaldi and Tesh, 1993). In Ecuador tegumentary leishmaniasis is a public health problem of large scale affecting as well the coastal area as the plains of the Amazon affluents, including areas of the Andean cordillera like Paute in the province of Azuay, Alausi and Huigra in the province of Chimborazo. In total 17 of the 22 provinces of Ecuador are endemic (Mouchet *et al.,* 1994; Hashiguchi *et al.,* 1987; Armijos *et al.,* 1997).

LTA is caused by protozoans of the genus *Leishmania* subdivided in three groups *i.e., L. (Leishmania) mexicana, L. (Viannia) braziliensis* and *L. (L.) donovani chagasi* (Grimaldi and Tesh, 1993; Harris *et al.,* 1998). The life cycle of the parasite requires vertebrate and invertebrate hosts. The promastigotes are found in the vector only (subgenus *Lutzomyia* and *Psychodopygus*). Once the parasites are inoculated into animals or humans by the sandfly bite and then they are phagocytosed by macrophages and transform into amastigotes, which reproduce by binary fission. The epidermis surrounding the insects bite ulcerates. In Ecuador different clinical symptoms of LTA have been observed suggesting that the lesions to be caused by different species and subspecies of the genus *Leishmania* (Armijos *et al.,* 1990, 1997;

Katakura *et al.,* 1992; Grimaldi and Tesh, 1992; Mimori *et al.,* 1989).

The efficiency and accuracy of the diagnosis of LTA are of increasing importance to improve the differential diagnosis of similar lesions of different etiology since the morbidity is rising at an alarming rate worldwide, and moreover because the therapy varies according to the identified species. In endemic areas of the New World the diagnosis is generally based on clinical criteria and few laboratories have the capacities of confirming the diagnosis. Specialized laboratories use the smear technique stained with Giemsa but its low sensitivity implicates a high percentage of false negative results (Lopez *et al.,* 1993; Navin *et al.,* 1990; Chico *et al.,* 1995). The low sensitivity and poor specificity of immunological techniques, due to cross reactions, especially in populations exposed to other parasitic infections of the *Trypanosomatidae* family like *Trypanosoma cruzi* and *T. rangeli* and other microorganisms such as *Plasmodium, Brucella* and *Mycobacteria tuberculosis* (Guevara *et al.,* 1989), account for their rare use as diagnostic tools. Alike the skin test of Montenegro using promastigote antigen does not differentiate between past and present infection and might turn out negative in early stages of infection or in patients with anergic diseases (Nonaka *et al,* 1990).

Specialized laboratories for the diagnosis of *Leishmania* use more than one test: the incubation of the parasite in specific cultures, the inoculation of aspirated material of biopsies in Golden hamsters is mandatory because of its high sensitivity. Nevertheless these examinations require trained personnel and adequate facilities for the preparation and incubation of the cultures as well as the laborious "care" of the Golden hamsters. Furthermore the multiplication of the parasite *in vitro* and the appearance of lesions in laboratory animals require days if not weeks. Moreover the outdoor work is often impeded by the contamination of the growth medium (Weigle *et al.,* 1987). The histopathological examination of fixed biopsies with formalin stands as an alternative for samples collected in rural areas but is only feasible in specialized laboratories.

Other alternative methods for identifying *Leishmania* species are biochemical techniques with isoenzymes, immunological techniques with monoclonal antibodies and molecular techniques. The progress of biomolecular research on DNA allows us today to characterize specific genomic sequences of many microorganisms (Eisenstein, 1990; Nutman *et al.,* 1994). In the case of *Leishmania* the characterization of DNA sequences of the kinetoplast minicircles has greatly simplified the identification of distinct species of *Leishmania* by amplification of those DNA fragments through PCR (Barker, 1987; Rodgers *et al.,* 1990; Eshita *et al.,* 1992). The subspecies of the subgenera *Viannia (panamensis, braziliensis* and *guyanensis)* and *Leishmania (mexicana* and *amazonensis)* are the most common in South American countries (Mimori *et al.,* 1998). In the coastal area of Ecuador *L. (V.) panamensis* is the most widely spread (Katakura *et al.,* 1992; Armijos *et al.,* 1997).

The present study demonstrates the utility of the PCR technique for the diagnosis of cutaneous leishmaniasis in endemic areas of Ecuadorian coast in comparison with standard diagnostic procedures employed widely.

Materials and Methods

Study area and patients

Research was realized in the Canton of El Carmen (province of Manabi) at the shore of the Pacific Ocean, northwest of Ecuador, at 500 to 800 m above sea level. The active recruitment of patients was realized during the months of January to July of 1997 in the cantonal hospital where persons with lesions suspected to be cutaneous leishmaniasis were involved in this study (Chico and Guderian, 1989). Following criteria for involvement of patients were used: age ranging from 4 to 60 years, no distinction of sex or ethnical background, consent of the patient previously given extensive explanations about the study on its advantages and contingent nuisance. Patients with infected/cont-

aminated lesions or a history of leishmaniasis treatment prior to three months from the study were excluded from the investigation. During the study 72 individuals were examined. Data regarding age, sex, ethnical background, employment, place and time of residency in endemic area were collected. The localization, size, number and persistence in time and morphological characteristics of the cutaneous lesions were registered.

Scraping

Samples were obtained after scraping the active border or raised part of a suspected lesion with a scalpel No. 20. The probe was then spread out on a clean slide, dried at ambient temperature and fixed with methanol prior to Giemsa coloration and microscope examinations with oil immersion enhancement (x1000) for 100 fields. Presence of *Leishmania* spp. amastigotes served as a positive diagnostic criterion of leishmaniasis. Smears were taken in duplicate and reviewed in Quito and Guayaquil laboratories.

Biopsy

After anesthetizing the area surrounding suspected lesions with xylocain 1%, a dermal fragment was obtained utilizing a 4 mm punch (Kai Japan) and consequently divided into three parts. The first sample, obtained for PCR examination, was collected in a tube containing 100 µl of TE solution (10 mM Tris-HCl, 0.1 mM EDTA) and was stored at -20℃ until DNA extraction. Another sample was ground into small fragments, to be used for cultures. The final specimen was placed in a 10% buffered formalin solution for histopathological studies.

Culture

Biopsy fragments were inoculated into tubes containing enriched NNN medium while under constant flame. Tubes were transported at 4℃ and incubated at 25℃. Every third day, microscopical evaluation of the culture was performed, searching for promastigote development of *Leishmania* spp. in order to find positive samples.

Histopathology

Sections and tinctures for microscopical examination of formalin-fixed probes were obtained *via* parafine immersion. Only recognition of *Leishmania* spp. amastigotes by an independent and experienced pathologist was considered to constitute a positive diagnosis.

DNA extraction

200 µl of lytic buffer [50mM NaCl, 50mM Tris-HCl (pH 8.0); 50mM EDTA; 1% Sodium Dodecyl Sulfate (SDS)] were added to the tube containing the TE buffered skin fragments before administration of a final concentration of 100 µg/ml of Proteinase K (Promega Corp., Madison Wis.). Then, samples were incubated for two hrs at 65℃ or, alternately, overnight at 37℃ prior to adding 4 µl of DTT, and then boiling for 30 min. The digested tissue was treated with double extraction *via* phenol/chloroform and single extraction with chloroform. DNA was precipitated in 2.5 times the sample volume of absolute ethanol and 0.1 times the sample volume of sodium acetate 3M. The pellet was washed with ethanol 70%, air dried and the DNA was finally resuspended in 50 µl of TE [10 mM Tris-HCl (pH 7.2), 1mM EDTA]. The product was stored at -20℃ until final usage. In order to avoid presence of reaction inhibitors in the probes as well as to conform to prior described dilution practice (Lopez *et al.*, 1993), we made a double extraction with phenol/chloroform.

Polymerase Chain Reaction (PCR)

DNA amplification reactions were made using the Perkin-Elmer 9600 turbo thermocycler (Perkin-Elmer, Emeryville, CA). A template of 3µl of purified DNA was added to a final reaction volume of 20µl, containing 1µM of each of the *L. (V.) braziliensis* complex specific oligonucleotide primers:MP1L: (5` TACTCCCCGACATGCCTCTG 3`) and MP3H: (5` GAACGG GGTTTCTGTATGC 3`). These amplify a specific DNA fragment of 70bp (base pairs or nucleotides) of *L. (V.) braziliensis* complex. A mixture of 250mM of each of the four deoxynucleotides with one unit of Taq DNA polymerase (Perkin-Elmer-Cetus, Norwalk, CT) was boiled at 94℃ for three minutes followed by 29 (denaturation) cycles of one minute at 94℃, one minute at 54℃ (alignment) and 30 seconds at 72℃ (elongation).

Visualization of PCR products

Amplification products were analyzed by means of 1.5% agar electrophoresis and visualized under ultraviolet light after coloration with ethidium bromide. The results were documented with Polaroid 655 photos.

Control of quality and contamination

Controls were made for each of the PCR assays: positive controls (DNA from biopsies of patients with confirmed leishmaniasis) as well as negative controls (biopsies of ulcers of a different etiology). Probes were processed in separate areas so as to avoid inter-sample contamination. All of the utilized material was either disposed or sterilized under UV light.

Results

Of the 72 dermal lesions that seemed conform to the clinical symptoms of *Leishmania* spp. infection, 33 were from female and 39 from male individuals of four to 60 years of age inclusive. 17 (38.6%) of the women and 27 (61.4%) of the men proved leishmaniasis positive, with a probable sex difference due to the nature of their daily occupation (agriculture and hunting *vs.* minor agriculture and housework). The age group most affected by the disease was 10 to 29 years of age. 95% originated from El Carmen region and the remaining 5% from the provinces of Esmeraldas, Pichincha and Morona Santiago.

Table 5.2.1. Prevalence of *Leishmania* spp. positive lesions detected by the four diagnostic methods

Diagnostic method	Positive	
	No.	%
PCR	40	90.9
Culture	20	45.5
Scraping	18	40.9
Histopathology	16	36.4

Of the 72 lesions suspected to be caused by *Leishmania* spp. only 44 (61.1%) proved to be positive for the disease in at least one out of the four diagnostic methods used in the study (Table 5.2.1). Using PCR, 40 out of 44 samples (90.9%) were positive. In contrast, detection of amastigotes barely reached a 40.9% sensitivity in scraping a recommended and widely utilized technique. Histopathological study of the biopsies only showed a 36.4% sensitivity. The relatively low sensitivity of the culture probes (45.5%) was partly related to contamination (19%), which required that the samples were discarded.

Table 5.2.2. Comparison of the four diagnostic methods for cutaneous leishmaniasis

Comparison of methods	No.	%		No.	%	p value
PCR *vs* culture	40	90.9	*vs*	20	45.5	p<0.001
PCR *vs* scraping	40	90.9	*vs*	18	40.9	p<0.001
PCR *vs* histopathology	40	90.9	*vs*	16	36.4	p<0.001
PCR *vs* 3 methods	40	90.9	*vs*	29	65.9	p<0.005
Culture *vs* scraping	20	45.5	*vs*	18	40.9	NS
Culture *vs* histopathology	20	45.5	*vs*	16	36.4	NS
Scraping *vs* histopathology	18	40.9	*vs*	19	36.4	NS

NS = Not significant.

Table 5.2.2 shows a comparison between the sensitivities of the four diagnostic methods. PCR proved to be far more sensitive than any of the other three techniques, alone or in combination. There was no statistically significant difference of sensitivity between the culture, scrapings, or histopathological methods.

Table 5.2.3 shows the frequency of positive samples utilizing combinations of the four diagnostic methods used in our study. In comparison with the other three methods, PCR identified eleven additional positive cases, which increased PCR sensitivity by an extra 25%. Only five lesions (11.4%) were concurrently identified by the four methods employed. On excluding PCR from the analysis, none of the detection methods alone or in combination presented a diagnostic positivity index greater than 65.9%. Four lesions appeared negative by means of PCR detection, although one proved to be positive by a combination of the three methods, and the others by histopathology, scraping or culture respectively. This apparent inconsistency in PCR sensitivity will be further analyzed in the discussion.

Table 5.2.3. Frequency of sample positivity of the four diagnostic methods employed in diagnosing cutaneous leishmaniasis

Scraping	Culture	Histopathology	PCR	No.(%)
-	-	-	+	11 (25.0)
+	+	-	+	7 (15.9)
+	-	-	+	6 (13.6)
+	+	+	+	5 (11.4)
-	+	+	+	4 (9.0)
-	+	-	+	4 (9.0)
-	-	+	-	4 (9.0)
-	-	+	+	3 (6.8)
+	-	+	+	0 (0.0)
+	+	+	-	0 (0.0)
+	+	-	+	0 (0.0)
+	-	-	-	0 (0.0)
-	+	-	-	0 (0.0)

The sensitivity of scraping and culture decreased if the lesions were chronic: after they had evolved for six months, only a small proportion of the lesions were detected *via* scraping (10%) or culture (40%). The sensitivity of the histopathological method to detect presence of the parasite in early lesions (ones having evolved for less than three months) was surprisingly low (20%). In contrast, PCR revealed itself to be highly sensitive in detecting the presence of *Leishmania* spp. regardless of the duration of evolution (Table 5.2.4).

Table 5.2.4. Index of positivity for *Leishmania* spp. utilizing the four diagnostic methods in relation to the stage of evolution of the cutaneous lesions

Evolution of lesions	Diagnostic method			
	Scraping %	Culture %	Histopathology %	PCR %
< 3 months (n=10)	40.0	60.0	20.0	100.0
3-6 months (n=23)	52.2	4.3	3.8	91.3
> 6 months (n=10)	10.0	40.0	60.0	80.0

Discussion

In diagnosing leishmaniasis, parasite detection is of extreme importance, as there is a great variety of etiological agents capable of causing cutaneous and mucocutaneous lesions similar to those that are characteristic of leishmaniasis, within the same endemic subtropical and tropical regions. Furthermore, leishmaniasis requires large and cost-intensive treatment with pentavalent antimonals, which are difficult to purchase and associated with a variety of adverse effects. In addition, identification of *Leishmania* (at least at the complex level) is important, as different species cause different clinical manifestations, from simple cutaneous lesions that resolve spontaneously, to disseminated forms that affect the

mucous and viscera, and require different therapeutic management.

In the rural areas of prevalence, diagnosis is based on clinical features, due to the absence either of laboratories or of trained staff. In medical centers, diagnosis is based on microscopical examination of Giemsa stained scrapings, whose sensitivity has been demonstrated to be very poor in various studies (Navin *et al.,* 1990; Matsumoto *et al.,* 1999; De Brujin *et al.,* 1993).Techniques like culture using special culture media, inoculation of animals and histopathological studies, can only be carried out in specialized centers or sites of research. Experience with various diseases demonstrated that molecular approaches have greater sensitivity, specificity and speed than conventional techniques in diagnosing the disease (Barker, 1994). One of the techniques using amplification of DNA is the polymerase chain reaction (PCR) that has proved to be a powerful technique for a great variety of applications in various areas of science, including medicine (Eisenstein, 1990; Saiki *et al.,* 1989).

Our findings demonstrate that sensitivity or security of clinical diagnosis of cutaneous leishmaniasis has been over estimated. Out of 72 suspected lesions, only 44 brought showed evidence of *Leishmania.* Similar observations can be found in other studies within endemic countries (Lopez *et al.,* 1993; De Brujin *et al.,* 1993), leading to inadequate treatment. Therefore, further studies are necessary to determine the exact etiology of leishmaniasis-like lesions.

In the present investigation, skin biopsies for PCR examination remained intact in TE medium, and did not necessitate refrigeration for at least 72 hrs while being transported, without impeding the efficiency of DNA amplification of the parasites.

In our study, the sensitivity of scraping to diagnose cutaneous lesions suspected of being caused by *Leishmania* was 40.9%. This percentage is in accord with previous reports concerning American cutaneous leishmaniasis, which range from 23% to 56% (Navin *et al.,* 1990; Chico *et al.,* 1995). Although this procedure appears to be simple in realization, inexpensive and readily available in regional and provincial hospitals, it requires first, a careful extraction and coloration of the sample. Second, experienced examiners, and third, the availability of a high resolution microscope. Reports of false positives lead to a variety of intensive examinations and/or inadequate treatment, including exposure of the patients to toxic side effects, and result in the loss of time and money. False positives can be due to the inexperience of the person performing microscopic examinations, due to coloration artifacts and due to the subjectivity of person examining the dermal lesions, leading to unnecessary and toxic treatment with antimonials drugs.

On the other hand, although culture in specific culture media does increase sensitivity, staff trained in cell and parasite culture is indispensable. Probes obtained in the field contain a high percentage of bacterial and/or fungus contaminants. In our study, 19% of the culture probes were contaminated, and had to be rejected. Another disadvantage of the culture method is the time required to obtain *Leishmania* promastigote growth. In our case, some of the strains developed within five days, whereas others took up to three weeks. Growth of other flagellates 'Kinetoplastidae' that could not be differentiated from *Leishmania* by microscopy have been reported: *C. fasciculata* and other trypanosomas, among others the so-called Eva (Harris *et al.,* 1998; Fu *et al.,* in press).

Although the observation rate for amastigotes was reported to vary between 14-16% in histopathologically examined biopsies, in our study, we found a sensitivity of 36.4%. Limited efficacy may be due to the scarce presence of amastigotes in chronic lesions, as well as in ulcers caused by *Leishmania* of the *braziliensis* complex. Moreover, 'shrinkage' and/or deformation of amastigotes as described by some authors, may reduce sensitivity of the histopathological technique. Given the limited utility of histopathological examinations in the present study, we conclude that this technique should be reserved only for selected patients.

Sensitivity of the PCR technique depends greatly upon the quality of the preparation of the DNA extrac-

tion sample; theoretically, PCR may amplify up to 0.14pg of purified *Leishmania* DNA, which is equivalent to half a parasite. This is of great interest in diagnosing chronic lesions or infections brought about by *L. (V.) braziliensis,* where parasites are scarce and not readily detectable by means of other techniques, as occurred in eleven (25%) of our patients. PCR sensitivity increases enormously when obtained from biopsies, compared to aspirates, which is certainly because of greater parasite number. This phenomenon may apply also for culture techniques (data not shown). It seems important to point out that four of the positive samples were not detected through PCR, although they were by the other methods. Lack of sensitivity is an unlikely explanation for these findings, a supposition that is corroborated by the successful detection of eleven additional positive probes. It is possible that these four probes contained other species (*mexicana* complex or of the *major*-like group), as previously described in the Ecuadorian coast (Katakura *et al.,* 1992; Armijos *et al.,* 1997), thus suggesting the need for further investigations that employing primers of the above-mentioned species. Recently, a set of primers capable of detecting the three complexes circulating in the Americas has been developed (Harris *et al.,* 1998). Although sensitivity varies between 0.001 and 100 parasites depending upon the species, its large scale use is limited to lesions produced by *L. (V.) braziliensis,* and to chronic infections, where parasite density is low.

From our experience with this protozoonosis as well as with others, like malaria, and other diseases, such as onchocercosis, and tuberculosis, we consider PCR to be applicable for the diagnosis and management of diverse pathological entities, of genetic anomalies and certain types of cancer, within the confines of an adequately equipped laboratory with experienced personnel. Furthermore, unidentified DNA may be extracted from any biological material, which favors the development of the technique for the detection of various pathologies.

Additional advantages offered by PCR in this and other pathological entities is its speed, its high sensitivity and specificity, plus the ease of discriminating between parasite complexes and species, thus allowing for better therapeutic orientation, and reducing social and economic cost in low income countries. Finally, the results of this study not only suggest a greater sensitivity of PCR compared to scraping, culture or histopathological techniques, but also demonstrate its applicability in zones of endemic prevalence.

PCR significantly increased the diagnostic index of leishmaniasis in the examined area and distinguished between cutaneous lesions due to *Leishmania* and those of other etiologies that are common in the tropics with high confidence. Nationwide standardization and implementation of this technique will surely prove to be of great benefit in the epidemiological understanding of the disease, as there are various species circulating in different geographical regions. Additionally, PCR would be of great help in discerning what are the vectors and reservoires of *Leishmania* spp.

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2. Differential Diagnosis of Cutaneous Leishmaniasis in Endemic Areas of Ecuador

ABSTRACT. Skin diseases found in endemic areas of cutaneous leishmaniasis in Ecuador were investigatedd whether there exists any skin disease and cutaneous changes that require to make differential diagnosis between the skin diseases and cutaneous leishmaniasis. Out of the various cutaneous changes observed, leg ulcers which were caused not by cutaneous leishmaniasis should be considered as a possibility of misdiagnosis and therefore properly examined.

Introduction

Cutaneous leishmaniasis (CL) is a parasitic disease caused by various strains of *Leishmania* spp. Man and domestic animals are infected by sandfly, a vector with *Leishmania* parasites. Clinically, CL is divided into localized, generalized and diffuse types. CL shows various cutaneous manifestations such as papules, nodules, ulcers with elevated borders and erythematous plaques. In generalized cutaneous leishmaniasis (GCL), the eruptions are disseminated throughout the entire body surface (Lazo, 1994). In diffuse cutaneous leishmaniasis (DCL), which associates with specific immunodeficiency against *Leishmania,* different clinical manifestations such as nodules, papules and erythematous plaques are observed throughout the entire body surface, with the exception of the scalp, axillary, inguinal, perineal and anal regions. Cutaneous manifestations in leishmaniasis are very similar to those of other infectious and skin diseases. Therefore, differential diagnosis between CL and other diseases including leprosy (Jopling 1984) and deep mycosis is very important, especially in countries where these diseases are relatively common. In Ecuador, CL, except in its GCL and DLC forms, is clinically divided into highland type (Andean type) and lowland type. The highland type of CL, observed in the highland of the Andes Mountains where the temperature and moisture is relatively low, occurs as miliary-to-pea-sized papules resembling insect bites and furuncles on the face and four extremities of children. The inflammation of the lesions is relatively minor compare to that of bacterial infection, although numerous *Leishmania* paresites are often detected within the lesions. It has been shown that, after a sanfly bite, the lesions gradually increase in size for several weeks. Most of the lesions heals spontaneously and form the small scars on the face and four extremities within half a year. The lowland type of CL, observed in the lowland with hot and humid forests, shows the above mentioned cutaneous changes, including ulcer with elevated border where induration is palpable at the margin. After the infection, the lesions gradually increased in size and formed relatively large and deep ulcers. A portion of each lesion heals spontaneously in about one year and leaves a relatively large scar. The inflammation of lesion is also minor. Therefore, the clinical symptoms of the lesions such as reddness and pressure pain, are much more minor than those of bacterial infection. As the positive rate of *Leishmania* parasites of this type is very small, diagnosis in the endemic areas of Ecuador has generally been performed by the history of the present illness and cutaneous changes of the patients. When bacterial infection is coexistent at the lesion site, the ulcers tend to become large and the lesions tend to endure longer (Edrissian, 1990). Therefore, distinction of the lesion is very important for treatment. Though various fungi have been isolated from CL ulcers, their role in the ulcerstion is still obscure (Nishimoto, 1992). Because CL lesions of the auricle and nose are likely to cause deformity, it is generally considered that lesions at

these sites should be treated aggresively at the early stages of the disease. In the examination for CL in Ecuador, we observed many non-cutaneous leishmaniasis cases diagnosed as CL and treated using meglumine antimonate, Gulcantime. In this paper, we show mainly ulcerative skin lesions, observed in the lowland area of Ecuador.

Materials and Methods

About 25 patients with ulcerative skin lesions and other cutaneous changes visited our outpatient clinic of the Multin y Casa Hospital in Babahoyo city, Province of Los Rios, Ecuador. The city is located on the Pacific coast of Ecuador. The patients were observed between July and September 1994. Each patient was thoroughly examined clinically and/or parasitologically. Nine patients were diagnosed to be CL and 16 patients were diagnosed to be non-cutaneous leishmaniasis (non-CL). In this preliminary study of differential diagnosis between CL and other skin diseases, the history of the present illness including the treatments performed at other hospitals and the cutaneous changes of the patients of 16 patients with non-CL observed at the outpatient clinic were mainly recorded in detail.

Result

The total patients in the survey were 25, 17 males and 8 females. Nine patients were diagnosed to be CL (Table 5.2.1). The mean age of 9 patients with CL was 15.0 year-old in males, 18.5 year-old in females, and 16.7 year-old in total. Two CL patients were treated by 2% parmomycine ointment. The treatment of 2% diamino-diphenyl-sulfone ointment for a CL patients was stopped because of a side effect of irritation and Mephaquin was prescribed for the patient. Oral administration of Mephaquin and Artesnate were performed for five and two patients respectively. Those treatments were effective. The mean age of patients with non-CL was 43.0 year-old in males, 36.4 year-old in females, and 39.7 year-old in total. The mean age of non-CL patients was 23.0 year older than that of CL patients. From the dermatological point of view, 16 patients with ulcerative skin lesions required differential diagnosis between CL and other skin diseases. Some of them, differential diagnosis of CL from other skin disease was very difficult. As shown in Table 5.2.2, most frequent cutaneous change of non-CL was ulcer on the leg (8/16, 50.0%). Out of 10 patients with ulcerative skin lesions, 6 patients (6/10, 60.0%) were misdiagnosed cases as to be CL and were treated by injections of meglumine antimonate (Glucantime). In

Table 5.2.1. Cutaneous leishmaniasis observed in an endemic area of Ecuador (Multin y Casa Hospital, Babahoyo, Los Rios)

No.	Age	Sex	Site of lesion	Treatment used
1	6	M	right hand	2% paromomycin (o)
2	10	M	left leg	Mephaquin (p.o.)
3	1	F	face, right thigh	2% paromomycin (o)
4	7	F	both forearm	2% DDS (o) -Mephaquin (p.o.)
5	22	F	left leg	Mephaquin (p.o.)
6	60	M	right hand	Artesunate (p.o.)
7	25	M	right forearm-hand	Artesunate (p.o.)
9	11	M	left cheek	Mephaquin (p.o.)
10	23	M	left ear	Mephaquin (p.o.)

O: ointment; p.o.: *per os* administration.

Table 5.2.2. Cutaneous changes of non-cutaneous leishmaniasis (non-CL) cases observed in an endemic area of Ecuador (Multin y Casa Hospital, Babahoyo, Los Rios)

No.	Age	Sex	Diagnosis	Past diagnosis (mis-diagnosis)	Cutaneous changes	Site of lesions	Treatment
1	44	M	ulcus cruris varicosum		ulcer, varix, stasis dermatitis	rt. leg	
2	60	F	ulcus cruris varicosum		ulcer, varix, stasis dermatitis	lt. leg	
3	24	M	ulcus cruris varicosum	CL	ulcer, dark-brownish pigmentation, schrach mark	legs	Glucantime (perilesional inj. x5)
4	63	M	ulcus cruris varicosum		erosion, dark-brownish pigmentation	leg	Glucantime (i.m. x14)
5	72	F	ulcus cruris varicosum	CL	yulcer, varix, induration	legs	Glucantime (i.m. x10)
6	33	F	leg ulcer		ulcer		
7	45	M	trauma	skin tumor (excision)	ulcer	leg	
8	48	M	trauma		ulcer, dark-brownish pigmentation	leg	
9	17	M	trauma + secondary infection	CL	ulcer, induration	rt. leg	Glucantime (perilesional inj. x19)
10	10	M	impetigo contagiosum		crusted lesion, exudation, erosion	rt. leg	
11	53	M	fruncle ?		fruncle-like lesion	cheek	
12	60	M	chromomycosis		verrucous nodule	rt. hand (dorsal)	
13	1	M	contact dermatitis ?	CL	reddish papules, erythemas	lt. cheek	Glucantime (2ml, i.m. x5)
14	20	F	CL		reddish papules, ulcer	lt. cheek - nose	Glucantime (i.m. x15)
15	40	M	B.C.C.		ulcer, black papules	nose	
16	69	M	B.C.C.	MCL	ulcer, black papules, nodule	face, oral cavity	operation, Glucantime (i.m. x30)

rt, right; lt, left; i.m., intramuscular injection; CL, cutaneous leishmaniasis; MCL, mucocutaneous leishmaniasis; Glucantime, meglumine antimonate; x, times of Glucantime injection; B.C.C., basal cell carcinoma.

addition, the Glucantime injection was performed for also two non-CL patients with non-ulcerative skin lesion, reddish papules and reddish plaques, and for two patients with basal cell carcinoma (BCC) on the face. A patient with BCC received about 30 intramuscular injections of Glucantime without any improvement. For those patients with secondary bacterial infection at the lesions, drugs and ointments of antibiotics and/or mercury chrome solution were prescribed.

Case 1 (No.1), a 44-year-old male. The thumb-sized ulcers on the legs were diagnosed as be ulcers cruris varicosum from the present history and the existence of typical varix on the lower extremities and atrophy blanch at the center of the ulcer. The induration was not palpable at the margin of the ulcer (Fig. 5.2.1A).

Case 2 (No.2), a 60-year-old female. The ulcer on the legs were relatively shallow without the elevated border. In addition, siderosclerosis was observed on the legs. Atrophy blanch was observed at the center of the ulcer. The lesion was diagnosed to be ulcus cruris varicosum (Fig. 5.2.1B, C).

Case 3 (No.8), a 48-year-old male. From the present history of the illness, it was suspected that the post traumatic lesion on the leg became the ulcer by secondary bacterial infection. The induration was not palpable at the margin of the ulcer (Fig.5.2.1D).

Case 4 (No.9), a 17-year-old male. The ulcer on the right leg was diagnosed at a rural hospital as CL ulcer. Even though the patient received 19 local injections of meglumine antimonate, he showed no improvement. This injection caused fibrosis around the lesion which was palpable as to be induration at the margin (Fig. 5.2.2A).

Case 5 (No.10), a 10-year-old male. Pea-sized shallow ulcers without the elevated border appeared on the lower extremities in about one week after the child was bit by insect. Scratch marks were also observed on his lower extremities. The lesion was diagnosed as impetigo contagiosum (Fig.5.2.2B).

Case 6 (No12), a 60-year-old male. About one year prior to examination, 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. The nodule was non-pedunculated, with a nearly flat top, and was raised about 1 cm. The surface was rough and irregular with a cauliflower-like appearance (Fig.5.2.2C). After various examinations, the lesion was determined to be a chromomycosis caused by *Fonsecaea pedrosoi* (type 4) (Kawasaki *et al.,* 1999; Hosokawa *et al.,* 1997).

Case 7 (No.13), a 1-year-old female. The reddish papules and erythema were located on the left cheek. The skin lesion was diagnosed to be CL at a rural hospital, and received five, 5 ml intramuscular injections of meglumine antimonate for a total of 25 ml. Two weeks after the treatment, however, no improvement was visible (Fig. 5.2.2D).

Case 8 (No.14), a 20-year-old female. The rice-grain-sized reddish papules and shallow ulcers were observed on the left cheek. Even though she received 15 intramuscular injections of meglumine antimonate, no improvement was observed (Fig. 5.3.3A).

Case 9 (No.15), a 40-year-old male. The shallow ulcer and black papules were strongly suspected to be basal cell carcinoma (BCC) (Fig.5.2.3B).

Case 10 (No.16), a 69-year-old male. The black papules and ulcers on the face were suspected to be relapsed BCC lesions (Fig. 5.2.3C). The maxilla was broken, and a metastatic lesion was observed at the oral cavity. The patient received about 30 intramuscular injections of meglumine antimonate without any improvement (Fig. 5.2.3D).

Case 11, a 22-year-old male. This case was observed in other city and not presented in the table. Six years prior to admittance at our clinic, he first noticed discrete papules on his left knee and right cheek; these began to increase in numbers and size, and to spread over the entire body; papules slowly evolved into infiltrated plaques and nodules without any ulceration. Over the course of about two years, he received different treatments from different physicians, but without improvement; consequently, he decided to visit a dermatological dispensary in Guayaquil. At the dispensary, he was diagnosed with lepromatous leprosy and medicated for about 5 years. Based on the

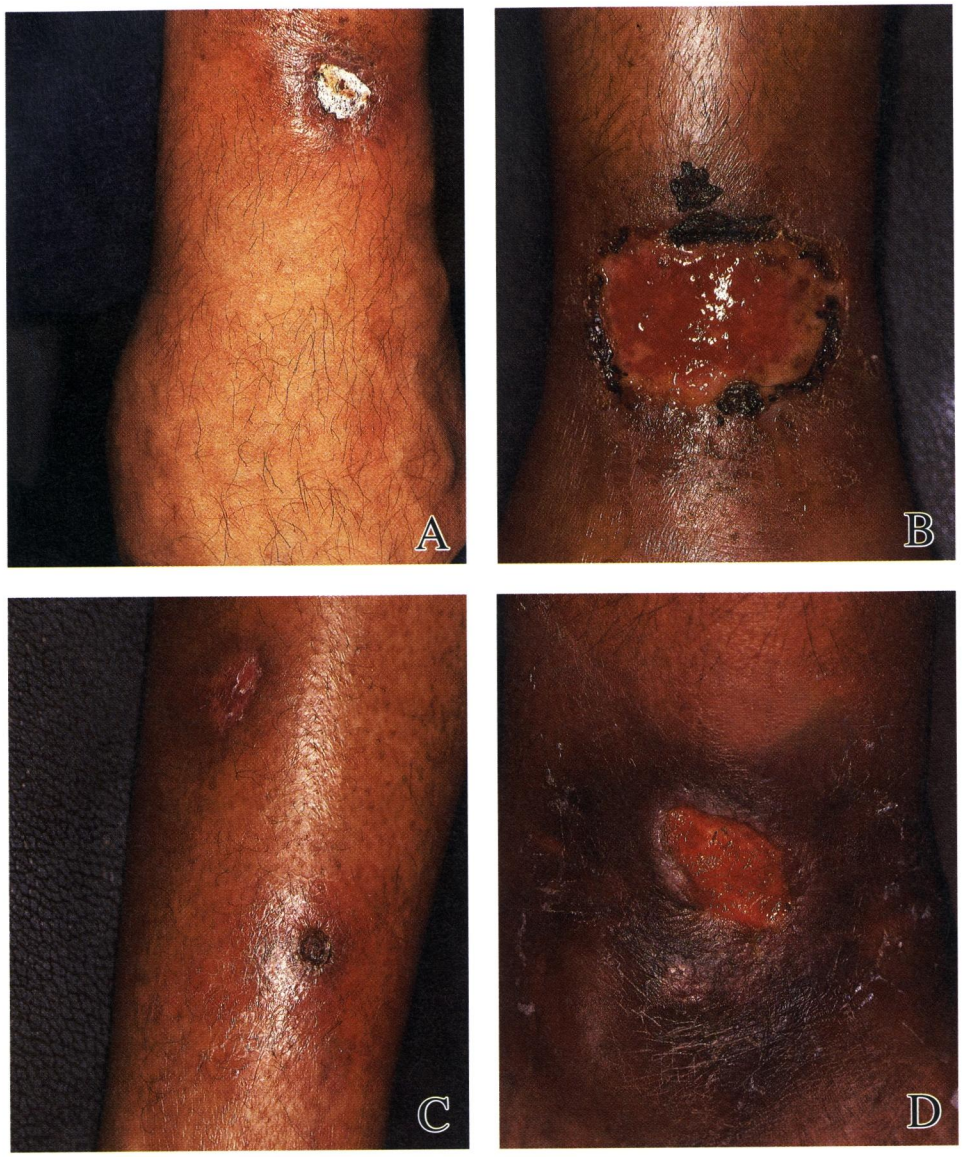

Figure 5.3.1. 5.3.1A. The thumb-sized ulcers on the legs of case 1 were diagnosed as be ulcers cruris varicosum. A typical varix was observed on the lower extremities and atrophy blanch was observed at the center of the ulcer. The induration was not palpable at the margin of the ulcer. **5.3.1B.** The ulcer on the legs of case 2 was relatively shallow without the elevated border. In addition, siderosclerosis was observed on the legs. This lesion was diagnosed to be ulcus cruris varicosum. **5.3.1C.** On the other site of the legs of case 2, pea to small finger's tip-sized ulcers were observed. Siderosclerosis was also observed around the ulcers. **5.3.1D.** The leg ulcer was considered to be the post traumatic lesion with secondary bacterial infection. The induration was not palpable at the margin of the ulcer. The black brownish pigmentation was observed around the ulcer.

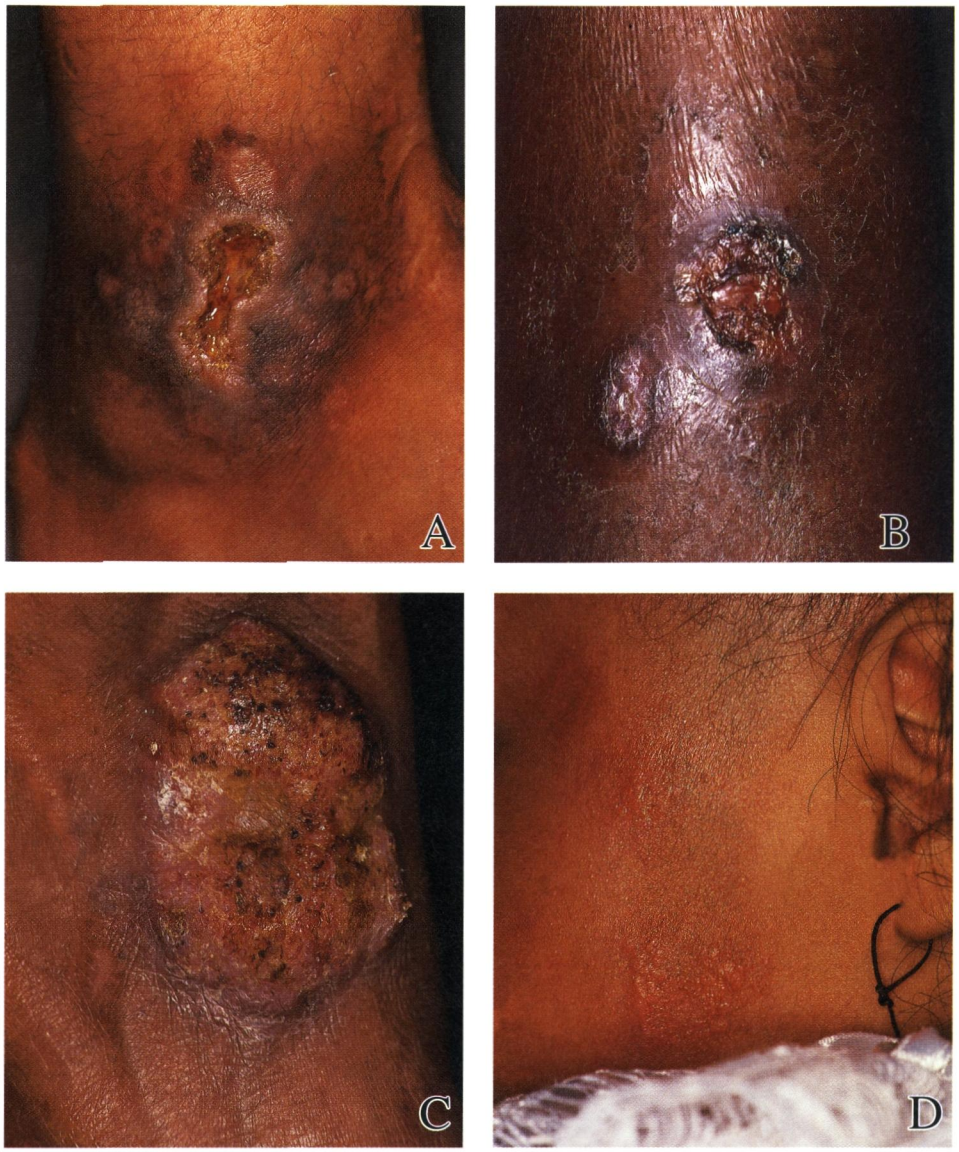

Figure 5.3.2. 5.3.2A. The ulcer on the right leg was diagnosed as CL ulcer. Even though the patient received 19 local injections of meglumine antimonate, no improvement was found. This injection caused fibrosis around the lesion which was paplable as to be induration at the margin. **5.3.2B.** Pea-sized shallow ulcers without the elevated border appeared on the lower extremities were diagnosed as impetigo contagiosum. **5.3.2C.** The verrucous lesions, its surface was rough and irregular with a cauliflower-like appearance, on the dorsal aspect of the right hand was suspected to be verrucous type of CL. The lesion was determined to be a chromomycosis caused by *Fonsecaea pedrosoi* (type 4). **5.3.2D.** The reddish papules and erythema on the left cheek was diagnosed to be CL and received five, 5 ml intramuscular injections of meglumine antimonate for a total of 25 ml. Two weeks after the treatment had ended, however, no improvement was visible.

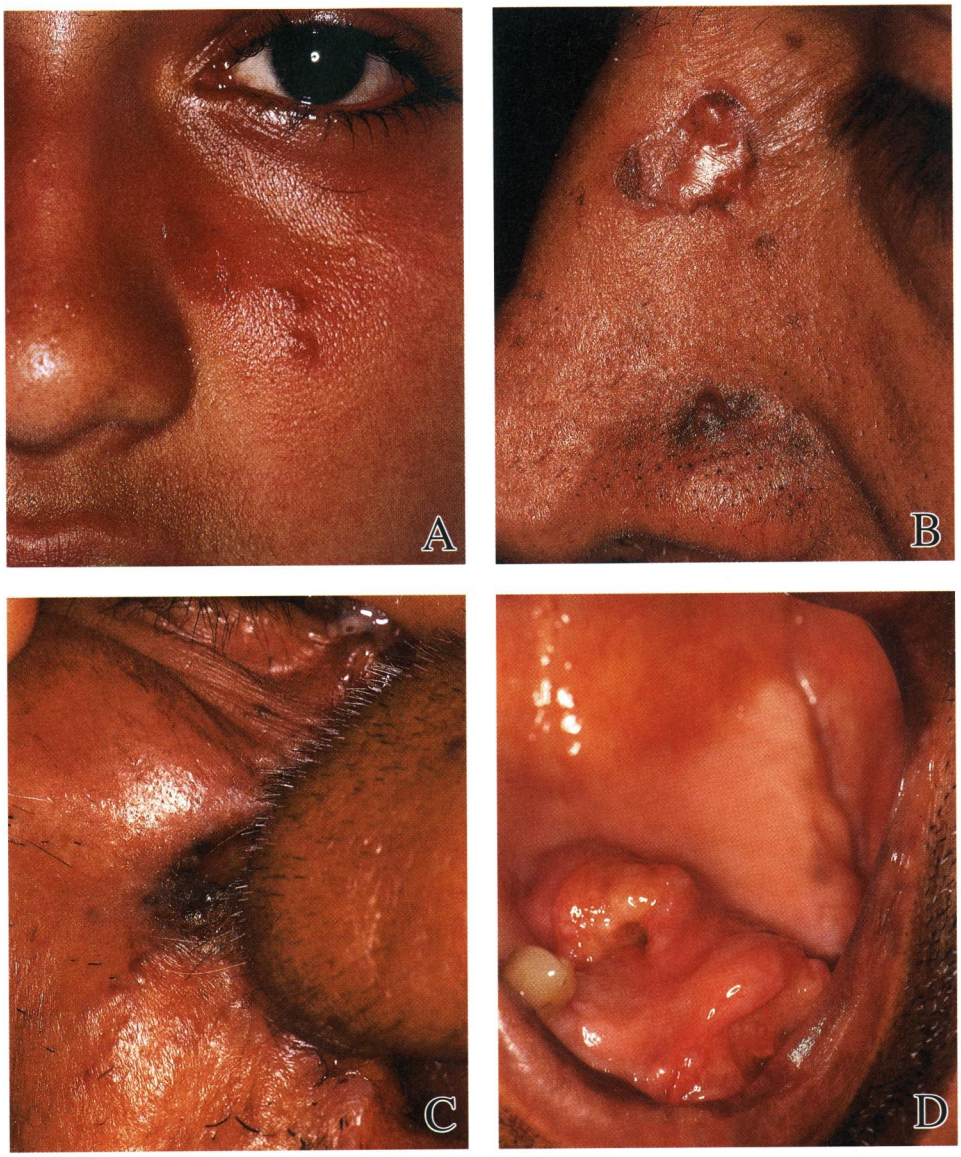

Figure 5.3.3. **5.3.3A.** The rice-grain-sized reddish papules and shallow ulcers on the left cheek were diagnosed to be CL. Even though the patient received 15 intramuscular injections of meglumine antimonate, no improvement was observed. **5.2.3B.** The shallow ulcer and black papules were strongly suspected to be basal cell carcinoma (BBC). **5.2.3C.** The black papules and ulcers on the face were suspected to be relapsed BCC lesions. **5.2.3D.** The maxilla was broken, and a metastatic lesion was observed at the oral cavity. The patient received about 30 intramuscular injections of meglumine antimonate without any improvement.

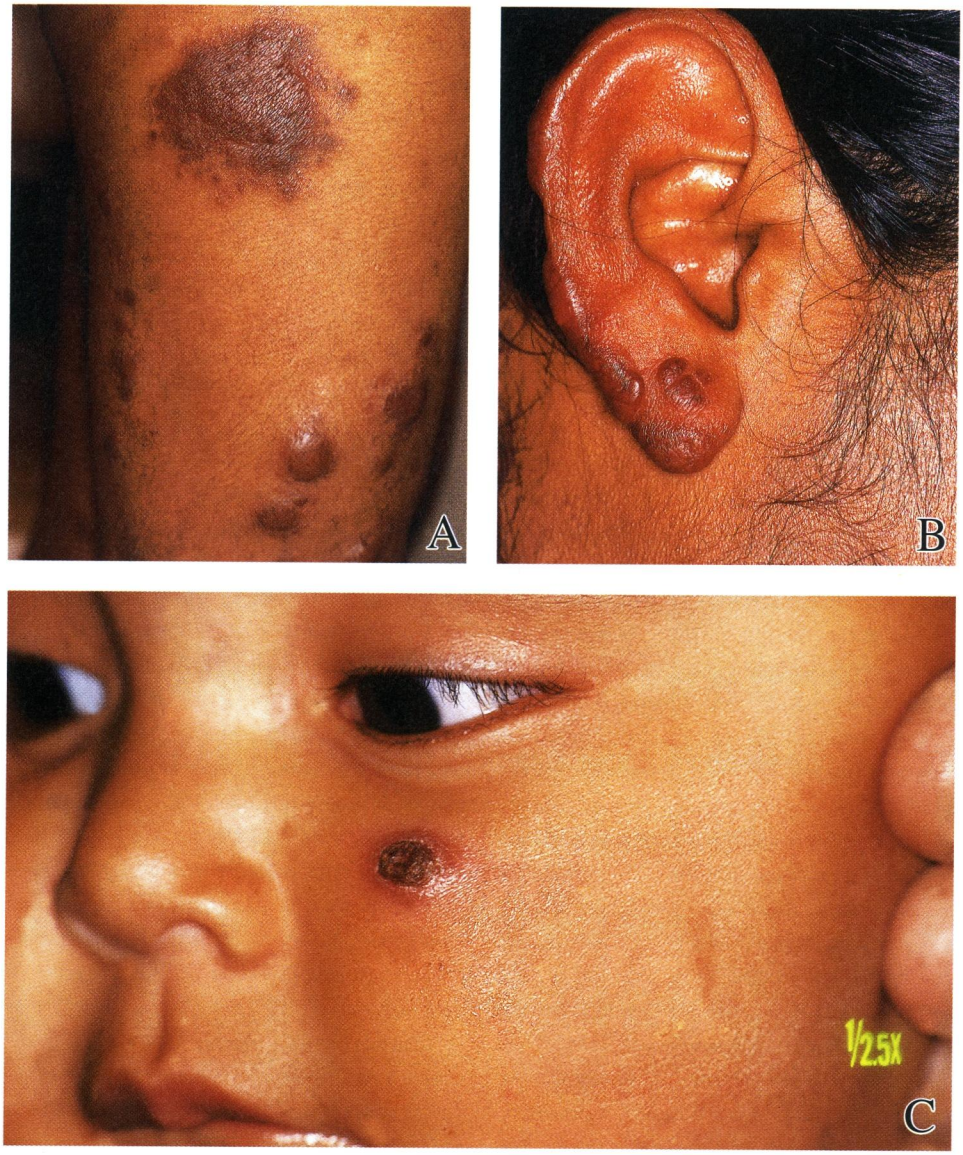

Figure 5.3.4. 5.3.4A. The papules, infiltrated plaques and nodules on the four extremities without any ulceration was diagnosed to be lepromatous leprosy and medicated for about 5 years. **5.3.4B.** The violet brownish papules on the ear were very similar with that of leprosy. The lesions were diagnosed to be diffuse cutaneous leishmaniasis (DCL). **5.3.4C.** In this case, typical lesions of the highland type of CL, insect bite-like and furuncle-like skin changes on the cheek were observed.

results of our examinations, however, he was finally diagnosed as DCL (Reyna *et al.,* 1994; Hosokawa *et al.,* 1997) (Fig. 5.2.4A, B).

Case 12, a one-year-old female. This case was observed in the highland area of the Andes and not presented in the table. In this case, typical lesions of the highland type of CL, insect bite-like and furuncle-like skin changes on the cheek were observed (Fig. 5.2.4C).

Comments

Case 1 (No.1) and Case 2 (No.2) were diagnosed to be ulcers of cruris varicosum from the present history and the existence of typical varix on the lower extremities and atrophy blanch at the center of the ulcer and/or siderosclerosis around the ulcer. In addition, the induration was not palpable at the margin of the ulcers or the elevated border of the ulcer was not observed. Case 3 (No.8) was diagnosed as be the post traumatic lesion because varix on the lower extremities and the induration at the margin of the ulcer was not observed. The pigmentation around the ulcer was suspected to be induced by a repeated bacterial infection and inflamation. Case 4 (No.9) was not responded to 19 local injections of meglumine antimonate. It was considered that this injection caused fibrosis around the lesion which was paplable as to be the induration of CL. Case 5 (No.10) was relatively easily diagnosed to be as impetigo contagiosum from the present history of the illness. At first, when we observed the eruption of case 6 (No.12), we suspected that of verrucous type of CL. 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 CL 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 CL has not been reported, the examination, especially detection of the microorganism, should be performed for confirming the diagnosis and adequate treatment. The eruptions of case 7 (No.13) and case 8 (No.14) were reddish papules and infiltrated erythemata on the face. The lesions were diagnosed to be CL at other hospitals. Even though they received five and 15 intramuscular injections of meglumine antimonate respectively, no improvement was observed. Diagnosis of Case 9 (No.15) with BCC was not so difficult for our dermatologists. It was suspected that the diagnosis of BCC might be difficult for general doctors without histopathological examination. Case 10 (No.16) with BCC was most miserable case experienced in Ecuador. The maxilla was broken and two perforated lesions were observed on his face. Until we examined the patient, he had received 30 intramuscular injections of meglumine antimonate without any improvement. If he received a dermatological examination at the early stage of the skin carcinoma, he surely might be healed completely. Case 11 with DCL had received different treatments from different physicians without improvement. For five years, he had been medicated as to be lepromatous leprosy. Some of the eruptions and their distribution were similar to that of lepromatous leprosy. If a bacteriological examination had been done, leprosy surely be ruled out. Though Case 12 showed typical lesion of the highland type of CL, sporotrichosis was also suspected from the cutaneous change and the site of the skin lesion. If a doctor did not experienced this type CL, the diagnosis might be very difficult.

Based on the observations of the cutaneous changes described and demonstrated in the result, special attention should be given to various infectious and noninfectious diseases, including skin carcinomas such as BCC and squamous cell carcinoma etc., for the differential diagnosis at the examination of patients with CL. Therefore, in order to ensure clinically the accuracy of CL diagnosis, it is very important to consider the history of the present illness of the patient and examine the margin of ulcers by palpation; these steps are particularly important in which these diseases are endemic but parasitological and histological examinations are not available. From our experience at the

Multin y Casa Hospital in Babahoyo city, the ulcers caused by stasis dermatitis were considered to be difficult to differentiate from CL. When the skin lesions were treated for a long term, differential diagnosis was especially difficult because of the fibrosis developed around the ulcers by treatments. The fibrosis was palpated as to be induration which is usually palpable at the margin of the ulcer of CL.

In case of highland type, the treatment is not needed for CL because the lesion is small and most of them heals spontaneously in a few months leaving a small scar. It was considered that secondary bacterial infection increased the term of the disease and the size of the lesion. Therefore the treatment for bacterial infection and for itching may be needed. In case of lowland type, though the lesion heals spontaneously in about half a year, the ulcer is relatively large and leave large scar. Therefore prevention of secondary bacterial infection is also important for a shortening the medication and for a cosmetic point of view. For a treatment of such a contaminated ulcerative lesion as above mentioned skin lesions, a treatment with meglumine antimonate solution (meglumine antimonate and mercury chrome solution) (Nonaka *et al.*,1997) was considered to be useful. When the lesion appeared on ear or nose, aggressive treatment is needed to prevent their deformity and perforation.

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Chapter 6

Clinical and Epidemiological Aspects

1. Clinical Survey of Cutaneous Leishmaniasis in Ecuador for 10 Years (1991 - 2000)

ABSTRACT. In this study, we analyzed clinical and epidemiological data on cutaneous changes of leishmaniasis recorded from 1991 to 2000 in Ecuador. The study areas were included Provinces of Manabi, Los Rios, Bolivar, Pichincha, Azuay and Esmeraldas located at the Pacific coast and the slope of Andean mountains. A total of 570 cutaneous leishmaniasis patients, 324 males and 246 females, living in Ecuador were examined for this study. Each patient was thoroughly examined clinically and parasitologically. The mean age of the patients was 19.82 years (±0.89 s.e.) in males, 17.45 years (± 1.00 s.e.) in females and 18.80 years (±0.67 s.e.) in total. Patients less than 20-year-old occupied more than half of all the patients examined. However, a total of 33 cases (5.8%) were senior patients of age more than 50-year-old, among those, 20 cases (6.2%) were males and 13 cases (5.2%), females. The total mean onset of cutaneous lesions was 6.17 months (±0.41 s.e.); 5.58 months (±0.39 s.e.) in males, 6.96 months (±0.56 s.e.) in females. The numbers of the patients, with the onset of lesions less than 1 month, were 49 cases (9.9%) in total, 35 cases (12.4 %) in males, 14 cases (6.7 %) in females. However, there were 17 cases (6.0 %) in males, 15 cases (7.1 %) in females and 32 cases (6.5 %) 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 popular was ulcer formation. The patients with ulcer occupied more than 50 % of the total. More than half of the lesions were located on the face and the extremities. The lesions on trunk were seen more frequently in male than female. On the other hand, the lesions on the legs were more popular in female. Almost half of the lesions were solitary, and the remainder were multiple. The cases with more than ten multiple lesions were seen in a total of 16 patients, 9 were male and 7 were female patients. Several species of *Leishmania* parasites and vectors were reported in the areas examined in this study. Cutaneous leishmaniasis in Ecuador may differ from the other areas because of the difference of the parasite species, various behaviours of the vectors and the different life styles of the inhabitants. Therefore, medical doctors assigned to the treatments for cutaneous leishmaniasis in Ecuador should have not only medical knowledge but also entomological, ecological, environmental and anthropological knowledge.

Introduction

There are three clinical types of leishmaniasis, *i.e,* cutaneous, mucocutaneous and visceral. Cutaneous leishmaniasises 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. That is, there were marked differences in clinical findings of cutaneous leishmaniasis between

the lowland and highland of Ecuador. The most popular cutaneous change was ulcer, but non-ulcerated cutaneous changes such as papules, plaques and nodules were also seen frequently (Nonaka *et al.*, 1990a, b; Gomez *et al.*, 1992; Reyna *et al.*, 1994). At that time, big ulcers more than 900㎟ were also seen in 21 (14.7%) of a total 143 cases of cutaneous leishmaniasis. Lymphnode swellings were also seen in half of the patients examined, and were more frequently in females. One case of generalized cutaneous leishmaniasis was also reported in Ecuador. In this study, we analyzed data on cutaneous changes of patients with leishmaniasis recorded from 1991 to 2000 in Ecuador.

Materials and Methods

A total of 570 cutaneous leishmaniasis patients living in Ecuador were analyzed for this study. The study areas were included Provinces of Manabi, Los Rios, Bolivar, Pichincha, Azuay and Esmeraldas, located in the Pacific coast and the slope of Andean mountains. The period of observation was 10 years from 1991 to 2000. Before each physical 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 immersion. 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 in our project (Furuya *et al.*, 1991) was also performed on a part of patients as an immunological examination.

Results

The results obtained are summarized in Table 6.1.1. to 6.1.5 and Figs 6.1.1. to 6.1.10. The total patients in this study were, 570, 324 males and 246 females (Table 6.1.1.). The total mean age of patients was 18.80 years (± 0.67 s.e.), 19.82 years (± 0.89 s.e.) were males, 17.45 years (± 1.00 s.e.) females. Young patients less than the age of 20-year occupied more than half of all the patients examined. However, a total of 33 cases (5.8%) were senior patients more than 50-year-old, 20 cases (6.2%) were males, and 13 cases (5.2%) females. The total mean onset time of cutaneous lesions was 6.17 months (± 0.41 s.e.), 5.58 months (± 0.39 s.e.) in males, 6.96 months (± 0.56 s.e.) in females (Table 6.1.2.). The total number of the patients with the onset of lesions less than 1 month was 49 cases (9.9%), 35 cases (12.4 %) were males, 14 cases (6.7 %) females. However, a total of 32 cases (6.5%), 17 cases (6.7 %) of males, 15 cases (7.1%) of females showed the duration time of lesions more than 13 months. The popular types of lesions included ulcer, nodule, erythematous plaque and papule (Table 6.1.3). The most popular was ulcer formation. The patients with ulcer occupied more than 50% in total. Distribution of the lesions is shown in Table 6.1.4. More than half of the lesions were located on the face and the extremities. The lesions on the trunk were seen more frequently in males as compared to female. On the other hand, the lesions on the legs were more popular in females. Almost half of the lesions were solitary, but the remainders were multiple (Table 6.1.5). A total of 16 cases, 9 male and 7 female pataients, were well multiple lesions.

Discussion

In 1990, we perfomred a comparative study of the skin changes due to 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 low-

Table 6.1.1. Summary of patients with cutaneous leishmaniasis

Age (years)	Male	(%)	Female	(%)	Total	(%)
- 5	59	18.2	56	22.8	115	20.2
6 - 9	48	14.8	45	18.3	93	16.3
10 - 19	77	23.8	58	23.7	135	23.7
20 - 29	75	23.1	37	15.0	112	19.6
30 - 39	27	8.3	23	9.3	50	8.8
40 - 49	18	5.6	14	5.7	32	5.6
50 - 59	6	1.9	6	2.4	12	2.1
60 -	14	4.3	7	2.8	21	3.7
Total	324	100.0	246	100.0	570	100.0

Table 6.1.2. The onset of cutaneous leishmaniasis lesions

Age (years) (months)	Male	(%)	Female	(%)	Total	(%)
- 1	35	12.4	14	6.7	49	9.9
2 - 3	116	41.0	76	36.2	192	39.0
4 - 6	74	26.1	49	23.3	123	24.9
7 - 12	41	14.5	56	26.7	97	19.7
13 -	17	6.0	15	7.1	32	6.5
Total	283	100.0	210	100.0	493	100.0

Table 6.1.3. The clinical forms of cutaneous leishmaniasis lesions

Types	Male	(%)	Female	(%)	Total	(%)
erythema	0	0.0	1	0.4	1	0.2
nodules	39	11.4	23	8.3	62	10.0
papules	39	11.4	47	16.9	86	13.8
plaques	32	9.3	23	8.3	55	8.9
scars	18	5.2	37	13.3	55	8.9
ulcers	215	62.7	147	52.8	362	58.2
Total	343	100.0	278	100.0	621	100.0

land, but those in highland were very small. The lesions were wet type in lowland, however dry type in highland. Mucocutaneous type of leishmaniasis was seen only in lowland. In 1991, we reported Andean leishmaniasis in Ecuador caused by infection with *Leishmania (Leishmania) 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 slope

Table 6.1.4. The site of 653 lesions

Areas	Male	(%)	Female	(%)	Total	(%)
Face	87	22.6	75	27.8	162	24.7
Ear	13	3.4	2	0.7	15	2.3
Neck	9	2.3	2	0.7	11	1.7
Back	14	3.7	7	2.6	21	3.2
Chest	5	1.3	0	0.0	5	0.8
Abdomen	1	0.3	0	0.0	1	0.2
Lumbal	1	0.3	3	1.1	4	0.6
Buttock	1	0.3	2	0.7	3	0.5
Trunk	3	0.8	0	0.0	3	0.5
Shoulder	3	0.8	3	1.1	6	0.9
Arm	42	11.0	30	11.1	72	11.0
Elbow	5	1.3	3	1.1	8	1.2
Forearm	80	20.8	39	14.4	119	18.2
Hand	21	5.5	6	2.2	27	4.1
Upper extremities	12	3.1	6	2.2	18	2.8
Thigh	13	3.4	11	4.1	24	3.7
Leg	68	17.8	79	29.4	147	22.4
Foot	1	0.3	0	0.0	1	0.2
Extremities	4	1.0	1	0.4	5	0.8
Whole body	0	0.0	1	0.4	1	0.2
Total	383	100.0	270	100.0	653	100.0

Table 6.1.5. The number of lesions per patient

Number	Male	(%)	Female	(%)	Total	(%)
1	163	51.6	105	44.9	268	48.8
2	70	22.2	54	23.1	124	22.5
3	41	13.0	31	13.2	72	13.1
4	14	4.4	12	5.1	26	4.7
5	7	2.2	10	4.3	17	3.1
6 - 9	12	3.8	15	6.4	27	4.9
10 -	9	2.8	7	3.0	16	2.9
Total	316	100.0	234	100.0	550	100.0

Table 6.1.6. The size of lesions

Size of lesion (mm^2)	Male	(%)	Female	(%)	Total	(%)
- 9	49	8.8	40	9.8	89	9.2
10 - 25	106	19.0	63	15.6	169	17.5
26 - 100	156	27.9	101	24.9	257	26.7
101 - 400	170	30.4	131	32.4	301	31.4
401 - 1600	64	11.5	61	15.1	125	13.0
1600 -	6	1.1	5	1.2	11	1.1
unknown	7	1.3	4	1.0	11	1.1
Total	558	100.0	405	100.0	963	100.0

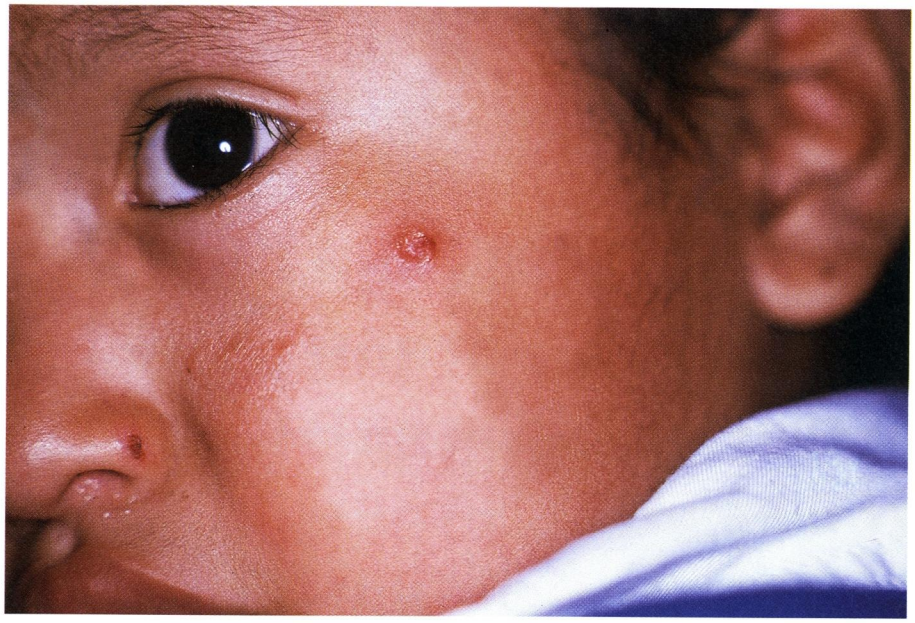

Figure 6.1.1. A clinical picture (papular type) of a 3-year-old boy in Huigra, an Andean endemic area. Insect bite like small papule was seen on his left cheek. The surface was slightly erosive.

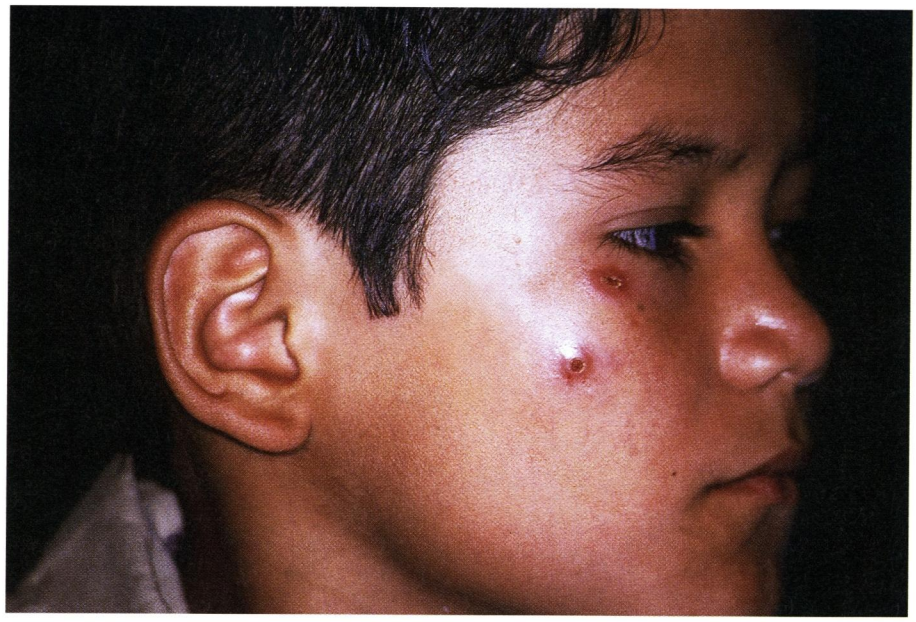

Figure 6.1.2. A clinical picture (papular type) of a 10-year-old boy in La Mana, a lowland endemic area. Two small papules were seen on his right cheek. The center of the papules had crusts.

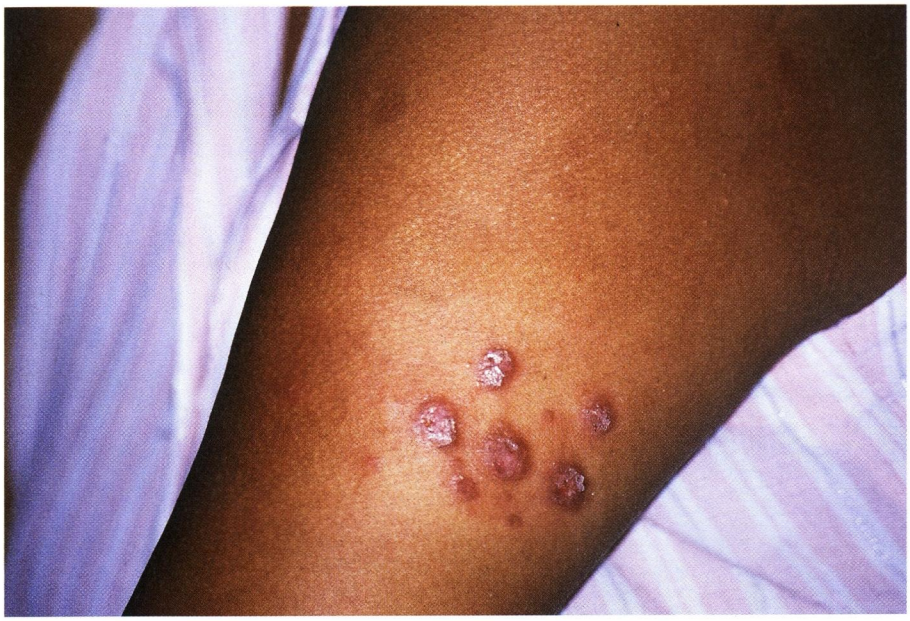

Figure 6.1.3. A clinical picture (papular type) of a 36-year-old female. Eight papules sized from small grain to pea were seen on her left arm.

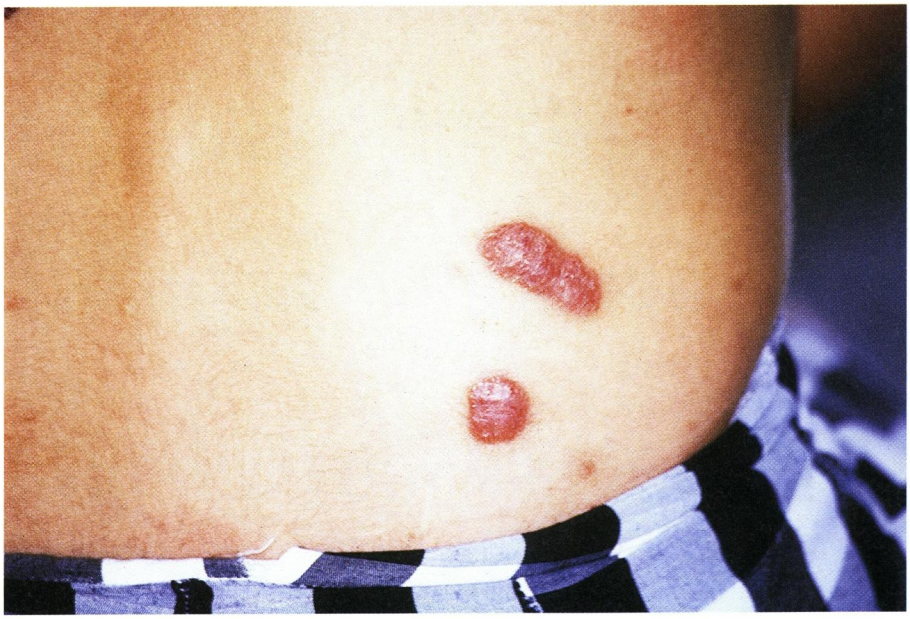

Figure 6.1.4. A clinical picture (plaque type) of a 16-year-old female. Two erythematous plaques were seen on her left iliac region. The surface showed granular.

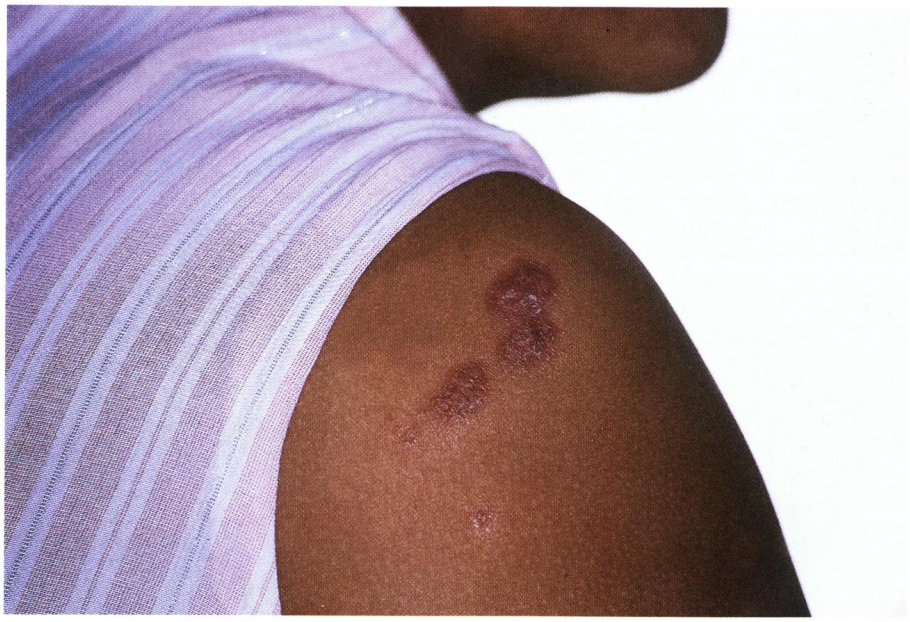

Figure 6.1.5. A clinical picture (plaque type) of a 26-year-old female. Three erythematus plaques were seen on her right deltoid region.

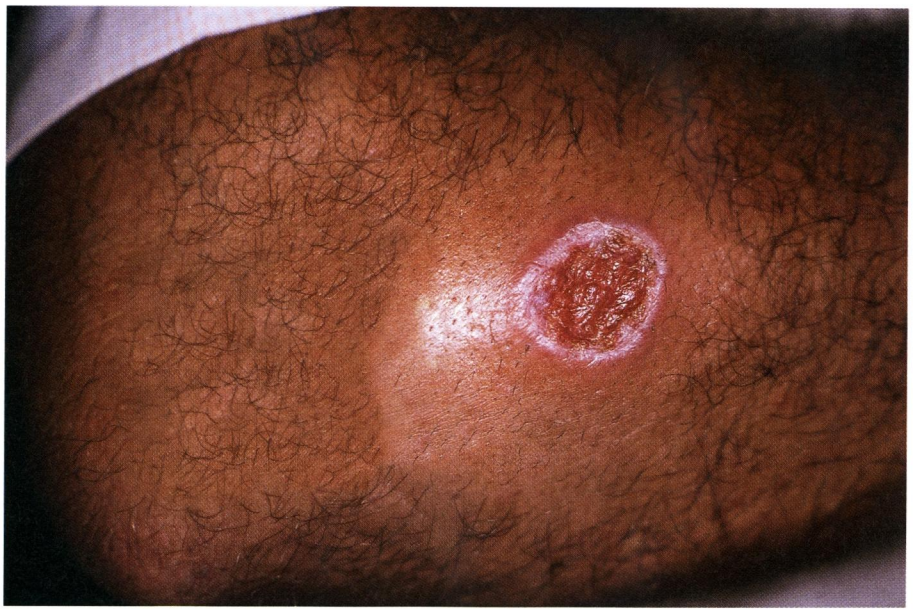

Figure 6.1.6. A clinical picture (ulcer type) of a 42-year-old male. A 20x20 mm sized ulcer with infected crust was seen on his left forearm. This picture shows a condition removed superficial crust.

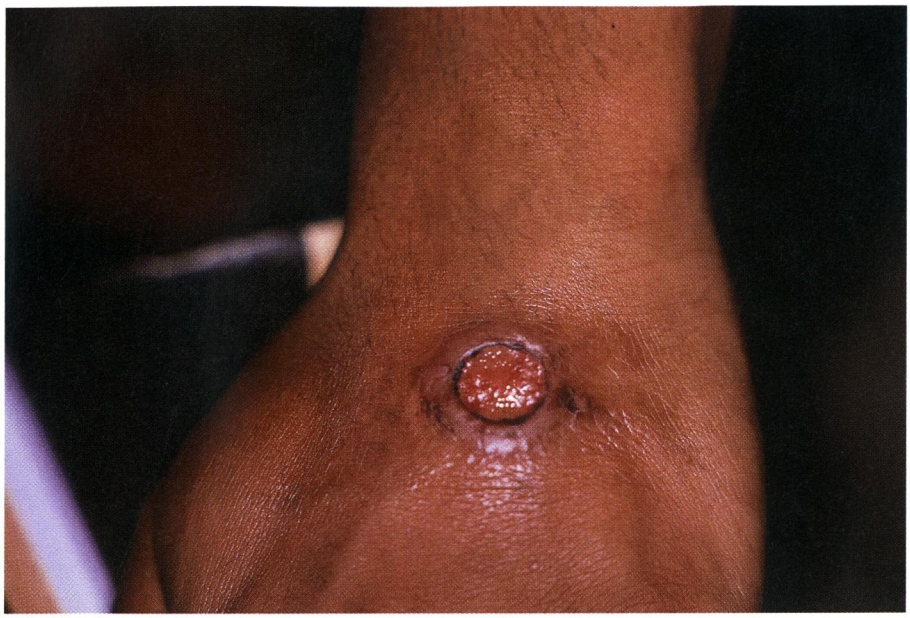

Figure 6.1.7. A clinical picture (ulcer type) of a 27-year-old male. A 10x10 mm sized ulcer with hard bank was seen on his left back of the hand.

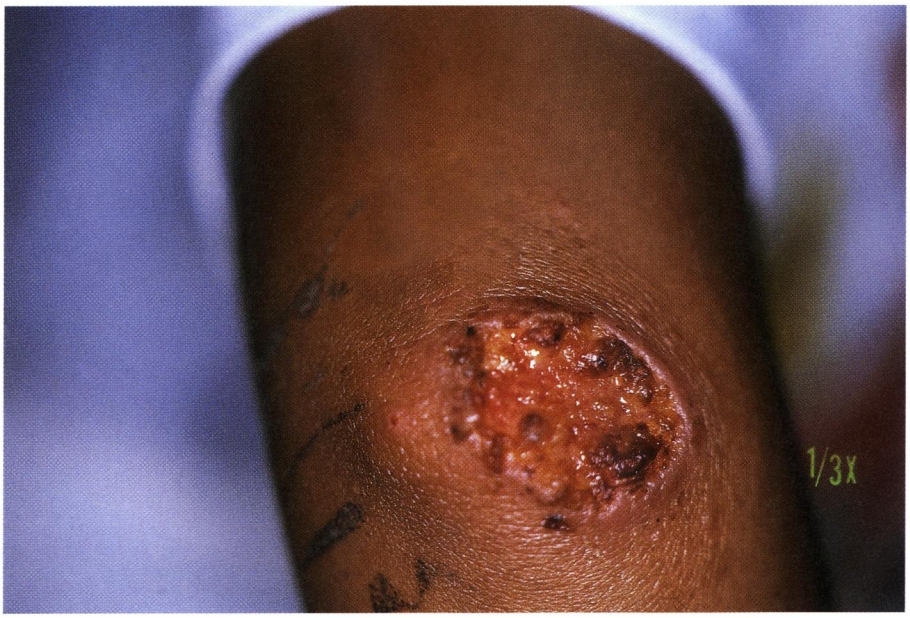

Figure 6.1.8. A clinical picture (ulcer type) of a 10-year-old girl. An ulcer sized 35x25 mm was seen on her left elbow. The surface of the ulcer had a secondary infection and was dirty and stinking.

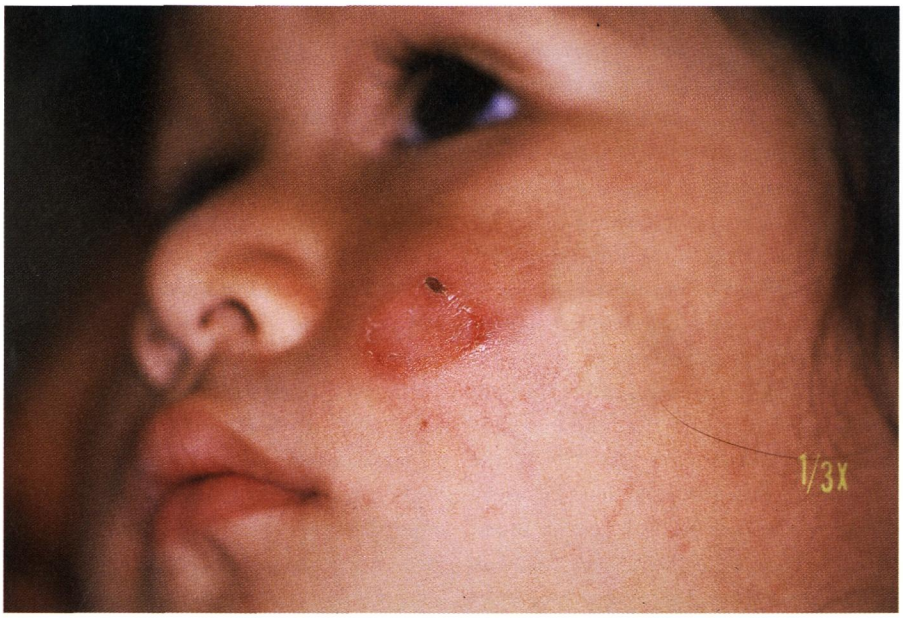

Figure 6.1.9. A clinical picture (scar type) of a 3-year-old girl. The lesion sized 13x16 mm was almost healed, but slight indurated erythematous erosion was still remained peripherally.

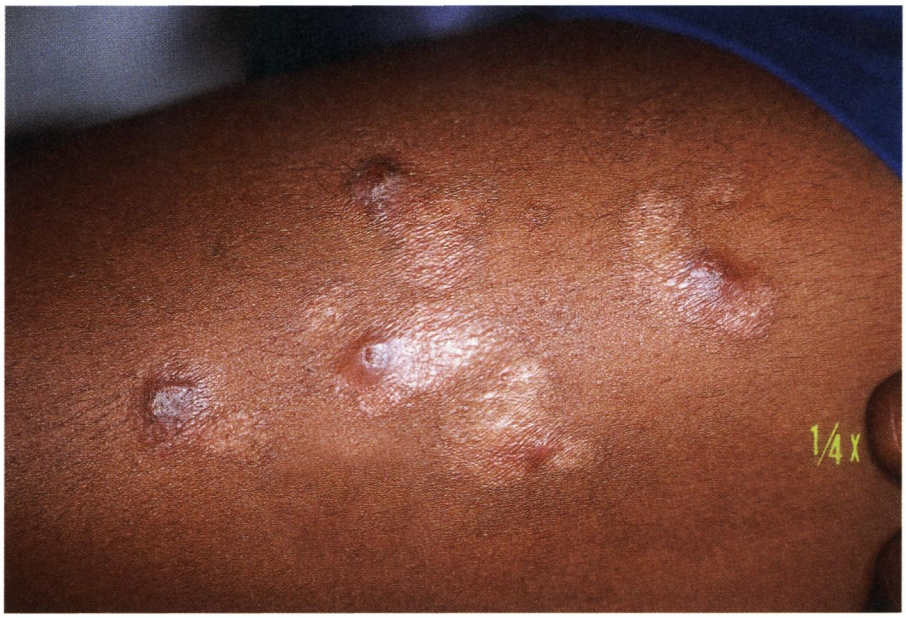

Figure 6.1.10. A clinical picture (scar type) of a 12-year-old boy. The lesions were almost healed, but new papules appeared again in the scars.

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 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. The most frequent duration time of lesions was three months (Nonaka *et al.,* 1992). 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% had 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 changes were also seen. The lesions with size of less than 400 ㎟ were frequent. There were 21 cases with lesions of more than 900 ㎟ and all of them were ulcerated. In this study, we analyzed 570 patients in total. The mean age of the patients was 2 years older than that in the previous study. Senior patients with more than 50 years of age were more frequent than those in reported previously (Nonaka *et al.,* 1990a). The mean duration period of cutaneous lesions was 1.5 months longer than in the previous study. The mean number of cutaneous lesions was 2.7. Seven cases possessed more than 10 lesions. This data showed a similar tendency as reported previously (Nonaka *et al.,* 1990a). The most frequent site was the face following the extremities, the exposed areas to various stimuli. This indicates that exposed body sites of human are possible sites for sandfly to bite for blood ingestion. 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 parasite species, various behaviors of the vectors and different life style of the inhabitants.

In comparison with other reports, a trial of paromomycin ointment to cutaneous leishmaniasis was reported in Tunisia (Ben Salah *et al.,* 1995). A total of 115 patients were compared, group one was treated with paromomycin ointment, while the other group with placebo. The mean age of their reported patients were 19.2 year old in treatment group and 18.2 year old in placebo. The most popular location of the lesions was extremities, 47.4% and 42.4% on upper limbs, 38.6% and 51.7% on lower limbs. The lesions were seen only 8.8% and 3.5% on face. The clinical appearance and site are different from the ours, and it may be due to a difference of clothing manner. Mean times from appearance of lesions were 39.7 days in treatment group and 33.5 days in placebo group. These data are shorter compared with our data, 5.58 months in male and 6.96 months in female. Furthermore, mean size of lesions were approximately 400 ㎟. A trial of topical paromomycin treatment to 251 patients with cutaneous leishmaniasis was reported in Iran (Asilian *et al.,* 1995). Their patients tended to be younger, and lesions were frequent on limbs. Onset of lesions was shorter as compared to ours. The most popular type of lesions was ulcer like same as of our data. The site of the lesions to be frequent on limbs was different from ours, but similar to Ben Salah's data (1995). A therapeutic trial with antifungal drugs to cutaneous leishmaniasis was performed (Larbi *et al.,* 1995). Velasco-Casttrein *et al.* (1997) treated 201 patients of cutaneous leishmaniasis with localized current field (radio frequency) in Tabasco, Mexico. They treated 201 lesions with localized current field. The time of evlution of lesions was common between 2 and five months, and tended to be longer than Tunisia and Iran. However, the lesions existed more than 3 years, and were seen in 4.97% in total, and 13.6% only were on the face. These data were quite different from ours, 24.7%. Alcais *et al.* (1997) reported an interesting study, in Bolivia describing the risk factors for onset of cutaneous and mucocutaneous leishmaniasis, showing a big difference of patient's age

between native and migrants. The highest frequency in natives was 15 to 19 years, but migrant patients 0 to 4 years. They described that significant risk factors for cutaneous leishmaniasis were gender, native/ migrant status, activity, and home-forest distance. Sousa *et al.* (1995) reported that, in Ceara State, Brazil, 77% of patients with parasitologically confirmed cutaneous leishaniasis had lymphadenopathy, furthermore, they confirmed cultures of lymph node aspirates yielded *Leishmania* more frequently (86%) than cultures of aspirates of skin (53%) or biopsies of skin (74%). We did not examine on lymphadenopathy in cutaneous leishmaniasis. *L. (V.) braziliensis* infection frequently accompany the lymphadenopathy in patients with cutaneous lesions. This means that, *L. (V.) braziliensis* parasites have to be killed not only in skin, but also in lymphnodes. There is an interesting report on mucosal leishmaniasis in Sudan (El-Hassan *et al.*, 1995). They examined 14 cases of mucosal leishmaniasis, and these patients had not only mucosal lesions, but also infection in lymph nodes, rarely bone marrow and spleen. The parasite was identified as *L. (L.) donovani* in 4 patients and as *L. (L.) major* in one, and unknown species in seven. They described that unlike American mucocutaneous leishmaniasis, mucosal leishmaniasis in Sudan was not preceeded or accompanied by cutaneous lesions and response to pentavalent antimony or ketoconazole was good. Lymphadenopathy may be different status by a different species of *Leishmania*. Lymphadenopathy in cutaneous leishmaniasis due to other species should be examine in detail. In Israel, sixty-seven cases of cutaneous leishmaniasis were examined for topical treatment of paromomycin sulphate and methylbenzethonium chloride (El-On *et at.,* 1986). Their patients had multiple, from 6 to 10 lesions due to *L. (L.) major.* In Iran, randomised vaccine trial of single dose of killed *L. (L.) major* plus BCG against anthroponotic cutaneous leishmaniasis was performed (Sharifi *et al.,* 1998). They used two groups, one (1839 children) was BCG and *L. (L.) major* antigen, and the other (1798 children) was BCG only. After 24 months of vaccination, they observed 53 cases in BCG with *L. (L.) major* antigen group, and 63 cases in BCG group. They did not recognize any difference of clincial severity between both groups. In summary, from our study, as previously reported, we described that the clinical symptoms may be not changed by only immune responses. Clinical symptoms may be different by life style, environmental factors, nutritional conditions, immunological status and etc.

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2. Clinical Features of Mucocutaneous Leishmaniasis in the Amazonian Region of Ecuador

ABSTRACT. Active and autochthonous 13 cases of mucocutaneous leishmaniasis from the Amazonian region of Ecuador are reported. The main clinical features were erythema, ulcerations, granulomas, septal perforation, swelling of upper lip and nose, bleeding and crusts. The mucosal tissues of nose, oral mucous and upper lip were the most affected. *L. (V.) braziliensis* was identified from two patient and others five are the subgenus *Viannia*, identified by polymerase chain reaction (PCR). 12 (92.3%) patients had typical scar appeared 4 to 38 years after healing of the primary cutaneous lesion. Seven anthropophilic *Lutzomyia* sandflies were identified, but none were incriminated as vector. This report emphasizes the importance of active search for mucocutaneous leishmaniasis in the Amazonian region and highlights the importance of early diagnosis and treatment.

Introduction

Mucocutaneous or mucosal leishmaniasis (MCL) is the most serious and destructive clinical form of American cutaneous leishmaniasis. Figures of skin lesions and facial deformities have been represented on pre-Inca pottery from Ecuador, Peru and Colombia dating back to the first century AD. These are evidences that cutaneous and mucosal forms of leishmaniasis prevailed in the New World as early as this period. Although MCL was reported in Ecuador since 1924 and is believed to be endemic in the Amazonian region, relatively little active research has been done. Heinert reported the first case of MCL who was admitted at the general hospital in Guayaquil (Heinert, 1924), after that in 1931 Trujillo reported a case of MCL form with osteoperiostitis (Valenzuela 1928). Leon (1954) made several considerations on MCL in Ecuador and other countries in South America. In 1979, Leon and Leon (1979) reported information on the diverse clinical aspects. Calero *et al.*(1986) reported two cases from the Amazonian region in 1986. The latest report that we are aware are from Amunarriz (1991) who reported 27 cases of MCL from the Amazonian region, during a period of 20 years. 260 cases were reported in Ecuadorian medical journals during 1920 and 1987. Of these, 239 (91.9%) were cutaneous form whilst 18 (6.9%) were MCL (Hashiguchi and Gomez, 1991). After that only clinical case reports have been made and most of them were diagnosed without parasitological confirmation.

MCL has been described as a chronic, severe and destructive disease that occurs several years after a primary skin ulcer has healed. Who have had multiple or chronic primary lesions, particularly above the waist, are at greatest risk. Between 2 and 40% of patients with cutaneous ulcers due to *Leishmania (Viannia) braziliensis,* and a much smaller proportion of those due to *L.(V.) guyanensis and L.(V.) panamensis,* will develop metastatic mucosal lesions. Recently in Colombia *L.(V.) panamensis* was attributed to cause the 76.6% of MCL of the Colombian Pacific Coast (Osorio *et al.,* 1998). Most commonly the nasal mucosa is affected anteriorly. If untreated, the lesions can perforate the septum and the nasal cartilage may collapse and the destructive lesion spreads to involve oronasopharyngeal mucosa down to and sometimes beyond the larynx, causing dysphonia and severe mutilation. Death may occur due to pneumonia, secondary sepsis or starvation.

In years 1999 and 2000, a collaborative research program was started between scientists of England, Japan, and investigators of Hospital Vozandes and Universidad Central de Quito y Catolica de Guayaquil.

One of the purposes of this study was to determine in an active search of the real incidence of MCL in indigenous population living at North East of Amazonian region. We report here the clinical characteristics of MCL among patients infected in the before mentioned area. The parasites strains causing the disease and the efficacy of treatment will be reported later on.

Materials and Methods

Biogeography of study place

Ecologically, Ecuador is an extremely rich and diverse country. Amazonian region is located to the east of the Andes mountain of the continental Ecuador. The place of study is situated in Provinces of Sucumbios and Orellana. The communities surveyed were Panduyacu, Dashino, Arenal and Km. 18 (Prov. Sucumbios); San Sebastian del Coca and Cotapino (Prov. Orellana) (Table 6.2.1). This area is covered by tropical rain forest with altitude of 200-300 m above sea level. The temperature varies between 23-24℃ and the humidity rises above 80% (Teran, 1984). The great variety of fauna and flora is similar to that found in the Amazon and Orinoco basins and would maintain an abundance of mammalian reservoirs and sandfly vectors.

Study population

Individuals suspected to have MCL were clinically examined and then recruited under a protocol study to be evaluated for parasitological confirmation. Demographic information, clinical characteristics of mucosal lesions, as well as time evolution and localization of cutaneous and mucosal lesions or scars were included. MCL was diagnosed by Giemsa-stained smear, culture of aspirate tissue fluid, PCR or histopathologic study of skin biopsy obtained from the lesion borders or granulomas. As well as using standard indirect ELISA and leishmanin skin test. House to house survey was also made in order to detect cases. Signed informed consent was obtained from each patient. The study protocol was approved by the ethical committee of the Hospital Vozandes, Quito, Ecuador.

Severity of lesions

The lesions were grouped by level of severity as follows: mild = limited to a single mucosal site; moderate = lesions in more than one mucosal site without perforation or mutilation; and severe = septal perforation and/or mutilation plus lesions in more than one mucosal site (Fig. 6.2.2).

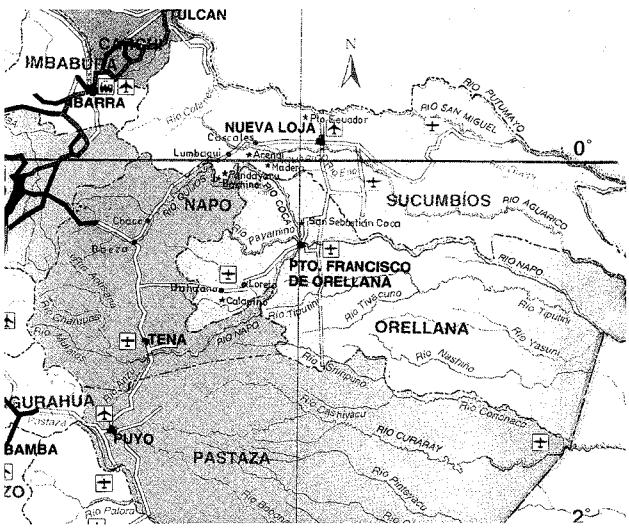
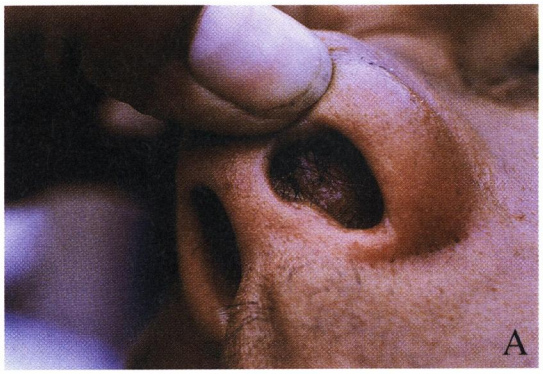
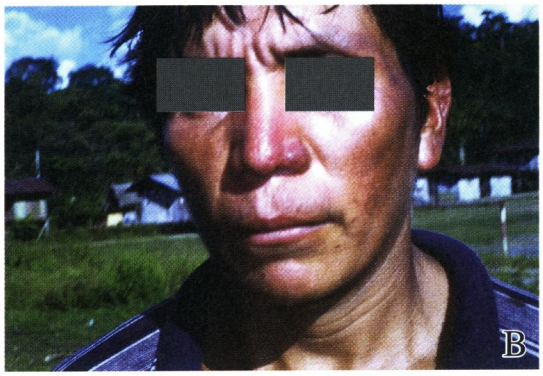


Figure 6.2.1. Map of the Northern Amazoian region of Ecuador. Showing the Provinces of Sucumbios and Orellana. Asterisks(*) indicate the communities from where patients came.





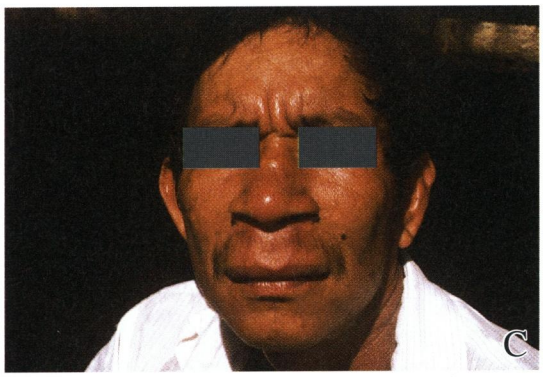


Figure 6.2.2. Pictures of patients with MCL. **A.** showing a granuloma in the nasal septum (Grade 1 = mild severity); **B.** showing mucosal lesions without perfolration or mutilation (Grade 2 = moderate severity); **C.** showing septal perforation with erythema on nose and upper lip etc. (Grade 3 = severe severity).

Sandfly collection and dissections

Sandfly collections were made by protected human bait placed in the vicinity of houses of confirmed cases in Cotapino as well as in the jungle during 18:00 and 21:00 hrs in the period of September 1999. A total of 43 sandflies were caught, dissected and examined microscopically for the presence of *Leishmania* in the gut.

Laboratory methods

Leishmania isolates were made by aspiration with fine-needle from active nodules, ulcers or erithematous nasal mucosa or upper lip. The aspirated material was then inoculated into NNN culture medium. Tube cultures were examined at regular intervals and were held for 3 or 4 weeks before being discarded. Serum specimens were analyzed by ELISA using *L.(Leishmania) amazonensis, L.(V.) panamensis* promastigotes crude antigen to detect IgG antibodies. Serum titers greater or equal to positive control were considered positive. The Montenegro skin test was performed on patients suspected of MCL by intradermal injection in the forearm of 0.1ml of a saline solution containing 5 x 10^5 killed *L.(V.) panamensis* and *L.(L.) amazonensis* promastigotes, the area of induration was measured 48 hr post-injection, and a diameter greater than or equal to 10 mm was considered positive. Smears were prepared from lesion scrapings, fixed with methanol, and stained with Giemsa for microscopy. Small 3 mm of diameter biopsy were taken using a sterile punch from nasal or lip lesions. These skin biopsies were divided in two, one fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histologic analysis. The second part of biopsy was stored in lysis buffer for PCR analysis.

Analysis by PCR

An aspirate or biopsy was taken from active nodules or erithematous nasal mucous and placed in 200 µl of lysis buffer (NaCl, EDTA, Tris). The DNA extraction and amplification was conducted as described by Mimori *et al.* (1998) using primers subgenus-specific L1-L2 and V1-V2; *L.(L.) mexicana, L.(V.) braziliensis,* and *L. (V.) panamensis*-specific

Table 6.2.1. Demographic aspects and localization of scars in patients with MCL in the Amazonian regions of Ecuador

Characteristics	No.	%
Gender		
Male	11	84.6
Female	2	15.4
Race		
Indigenous	10	76.9
Mestizo	3	23.1
Age (years)		
0 - 20	0	00.0
20 - 30	3	23.1
31 -40	4	30.8
41 - 50	2	15.4
51 - 60	3	23.1
60 - plus	1	7.7
Localization of cutaneous scars		
Face	2	15.4
Upper extremities	8	61.5
Lower extremities	3	23.1
Trunk	0	00.0

Table 6.2.2. Frequency of affected mucosa and clinical presentations of the mucosal lesions in patients with ML from Amazonian regions of Ecuador

Characteristics	No.	%
Localisation of lesions		
Nasal tissue	13	100
Oral mucosa	4	30.8
Upper lip	5	38.4
Characteristics of lesions		
Erythema	13	100
Bleeding and crusts	8	61.5
Septal perforation	7	53.8
Granuloma	6	46.1
Swelling of upper lip	6	46.1
Enlargement and protuberant nose	5	38.4
Voice disturbance (dysphonia)	4	30.7
Ulcer	3	23.0
Mutilation	3	23.0
Crusts	2	15.3
Erosion of septum without perforation	2	15.3

PCR assay. The products were gel purified in agarose 1.5% and stained with ethidium bromide.

Results

Demographic aspects of patients

The majority of the 13 patients with MCL were adults (age range = 22-67 years old, median = 42.4 years old), indigenous 80% (10 out of 13), 11 males and 2 females. All patients were infected in the provinces of Orellana and Sucumbios (Table 6.2.1).

Clinical characteristics

The 13 patients presented a total of 64 mucosal lesions. All patients had lesions in nasal mucosa tissue, septum and turbinate, 5 in upper lip and 4 in pharynx. Larynx was not examined, but 4 patients suffered of dysphonia. Mucosal lesions appeared more frequently as an erythema (13), bleeding and crusts (8), septal perforation (7), granuloma and swelling of upper lip (6), protuberant nose (5), dysphonia (4), and etc. (Table 6.2.2).

The severity of mucosal lesions showed thus: mild (2 cases), moderate (4 cases) and severe (7 cases) manifestations. The mean duration of mucosal lesions at diagnoses was 11.6 years (evolution range 1 to 25 years). Severe lesions always had a longer time of evolution (17.3 years) and conversely for mild lesions. No active cutaneous lesion was found concomitantly.

The majority (92.3%, 12 of 13) of the patients had evidence of previous typical scars of cutaneous leishmaniasis, which were located on face (2), upper extremities (8). Only one scar was located contiguous to the nasal mucosa tissue.

Diagnosis and species identification

Of the 13 cases with MCL, in 3 (23%) we could isolate the parasite in culture and 2 were identified to be *L (V.) braziliensis* by PCR. The others 5 were determined by PCR from biopsy/aspirate to be subgenus *Viannia* group and 1 to be subgenus *Leishmania.* All 9 patients had strong response to Montenegro skin test and high titeres of antibodies by ELISA

Entomologic data

Seven antrophophilic species *of Lutzomyia* could be identified in Cotapino and surroundings: *Lu. tortura, Lu. flaviscutellata, Lu. olmeca bicolor, Lu gomezi, Lu. carrerai thula, Lu. geniculata* and *Lu. yuilli yuilli.*

Discussion

The information on geographical distribution and prevalence of MCL in Ecuador has been scarce and fragmented, even it has been reported since 1924 (Heinert, 1924). Parasitologically confirmed cases has been reported only from the Amazonian region and the most important casuistic is from Napo basin, province of Orellana (Amunarriz, 1991). Rare cases have been recorded from the Pacific coast by only clinical diagnosis, the case with osteoperiostitis coming from province of Esmeraldas (Valenzuela, 1928) could be yaws, because its complication never after has been reported with MCL, another supposed case of MCL from Puerto Quito was later confirmed to be produced by fungus *Blastomyces dermatitis* and never recovered after received Glucantime longer. An important point to clear is that several cases have nasal mucosal lesions by the ability of *Leishmania* parasite to invade mucosal tissue due to contiguous localization of skin lesions, it is not real MCL.

The knowledge about the time and form of evolution and, clinical presentation is important in early recognition and correct treatment of MCL. This active search favors the description of early and late events in the natural history of MCL and we could indicate that mucosal lesions start with symptoms in the nasal tissue with pruritus, discharge, bleeding, crusts and obstruction. Signs observed initially are nasal mucosal erythema, bleeding-crusts, erosion of septum and turbinate of nasal tissue, nodules and granulomas. It continues to invade nasal tissue and produce hypertrophy protuberant nose, ulcers. Finally it goes to septal perforation, invade upper lip, pharynx, larynx producing dysphonia and mutilation because the nasal cartilage may collapse, predisponing to pneumonia or

secondary sepsis. This sequence is not always true, laryngeal lesions in the absence of other mucosal lesions have been reported (Jones *et al.,* 1987).

Only 1 (7.7 %) out of 13 did not present previous scar of cutaneous leishmaniasis and nobody presented active cutaneous lesions simultaneously. This finding correlates with other reports (Marsden, 1986; Llanos-Cuentas *et al.,* 1984, Saenz *et al.,* 1989), but contrasts with findings in Colombian MCL reported in two Colombian studies where 61% and 63% presented cutaneous and mucosal lesions simultaneously and only four (17%) had typical scar (Osorio *et al.,* 1998, Weigle *et al.,* 1993). Such a difference could be explained by because Colombian cases are coming from the Pacific coast and the majority are produced by *L. (V.) panamensis.* However, still the anxiety to find MCL in the Pacific coast of Ecuador where *L. (V.) panamensis* are prevalent (Armijos *et al.,* 1997; Hashiguchi *et al.,* 1991) as been found in Colombia and Panama (Saenz *et al.,* 1989; Osorio *et al.,* 1998). Such difference between Ecuador and Colombia or Panama could reflect particular characteristics of the population or parasite strains in the region rather than a species related difference as pointed by Osorio *et al.* (1998). And in our cases two of them were identified as *L. (V.) braziliensis* and the others belong to the subgenus *Viannia* determined by PCR.

The mild severity of lesions and their interval between mucosal symptoms and healing of the skin lesions found here suggests that mucosal involvement occurs soon after a skin lesion appeared without a long latent period and seems to be shorter than reported by Marsden *et al.* (1991) and Jones *et al.* (1987). But the period between the parasite's invasion and becoming sever disfigurings is longer, it may take 10 to 15 years.

In accordance to previous studies we found one patient without previous scar suggesting that subclinical infection could occur as reported from patients with MCL (Wiegle *et al.,* 1986), furthemore, the ability of *Leishmania* parasites to remain latent during long periods of time and became active and invasive after exogenous factor such as malnutrition, to age or a concomitant unrelated illness or altered immunocompetence is also feasible (Marsden, 1986).

Localisation of mucosal lesion was similar to that reported elsewhere (Marden *et al.,* 1991; Jones *et al.,* 1987; Osorio *et al,* 1998; Llanos Cuentas *et al.,* 1984); those findings indicated the deep mucosa of the nose as the area most frequently and primarily affected. Hence a complete clinical examination helped with rhinoscopy of the upper respiratory tract is mandatory in suspected patients with MCL, and it is necessary to establish the extension and severity of the disease.

In our study 7 (53.8%) patients were found with severe form of MCL as reported by other authors (Llanos-Cuentas *et al.,* 1984; Marsden, 1986), it could be explained because of the long time of evolution (median =17.3 years) and also because of major virulence and the capability of inducing a destructive immune response of *L.(V.) braziliensis* (Saravia *et al.,* 1989), compared with *L. (V.) panamensis* or *L.(V.) guyanensis* as reported in Colombia and Panama which limited to one mucosal surface (mild) in the majority of patients (Osorio *et al.,* 1998; Saenz *et al.,* 1991).

Walton *et al.* (1979) reported that patients of African ancestry suffered more aggressive mucosal lesion than indigenous people. In this report 10 of 13 patients were indians and the severities of lesions were similar to mestizo according to evolution time. So, the pathogenesis of MCL has not yet been elucidated depending on several external and internal factors accomplished by the parasite and host and probably by the vector.

Young adult males who have had multiple or chronic primary lesions, particularly above the waist, are at greatest risk (Llanos-Cuentas *et al.,* 1984). In the present cases 83.3% (10 of 12) of scars were located on the face and upper extremities.

In conclusion, the incidence of MCL in the Amazonian region of Ecuador is present and seems to be highly prevalent in Indians groups. Active research focused in the communities will reveal the real prevalence and the severity of the disease. Furthermore it will improve detecting mild cases with early diagnosis and treatment consequently avoiding severe forms and mutilation, which is a social stigma. And, indi-

viduals consulting for active cutaneous form should be examined and carefully evaluated for mucosal lesions in order to detect early manifestations of MCL.

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3. A Comparison of Ultraviolet Radiation Energy between Lowland and Highland in Ecuador - Is the Skin Manifestation of Cutaneous Leishmaniasis Related to Ultraviolet Radiation ?

ABSTRACT. Ultraviolet radiation has a tremendous influence on immune system, especially to skin. It is speculated that ultraviolet exposure plays a role in infectious condition. However, a few studies describing relationship between infectious disease and ultraviolet radiation have been reported. In our previous report, we described a relationship between cutaneous leishmaniasis and ultraviolet light. We observed that there was a marked difference in clinical appearance between the two areas, the highland and lowland of Ecuador. In this study, we tried to analyze ultraviolet radiation energy in Ecuador, and to compare the difference of radiation energy between lowland and highland. The energy of UVB and UVA irradiation was measured using an UV-radiometer UVR-305/365D(II) (Topcon, Tokyo, Japan). It was measured at different places in Ecuador and at a top of the building of University Hospital, University of the Ryukyus, Okinawa, Japan. Meteorological data in Ecuador, obtained from the annual meteorology 1994, No.34 (National Institute for Meteorology and Hydrology, Quito, Ecuador) was also used. From the data, the daylight time UV energy was picked up. A total of eight places in highland and ten in lowland were selected. The mean altitude was 2669.8m in highland, and was 139.5m in lowland. Annual daylight time in highland was 1801.7 hrs and was 904.1 hrs in lowland, while it was 1876.4 hrs in Okinawa. The mean values of UVB and UVA energy in lowland were 0.433, and 3.544 mW/cm^2 respectively, and in highland were 0.655 and 4.958 mW/cm^2 respectively. The mean value of UVB and UVA in Okinawa was 0.303 and 2.698 mW/cm^2 respectively. From these data, the annual total UVB and UVA values were calculated and estimated. Annual UVB and UVA energy level in lowland was 0.56KJ/m^2 and 4.61KJ/m^2 respectively. In highland, those annual energy levels were 1.70KJ/m^2, and 12.86KJ/m^2 respectively. Therefore, highland/lowland ratio of UVB energy was 1.70/0.56 = 3.04 and the ratio of UVA was 12.86/4.61 = 2.79. This indicates that the UV energy in lowland was approximately one-third times less than in highland. Annual energy in Okinawa was 0.82 KJ/m^2 in UVB, and 7.29 KJ/m^2 in UVA. These values were located between those in lowland and highland. Definitely, every individual including human have a different susceptibility to the UV radiation, therefore, every individual will have a different immunity. If different individuals are exposed to some energy of UV radiation, the immunity of one individual may be up-regulated, but that of other may be down-regulated. So, it is extremely difficult to predict that UV radiation causes up-regulation or down-regulation of immune system. The influence of UV radiation may be different by the individuals. Therefore, clinical symptoms of infectious diseases such as cutaneous leishmaniasis may be various. We have to further investigate in response to elucidate what kind of factors affects immune system of cutaneous leishmaniasis.

Introduction

Ultraviolet radiation has a tremendous influence on immune system, especially on the skin. Ultraviolet radiation is divided into three main wavelengths, short-wave (UVC, 200 to 280 nm), mid-wave (UVB, 280 to

320 nm) and long-wave (UVA, 320 to 400 nm). UVC radiation does not reach the earth's surface because of totally absorbed by the atmospheric ozone layer (Granstein, 1999). It is a well-known fact that ultraviolet radiation induces skin cancer (Ichihashi *et al.,* 1999), DNA damages and alterations of immune regulation (Horio, 1999). It is speculated that there is an important role of ultraviolet exposure in infectious diseases. However, a few reports of a relationship between infectious disease and ultraviolet radiation have been reported. Giannini (1986) reported the relationship between UVB and cutaneous leishmaniasis. In our previous study, we described a relationship between cutaneous leishmaniasis and UVA (Khaskhely *et al.,* 2001). On the other hand, we observed that there was a marked difference of disease outcome in highland and lowland of Ecuador (Nonaka *et al.,* 1990). The mean age of the patients in lowland was 20 years, and of highland was 2 years. The number of the lesions was more in lowland than in highland. Large ulcers were frequently seen in lowland, but those in highland were very small. The lesions were wet types in lowland, however dry ones in highland. Different factors such as difference of vectors and species of *Leishmania,* with or without secondary infection, the life style of patients and etc., were suspected for these clinical difference between two areas. In this study, we tried to analyze ultraviolet radiation energy level in Ecuador, comparing the difference of radiation energy between lowland and highland.

Materials and Methods

Measurement of ultraviolet light

The energy of UVB and UVA irradiation was measured by using an UV-radiometer UVR-305/365D (II) (Topcon, Tokyo, Japan). It was measured at different places in Ecuador (Guayaquil city, and Andean places) and the top of university hospital building, University of the Ryukyus, Okinawa, in Japan.

Estimation of energy of UVB and UVA per day

Meteorological data in Ecuador were used from the annual meteorology 1994, No. 34 (National Institute for Meteorology and Hydrology, Quito, Ecuador). From these data, the daylight time was picked up. Eight places in highland and ten places in lowland were selected (Tables 8.3.1 and 8.3.2).

Table 8.3.1. The daylight time in Andean highland of Ecuador

Locality	La Tola	Izobamba	Rumipanba	La Argelia	San Gabriel	Otavalo	Querochaca	Tomalon	Mean
Altitude (m)	2480.0	3058.0	2680.0	2160.0	2860.0	2550.0	2940.0	2790.0	2689.8
Jan	183.3	149.2	155.9	96.7	128.2	158.9	161.9	185.3	152.4
Feb	144.7	126.1	130.9	94.3	112.8	119.1	120.1	147.3	124.4
Mar	123.0	111.6	136.8	110.7	85.6	106.3	131.1	122.8	116.0
Apr	134.9	123.1	124.0	119.6	95.1	134.0	127.1	151.9	126.2
May	150.2	133.5	134.3	134.1	113.8	149.3	125.9	162.5	138.0
Jun	189.5	172.7	154.5	112.3	109.5	180.2	146.7	221.0	160.8
Jul	240.2	223.8	146.7	133.7	110.0	205.8	133.7	268.3	182.8
Aug	211.5	186.0	142.7	115.9	102.6	168.8	109.9	230.5	158.5
Sep	211.0	175.6	149.8	135.0	111.1	146.3	119.7	237.2	160.7
Oct	198.8	185.3	195.7	170.4	134.5	178.2	184.2	206.2	181.7
Nov	155.7	142.3	151.2	121.6	135.4	149.0	155.2	161.3	146.5
Dec	161.9	155.9	169.5	153.0	128.4	149.6	139.0	173.8	153.9
Total	2104.7	1885.3	1792.0	1497.3	1367.0	1844.5	1654.5	2268.1	1801.7

Table 8.3.2. The daylight time in lowland of Ecuador

Locality	Portviejo	La Concordia	Milagro	Bucay	Babahoyo	Julcuy	Machala	El Vergel	Vinces	Guayaquil	Mean
Altitude (m)	48.0	380.0	13.0	480.0	7.0	240.0	25.0	155.0	41.0	6.0	139.5
Jan	71.3	59.6	87.3	25.8	74.9	49.5	51.2	50.1	75.4	69.6	61.5
Feb	80.7	59.9	74.4	27.0	78.3	72.0	52.5	59.9	84.1	72.6	66.1
Mar	118.2	76.1	107.2	12.9	104.6	104.6	55.2	56.1	105.6	104.4	84.5
Apr	105.4	72.8	92.7	57.6	91.9	93.2	63.2	60.4	98.1	96.0	83.1
May	120.9	62.5	99.0	44.7	81.9	115.8	67.9	52.4	86.1	100.2	83.1
Jun	107.4	47.8	49.3	46.3	28.5	82.9	36.1	24.7	51.6	91.5	56.6
Jul	148.5	57.0	71.2	68.1	61.2	141.1	37.3	33.0	91.2	136.1	84.5
Aug	133.8	55.2	66.2	52.4	43.7	155.9	21.1	39.9	62.0	135.1	76.5
Sep	200.1	87.8	119.1	72.7	99.9	212.5	44.4	80.9	136.7	191.9	124.6
Oct	122.2	69.4	54.3	49.9	57.3	126.0	17.5	49.3	89.2	105.3	74.0
Nov	99.5	42.5	43.5	33.1	45.4	91.8	10.6	39.0	74.6	95.4	57.5
Dec	90.0	45.7	79.6	31.8	58.1	81.0	40.1	51.0	89.7	52.5	62.0
Total	1398.0	736.3	843.8	522.3	825.7	1326.3	497.1	596.7	1044.3	1250.6	904.1

Table 8.3.3. Daylight time in Okinawa, Japan (mean value between 1961 and 1990)

Locality	Naha city	Minami Daito	Miyako jima	Ishigaki jima	Yonaguni jima	Kume jima
Altitude (m)	28.1	14.4	39.9	5.7	30	4
Jan	95.6	117.3	91.5	84.5	56.0	81.2
Feb	89.1	114.0	84.5	78.8	52.3	76.6
Mar	114.3	141.4	114.9	107.9	80.4	101.4
Apr	148.8	171.5	146.9	141.1	119.4	138.6
May	150.8	177.0	157.3	157.6	139.0	139.0
Jun	178.9	200.2	194.1	199.7	185.1	169.7
Jul	258.1	262.4	261.0	266.2	264.8	261.3
Aug	230.9	233.4	236.5	235.9	241.5	240.9
Sep	207.6	227.4	204.5	204.9	199.9	211.1
Oct	174.0	181.3	167.6	164.0	151.1	172.6
Nov	120.3	122.7	108.6	108.9	80.2	107.3
Dec	108.0	106.5	97.4	95.9	64.1	94.2
Total	1876.4	2055.1	1864.8	1845.2	1633.7	1793.9

Table 8.3.4. The energy level of ultraviolet radiation in lowland and highlamd of Ecuador

Date	Locality	Altitude	Time	Volume of Cloud	Weather	UVB	UVA
19990113	Guayaquil	30	900	5	fine	0.507	4.340
			1000	3	fine	0.605	4.730
			1100	2	fine	0.765	5.310
			1200	10	partly cloudy	0.556	4.449
19990114	Guayaquil	30	930	10	partly cloudy	0.325	2.960
			1000	10	cloudy	0.222	2.100
			1100	10	cloudy	0.230	1.990
19990115	Guayaquil	30	900	10	partly cloudy	0.343	3.070
			1000	10	partly cloudy	0.543	4.560
			1100	10	cloudy	0.322	2.570
19990115	San Herald		1200	10	partly cloudy	0.387	4.570
19990118	Guayaquil	30	900	10	cloudy	0.153	1.562
			1000	10	cloudy	0.198	1.903
			1200	10	cloudy	0.164	1.513
19990119	Guayaquil	30	1130	10	cloudy	0.293	2.360
19990120	Guayaquil	30	1000	3	fine	0.565	5.100
			1110	5	fine	0.656	5.500
			1200	7	fine	0.787	5.890
19990121	Guayaquil	30	1000	1	fine	0.610	4.700
			1100	1	fine	0.801	5.810
			1200	1	fine	0.763	5.420
			1300	5	fine	0.719	5.200
19990122	Guayaquil	30	1000	4	fine	0.648	4.810
			1100	2	fine	0.765	5.580
			1200	4	fine	0.718	5.140
19990123	Troncal	300	1000	5	fine	0.750	6.140
			1100	10	cloudy	0.205	1.853
			1200	10	cloudy	0.327	2.58
			1300	10	cloudy	0.272	2.54
			1400	10	cloudy	0.216	1.976
			1530	10	cloudy	0.148	1.619
			1300	10	cloudy	0.047	0.65
19990124	Troncal	300	1100	10	cloudy	0.137	1.3
			1200	10	cloudy	0.169	1.609
			1300	10	cloudy	0.33	2.71
			1400	7	fine	0.648	4.8
19990125	Guayaquil	30	1100	10	cloudy	0.107	1.002
			1200	10	cloudy	0.081	0.748

Table 8.3.4. (continued)

19990126	Guayaquil	30	900	10	cloudy	0.076	0.866
			1000	9	partly cloudy	0.358	3.35
			1100	10	partly cloudy	0.309	2.62
			1200	9	cloudy	0.427	3.8
19990128	Guayaquil	30	1000	4	fine	0.6	4.91
			1100	5	fine	0.916	6.4
			1200	5	fine	0.873	6.52
19990129	Guayaquil	30	1430	7	partly cloudy	0.33	3.23
19990130	La Mana	500	1200	10	cloudy	0.406	3.29
			1230	10	cloudy	0.628	5.03
			1330	10	cloudy	0.408	3.4
19990131	La Mana	500	915	10	cloudy	0.077	1.053
			1100	10	cloudy	0.433	3.81
			1200	10	cloudy	0.343	2.98
			1300	10	cloudy	0.143	1.41
			1400	10	cloudy	0.303	3.01
19990201			1000	9	cloudy	0.336	3
			1100	7	partly cloudy	0.374	3.1
19990206	Km20	100	1030	3	fine	0.778	6.03
	Km26	100	1100	4	fine	0.91	7.02
		850	1200	10	cloudy	0.431	3.61
Mean		158.4				0.433	3.544
19990207	Alausi	1200	1230	9	cloudy	1.102	7.5
		2500	1300	10	cloudy	1.603	10.05
		2700	1400	10	rain	0.224	2.35
		2200	1445	9	partly cloudy	1.225	8.25
		2300	1530	10	fog	0.332	2.91
		2200	1600	10	cloudy	0.544	5.58
		2200	1700	10	cloudy	0.073	0.853
		2300	830	9	cloudy	0.301	2.8
		2300	900	10	cloudy	0.489	4.33
Mean		2211.11				0.655	4.958

Table 8.3.5. The energy level of ultraviolet radiation in Okinawa, Japan

Date	Time	Volume of Cloud	Weather	UVB	UVA
19990215	930	3	fine	0.359	3.950
	1030	7	cloudy	0.245	2.220
	1500	8	cloudy	0.263	3.160
19990216	1200	0	fine	0.725	6.030
19990217	1000	0	fine	0.501	4.780
	1100	0	fine	0.668	5.400
	1200	0	fine	0.760	5.400
	1300	0	fine	0.785	5.860
19990218	900	0	fine	0.298	3.160
	1000	7	cloudy	0.189	1.760
	1100	0	fine	0.673	5.560
	1200	0	fine	0.760	5.950
	1300	7	fine	0.653	5.040
	1400	5	fine	0.679	5.450
	1500	8	cloudy	0.243	2.510
	1600	3	fine	0.287	3.050
	1700	8	cloudy	0.006	0.252
19990219	900	10	cloudy	0.009	0.073
	1200	10	cloudy	0.188	1.413
	1300	5	fine	0.566	4.870
	1400	9	cloudy	0.150	1.043
	1500	9	cloudy	0.334	3.390
	1600	10	cloudy	0.056	0.555
	1700	10	cloudy	0.000	0.088
19990220	1000	10	cloudy	0.034	0.340
	1200	10	cloudy	0.016	0.267
	1400	10	cloudy	0.036	0.351
	1700	10	cloudy	0	0.034
19990221	900	1	fine	0.431	3.78
	1100	0	fine	0.471	4.66
	1200	6	fine	0.588	4.78
	1300	5	fine	0.635	5.01
	1400	4	fine	0.533	4.34
	1500	9	cloudy	0.122	1.21
	1600	10	cloudy	0.052	0.618
	1700	10	cloudy	0	0.17
19990222	900	10	cloudy	0.062	0.837

Table 8.3.5. (continued)

	1000	10	cloudy	0.243	2.87
	1100	9	cloudy	0.41	3.62
	1200	10	cloudy	0.291	3.06
	1300	10	cloudy	0.354	3.64
	1400	8	cloudy	0.287	2.74
	1500	8	cloudy	0.246	2.31
	1600	8	cloudy	0.072	1.212
	1700	10	cloudy	0.012	0.301
19990223	900	0	fine	0.229	2.39
	1000	0	fine	0.418	3.65
	1100	0	fine	0.584	4.71
	1200	0	fine	0.643	5.07
	1300	0	fine	0.665	5.07
	1400	5	fine	0.411	3.64
	1500	5	fine	0.246	3.46
	1600	10	cloudy	0.07	0.773
	1700	10	cloudy	0.019	0.335
19990224	900	7	fine	0.235	2.75
	1000	10	cloudy	0.294	2.08
	1100	10	cloudy	0.193	1.493
	1200	10	cloudy	0.119	1.08
	1300	10	cloudy	0.23	2.67
	1400	10	cloudy	0.091	1.117
19990225	900	10	cloudy	0.104	1.204
	1000	10	cloudy	0.117	1.266
	1100	10	cloudy	0.228	2.15
	1200	0	fine	0.678	5.27
	1300	10	cloudy	0.462	3.63
	1400	10	cloudy	0.268	2.23
	1500	10	cloudy	0.159	1.566
	1600	10	cloudy	0.109	1.098
	1700	10	cloudy	0.012	0.357
Mean				0.303	2.698
Median				0.246	2.670

Results

The mean altitude in highland was 2669.8m, but that in lowland was 139.5m. Annual daylight time in highland was 1801.7 hrs, and that in lowland was 904.1 hrs. In Okinawa, the data of daylight time was shown in Table 8.3.3. Annual daylight time in Naha was 1876.4 hrs. The energy of ultraviolet irradiation measured in Ecuador and Okinawa was shown in Tables 8.3.4 and 8.3.5. Mean value of altitude examined in lowland was 158.4m, and 2211.1m in highland. The altitude in the building of Ryukyu University Hospital in Okinawa was approximately 78.0m. The mean UVB and UVA energy level in lowland of Ecuador was 0.433, and 3.544 mW/cm^2 respectively. That in highland was 0.655 and 4.958 mW/cm^2 respectively. The mean UVB and UVA energy level in Okinawa was 0.303 and 2.698 mW/cm^2 respectively. From these data, annual total UVB and UVA values were calculated and estimated respectively. Annual energy in lowland of Ecuador was 0.56KJ/m^2 in UVB, and 4.61KJ/m^2 in UVA. That in highland was 1.70KJ/m^2 in UVB, and 12.86KJ/m^2 in UVA. Therefore, highland/lowland ratio of UVB energy was 1.70/0.56=3.04 and those of UVA was 12.86/4.61= 2.79. This means that UV energy in lowland was approximately one-third times less than in highland. In Okinawa, the annual energy level of UVB and UVA was 0.82 KJ/m^2, and 7.29 KJ/m^2 respectively.

Discussion

Our data showed that UVB and UVA energy level in lowland was approximately one-third times less than in highland. Annual energy in Okinawa was located between those in lowland and highland. It is a question, is there any relationship between UV energy difference and clinical symptoms of cutaneous leishmaniasis ? It is very difficult to open a lock of this question. In general, UV radiations suppress an immune system, and aggravate cutaneous disease such as herpes simplex. Similarly, UVB-irradiated mice, before infection with *Candida albicans* (Denkins *et al.,* 1981), *Mycobacterium bovis* (Jeevans *et al.,* 1990), and herpes simplex virus (Howie *et al.,* 1986), showed decreased delayed type hypersensitivity response and more severe disease outcome. After multiple higher doses of UVB radiation, delayed type hypersensitivity response to reovirus type 1 was inhibited in mice injected intraperitoneally; although clearance of the virus was not changed (Letvin *et al.,* 1981). There is a very interesting experimental report on a relationship between UV and cutaneous leishmaniasis. Giannini (1986) studied using mice whether suberythematous levels of UVB radiation affects on the development of cutaneous leishmaniasis or not. It is important to note that the effective dose of radiation (15mJ/cm^2) used was well below of minimum erythema dose (MED) (Parrish, 1983), and was the minimum amount of radiation shown by others, selectively to damage Langerhans cells (Aberer *et al.,* 1981). This dose was considerably lower than those used in earlier studies of suppression of contact hypersensitivity by UVB irradiation. Low dose of UVB applied locally to the injection site suppressed the development of skin lesions after the inoculation of *L. (L.) major* promastigotes. Aberer *et al.* (1981) suggested that the primary targets of UVB radiation is the host cells and not *Leishmania* parasites. Because UVB irradiation of parasites cultured *in vitro* did not affect their viability, but did kill host cells. It was also suggested that local perturbations in the functions of the skin-associated lymphoid tissue during the initial phases of *Leishmania* infection could profoundly influence immunological response and the subsequent development of clinical disease. On the other hand, post kala-azar dermal leishmaniasis (PKDL) is most severe in the sun-exposed skin (El Hassan, 2000). We conducted to determine whether exposing mice to UV radiation would affect the pathogenesis of infection with *L. (L.) amazonensis* which causes progressive cutaneous disease in susceptible mouse strain (Khaskhely, 2001). We, in our previous study, demonstrated that low-dose UVA-irradiated mice can suppress lesion development of *L. (L.) amozonensis* through the induction of INF-γ, TNF-α and IL-12,

which are known to be crucial for host protection, as well as effective in containing and killing *Leishmania* parasites. Whereas, IL-4 and IL-10 cytokines which are known to cause susceptibility of BALB/c mice to leishmaniasis, were down-regulated. By contrast, non-irradiated mice could not control infection at the site of inoculation, and developed cutaneous ulcers. Moreover, skin samples of irradiated mice also showed positive mRNA expression of INF-γ, but not of IL-4. This indicate that the cell-mediated response switch from Th2 to Th1 pattern suppressed the cutaneous lesions of *L. (L.) amazonensis*. It has been reported that parasitophorus vacuoles resulted from the inability of macrophages to kill the *Leishmania* parasites, and were a morphological sign of intracellular parasite survival (Veress *et al.*, 1981). Our light and electron microscopic observations in UVA-irradiated mice, of macrophages containing fewer phagocytozed parasites, smaller sized parasitophorus vesicles and degenerated *Leishmania* parasites, as compared to controls, supported the idea that the activation of macrophages is leishmanicidal, possibly augmented by UVA irradiation. Granstein (1999) described that ultraviolet radiation clearly alters a number of parameter of immunologic function after exposure both *in vitro* and *in vivo*. Ultraviolet radiation effect *in vitro* are, to alter ability of antigen-presenting cells (including Langerhans cells) to present antigen, to alter ability of lymphocytes to respond to mitogen or antigen, and to alter cytokine production and to induce the release of immunosuppressive factors. Furthermore, ultraviolet radiation effects *in vivo* are, to induce skin cancer formation, to alter Langerhans cell morphology and function, suppress the induction of contact hypersensitivity, suppression of the induction of delayed-type hypersensitivity, to alter cell trafficking, to increase circulating levels of cytokines (IL-1, IL-6 and TNF-α), and to alter proportions of lymphocyte subtypes in peripheral blood. Almost hundred years ago, there was a first report on the relationship between skin cancer and exposure to sunlight (Unna, 1894). In 1928, it is already reported that ultraviolet radiation can cause skin cancer in mice (Findlay, 1928). Epidemiological studies on skin cancer in light skinned people indicated that sunlight is the most harmful cause of photoaging and cancer development of human skin (Urbach *et al.*, 1974). Definitely, every individual including human has a different susceptibility to the UV radiation, therefore, every individual may have a different immunity. If different individuals are exposed to some energy of UV radiation, the immunity of one individual may be up-regulated, but that of other may be down-regulated. So, it is extremely difficult to predict that UV radiation causes up-regulation or down-regulation of immune system. The influence of UV radiation may be different in the individuals. Therefore, clinical symptoms of infectious diseases such as cutaneous leishmaniasis may be various. We have to further investigate in response to elucidate what kind of factors affects immune system of cutaneous leishmaniasis.

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Chapter 7

Experimental Leishmaniasis

1. Effects of Ultraviolet A Irradiation on the Mice Infected with *Leishmania (L.) amazonensis*

ABSTRACT. This study was aimed at the determination of the effects of ultraviolet light on the pathogenesis of cutaneous leishmaniasis. BALB/c mice were irradiated with UVA 10 and 30 J/cm^2/day for 4 consecutive days, to test the infectivity of *Leishmania (Leishmania) amazonensis*. Disease parameters were observed for 12 weeks of inoculation. The lesion development was significantly suppressed in UVA irradiated mice as compared to the control. Light and electron microscopic examination revealed a few *Leishmania* parasites phagocytozed by the dermal macrophages in UVA irradiated mice. Sandwich enzyme-linked-immunosorbent-assay (ELISA) disclosed the up-regulation of IFN-γ cytokine time and dose dependently, while, IL-4 cytokine level was down-regulated. Reverse transcription polymerase chain reaction (RT-PCR) examination also showed the positive signal for IFN-γ mRNA in UVA irradiated subjects, while, mice in the control group showed negative results. None of the samples showed signal for IL-4. Our results showed that both systemic and local IFN-γ cytokine responses were prominent after UVA irradiation. IFN-γ, an important cytokine underlying the innate against the disease, was up-regulated and IL-4 was down-regulated. This indicates that cytokine response shift from Th2 to Th1 pattern, which possibly protected UVA-irradiated mice from *L. (L.) amazonensis* infection.

Introduction

Leishmaniasis is a vector-borne disease and largely zoonotic disease caused by parasitic protozoans in the genus *Leishmania*. It is endemic from warm to tropical regions of both Old World and New World. The causative parasites are transmitted by blood-sucking sandflies and exist extracellularly in the gut of these vectors mostly as the motile flagellated promastigote stage. While, infection to the mammalian hosts, promastigotes infect macrophages. In these cells, the parasites differentiate into the amastigote form and multiply as such in the phagolysosome. Infection of mammalian hosts by different *Leishmania* species culminates in different forms of leishmaniasis. Cutaneous leishmaniasis is marked by the skin lesions, which may evolve from small erythematic or nodular induration to creater-like with dry or wet center, but some times, all are self-limiting and self-healing in the course of several months. In all cases of leishmaniasis, macrophages are the exclusive host cells. Where symptoms are manifested or in other animals, the pathology is due not just to the destruction of macrophages by the infection but, infect, largely to the immunopathological responses of the host to parasite antigens. These antigens are present in circulation and on the surface of the infected macrophages and other cells. Antibodies produced by the host are mostly ineffective against intracellular parasites, but mostly damage cells and tissues, to which these antigens adhere. The interactions of these or other antigens with immune cells possibly also result in the production of cytokines, which

may exacerbate or suppress the disease.

Cure of leishmaniasis spontaneously or after chemotherapy is thus thought to be CD4+ cell-mediated. Immunity to leishmaniasis has been studied mainly in mouse models. Disease protection or exacerbation is associated with the type of T-cells mediated immune response mounted by the mouse. Disease-exacerbation in BALB/c mice is associated with expression of Th2 (IL-4) response, and protection in C57BL/6 mice is associated by Th1 (IFN-γ) response (Heinzel *et al.,* 1989 and Scot *et al.,* 1991).

Ultraviolet radiation is a ubiquitous component of our environment, and its amount on earth surface is day by day increasing due to stratospheric ozone layer depletion (Hofman *et al.,* 1987). UVA (320-400) is one of the more important agents, representing the major fraction of solar UV radiation reaching the earth surface. It is well known that UVA may induce deleterious effects in bacterial cells. It has been reported that UVA irradiation induces damage to *Escherichia coli* either by lethal or sub-lethal doses (Moss *et al.,* 1981 and Jagger *et al.,* 1987). Effects of UVA on *Salmonella typhimurium* (Kramer *et al.,* 1987) and *Bacillus subtilis* (Taber *et al.,* 1978) has also been reported, but no any study has been reported about the effects of UVA on *Leishmania* infection.

The effects of low-doses UVA (10 and 30 J/㎠) irradiation on *L. (L.) amazonensis* were examined in the present study. The results obtained by exposing mice, prior to *Leishmania* infection, significantly suppressed the cutaneous lesion development.

Materials and Methods

Animals

Six to eight week-old-BALB/c male mice, obtained from the animal center of the Faculty of Medicine, University of the Ryukyus, Okinawa, Japan, were used throughout. All animals were routinely screened and found to be negative for bacterial pathogens and for sub-clinical viral infections. Mice were given free access to NIH 31-mouse food and sterilized water.

Parasites and in vitro *culture conditions*

L. (L.) amazonensis (MHOM/BR/73/M2269) were used in this study. To ensure high infectivity, the strain was passed through the BALB/c mice once. Suspension of viable amastigotes, derived from cutaneous lesion of infected mouse, were cultured *in vitro* in RPMI 1640 (Nakaraitesuka, Japan) medium containing 10% inactivated fetal bovine serum (FBS) (Bio Whittaker, USA) supplemented with antibiotics (30 μg/ml Ampicillin + 100 μg/ml Gentamycin) at 24℃. Parasites were counted and adjusted to 2×10^6 viable organisms per 0.05 ml in normal saline solution.

UV-irradiation

The mice were irradiated at a distance of 30 cm by a Dermaray M-DMR-100 (SBLB, Toshiba, Co, Tokyo, Japan) with a planer bank of 10 UVA tubes (Torex, FL20S-BLB/DMR, Toshiba, Medical, Supply, Tokyo, Japan) filtered through a sheet of 3 mm window glass, providing 0.49 mW/㎠. The irradiation was monitered using a Torex UV Radiometer (UVR-305/365-D II, Topcon, Corp, Tokyo, Japan) with appropriately calibrated detectors for the UVA and UVB wave bands. Mice were divided into 3 groups: A was irradiated with UVA 10 J/㎠; B, UVA 30J/㎠ per day for 4 consecutive days; and group C served as a control. All experimental animals were restrained in specially made individual wire cages, and exposed on their shaved dorsum. Temperature was controlled with an electric fan.

Leishmania *infection and disease parameters*

After 12 hrs of UV-irradiation, experimental and control group mice were anaesthetized with diethyl ether (Cica-Reagent, Kanto Chemicals Co, Inc. Tokyo, Japan). 2×10^6 *L. (L.) amazonensis* promastigotes in a 0.05 ㎖ were injected intradermally. The extent of cutaneous lesion development was recorded for a total of 12 weeks. Lesion size was calculated by measuring length multiplied by width. All mice were sacrificed after 12 weeks of inoculation and biopsy specimens were collected for routine light and electron microscopy.

Histopathology

Biopsy samples were immediately fixed in 10%

formalin (pH 7.2) for at least 72 hrs, dehydrated in graded alcohol and xylene series, embedded in paraffin and sectioned at 4-6μm. Coded slides stained using hematoxylin and eosin (H & E) were examined by light microscopy to determine the extent of inflammatory cell infiltration and presence of intracellular parasites.

Electron microscopy

Tissue samples, immediately after biopsy, were fixed in cold 2% glutaraldehyde and 1% Osmium tetroxide, standard acetone dehydration and Epoxy resin embedding were followed by thick and thin sectioning. Semithin sections (1μm) were stained with toludine blue and observed by light microscope as a conventional reference for thin sectioning. Ultrathin sections (60-80 nm) cut by a Diatome® diamond knife were subjected to uranyl acetate/lead citrate contrasting solutions and examined in a JEOL 2000 EX transmission electron microscope (JEOL, Tokyo, Japan) at 80 KV.

Enzyme-linked-immunosorbent-assay (ELISA)

To observe the effects of low-dose UVA irradiation, prior to *Leishmania* infection, expression of IFN-γ and IL-4 was examined. Six to eight week-old BALB/c male mice, obtained from the animal center of the Faculty of Medicine, University of the Ryukyus, Okinawa, Japan, were irradiated with identical doses (10 and 30 J/㎠) of UVA. Blood and fresh skin samples were taken at different time points. Blood samples were kept at room temperature for two hrs. Serum was obtained after centrifuge at 2000×g for 20 min at 4℃. The cytokines level in the serum was evaluated by sandwich ELISA, according to protocol provided by the manufacturer, using commercially available, IFN-γ murine Quantikine M kits (R & D Systems, Minneapolis, MN, USA) and IL-4 Endogen Mouse Interlukin-4 kit (Endogen, Inc, Woburn, MA USA). All measurements were carried out in duplicate.

RNA extraction

mRNA was isolated from 200 ㎎ fresh skin tissues by means of acid guanidiniumthiocynate-phenol-chloroform (AGPC) extraction method, by using RNA ZOL B (TEL TEST 'B', Friedswood, TX, USA) according to manufacturer's protocol.

Reverse transcription polymerase chain reaction (RT-PCR)

cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Toyobo, Osaka, Japan.) and oligo (dT) 12-18 primers (Gibco-BRL Products, Gaithersburg, MD). We added 5 μℓ of each cDNA solution to 47 μℓ reaction mixture that contained 5 μℓ 10×reaction buffer (500 mM KCl, 100 mM Tris-HCl, [pH 8.3], and 1.5mM $MgCl_2$), 4 μℓ of dNTP (2.5mM), 0.25 μℓ of Taq DNA polymerase (Takara Co., Ltd., Japan), 100 pmol (1 μℓ) of each forward and reverse primers and 35.75 μℓ of sterile water. The reaction mixture was subject to 50 cycles GAPDH, IFN-γ and 45 cycles for IL-4, of amplification in a DNA programmable thermal controller PTC-100 (MJ Research, Inc., Water town, MA, USA). An amplification cycle consisted of denaturation at 94℃ for 3 min, primer annealing the template at, for IFN-γ and GAPDH was 60℃ and for IL-4 was 45℃, for 1 min. The extension was carried out at 74℃ for 4 min.

After amplification, PCR products were subjected to electrophoresis in a 2.5% agarose gel contained 0.8 ㎎/㎖ ethidium bromide. A 100 base pairs ladder (Toyobo, Osaka, Japan) was used as a size marker. It was confirmed that the amount of PCR products increased proportion to the amount of cDNA template used under these conditions. The amount of cDNA products was standardized on the basis of GAPDH cDNA concentration.

Statistical analysis

When appropriate, student's unpaired *t*-test was performed using Statview software (Cricket Software, Philadelphia, PA), to determine the statistical significance of differences between groups. P values of <0.05 were considered significant.

Results

Effects of UVA irradiation on lesion development

Two different doses of UVA 10 J and 30 J/㎠/day

for 4 consecutive days were applied. The development of cutaneous lesions in relatively susceptible host, BALB/c, mice was indeed affected. Lesion development was suppressed in UVA irradiated mice. After 3 weeks of inoculation all irradiated mice had no visible lesions while as 33.3% of control mice were with lesions. As early as 6 weeks of inoculation, most of the irradiated mice had no visible lesions. After 12 weeks of inoculation, 33.3% of mice, irradiated with UVA 10 J and 30 J/cm²/day, respectively, had lesions ($p<0.001$). Whereas, all (100%) of control mice were with ulcers (Fig.7.1.1). Statistically significant difference of lesion size was observed in, 10 and 30 J/cm²/day, UVA-irradiated mice ($p<0.002$ and $p<0.001$) as compared to controls.

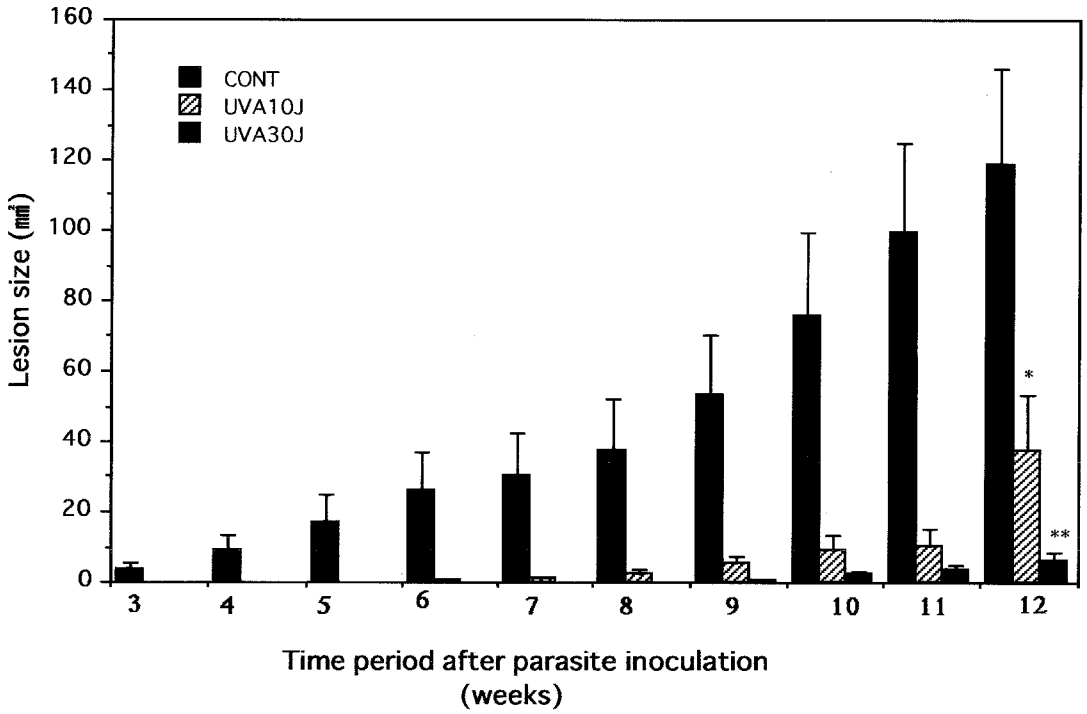

Figure 7.1.1. Lesion size measurement results of UVA, 10 and 30 J/cm²/day, irradiated and control mice. *: $p<0.002$, **: $p<0.001$.

Light microscopy

On routine histopathology, UVA-irradiated mice showed cell infiltration composed of lymphocytes, monocytes, eosinophils and macrophages at injection site. A few *Leishmania* parasites phagocytozed by macrophages were seen in UVA irradiated mice. Control subjects revealed cell infiltration composed of lymphocytes, eosinophils, macrophages and a few monocytes. Dermal macrophages showed numerous phagocytozed *Leishmania* parasites (Fig. 7.1.2a-d).

Electron microscopy

Ultrastractural examination of control mice

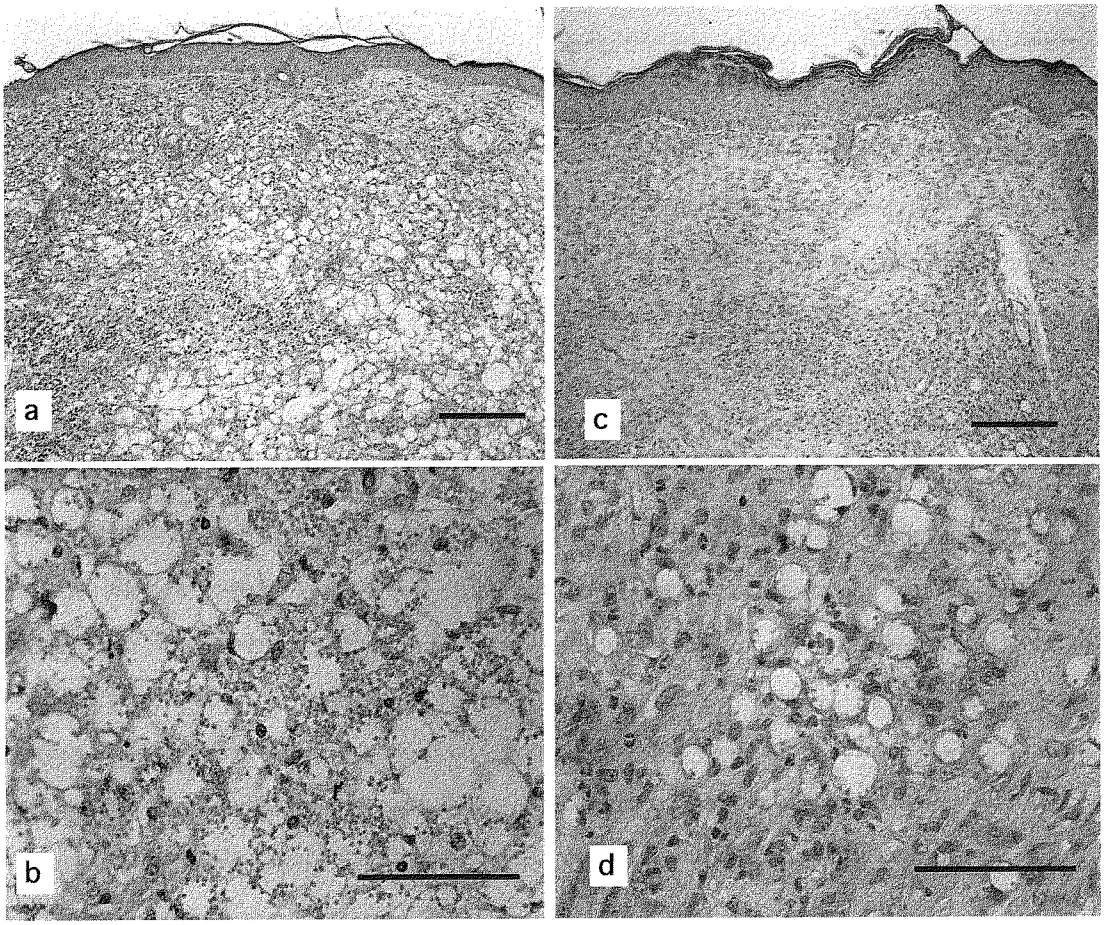

Figure 7.1.2. H. E. staining of control and UVA irradiated mice. (a) Control mice skin, (b) higher magnification of (a). (c) UVA irradiated mice skin, (d) higher magnification of (c). A few *Leishmania* parasites are phagocytozed by macrophages in irradiated mice as compared to control. Scale bar=100 μm.

revealed that the dermal macrophages had large PVs and contained many *Leishmania* parasites. These parasites were round in shape and had well-developed cell organelle. Numerous *Leishmania* parasites were seen outside of macrophages. In specimens obtained from UV-irradiated subjects, PVs of parasitozed macrophages were smaller as compared to controls. *Leishmania* parasites were observed inside the macrophages, but they were few in number and variable in size, and shape, and some of them showed degenerated change. None of the parasite was observed outside of the macrophages. In deep dermis, parasitized macrophages also formed a nest-like structure attached with dermal lymphocytes (Fig. 7.1.3a, b).

Effects of UVA irradiation on systemic IFN-γ and IL-4 expression

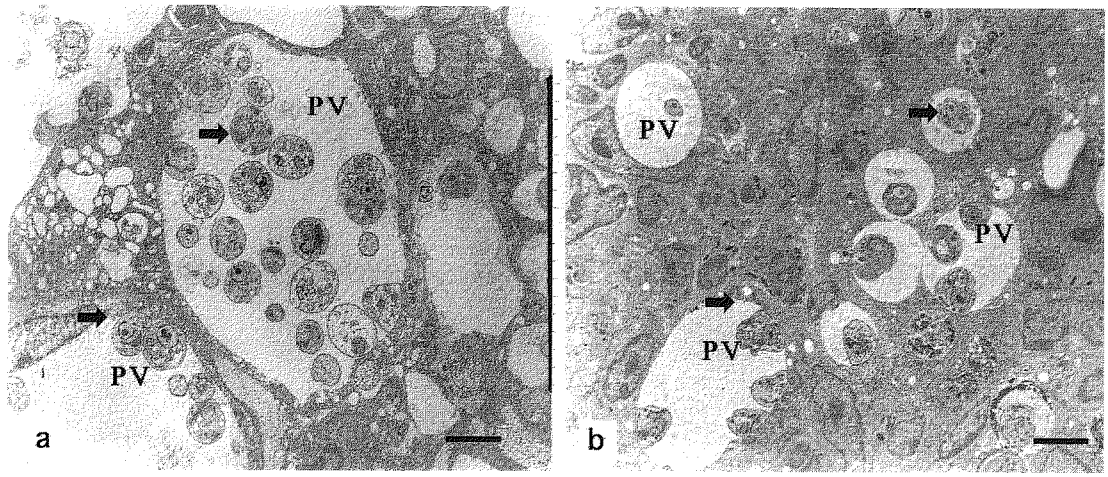

Figure 7.1.3. a. Electron micrograph of control mice showing bigger size parasitophorous vacuoles *(PV)* containing many *Leishmania* parasites *(arrows)*. Bar=5 mm. **b.** Electron micrograph of UVA irradiated mice showing smaller size parasitophorous vacuoles *(PV)* and some of them showing a degenerated change *(arrows)*. Bar=5 μm.

Figures 4 and 5 shows the IFN-γ and IL-4 expression in the blood serum of experimental and control subjects. The IFN-γ response was prominent at 4 hrs and peaked at 6 hrs of last exposure. While, IL-4 level was down-regulated. The increased level of serum IFN-γ was UVA irradiation dose dependent. Mice group, after 6 hrs of UVA 10 J/cm^2 irradiation, showed 1.2 times increased level of IFN-γ compared to control but the difference was statistically significant ($p<0.01$). While, the mice group irradiated with UVA 30 J/cm^2, after 6 hrs, showed almost 1.5 times increased level of IFN-γ, the difference was statistically significant ($p<0.0006$). Present results revealed that the expression of IFN-γ cytokine in blood sera was dose dependent, higher the dose of UVA irradiation increased level of IFN-γ. The difference between two applied doses of UVA 10 and 30 J/cm^2 was also statistically significant ($p<0.01$) (Fig. 7.1.4). In contrast to IFN-γ, the level of IL-4 cytokine was suppressed. Suppression was, UVA dose dependent. Mice groups, after 6hrs of UVA 10 J/cm^2 irradiation, showed 1.1 times suppressed level of IL-4 compared to control, but the difference was significant ($p<0.002$). While as, the mice group irradiated with UVA 30 J/cm^2, after 6 hrs, showed 1.7 times suppressed level of IL-4, and the difference was statistically significant ($p<0.0000005$) (Fig.7.1.5). Our ELISA results revealed that the expression of IL-4 (Th2) cytokine was dose- dependent, higher the dose of UVA, suppressed the expression of IL-4. The difference between two doses of UVA 10 and 30 J/cm^2 irradiation was statistically significant ($p<0.0005$).

Effects of UVA irradiation on local IFN-γ and IL-4 expression

Effects of UVA irradiation on local (skin) induction of IFN-γ and IL-4 was examined. RT-PCR was then applied to assess cytokine mRNA expression in UVA irradiated and control mice skin. We examined the mRNA level of IFN-γ (Th1) and IL-4 (Th2) cytokines. A significant positive signal at 426 base pairs for IFN-γ was obtained in UVA irradiated mice (Fig. 7.1.6). While, the control showed negative results. None of the samples, taken from irradiated and control mice, showed positive signal for IL-4.

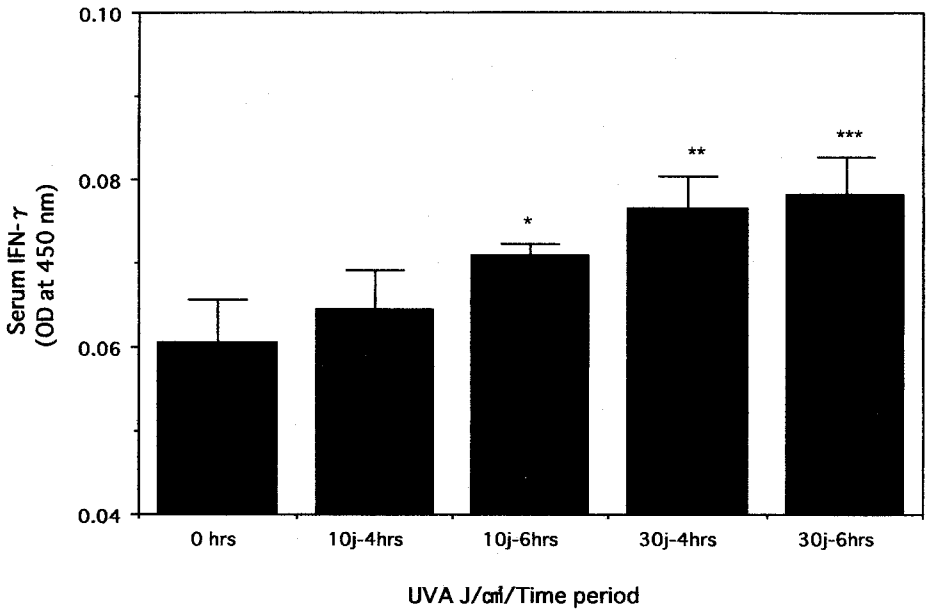

Figure 7.1.4. Serum IFN-γ expression in UVA, 10 and 30 J/cm^2/day, irradiated and control mice, at different time points. Elevated level for IFN-γ was observed at 4 hrs and peaking at 6 hrs of last exposure, time and dose dependently as compared to control.

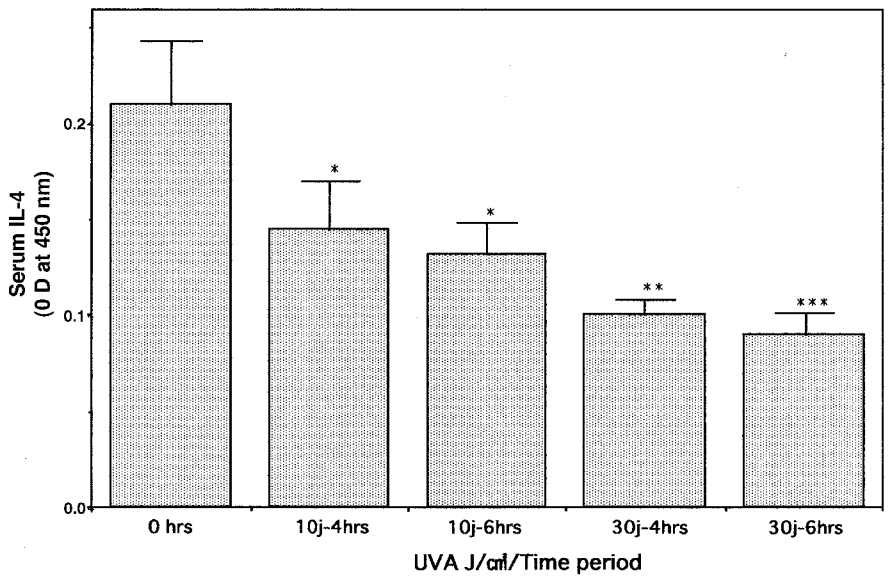

Figure 7.1.5. Expression of serum IL-4 in UVA, 10 and 30 J/cm^2/day, irradiated and control mice, at different time points. IL-4 level was down-regulated by UVA irradiation time and dose dependently as compared to the control.

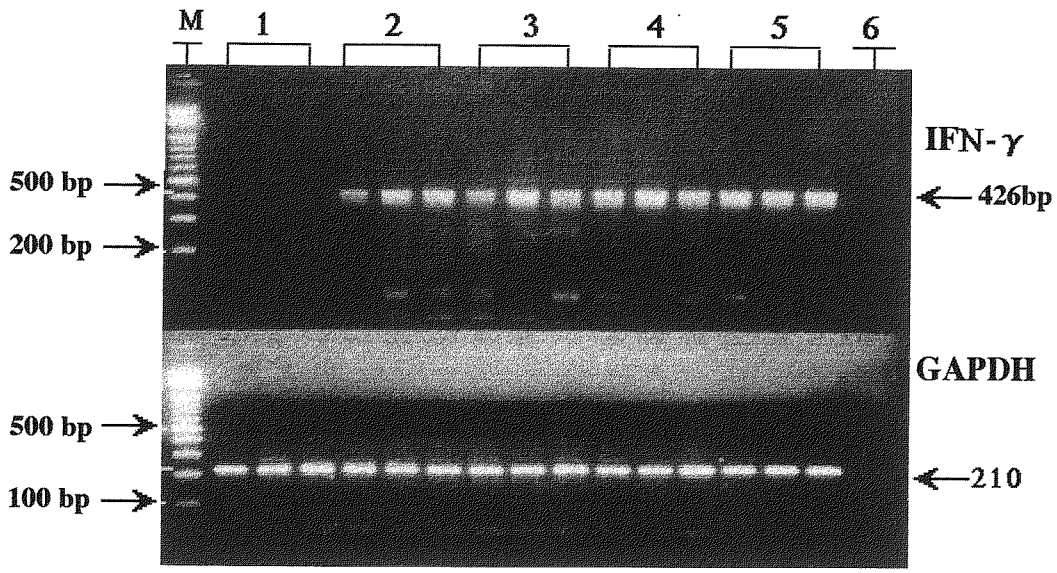

Figure 7.1.6. Results of RT-PCR with ethidium bromide stained 2.5% agarose gel after electrophoresis using cDNAs from control and UVA irradiated mice. No. 1: control mice, No. 2: mice irradiated with UVA 10 J/cm²/day at 4 hrs, No. 3: at 6 hrs, No. 4: mice irradiated with 30 J/cm²/day at 4 hrs, and No. 5: at 6 hrs of last exposure. No. 6: negative control (no cDNA), M: size marker.

Discussion

L. (L.) amazonensis is a well-characterized macrophage parasite that clearly requires a strong Th1 cell-mediated response to resolve the infection. Present study evaluated whether low-doses of UVA irradiation prior to parasite inoculation exerts a systemic and local influence that deters a successful immune response against *L. (L.) amazonensis*. Our results disclosed that UVA radiation markedly suppressed the cutaneous lesion development in susceptible BALB/c mice, whereas, infection was rapidly progressive and more severe in control mice. For further clarification we examined the effects of identical doses of UVA irradiation on systemic and local expression of IFN-γ and IL-4 cytokines. UVA irradiation, prior to *Leishmania* infection, resulted in up-regulation of IFN-γ and down regulation of IL-4 cytokines.

In scientific literature, only one study had been reported describing the effects of UVB irradiation on *Leishmania* infection in murine model by Giannini (1986). He observed a significant suppression of lesion development after UVB irradiation in mice infected with *L. (L.) major*. A reduction in the lesion size after UVB irradiation also has been reported in BCG infection in mice (Jeevan and Kripke., 1990). Nonaka and co-workers in their clinical comparative study in Ecuador, reported that the size of cutaneous lesions in the lowlands was larger than the highlands. A number of etiological factors, including UV-light, were suggested to explain this clinical difference (Nonaka *et al.*, 1990). Present study is the first time, in which effects of UVA irradiation were examined on murine model of *L. (L.) amazonensis*. It showed that disease outcome irradiated with UVA 10 and 30 J/cm² prior to injection of *Leishmania* promastigotes, delayed the disease outcome. At the termination of this experiment (after 12 weeks), 33.3% of mice irradiated with 10 and 30 J/cm² developed small nodules, while, 100% of control mice were with ulcers. The size and frequency

of development of cutaneous lesions was significantly suppressed, the difference was statistically significant ($p<0.002$, $p<0.001$), respectively as compared to controls. Light microscopy of irradiated mice showed that a few macrophages had small cytoplasmic vacuoles and contained fewer *Leishmania* parasites. It is believed that *Leishmania* promastigotes are changed into amastigotes forms inside the macrophages. Veress *et al.* (1981) reported that the PVs were the result of inability of macrophages to kill the *Leishmania* parasites, and are thought to be the morphological sign of intercellular parasite survival. Our electron microscopic observations of macrophages with phagocytozed *Leishmania* parasites, smaller PV and degenerated parasites in UVA irradiated mice, supports the idea that the activation of macrophages is leishmanicidal, possibily augmented by UVA irradiation.

Murine model of leishmaniasis exemplifies the Th1/Th2 paradigm, in which the outcome of the disease is determined by the nature and magnitude of T-cells and cytokine response early in infection. In infected inbread mice, production of IFN-γ by Th1 and natural killer cells mediates resistance, whereas, expansion of IL-4-producing Th2 cells confers susceptibility (Reed and Scott, 1993). *Leishmania* infection in BALB/c mice usually leads to dominant Th2 response, and uncontrolled lesions develop with occasional metastasis, whereas other strains of mice, C3H/HeN and C57BL/6, develop Th1 response, control parasite multiplication, heal and sometimes resist to disease. IFN-γ with IL-12, before or after infection, has an important role in the Th1 cell development, in resistant C3H/HeN and susceptible BALB/c mice in *L. (L.) major* disease outcome (Afonso *et al.*, 1994). It has been reported that UVA irradiation resulted in a marked increase of expression of IFN-γ and IL-12 (Shen *et al.*, 1999). In present study, the mice groups irradiated with UVA 10 and 30 J/㎠ showed a hyper stimulated immunological state, compare to that of control. The serum level of IFN-γ was elevated at 4 hrs and was at peak at 6 hrs of last UVA exposure, as compared to control, and the difference was statistically significant ($p<0.01$ and $p<0.0006$ respectively). Contrary, the level of IL-4 was down regulated in irradiated subjects. Our study also showed, for the first time, that the up regulated level of IFN-γ and down regulated level of IL-4 were UVA dose dependent. The effect of two doses of UVA, 10 and 30 J/㎠, on IFN-γ expression was statistically significant ($p<0.01$). While the suppression of IL-4 was also dose dependent, and the difference between UVA 10 and 30 J/㎠ was statistically significant ($p<0.0000005$).

Skin is particularly relevant to immunity to *L. (L.) amazonensis,* as the cutaneous form of the disease is the most common, and the capability to control infection is much greater in the skin. To observe the local effects of UVA irradiation, mRNA levels for IFN-γ and IL-4 were examined by RT-PCR. Our results revealed induction of mRNA levels for IFN-γ in UVA-irradiated mice skin, but the signal was negative in control mice. This indicated that IFN-γ was also induced in skin by UVA irradiation. Previous studies have documented a critical role for TNF-α, IL-12 and IFN-γ to protect against *Francisella tularensis* (Sjosted *et al.*, 1996), *listeria* (Tripp *et al.*, 1993) and leishmaniasis (Roach *et al.*, 1991). IL-12 together with TNF-α, stimulates the release of the macrophage-activating agent IFN-γ by NK cells, enhancing parasiticidal activity (Tripp *et al.*, 1993). Thus, each of these cytokines, specially increased IFN-γ plays a most significant role in the control of experimental *L. (L.) amazonensis* infection. Early appearance of the IFN-γ, after UVA irradiation at the site of inoculation, as observed in the present study, is likely to benefit host protection. The level at which UVA irradiation may up regulate IFN-γ in skin as well as in blood serum could involve other cytokines. In summary, our study demonstrated the UVA irradiation, prior to infection, alters the equilibrium of cytokines towards Th1 and away from the Th2 pattern. The cytokines from Th1 cells mediate other immune cells to eliminate parasite through several possible mechanisms. Activation of macrophages by IFN-γ, for example, is known to stimulate their respiratory burst and other antimicro-

bicidal activities of the macrophages, thereby killing the amastigotes therein.

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2. An Influence of Delayed Type Hypersensitivity Reaction and Ultraviolet Light to Experimental Leishmaniasis

ABSTRACT. The aim of present study was to determine whether prior induction of non-specific delayed type reaction and ultraviolet B irradiation, at the site of *Leishmania* infection would modify the pathogenesis of infection. A mice group was made hypersensitive to 0.5% 2,4-dinitrofluorobenzene DNFB, and other group was irradiated with different doses of UVB, at shaved skin of the back. After 12 hrs an infective dose of *L. (L.) amazonensis* was injected. The lesion development was observed for 12 weeks of post-infection. Experiment was repeated three times and following conclusions were made; 1) induction of delayed reaction by DNFB significantly inhibited the cutaneous lesion development, 2) UVB irradiation suppressed the development of leishmanial lesion, 3) pathogenesis was more effective only in the absence of delayed reaction and UV-irradiation to the control animal.

Introduction

Cutaneous leishmaniasis is a widespread parasitic disease through the much of the Third World. The disease is caused by flagellate protozoan *Leishmania* parasites, an obligate intracellular parasite in human that resides and multiplies within macrophages. *Leishmania* parasites are naturally transmitted by blood-sucking, phlebotomine flies (sandfly) to human and reservoir animals. Cutaneous lesions, generally appears on the exposed body parts that are naturally exposed both to the bites of sandfly and the solar radiation. Little is known about the development of the pathology, during the cutaneous phase of infection, certainly the immunogenetic background of the host, is a major factor in the response to cutaneous *Leishmania* infection.

Delayed-type hypersensitivity (DTH) is one of the principal, immunological features of cutaneous infection. Sensitization is induced by topical application of reactive antigens which couple self-tissue. It is generally accepted that DTH is a cell-mediated immune response and has been shown to accompany the appearance, in *Leishmania*-infected guinea-pigs, of sensitized lymphocytes capable of responding *in vitro* leishmnial antigen by producing lymphokines and mitogenic factor (Blewett *et al.,* 1970). In addition, since protection following transfer of serum from immune animals to normal recipients has not been achieved. Activated macrophages are known to exhibit non-specific resistance to a variety of intracellular pathogens and they have been shown to occur at the site of delayed reaction (Dannenberg *et al.,* 1974). The mechanism of recovery and resistance to infection in the host-parasite model was to be primarily cell-mediated (Turk *et al.,* 1970).

Irradiation with UVB causes immunosuppression of cell mediated immunity (Noonan *et al.,* 1990). The role of UV immune suppression in other diseases is however, less well defined. As UVB levels in sunlight are sufficient to predict significant UV-induced immunosuppression at most latitudes. The first indication that UV-induced immunosuppression may play a role in the outcome of an infectious disease came from the studies of Giannini (1986). He found that UV-irradiation inhibited the cutaneous lesion development in *L. (L.) major* infected mice.

The present study was therefore aimed at examining the evaluation of leishmanial lesions at the site of a strong and sustained delayed reaction and UVB irradiation, prior to *L. (L.) amazonensis* infection. We here report the development pattern of leishmanial lesions after inoculation of *L. (L.) amazonensis* at the site of DTH reaction caused by 2,4-dinitrofluorobenzene (DNFB) and UVB irradiation.

Materials and Methods

Animals

Six- to eight-week-old BALB/c male mice were purchased from University of the Ryukyus, Animal Center (Okinawa, Japan). Mice were divided into four groups. Group one was sensitized with 0.5% DNFB, the second was irradiated with UVB 25 mJ/㎠ per day for four consecutive days, the third was irradiated with UVB10 mJ/㎠ per day, single dose and the fourth was non-sensitized, non-irradiated control group.

Parasites and in vitro *culture conditions*

L. (L.) amazonensis (MHOM/BR/73/M2269) were used in this study. To ensure high infectivity, the strain was passed through the BALB/c mice once. Suspension of viable amastigotes, derived from cutaneous lesions of infected mice, were cultured *in vitro* in RPMI 1640 medium (Nakaraitsuka, Japan) containing 10% fetal bovine serum (FBS) (Bio Whittaker, USA) supplemented with antibiotics, 30 μg/㎖ Ampicillin and 100 μg/㎖ Gentamycin, at 24℃. After 7-10 days, promastigotes were harvested by centrifugation (1800 x g for 15 min) and washed with normal saline. Parasites were adjusted to $2x10^6$ viable organisms per 0.05 ml in normal saline.

Reagents and induction of hypersensitivity

2,4-dinitrofluorobezene (DNFB) was supplied by WAKO (WAKO, Pure Chemical Co. Osaka, Japan). Experimental group mice were sensitized with 25 μl of 0.5% DNFB in 4:1 acetone: olive oil solution by painting at the shaved back skin. Left ear was painted with 20 μl of 0. 2% DNFB in the same vehicle. The ear swelling was measured 24 hrs later with a dial thickness gauge.

UV-irradiation

Experimental group mice were irradiated with UVB 25 mJ/㎠/day for four consecutive days, and 100 mJ/㎠/day as a single dose. The source for irradiation was Dermaray M-DMR-100 with a bank of five fluorescent sunlamps (FL20SE-30, Toshiba Co., Tokyo, Japan) with emission spectrum of 0.49 mW/㎠/sec. Mice were kept in specially made wire cages and were irradiated from a distance of 30 cm.

Parasite inoculation and disease parameters

After 12 hrs of UVB irradiation and contact sensitized with DNFB, all mice groups, including normal control mice, were injected with $2 x10^6$ promastigotes of *L. (L.) amazonensis* promastgotes suspended in 0.05 ㎖ of normal saline. Cutaneous lesion size was measured weekly for 12 weeks of inoculation. All mice were sacrificed after 12 weeks and biopsy specimens were collected. Formalin-fixed paraffin-embedded samples were processed for light microscopy to determine the presence of intracellular parasites and cell infiltration.

Electron microscopy

Biopsied tissue samples were cut into small pieces and fixed in phosphate buffered cold 2% glutaraldehyde solution for electron microscopy. The samples were washed with phosphate-buffered 1% Osmium tetraoxide, and then dehydrated with ethanol series and propylene oxide, and embedded in Epon 812 resin. Ultrathin sections were cut with a diatome diamond knife and counter stained with uranyl acetate and lead citrate, and observed under a JEOL 2000 EX transmission electron microscope (JEOL, Tokyo, Japan) at 80 KV.

Results

Comparative results of lesion development and size are shown in Table 7.2.1.

Effects of DNFB on development of contact hypersensititvity and cutaneous leishmaniasis

To observe the effects of DTH, on *Leishmania* infection, BALB/c mice were painted with 0.5% DNFB, prior to *Leishmania* infection, for 4 consecutive days. *L. (L.) amazonensis* promastigotes were injected at sensitized shaved back skin. Visible cutaneous lesions, at inoculation site, were measured weekly. In three time repeated experiment, 0.5% DNFB painted group mice showed a significant suppression of lesion development (Fig.7.2.2).

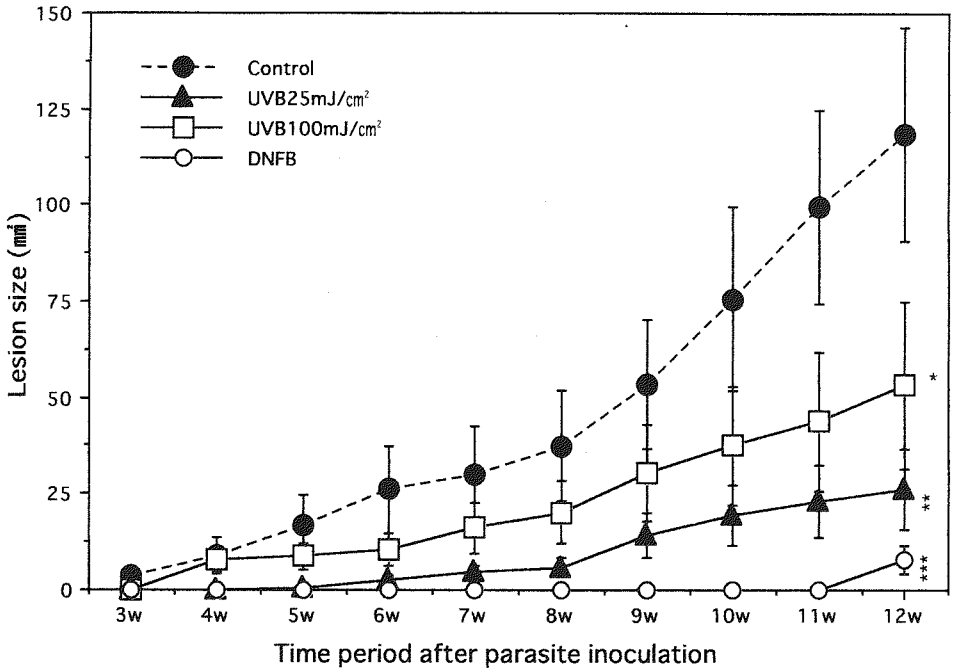

Figure 7.2.1. Cutaneous lesion size measured weekly. Lesion size difference observed between the groups was statistically significant. *: $p<0.001$, **: $p<0.0002$, ***: $p<0.0001$.

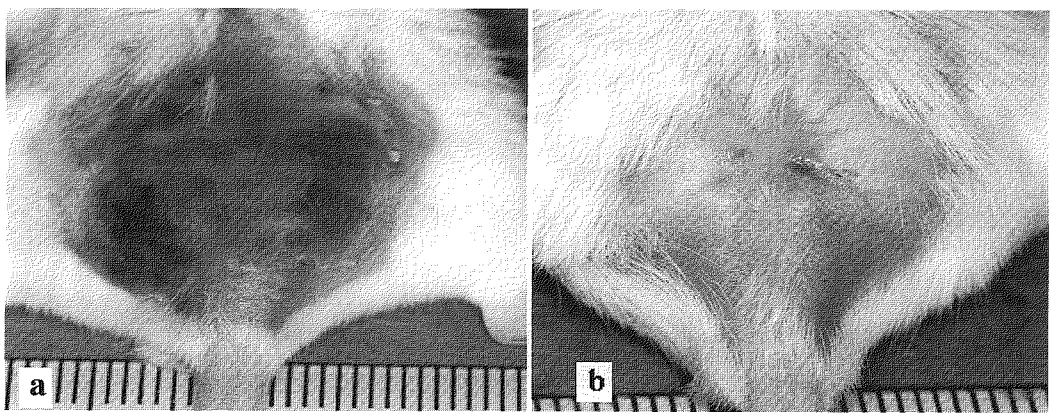

Figure 7.2.2. Clinical photograph of mouse sensitized with 0.5% DNFB and then infected with *L. (L.) amazonensis* promastigotes. *a:* before, *b:* 12 weeks after *Leishmania* parasite inoculation.

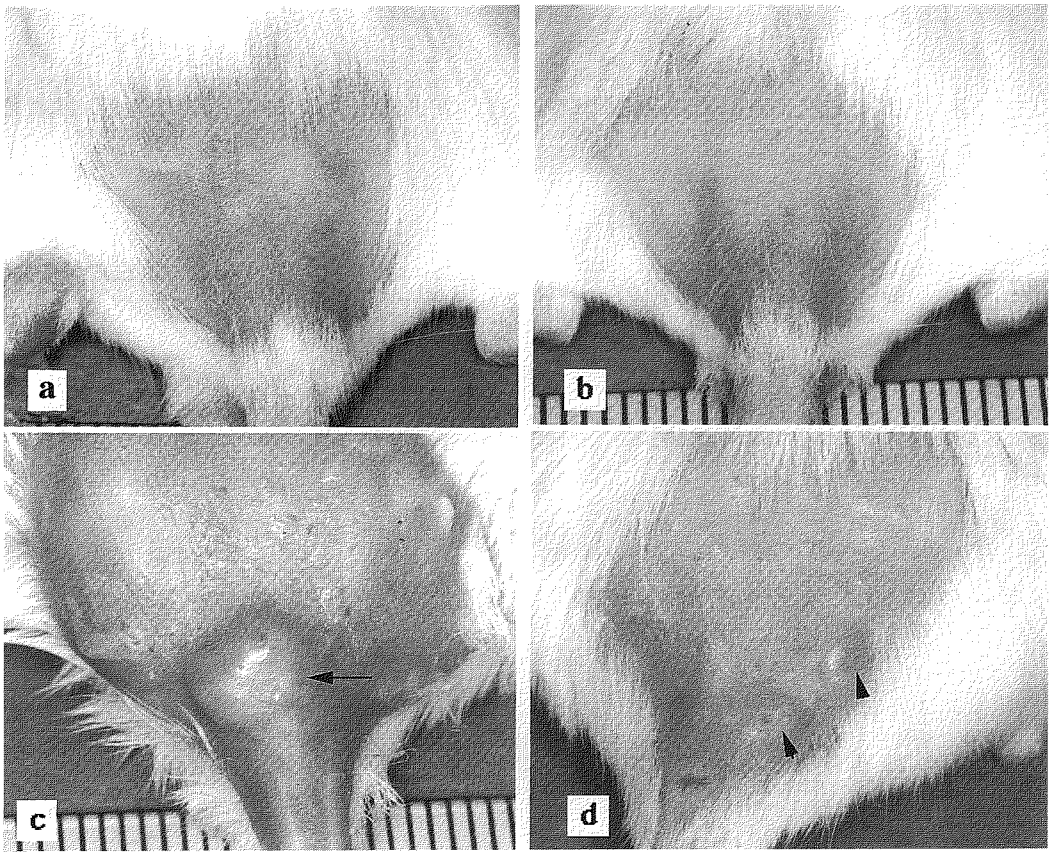

Figure 7.2.3. Clinical photograph of mouse pre-irradiated with UVB. Mice in *a* and *c* irradiated with 25 mJ/day for 4 consecutive days. Mice in *b* and *d* irradiated with 100 mJ/day/single dose. *a* and *b*: before *Leishmania* inoculation; *c* and *d*: after 12 weeks of *Leishmania* inoculation. Nodule of 2x3 mm size (arrow) and 5x6 mm size (arrow heads) can be seen.

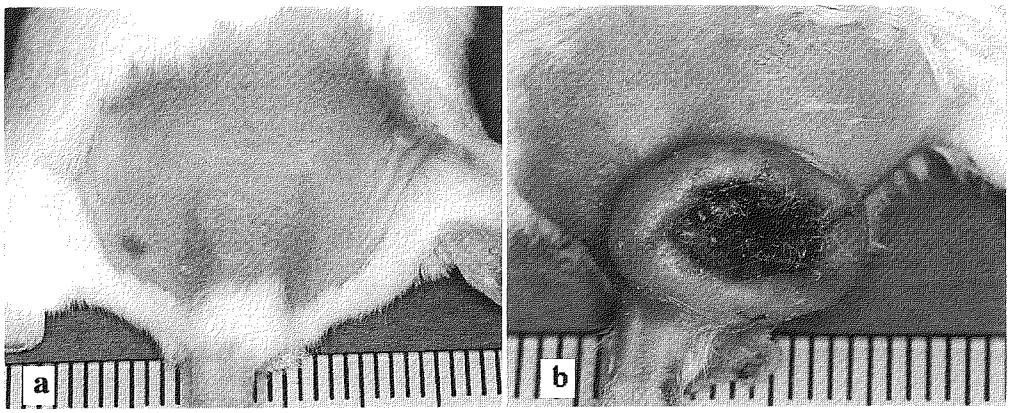

Figure 7.2.4. Clinical photograph of control mouse. *a*: before, *b*: 12 weeks after *Leishmania* inoculation. An ulcer of 14 x11 mm size can be observed in b.

Effects of UVB irradiation on cutaneous leishmaniasis

Doses of 25 and 100 m/㎠/day of UVB radiation applied to the site of *Leishmania* parasites inoculation, surprisingly, showed suppression of lesion development. As early as 12 weeks of post infection, 50% mice, irradiated with UVB 25 mJ/㎠/day, showed lesions at inoculation site (Fig.7.2.3), 75% of the mice were with small papules and 25% showed ulcer. Whereas 66.6% mice, irradiated with UV B100 mJ/㎠ /day showed lesions, 50% mice were with ulcers and 50% with small nodules. All control mice had big ulcers (Fig.7.2.4).

Histopathology

DNFB sensitized mice, on routine histopathology, 60% specimens were positive for *Leishmania* parasites, while 40% were negative. Moderate cell infiltration mainly of lymphocytes, monocytes and eosinophils was observed at the upper dermis (Fig.7.2.5). The mice irradiated with UVB 25 and100 mJ/㎠, showed 75% and 100% positive for *Leishmania* parasites on routine histopathology, respectively. Cell infiltration, mainly of lymphocytes, monocytes and eosinophils was seen at the upper dermis (Fig. 7.2.6). All control specimens were positive for *Leishmania* parasites (Fig.7.2.7).

Ultrastructural examination

Macrophage infiltration was prominent in all groups. Amastigote forms of *L. (L.) amazonensis* were found within a high portion of the macrophage profile in control as compared with experimental groups. The organisms were round or oval in outline. Bigger size parasitophorous vacuoles (PV) with numerous *Leishmania* parasites were observed within the macrophages of control mice. The size of parasitophorous vacoule and the number of phagocytozed parasites were smaller in DNFB sensitized and UVB irradiated mice groups as compared to control (Fig.7.2.8a-c). Degenerated or de-gradated *Leishmania* parasites were observed in UVB-irradiated group. In dermis of the experimental mice the eosinophils were present close to the macrophages and formed a nest-like structure (Fig. 7.2.8d).

Discussion

Because of progressive thinning of stratospheric ozone layer, an ever-increasing proportion of solar energy reaching the earth surface is composed of highly active UVB radiation. Increasing UVB flux is projected to have a major impact on skin cancer health expenditures in the near future. Our *in vivo* study was aimed to investigate the effects of UVB irradiation and DTH caused by DNFB, at inoculation site, prior to infection with *L. (L.) amazonensis,* would influence the cutaneous lesion development. It is of importance that the dose of UVB-irradiation 25 mJ/㎠, used in this study, is well below the minimum erythemal dose of 40 mJ/㎠. (Parrish *et al.,* 1983), and is also the minimal dose of radiation selectively to damage the Langerhans cells as reported previously (Aberer *et al.,* 1981). The level of UVB-irradiation, similar to those used in present experiment, would likely be encountered by population at risk for leishmaniasis, specially, rural population. Our working assumption was the lesion size in UVB-irradiated group mice will be more severe, but, surprisingly, it was significantly suppressed.

Repeated application of DNFB on skin induce topical contact dermatitis and is mainly due to delayed hypersensitivity, mediated by Th1 type T cells (Gauchat *et al.,* 1993). Hiroichi *et al.* (1997) reported the strong expression of IFN-γ and IL-2 mRNA in the skin lesion of mice treated with DNFB. A non-specific factor, which has demonstrable adverse effects *in vitro* on survival of *Leishmanmia* parasites, is so-called natural antibodies. Schmunis *et al.* (1970) found lytic and agglutinating factors to promastigotes of several *Leishmania* species in normal sera of murine models. It is conceivable, therefore, that increased exudation of serum with its lytic factors, as the result of inflamatory response to DNFB could eliminate a considerable amount of parasites from the host tissue. The cellular component of DTH, are rather defined, consisting primarily of mononuclear phagocytes, lymphocytes and some basophilic cells in contact sensitivity. Among those macrophages are known to have

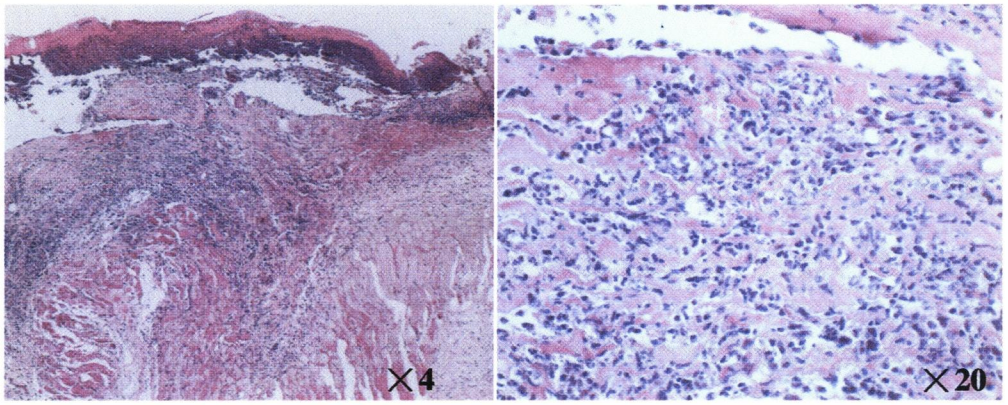

Figure 7.2.5. Low (x4) and high (x20) magnifications of histopathology of 0.5% DNF sensitized mouse, after 12 weeks of *Leishmania* inoculation.

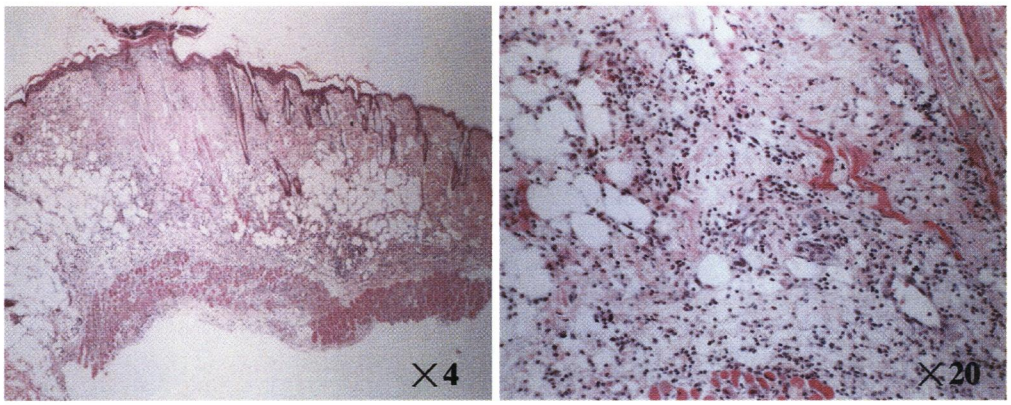

Figure 7.2.6. Low (x4) and high (x20) magnifications of histopathology of mouse pre-irradiated with UVB, after 12 weeks of *Leishmania* inoculation.

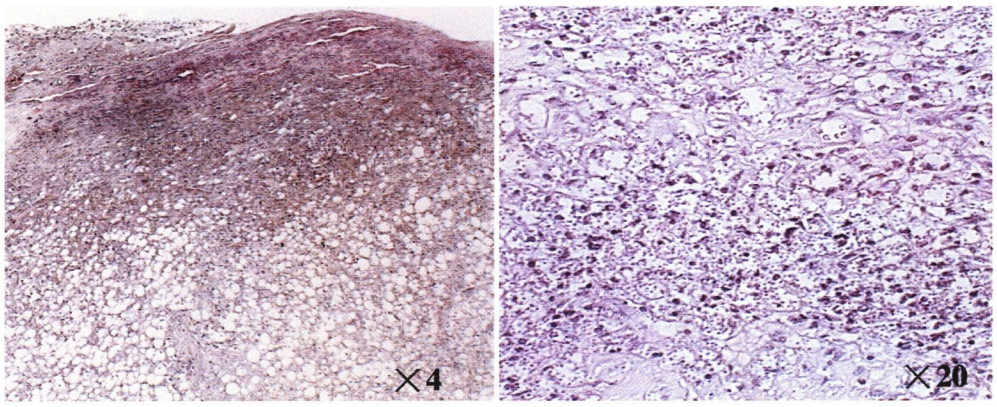

Figure 7.2.7. Low (x4) and high (x20) maginifications of histopathology of mouse in control group, after 12 weeks of *Leishmania* inoculation.

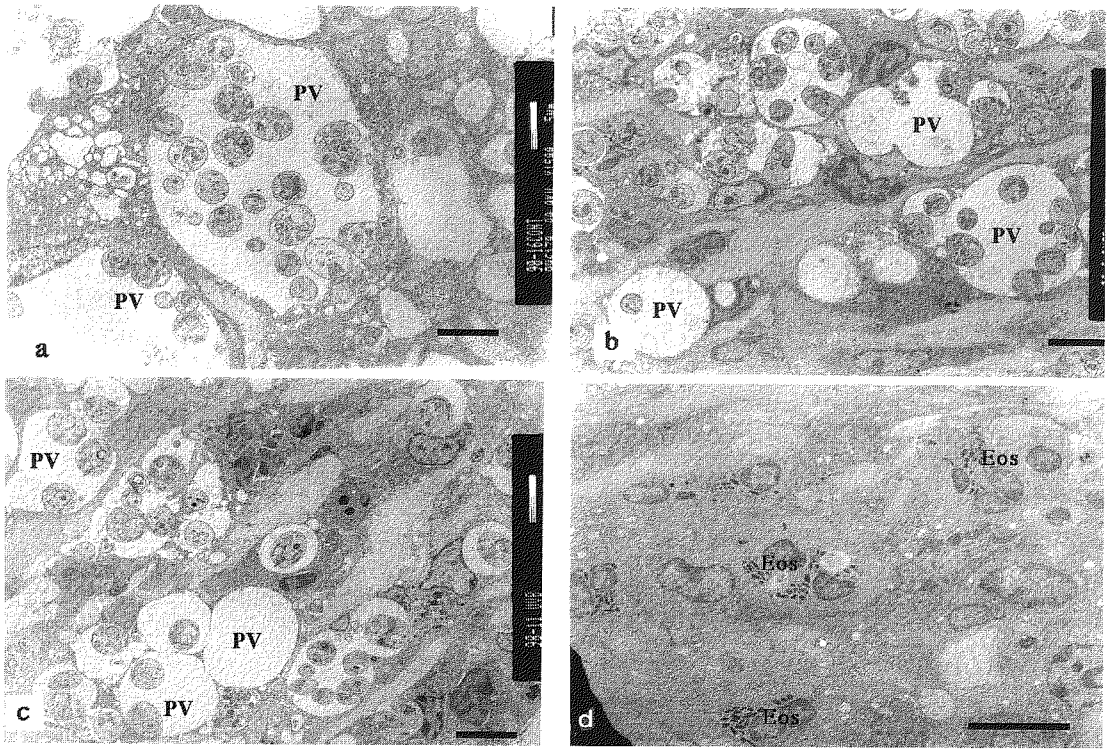

Figure 7.2.8. Photomicrograph of ultrastructural observations at parasite inoculation sites, after 12 weeks of inoculation. *a*: control, *b*: irradiated with UVB, *c*: sensitized with DNFB, *d*: showing eosinophils (Eos) around macrophages in mice of experimental groups. PV: parasitophorous vacuoles. Bar = 5 μm.

considerable microbial capacity. This capacity is non-specific and is enhanced in activated macrophages. Activated macrophages, at the site of DTH, have been postulated to be microbial and non-microbial. Convit *et al.* (1974) reported the destruction of intercellular *Mycobacteria leprae* in lepromatous leprosy patients, which were inoculated with a mixture of *M. leprae* and *M. tuberculosis*. Local macrophage activation as a result of BCG injection was considered as the mechanism responsible for elimination of *M. leprae,* a situation somewhat analogous to the present results.

UVB irradiation interferes with immune responses in skin-associated infections (Patki *et al.,* 1991). Studies in a mouse model of infection with herpes simplex virus have shown decreased DTH responses to virally infected cells after UV-radiation of the host (Ross *et al.,* 1986). Other studies with *Candida* and *Mycobacteria* sp. infection of mice have also found a UV-induced immuno-suppression of DTH responses to these organisms and an associated increase in numbers of infectious organisms in the spleen, lymphnodes and at the site of infection (Jeevan *et al.,* 1989a, and Jeevan *et al.,* 1992b). The indication that UV induced immune suppression may play a role in the outcome of an infectious disease came from the studies of Giannini (1986), who found that irradiation with UVB decreased the DTH response and the pathology of skin lesions in mice infected with *L. (L.) major.* The observed effect of UVB irradiation was appeared to be on the host not on the parasites, since the parasite load was not decreased. The present results are in agreement with above study, and we found a sig-

nificant suppression of lesion development in UVB-irradiated mice. The mechanism by which UVB irradiation suppresses the lesion development in leishmaniasis is still not clear. A more likely possibility for the dichtomy between UV-mediated immunosuppression and the effects of UV irradiation on *L. (L.) amazonensis* is maybe the nature of the immune responses elicited. Studies indicate that recombinant tumor necrosis factor (TNF) and purified TNF were both able to activate macrophages to destroy intracellular *L. (L.) major* parasites (Theodos *et al.*, 1991). Kibitel *et al.* (1998) reported that UV light induces the expression of TNF-α. We hypothesize that, UVB irradiation caused the induction of TNF-α which activated the macrophages to kill *Leishmania* parasites in present experimental model.

In summary, our study disclosed that, DTH caused by DNFB and UVB-irradiation, through the non-specific and anti-microbial action of activated macrophages or through hitherto unrecognized tissue changes, which may occur at the site of inflammation reaction and maybe through release of cytokines, protected BALB/c mice from *L. (L.) amzonensis.*

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3. Immunohistochemical Investigation of the Human Skin Lesion after Sandfly *(Lutzomyia hartmanni)* Bite

ABSTRACT. A case of a thirty eight-year-old healthy man bit by sandflies was reported. The skin lesion was biopsied and examined by using immunohistochemical methods. The results of the study showed that sandfly bite induced T-lymphocytes, macrophages, mast cells and Langerhans cells at the site of sandfly bite, which indicated that *Leishmania* infection might be easily completed through Th2 and delayed type hypersensitivity response.

Introduction

Leishmania protozoan, a causative agent of leishmaniasis, is transmitted through saliva of sandfly to host skin, when it sucks blood from mammalian host. It has been reported that the sandfly saliva enhanced *Leishmania* infectivity (Titus *et al.*, 1988; Theodos *et al.*, 1993; Hall *et al.*, 1995). Titus *et al.* (1988) have reported that mice infected with *L. (L.) major* promastigotes with sandfly salivary gland lysate had five to ten times larger skin lesions and contained 5000 times more parasites, when compared to mice injected with only promastigotes. They also have described that parasites were detected at the site of infection only when injected together with the lysates. Though it is still unclear how *Leishmania* parasites complete the infection or eliminated from human at initial stage of the infection, sandfly saliva may have an important role during *Leishmania* infection.

Insect bite, including sandfly bite, causes variable skin reactions, such as red papules, vesicles and erythema on human individuals. It is considered that the skin reaction is closely related to host immunity. In science literature, none has been describing the sandfly bite case investigated histochemically. Here, for the first time, we report a case, which showed a lesion bit by sandfly, and was examined by using histochemical methods.

Subject and Methods

Case report

A thirty eight-year-old healthy male investigator (dermatologist: A.T.) has been to Ecuador, South America, in order to study cutaneous leishmaniasis for about 2 months in 1998. He was involved in trapping sandflies several times by method of protected human bait, but using partially exposed his lower legs. At the first two weeks, he never showed the skin lesion at the site of sandfly bite. On the third week, he noticed bite-site showing strong reaction with red-colored papules. The skin lesion, caused by sandfly bite, was examined by using immunohistochemical techniques.

Materials

At the time of sandfly trapping, he marked the site of sandfly bite on his left lower leg and observed one week. The sandflies were identified to be *Lutzomyia hartmanni* and no *Leishmania* promastigotes were detected on a dissection. The two different site showed erythema each, and 24 hrs after the bite changed into 2-3 mm sized red papules (Fig. 7.3.1). These lesions remained being papules for one week. Then, skin samples were biopsied and fixed in 10% formalin solution for histochemical investigation.

Immunohistochemistry

Immunohistochemical study was performed on CD45R0, lysozyme, S-100 and tryptase using DAKO LSAB kit (DAKO, Kyoto, Japan) following manufacturer instruction.

Results

Epidermis showed slight spongiosis and loss of normal polarity with many small round-shaped cell

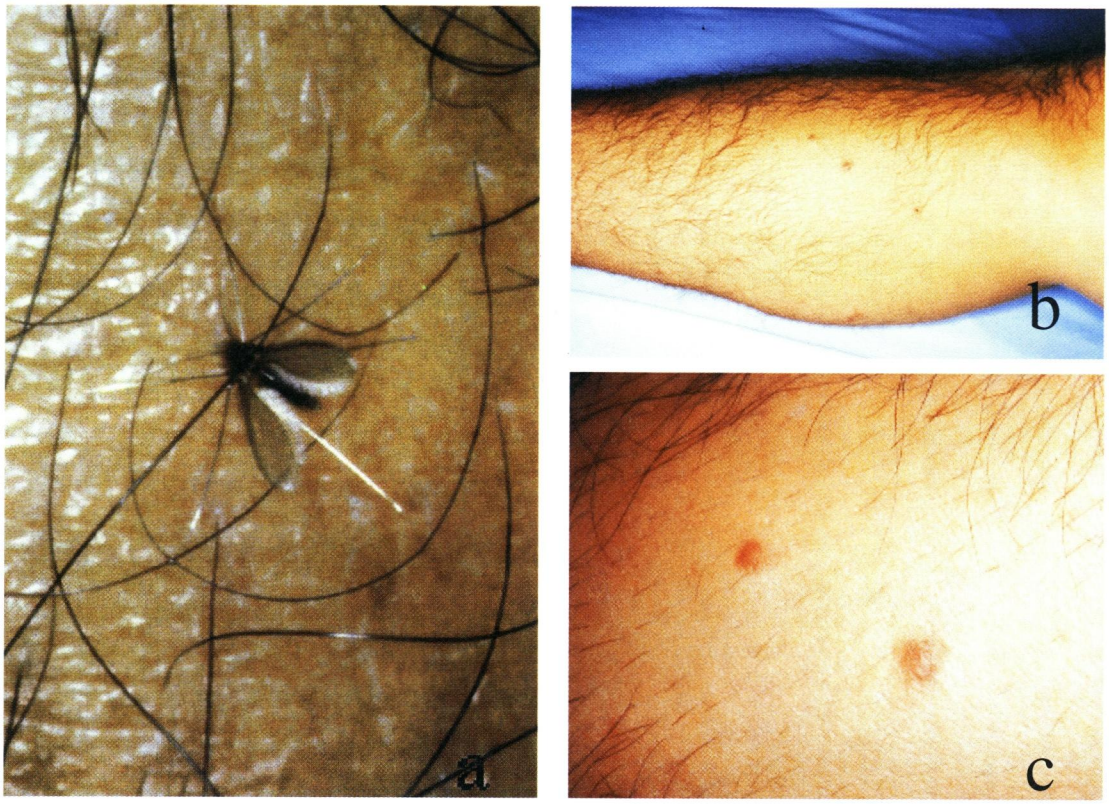

Figure 7.3.1. Sandfly *Lutzomyia hartmanni* is sucking blood (a). The lesion shows red papules margined by erythema (b, c).

infiltration on H.E section. These small round-shaped cells also infiltrated throughout the dermis (Fig.7.3.2). One-third of these cells were stained by CD45R0 antibody, demonstrating positive reaction to T lymphocytes (Fig.7.3.3a). Dense infiltration of lymphocytes and comparatively large round-shaped cells were observed from upper to deep dermis (Fig.7.3.2). The round-shaped cells showed positive result to lysozyme, reacting to macrophages (Fig.7.3.3b). S-100 protein staining revealed that positive dendritic cells, probably Langerhans cells, were increased in number in the epidermis (Fig.7.3.3c). Many tryptase-positive cells were observed in dermis, indicating that mast cells were abundant there (Fig.7.3.3d).

Discussion

Leishmania promastigotes change their form to amastigotes after entering host macrophage and proliferate there by binary fission. In human, the parasites cannot survive outside of the macrophages because human immune systems, represented by neutrophils, lymphocytes and immunogloblins, eliminate the pathogenic agents from the human body. Thus, at initial stage of *Leishmania* infection, human-host immune reaction to sandfly bite must contribute whether the infection still continues or not. This case was bit by the sand flies without *Leishmania* promastigotes in their gut, which indicated that the skin reaction might be influenced by only sandfly bite.

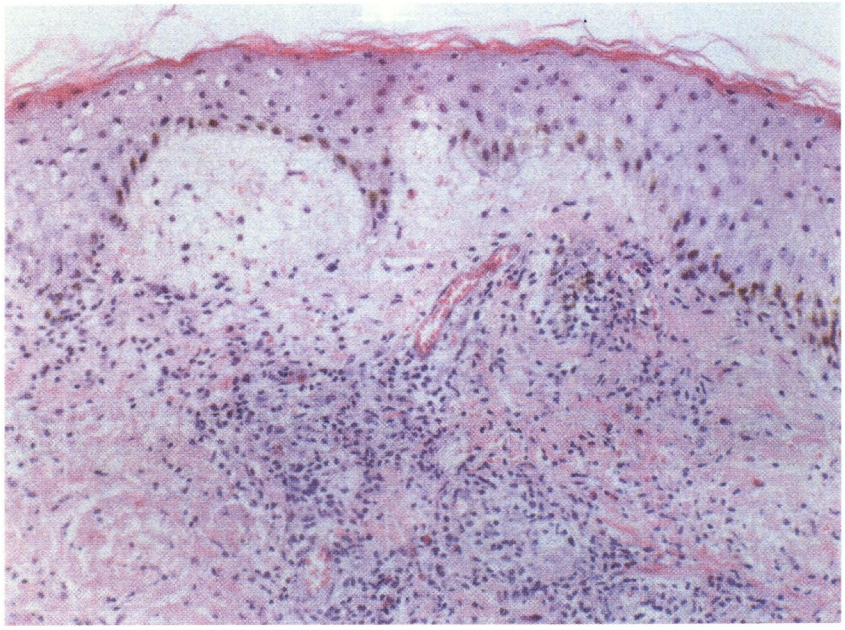

Figure 7.3.2. H.E. staining shows that small round-shaped cells infiltrate from epidermis to deeper dermis. Larger round-shaped cells are also observed in the dermis.

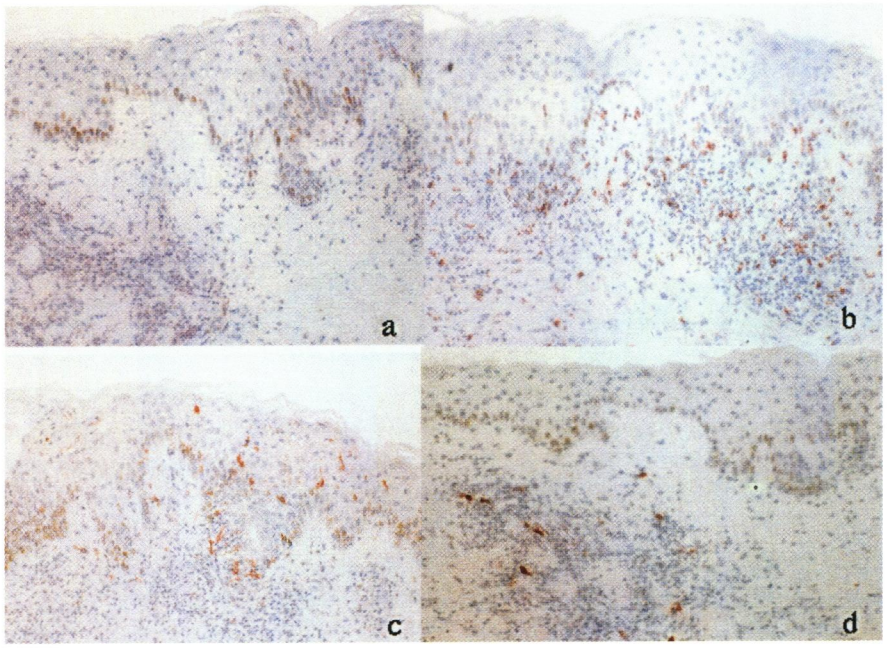

Figure 7.3.3. CD45R0 positive cells, indicating T-lymphocytes, are much observed throughout the dermis with epidermal infiltration (a). Lysozyme staining shows many positive cells in the dermis, which indicates macrophages are rich there (b). S-100 protein reacting Langerhans cells are increased in the epidermis (c). Tryptase positive cells, indicating mast cells, are also increased in the dermis (d).

Immunohistochemical study of the present case showed epidermal Langerhans cells and dermal T-lymphocytes were increased. Macrophages were also increased in the dermis. These findings may be due to delayed type hypersensitivity (DTH) reaction, most probably due to sandfly saliva. In facts, the subject case never showed skin reaction like as erythema at the first two weeks of the trap, which indicated that he was sensitized during the trap. Mast cells were much observed in the dermis. Increase of cutaneous mast cells may represent that Th2 response was dominant at the site of sand fly bite. Th2 response is considered to be the important factor that makes the disease progressed in murine leishmaniasis (Sadick *et. al.,* 1986, Heinzel *et al.,* 1989, Scott *et al.,* 1991, Locksley *et al.,* 1992). The results of this study give us the ideas that, 1) the saliva may induce Th2 response at the site, 2) DTH response may make the chance of the parasites to infect macrophage, 3) these responses make advantage to complete the infection. Further investigations are needed.

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4. Pre-Injection of Homogenates of Sandfly *Lutzomyia hartmanni* Heads with Salivary Glands Enhances *Leishmania* Infection in BALB/c Mice

ABSTRACT. BALB/c mice infected with *Leishamania (Leishmania) amazonensis* were studied in skin lesion development, treated by pre-injection of homogenates of heads with salivary gland of sandfly *Lutzomyia hartmanni.* The lesion was markedly developed by the treatment, compared to control mice. Electron microscopic examination revealed many *Leishmania* amastigotes survive outside of the macrophages in the lesion of pre-injected mice. The present results were consistent with the previous findings that sandfly saliva enhances *Leishmania* infectivity.

Introduction

Leishmania parasites exist as flagellated extracellular promastigotes in their vector, phlebotomine sandflies. The mammalian host is infected with the parasite when the sandfly sucks blood and injects the parasite in the saliva. The parasites are found as aflagellate amastigotes in the mammalian host macrophages (Neva and Sacks, 1990). Titus *et al.* (1988) have reported that mice infected with *L. (Leishmania) major* promastigotes with sandfly salivary gland lysates had five to ten times larger skin lesions and contained 5000 times more parasites, when compared to mice injected with promastigotes only. They also have described that parasites were detected at the site of infection only when injected together with the lysates. Theodos *et al.* (1993) have demonstrated that sandfly saliva inhibited the ability of macrophages to present leishmanial antigens to parasite-specific T cells using *L. (L.) major* antigens. These *in vivo* and *in vitro* studies suggest that sandfly saliva enhances *Leishmania* infectivity at the infection. In BALB/c infected with *L. (V.) braziliensis,* infection progressed to extensive, poorly organized macrophages and many amastigotes in the presence of sandfly salivary gland lysates, but few parasites were seen in the absence of the lysates on light microscopy (Donnelly *et al.*, 1998).

In the present study, we examined the development of skin lesions, by light and electron microscopic observations in BALB/c mice infected with *L. (L.) amazonensis,* in order to check whether pre-injection of homogenated solution of heads with salivary glands of sandfly *Lutzomyia hartmanni* would change the disease outcome.

Materials and Methods

Animals and parasite infection

A total of 8 male BALB/c mice at the age of 4 weeks were used in this study. The mice were handled under the regulation of the animal center, Faculty of Medicine, University of the Ryukyus, Okinawa, Japan. The mice were divided into two groups. One was the control group (n=4), and the other was the experimental group (n=4). The mice were injected with 2×10^6 of *L.(L.) amazonensis* promastigotes at the shaved skin of the back. Twenty two heads of the sand flies with salivary glands were homogenated in 0.8 ml of normal saline for the lysates. Twenty four hrs before the parasite inoculation, the experimental mice were pre-injected with 50 μl of lysates at the inoculation site. On the other hand, the control mice were injected with the same numbers of the parasites in 50 μl of normal saline without the lysates.

Assessment of lesion development

Lesion development at the inoculation site was observed and measured for 8 weeks. The size was expressed as long x short axis (mm^2) of the lesion.

Light and electron microscopy

Eight weeks after the infection, skin lesion was biopsied and observed by light and electron microscopes. For light microscopic examination, the specimens were fixed in 10% formalin, cut and stained with hematoxyline-eosine. For electron microscopy, the specimens were cut into small pieces and fixed with 2% glutaraldehyde. Samples were fixed with phosphate-buffered 1% osmium tetroxide, and then dehydrated with an ethanol series and propylene oxide, and embedded in Epon 812 resin. Ultrathin sections were cut and stained with uranyl and lead acetate and, then observed by JEOL 2000 EX transmission electron microscope (JEOL, Tokyo, Japan).

Results

Lesion development

After 8 weeks of parasite inoculation, the control mice had small sized lesions, while mice pre-injected with saliva lysates showed bigger sized ulcers. The mean lesion size of control mice was 115 mm^2 8 weeks after the infection. In mice pre-treated with lysates of sandfly head with saliva, the mean lesion size was 750 mm^2 at that time (Figs.7.4.1 and 7.4.2). The lesion development was significantly enhanced by the saliva lysates treatment ($p< 0.01$)(Fig.7.4.2).

H.E staining

Numerous vacuolar cells were observed with dense lymphocytes-like cell infiltration extending from mid to deep dermis in both the control and experimental mice. The vacuolar formation in the experimental mice was a little bit larger as compared to the controls (Fig. 7.4.3).

Electron microscope

The control and experimental mice showed that several amastigotes present in the macrophage vacuoles called parasitophorous vacuole (PV), and that the cell organelles of the amastigotes were well developed. In the experimental mice, many amastigotes were observed outside the macrophages as compared to the control (Fig.7.4.4).

Discussion

The results presented here demonstrated that the skin lesion was significantly enlarged in *L. (L.) amazonensis* -infected BALB/c mice pre-injected with the lysates containing head with salivary gland of sandfly *Lu. hartmanni.* Our results are in consistent with the previous workers' reports, who examined the skin lesion development in the mice infected with *L. (L.) major* and/or *L. (L.) brasziliensis* and co-injected with salivary gland lysates of sandfly *Lu. longipalpis* (Titus *et al.,* 1988; Samuelson *et al.,* 199; Theodos *et al.,* 1993; Donnelly *et al.,* 1998). These results ensure that sandfly saliva must cooperate with *Leishmania* parasites to progress the disease in a mammalian host. Whereas, salivary gland homogenates of *Lu. longipalpis* inhibited the *in vitro* multiplication of *L. (L.) amazonensis* promastigotes, but did not inhibit the growth in the gut extracts of *Lu. longipalpis.* The promastigotes bodies were slender and longer shape in the presence of the salivary gland homogenates (Charlab and Ribeiro, 1993). Those mechanisms are very advantageous to accomplish the infection in a target mammalian host. Because, when the fully developed promastigotes present in the gut of the sandfly are transmitted to the mammalian host, the promastigotes become slender and longer shaped cells, and their multiplication is inhibited by the saliva, which seems to be favorable to insert the mammalian macrophages. *Leishmania* promastigotes change their form to amastigotes after entering the macrophages and proliferate there by binary fission. This may be one of the reasons why lesion development was markedly enhanced in *Leishmania*-infected mice co-injected with sandfly salivary lysates.

In human, the parasites cannot survive outside of the macrophages because human immune systems, represented by neutrophils, lymphocytes and immunoglobulins, eliminate the pathogenic agents from the human body. Even in BALB/c mice, the parasites are hard to survive outside of the macrophages since the present electron microscopic examination showed few parasites outside of the macrophages in the control

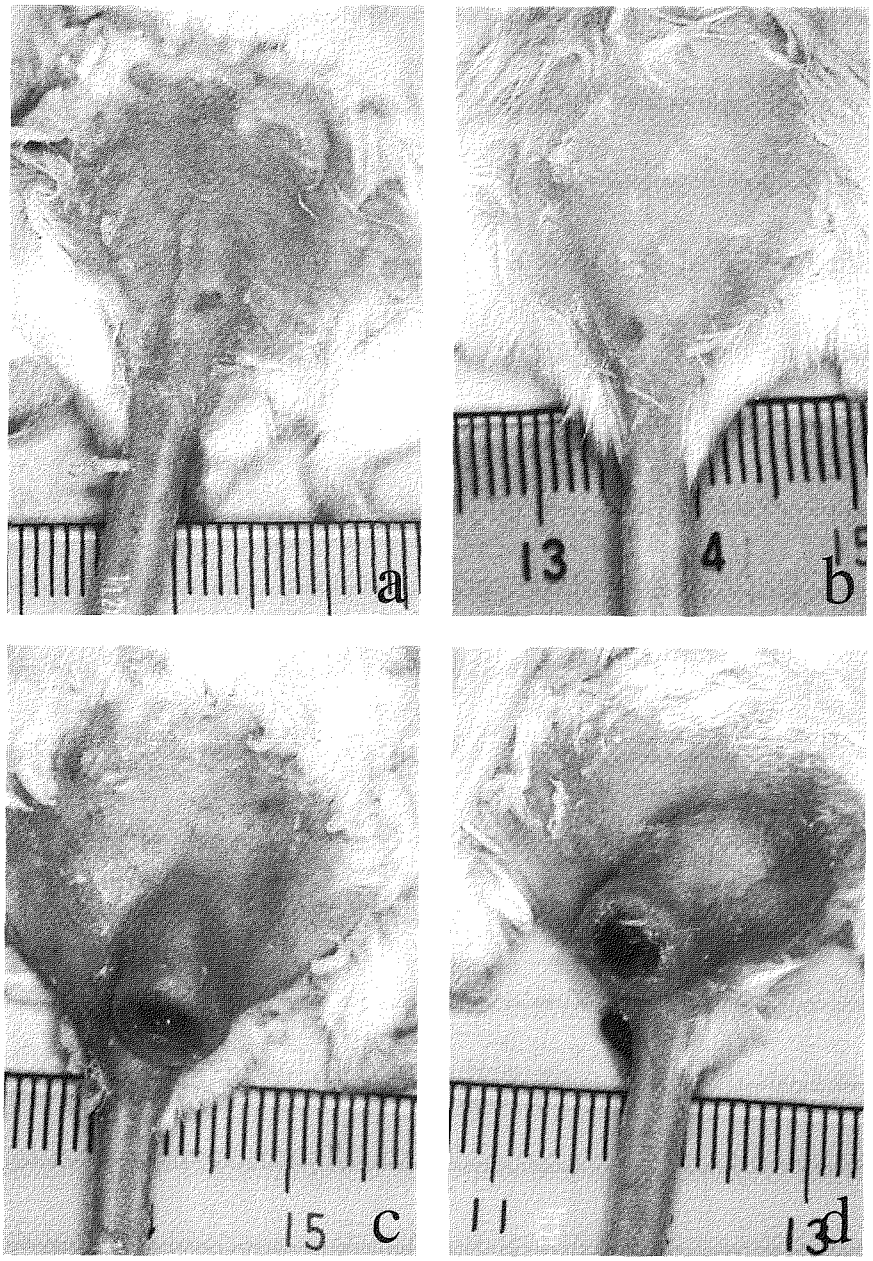

Figure 7.4.1. Lesion development of BALB/c mice infected with *L. (L.) amazonensis* promastigotes 8 weeks after infection. **a** and **b**: small lesions found in the control mice received the parasites in the normal saline without salivary homogenates. **c** and **d**: marked enlargement of skin nodules with ulcer found in the experimental mice pre-injected with salivary homogenates before the parasite inoculation.

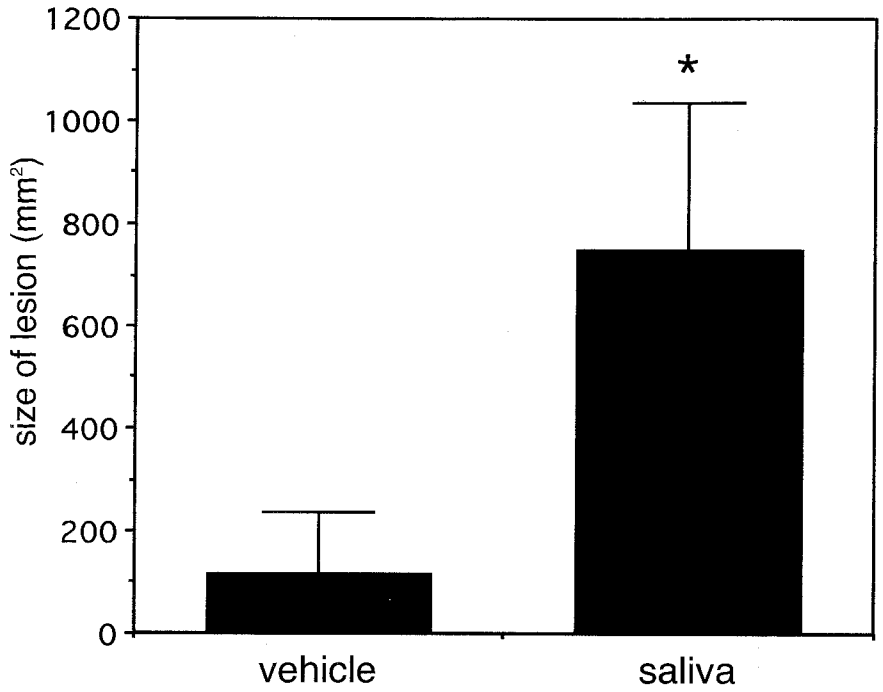

Figure 7.4.2. The mean skin lesion size of the mice pre-injected with/without the sandfly salivary homogenates. The skin lesion development is significantly accelerated in the mice pre-injected with the homogenates ($p < 0.01$).

mice. In contrast, in the mice pre-injected with the salivary lysates, many parasites (amastigotes) were observed outside the macrophages. This fact indicates that immunoprotection in the lysates pre-injected mice might be insufficient to inhibit parasites survival by the present treatment. Th2 response is considered to be the important factor that makes the disease progress in murine leishmaniasis (Sadick *et al.*, 1986, Heinzel *et al.*, 1989, Scott *et al.*, 1991, Locksley *et al.*, 1992). By the injection with the sandfly head homogenates, Th2 response may be accelerated in the skin. Further investigations are needed to examine the immune responses, in relation to the skin lesion development of the mice co-injected with the salivary lysates and the parasites.

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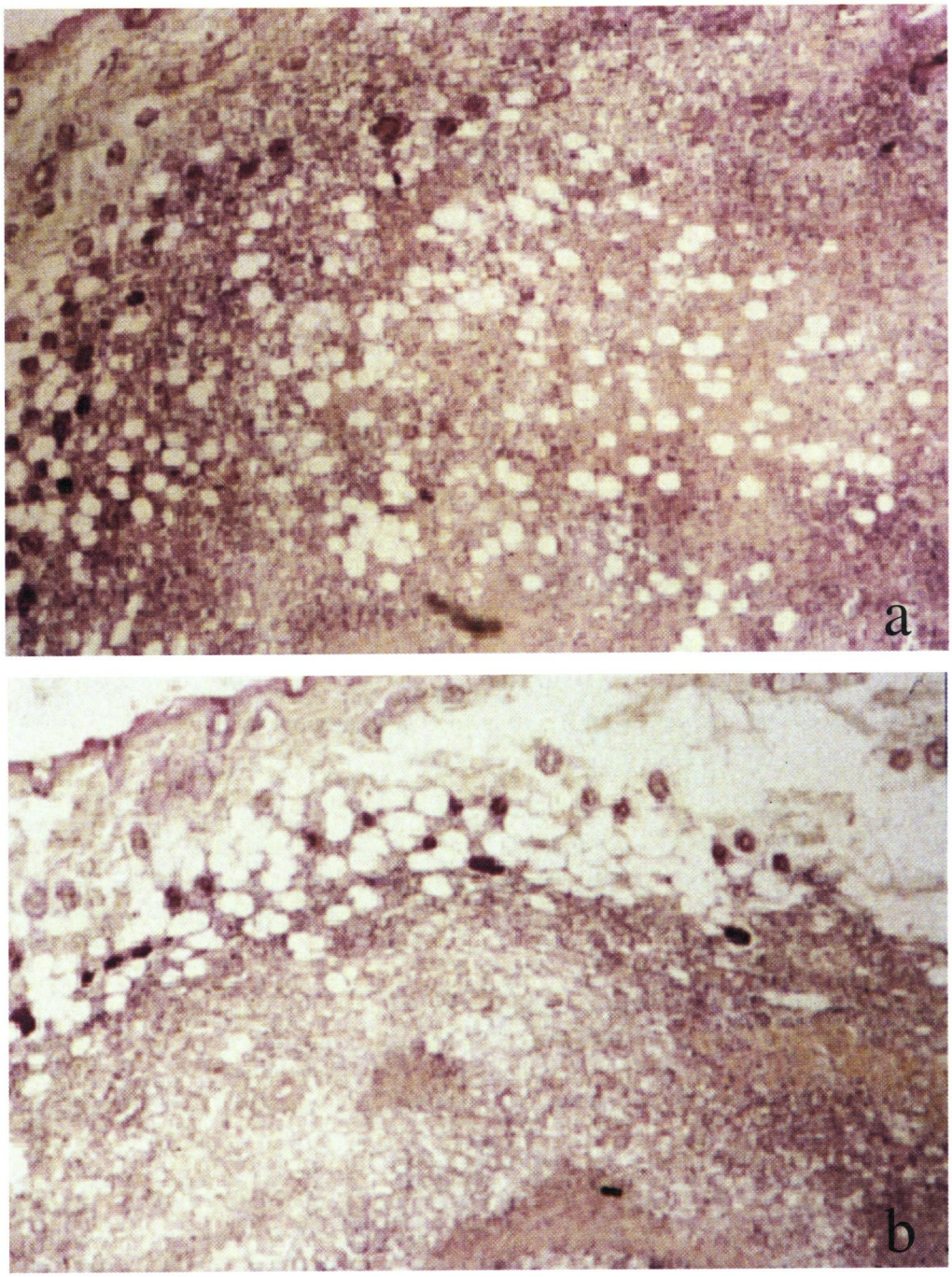

Figure 7.4.3. H.E. stainings of the lesion in the mice pre-injected with/without the sandfly salivary homogenates. Vacuolar cells are markedly enlarged in size in the mice pre-injected with the homogenates (**a**) compared to the mice without the homogenates (**b**).

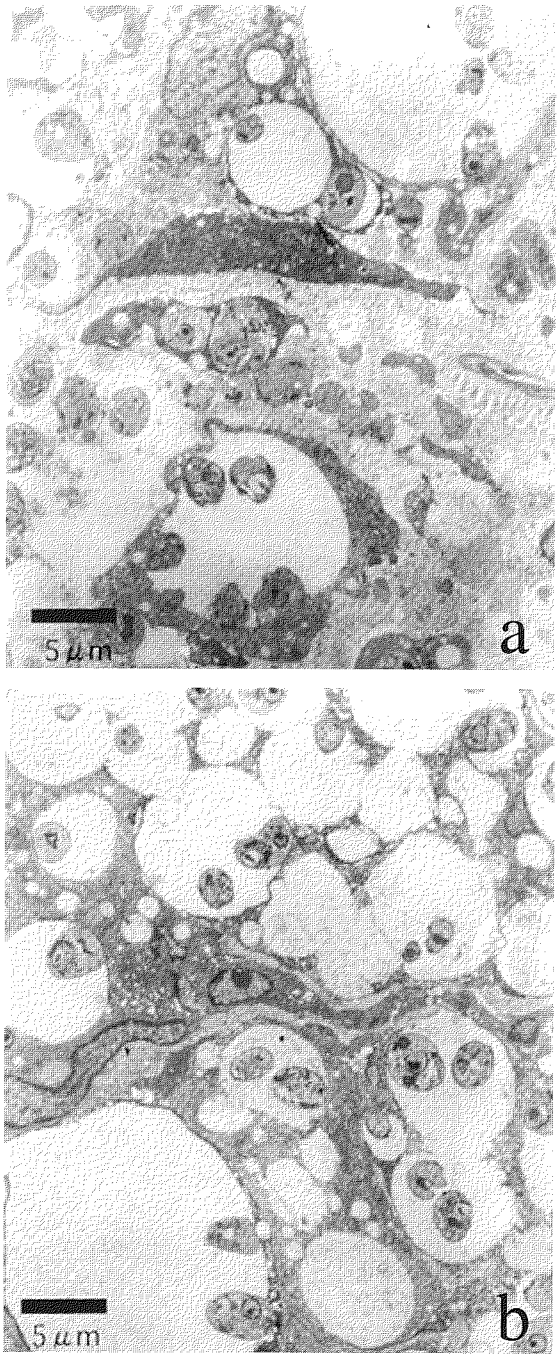

Figure 7.4.4. Electron microscopic findings of the lesion in the mice pre-injected without (**a**)/with (**b**) the sandfly salivary homogenates. Cell organelles of amastigotes are well developed in both groups (**a** and **b**). Note many amastigotes outside of the macrophages in the mice pre-injected with the homogenates (**b**).

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Chapter 8

Experimental Treatment

1. Anti-Leishmanial Effect of Glucantime® against *Leishmania* Promastigote and Amastigote Form

ABSTRACT. Meglumine antimoniate (MA), an anti-leishmanial agent, has been used for the treatment of all forms of leishamaniasis. The present study demonstrated the efficacy of the drug against both promastigote and intracellular amastigote of *Leishmania (Leishmania) major* and *amazonensis in vitro* study. Two species of *Leishmania* promastigotes were treated with 0.085-850 μg/ml concentrations of meglumine antimoniate. Anti-promastigote proliferation of the drug was observed among three (0.085-850 μg/ml) concentrations, but the promastigotes were observed still alive by neutral red staining in those concentrations. Ultrastructural observation revealed a few well-developed amastigotes and presence of small, thin parasitophorous vacuoles (PV) in macrophages treated with MA compared to macrophages treated without MA. In Giemsa staining, the macrophages treated with MA revealed many promastigotes outside and a few amastigotes inside of the cells compared to macrophages treated without MA. From the data of the present study, it was suggested that MA inhibits directly the proliferation of promastigote, and may have inhibitory effect to interfere the entry of promastigotes into macrophages. Anti-leishmanial activity of MA is probably mediated *via* promastigotes proliferation, and also *via* inhibitory effects on macrophages to suppress the pathogenesis of *Leishmania* infection.

Introduction

The chemotherapeutic agents that include pentavalent antimonial compounds such as sodium stibogluconate (Pentostam®) or meglumine antimoniate (Glucantime®) remains the drug of choice for all forms disease inspite of their reported severe toxicity of liver, heart and kidney (Ollario *et al.*, 1993). Though it is not clear that how antimonial drug acts as antileishmanial drug.

The characteristic of all leishmanial infection is the intracellular parasitism of macrophages by amastigotes, the mammalian stage of this parasite responsible for all symptoms and pathology. Parasitic survival depends on a number of factors including manipulation of the host immune system such as the host cells; macrophages either do not receive or do not act upon appropriate signals (Liew *et al.*, 1993; Reiner *et al.*, 1995). Recent studies have been reported that resistant strains of *Leishmania (L.) major* promastigotes was quite sensitive to the drug at much lower concentrartion as compared to amastigotes form in macrophage, suggesting that Pentostam® inhibitory action is mediated through the macrophage rather than through a direct toxic effect on the parasite (Ibrahim *et al.*, 1994). However, a few reports have been described about how MA acted on *Leishmania* promastigotes and intracellular amastigotes. To elucidate the action of MA on *Leishmania* parasites, we investigated *L. (L.) major* promastigotes proliferative activity in incubation with MA, and also the morphological changes of *L.(L.) major*-infected macrophage before

and after treatment of MA.

Materials and Methods

Parasites and administration of drug

Two species of *Leishmania* promastigotes; *L. (L.) major* (MHOM/SU/73/5ASKH) and *L. (L.) amazonensis* (MHOM/BR73/M2269) were cultured in RPMI 1640 (GIBCO BRL, Tokyo, Japan) medium supplemented with 10% FBS, 50 μg/ml of streptomycin and 50 U/ml of penicillin. The promastigotes form of both strains was incubated with meglumine antimoniate (MA) with three different concentrations as follows: 0 μg/ml, 0.085 μg/ml, 8.5 μg/ml and 850 μg/ml for 48 hrs. The parasites were counted by haemocytometer after 1, 6, 24 and 48 hrs incubation with MA, and stained with neutral red accordingly.

Macrophage co-cultured with Leishmania *promastigotes*

The macrophage cell line J774 was purchased from Dainippon Pharm. Co. (Osaka, Japan), and cultured in DMEM (GIBCO BRL, Tokyo, Japan) medium supplemented with 10% FBS, 50 μg/ml of streptomycin and 50 U/ml of penicillin, kept in incubator at 37℃ with 5% CO_2 and 95% air humidity environment. The Lab-Tek® tissue culture chamber slides (Nalge Nunc, International Corp., Napeville, IL) were used for macrophage culture and allowed to confluent growth for three days, following infection with *Leishmania* promastigotes. Different concentrations (0, 0.085, 8.5 and 850 μg/ml) of MA, were administered to macrophages and incubated for 48 hrs. The parasites in medium were counted by haemocytometer. Macrophages in the chamber slide were washed with PBS and fixed with 100% ethanol, and then stained with Giemsa staining.

Electron microscopic study of infected macrophage

The macrophage cell line J774 was cultured in 35 mm culture dishes and allowed to confluent growth for three days. Then infection of macrophages with *L. (L.) major* was done for 24 hrs. Then, MA was added at concentration of 20 μg/ml and incubated for 24 hrs. The macrophages were harvested and fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, followed 1 hr fixation of 1% Osmium tetraoxide. The samples were dehydrated with a series of ethanol and embedded in Epon 812 resin. Ultra thin sections were cut and stained with uranyl followed by lead citrate, then observed by JEOL 2000 EX electron microscope (JEOL, Tokyo, Japan).

Results

Drug susceptibility on promastigotes growth

The effectiveness of the drug on promastigotes growth was observed at three different concentrations of MA. The promastigotes of *L. (L.) major* were treated with MA showed the gradual reduction in number, time dependently among 0.085-850 μg/ml concentrations. In contrast, promastigotes treated without MA proliferated time dependently. The reduction rate was similar among three concentrations of MA (Fig. 8.1.1A). *L. (L.) amazonensis* promastigotes showed almost same result by treatment with MA (Fig. 8.1.1B).

Promastigotes co-cultured with macrophages

The macrophages were cultured with two species of *Leishmania* promastigotes, treated with different concentrations (0, 0.085, 8.5, 850 μg/ml) of MA for 48 hrs. The both species of *Leishmania* promastigotes revealed the same reduction in number (Fig.8.1.2A, 2B).

Neutral red and Giemsa staining

Neutral red staining was performed to assess the viability status of promastigotes, showing alive promastigotes as red-colored. The *L. (L.) major* promastigotes treated at 8.5 and 850 μg/ml concentrations of MA showed red colored promastigotes as well as promastigotes treated without MA. However, MA-treated promastigotes showed round shaped and aggregated in their forms, different from spindle-like shaped form in control (without MA) (Fig. 8.1.3A-C). A few amastigotes were observed outside of macrophages, and amastigotes could be observed inside of the macrophages treated without MA (Fig. 8.1.4A), while, macrophages treated with MA revealed many pro-

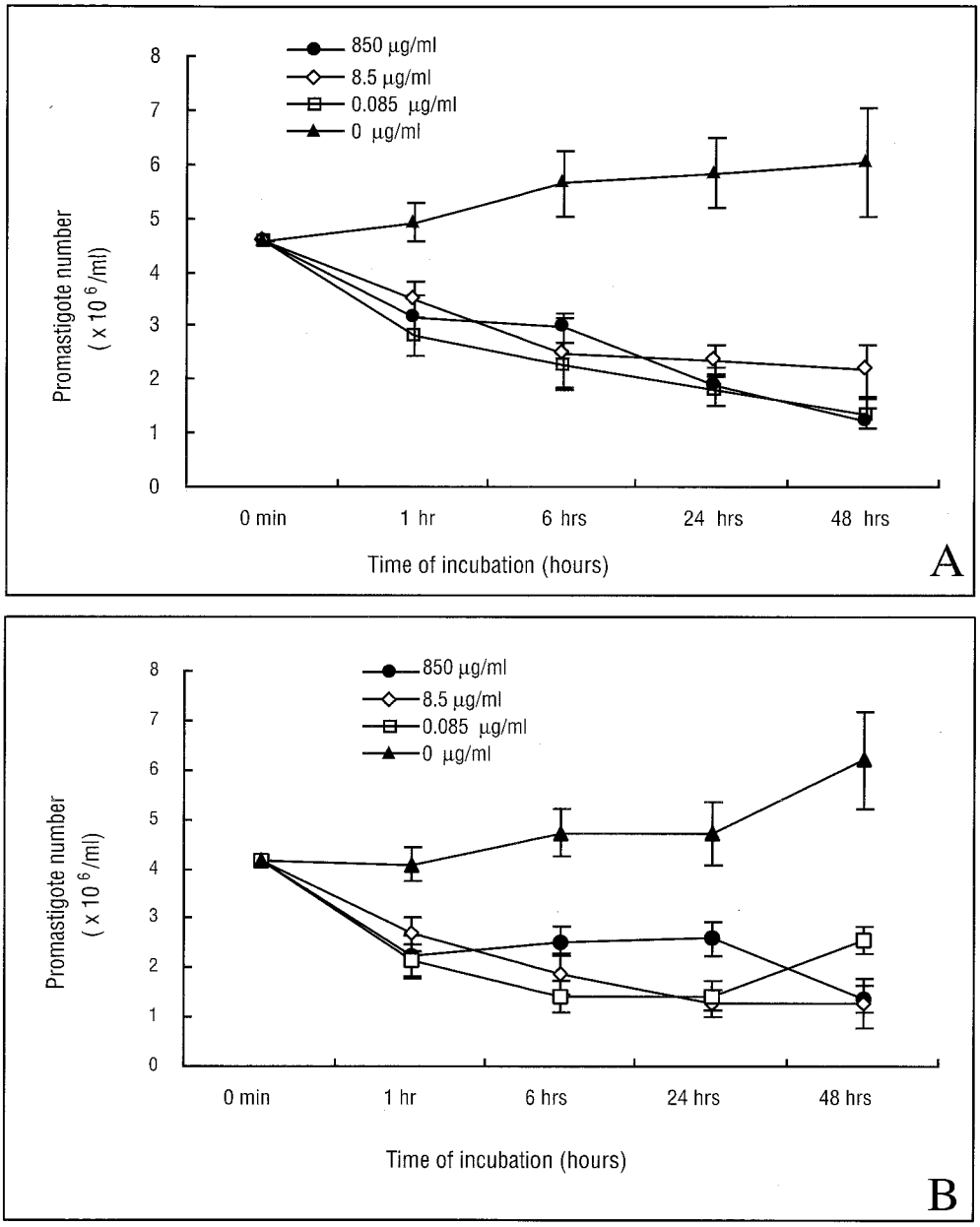

Figure 8.1.1. The number of *L. (L.) major* **(A)** and *L. (L.) amazonensis* **(B)** promastigotes in culture medium. Both *Leishmania* species of promastigotes were treated without MA showed the gradual increase in number, while those treated with MA were remarkably reduced in number among three different concentrations (0.085, 8.5 and 850 μg/ml). Each point is representing mean ± standard deviations.

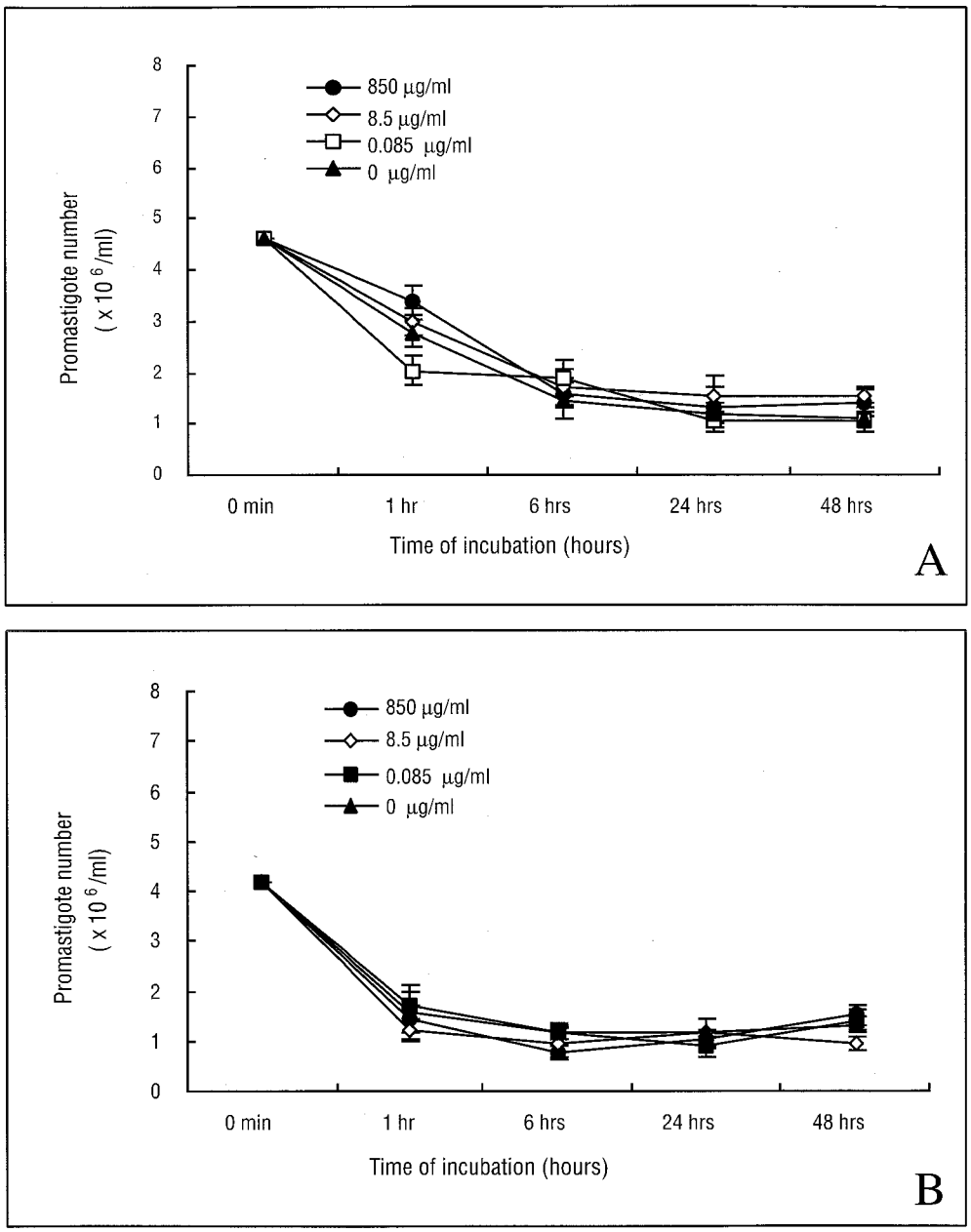

Figure 8.1.2. The number of *L. (L.) major* **(A)** and *L. (L.) amazonensis* **(B)** promastigotes co-cultured with macrophage. Both strains of promastigotes showed a gradual reduction in number treated with or without MA. Each point is representing mean ± standard deviations.

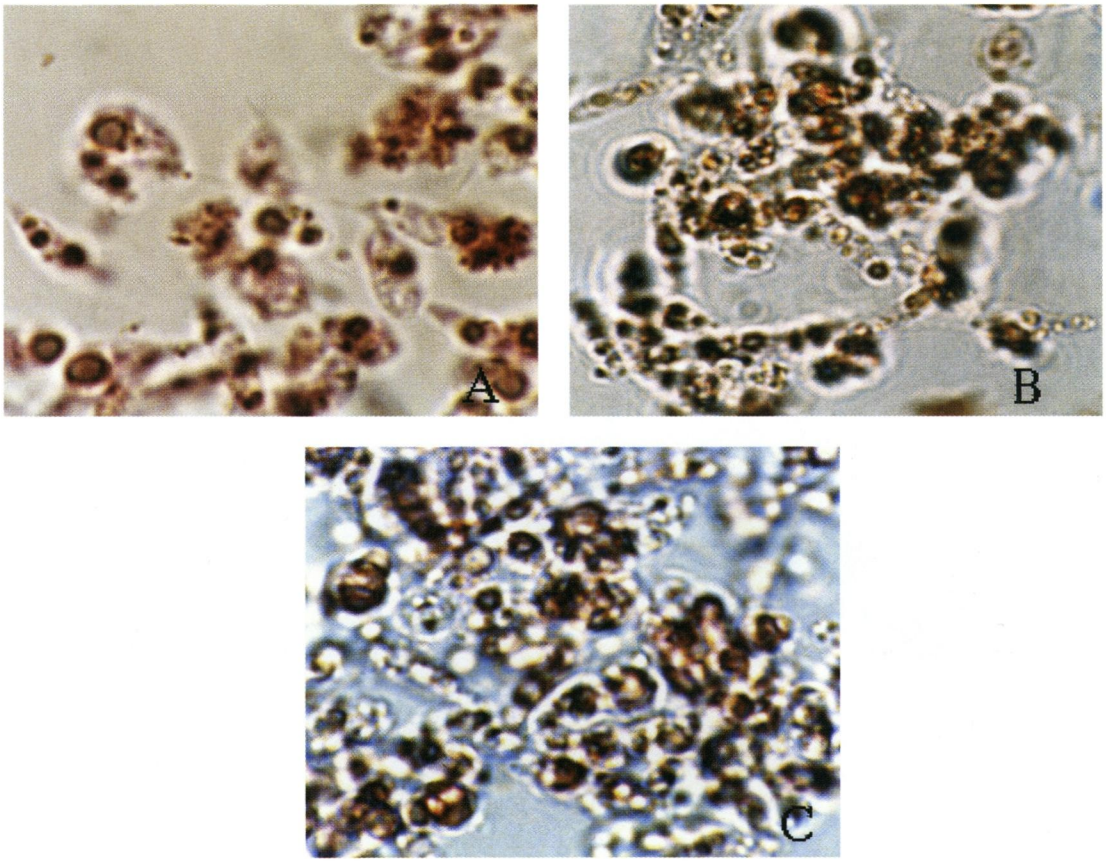

Figure 8.1.3. Neutral red staining of *L.(L.) major* promastigotes. **A**: promastigotes treated without MA, showed spindle-like form colored in red. **B**: promastigotes treated with 8.5 µg/ml concentration of MA showed red colored, round-shaped and aggregated. **C**: promastigotes treated with 850 µg/ml of MA were still alive.

mastigotes outside in Giemsa staining (Fig. 8.1.4B).

Electron Microscopic Study

The *L. (L.) major* infected macrophages treated without MA revealed that the intracellular amastigotes were multiple in numbers within parasitophorous vacuoles (PVs). The multiple numbers of amastigotes were circular or round shaped having well developed cell organelles. The cytoplasm of macrophage showed vacuolar change, suggesting the macrophage was in degeneration (Fig. 8.1.5A). In contrast to the macrophage treated with MA revealed a single amastigote in PV and comparatively preserved cell organelles of the cytoplasm (Fig. 8.1.5B).

Discussion

The recommended treatment for leishmaniaisis is considered Pentostam® or MA (Glucantime®). Although there are several reports of variation in efficacy between the two main commercially available formulations, these two have been considered as mostly equivalent. The exact mode of mechanism of antimonial compounds is not well known. It has been speculated that pentavalent antimony compound inhibit the glycolysis and β-oxidation of fatty acids of the parasites resulting in a net reduction in the generation of ATP to GTP (Berman *et al.*, 1988). Goodwin and Page

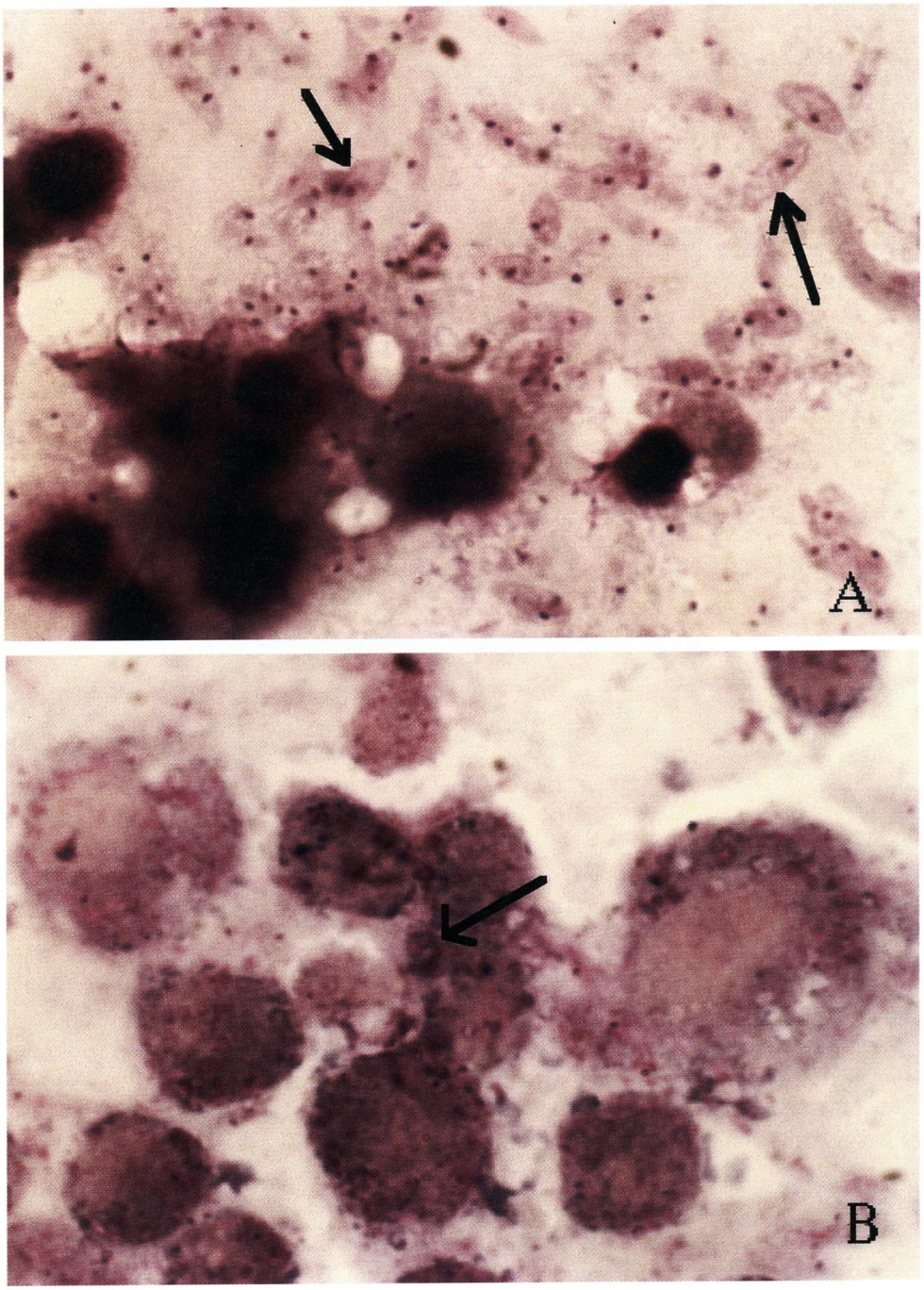

Figure 8.1.4. Giemsa staining of *L. (L.) major* infected macrophages. **A**: macrophages treated without MA showed few promastigotes outside and amastigotes (arrows) inside of them. **B**: macrophages treated with MA at the concentration of 850 µg/ml showed many promastigotes (arrows) inside of them.

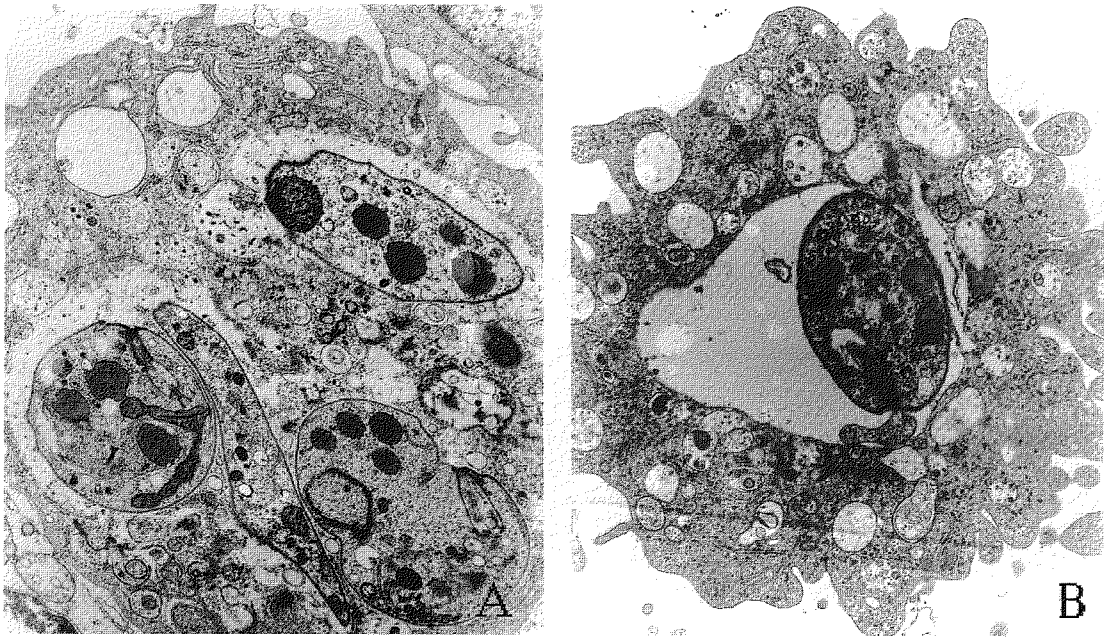

Figure 8.1.5. Ultrastructural findings of *L. (L.) major* infected macrophages. **A**: infected-macrophages treated without MA revealed that multiple numbers of amastigotes in PVs, the cytoplasm of macrophages was in degenerative change. **B**: infected-macrophages treated with 20 μg/ml of MA showed a single amastigote and comparatively well preserved cell organelles of cytoplasm.

(1943) suggested that the net reduction of pentavalent Sb^{+5} to an active trivalent Sb^{+3} form in the host organism might be responsible for its parasiticidal activity.

The present study provided the information that anti-promastigote proliferation of MA was observed at 0.085-850 μg/ml concentrations, but the parasites were still alive on neutral red staining observation. The living cells take up neutral red and sequester it in the lysosomes, but the stain is not retained by non-viable cells (Freshney *et al.,* 1994). Our *in vitro* data suggested that MA had not leishmaniacidal effect to promastigotes form at least the range from 0.085 to 850 μg/ml of concentration.

On the observation of Giemsa staining of *L. (L.) major* infected macrophages treated with MA, many promastigotes outside and a few amastigotes inside were observed on the chamber slide. That finding was definitely different from the observation of infected macrophages treated without MA that revealed a few promastigotes were seen outside of the cells, despite that the number of living promastigotes in culture medium were almost same among 0–850 μg/ml concentrations of MA. From the present results, it was suggested that MA might have inhibitory effects to interfere the entry of promastigotes into macrophages.

Ultrastructural observation of this study disclosed that macrophages contained well-developed amastigotes in PV. The macrophages treated without MA showed multiple numbers of amastigotes. The cytoplasm of the macrophages also showed degenerative change as compared to that with MA treatment. The degenerative cytoplasm may manifest the cells on the course to burst by excessive parasites multiply. These findings suggested that MA might inhibit the intracellular proliferation of *Leishmania* parasites.

The present study demonstrated that MA might act as an anti-leishmanial agent through the inhibitory effects on not only *Leishmania* promastigotes proliferation but also intracellular amastigotes proliferation. In addition, it is noteworthy that MA may interfere the promastigotes to enter macrophage. In previous reports, Pentostam® were found more effective against amastigotes in host cells *in vitro* than against promastigotes in culture (Berman *et al.,* 1982; Neal *et al.,* 1987). It has also been reported that the resistant strain of *L.(L.) major* promastigotes was quite sensitive to Pentostam® at much lower concentration in the amastigotes in macrophage, suggesting that Pentostam' s inhibitory action is mediated through the macrophage rather than a direct toxic effect of the drug on the parasite (Ibrahim *et al.,* 1994). The further information about MA should be necessary to evaluate the relationship of the efficacy of the drug *in vivo*.

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2. Anti-Leishmanial Effects of LPS Derivative and IFN-γ in Experimental Leishmaniasis: A Preliminary Study of Combination Therapy with Meglumine Antimoniate

ABSTRACT. ONO-4007, a novel synthetic LPS derivative, and IFN-γ were used against experimental leishmaniasis in combination with meglumine antimoniate. In BALB/c mice treated, the leishmanial lesion development was inhibited compared to control. Specially, combination therapy using meglumine antimoniate and IFN-γ completely suppressed the lesion development. In *in vitro* study, *Leishmania* parasite proliferation in macrophages was markedly reduced in drug-administrated group compared to control. Both ONO-4007 and IFN-γ exerted antileishmanial effect when used with antimoniate as combination therapy.

Introduction

Antimonials have been globally used for leishmaniasis as the most effective drug. But antimonials have some side effects, such as liver, renal and heart disfunction. Till to date, no suitable drug alternatives have been developed. Even for localized cutaneous leishmaniasis, single use of antimoniate can't lead the disease to complete cure (Garcia *et al.*, 1998). Thus, other new drugs or methods are needed to be developed for treatments.

Promastigotes form of *Leishmania* parasites, are taken into host macrophages and change their form to amastigotes. They proliferate by binary fission in the cytoplasm of the macrophage against the host immunity. In murine model, intracellular killing of *Leishmania* parasites within macrophages depends on the toxic activities of nitrogen oxidation products released from the activated cells (Mauel *et al.*, 1997; Assreuy *et al.*, 1994; Cunha *et al.*, 1993). Several reseachers have reported that the macrophage activating factor, such as tetrapeptide tuftsin and tumor necrosis factor (TNF), induced nitric oxide (NO) synthesis to kill *Leishmania* parasites *in vitro* in presence of interferon-γ (IFN-γ) (Cillari *et al.*, 1994; Green *et al.*, 1994; Liew *et al.*, 1990). It has been reported that a therapy with antimoniate in combination with IFN-γ was more effective than conventional drug therapy in leishmanial mice (Li *et al.*, 1997), and also reported that lipopolysaccharide (LPS) was a necessary secondary stimulus to kill *Leishmania* amastigotes *in vitro* (Roach *et al.*, 1991). ONO-4007, a novel synthetic LPS derivative, stimulated glycogen-elicited peritoneal macrophage not only to induce TNF but also to exert tumoricidal activities (Fig. 8.2.1) (De Yang *et al.*, 1994). Based on these reports, ONO-4007 and IFN-γ were used in combination with meglumine antimoniate against experimental leishmanial mice and *Leishmania* parasitized macrophages, in order to examine the anti-leishmanial efficacy.

Materials and Methods

Administration of drugs to mice

Four to eight weeks old male BALB/c mice were inoculated subcutaneously with 2×10^6 *L. (Leishmania) amazonensis* promastigotes on their back. Two weeks after the inoculation, drugs were administrated for the next two weeks as shown in Table 8.2.1. We observed the lesion development in the inoculated site for the next 12 weeks. Seven groups including the control (without drug administration) were used; each group was composed of two mice.

Culture of Leishmania *parasites and macrophages*

Murine macrophage cell line J774 was purchased

Table 8.2.1. Drugs and their administration

Group	Drugs used
1	without drug
2	meglumine antimoniate (250 mg/kg, for 14 days)
3	meglumine antimoniate (250 mg/kg, for 14 days) + ONO4007 (30mg/kg, 3 times/wk, for 2 wks)
4	meglumine antimoniate (250 mg/kg, 3 times/wk for 2 wks) + IFN-γ (15000 U/mouse, 3 times/wk for 2 wks)
5	meglumine antimoniate (250 mg/kg, for 14 days) + IFN-γ (15000U/mouse, 3 times/wk for 2 wks)
6	meglumine antimoniate (250 mg/kg, 3 times/wk for 2 wks) + IFN-γ (15000 U/mouse, 3 times/wk for 2 wks)
7	meglumine antimoniate (250 mg/kg, for 14 days) + IFN-γ (15000 U/mouse for 14 days)

Table 8.2.2. Treatment for *Leishmania*-infected macrophages

Group	Drugs used
A	without drug (as control)
B	85 μg/ml of meglumine antimoniate + 20 μg/ml of ONO4007
C	85 μg/ml of meglumine antimoniate + 1,000 IU/ml of IFN-γ
D	85 μg/ml of meglumine antimoniate
E	20 μg/ml of ONO4007
F	1,000 IU/ml of IFN-γ

from Dainihon Pharma. Co. (Tokyo, Japan) and cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 units/ml of penicillin and 100 μg/ml of streptmycin at 37℃. *L. (L.) amazonensis* was cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum, 100 units/ml of penicillin and 100 μg/ml of streptomycin at 24℃.

Administration of drugs to parasitized macrophages

Cultured macrophages were infected with *L. (L.) amazonensis*. 48 hrs after the infection, the cells were incubated for 24 hrs with the drugs as Table 8.2.2. The infected macrophages were harvested and washed with PBS. They were fixed for 1 hr with 2% glutaraldehyde in 0.1M cacodylate buffer. The samples were fixed with phosphate-buffered 1% osmium tetroxide, and then dehydrated with an ethanol series and propylene oxide. After embedded in Epon 812 resin, semi-thin sections of the samples were stained with toluidine blue.

Results

Lesion development of infected mice

The lesion size at inoculation site was measured, and expressed as length x width (mm^2). Mean lesion size of each group after the innoculation was shown in Fig. 8.2.1. The lesion sizes of each group at 14 weeks were as follows: Group 1 =190.5 mm^2, Group 2 = 8 mm^2, Group 3 = 59 mm^2, Group 4 = 61 mm^2, Group 5 = 28 mm^2, Group 6 = 28 mm^2, Group 7 = 0 mm^2. No lesion was developed in meglumine antimoniate + IFN-γ daily injected mice (Group7). Though other experimental mice developed the lesions, the lesion development was smarkedly suppressed compared to control group (Figs. 8.2.2 and 8.2.3).

Leishmania-*infected macrophages treated with drugs*

Infected macrophages without drug treatment showed large vacuoles including *Leishmania* amastigotes. Many amastigote-form parasites and collapsed macrophages were observed in the field (Fig.8.2.4A). In contrast, in the macrophages treated with meglumine antimoniate, ONO-4007, IFN-γ, meglumine antimoniate + ONO-4007, and meglumine antimoniate + IFN-γ, a few parasites were shown within the cells. Collapsed macrophages were observed in the field less than control. Few large intracellular vacuoles were formed in macrophages (Fig.8.2.4B-D).

Discussion

In experimental murine leishmaniasis, the over-

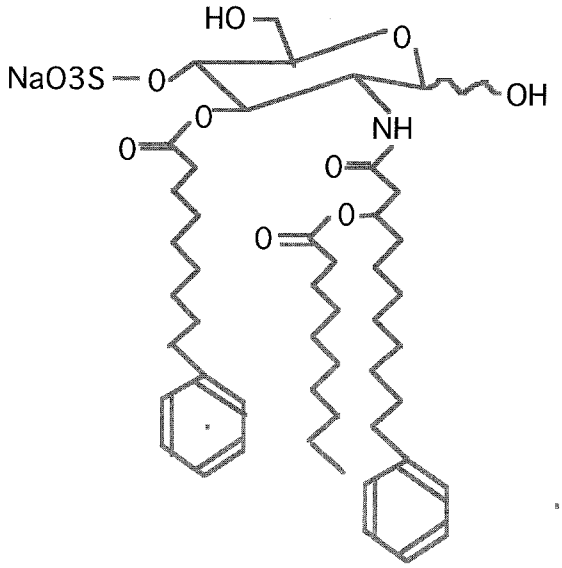


Figure 8.2.1. Chemical structure of ONO-4007.

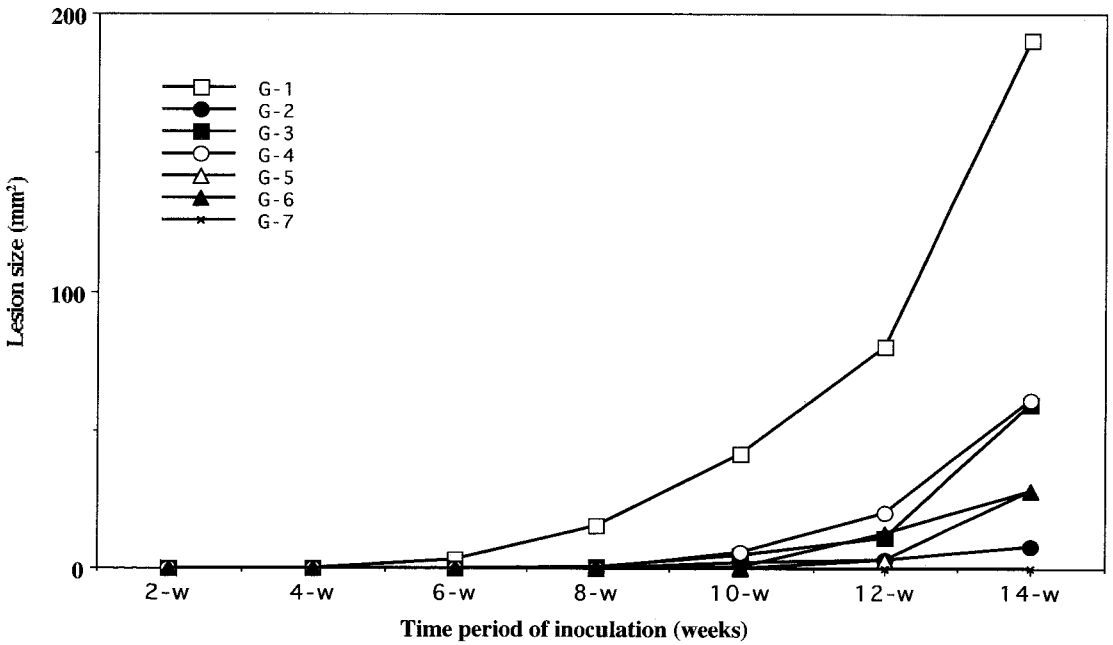
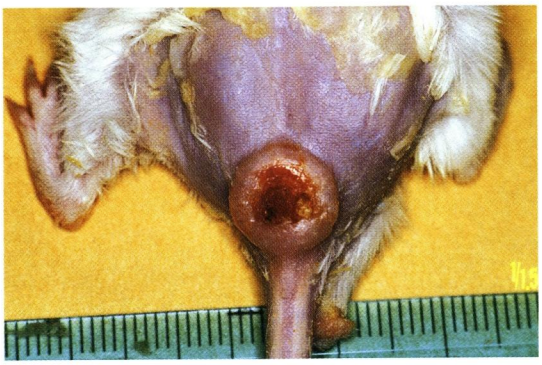
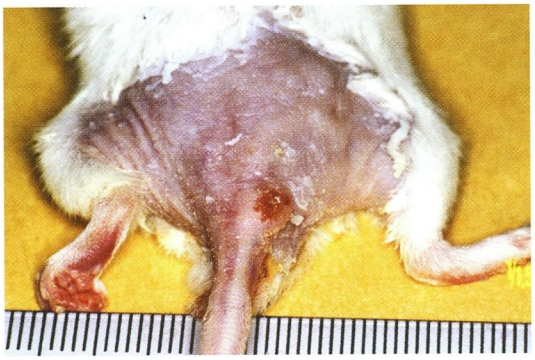


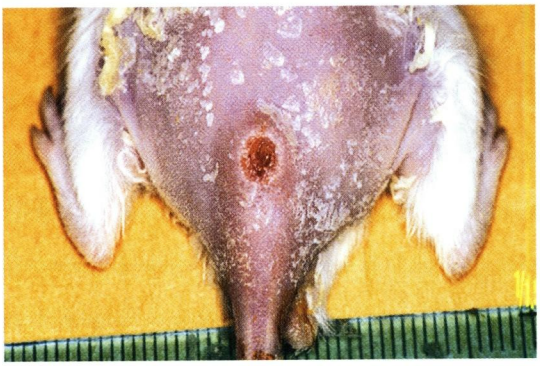
Figure 8.2.2. Lesion size after *Leishmania* promastigote inoculation. In all drug used groups (G2-7), the lesions were markedly suppressed compared to the control (G-1).



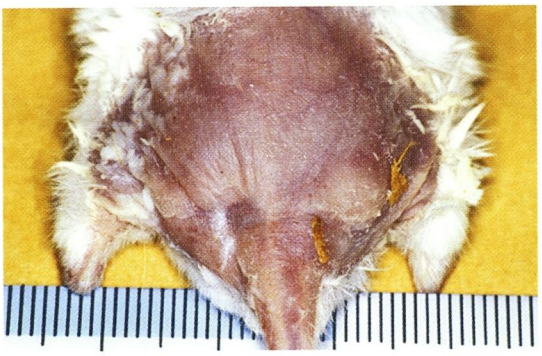
Group 1



Group 2



Group 5

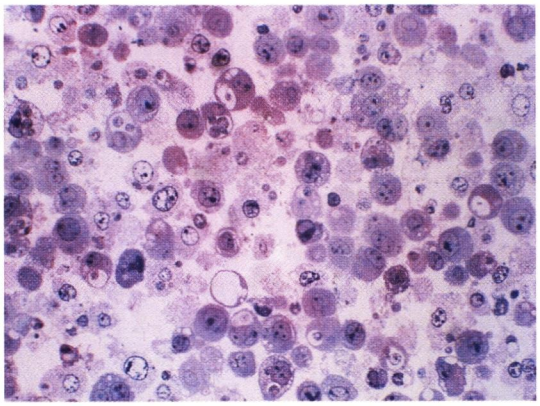


Group 7

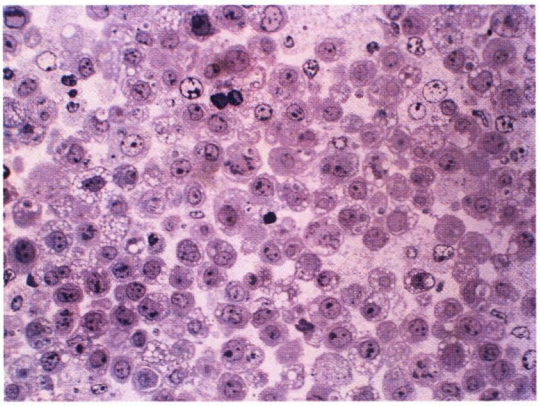
Figure 8.2.3. Comparison of lesion sizes 14 weeks after *Leishmania* inoculation. Group 1: showing a huge nodule with ulcer in the control mouse; Groups 2, 5 and 7: showing markedly suppressed lesions (2 and 5) or no lesion (7) in drug administered mice.

all control of the immune response depends on the activation of one of the two subsets of T lymphocytes, Th1 and Th2, and the cytokines play important roles on the course of the disease (Scott *et al.*, 1988; Heinzel *et al.*, 1989, 1991). IFN-γ and interleukin-2 (IL-2) secreting Th1 cells are expanded in the strains of mice that develop cell mediated immunity leading to self limiting disease, while strains of mice that develop fatal disseminated infection exhibit strong responses to IL-4 and IL-10 secreting Th2 cells. Nabors *et al.* (1996) have studied the lesion development of leishmanial BALB/c mice treated with sodium stibogluconate (SS). Accoding to their report, leishmanial BALB/c mice developed the lesion during SS treatment when pretreated with anti-IFN-γ, while the lesion development was inhibited without anti-IFN-γ pretreatment. The mice pretreated with anti-IL-4 showed IFN-γ up-regulation in contrast to untreated mice. Those data indicate that antimoniate would be more effective in combination with IFN-γ or macrophage activating chemicals.

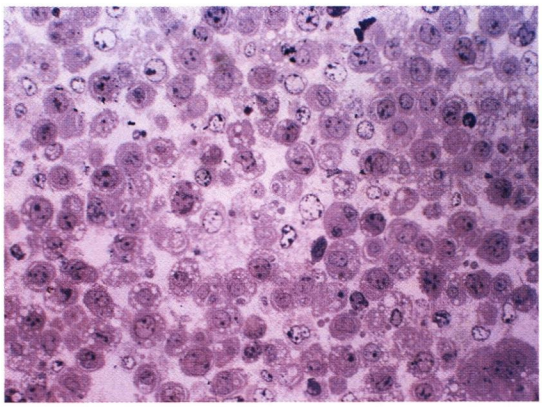
In this study, treatment with meglumine antimoniate alone could not completely suppress the leishmanial lesion development in mice. However, the mice treated with meglumine antimoniate plus IFN-γ never developed the lesion on the inoculation site. This corresponded to the results of Li *et al.* (1997). ONO-4007 and IFN-γ, even when used alone, suppressed the



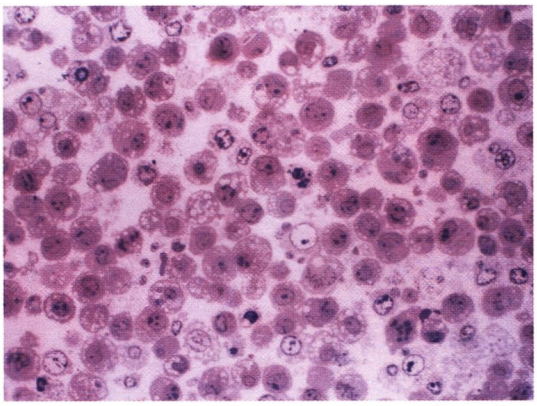
Group A



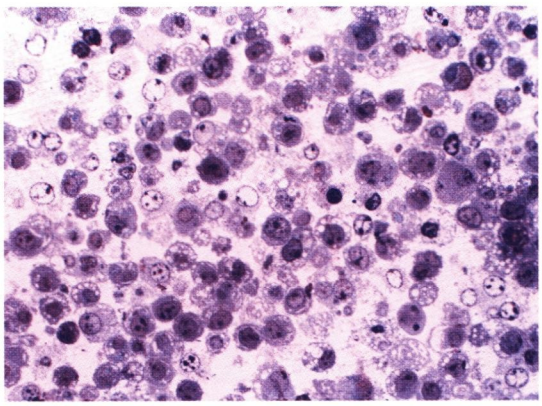
Group B



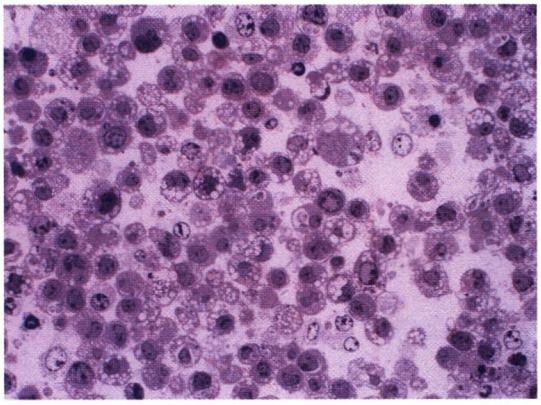
Group C



Group D



Group E



Group F

Figure 8.2.4. *Leishmania* parasitized macrophages. Control (Group A) shows large vacuolar formation including many amastigotes, in contrast, drug administered groups (Groups B-F) show few amastigotes or poor vacuolar formation.

lesion development compared to the control mice. In *in vitro* study, both IFN-γ and ONO-4007 inhibited intracellular proliferation of *Leishmania* amastigotes compared to the control. Therefore, ONO-4007 may be as effective as IFN-γ when used with meglumine antimoniate. Further investigation should be carried out to explore the efficacy of ONO-4007 as an antileishmanial agent as a single treatment and in combination therapy on other *Leishmania* species.

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Chapter 9

Related Papers

1. Detection of New Endemic Areas of Cutaneous Leishmaniasis in Pakistan

ABSTRACT. Cutaneous leishmaniasis (CL) is endemic in Pakistan and is widely spreading. We report some new endemic areas of CL in the country. A total of 950 cases of CL visited our department from 1996 to 2000. Among them, 450 were the residents of Jacobabad, Larkana, and Dadu districts of Sindh province and had never been traveled to the endemic areas before. These districts have never been reported/recognized as endemic for CL. Others were the residents of endemic areas of Balochistan province. All the patients were between 3 months and 60 years of age. Two hundred and five patients were females and 235 were males. Duration of the disease ranged from 2 months to one and half year. Most of the patients had single lesion on the face and/or extremities. Clinically the disease was classified as dry papular type, 305 cases; dry ulcerative type, 122 cases; and wet ulcerative type, 13 cases. No case of mucocutaneous or visceral leishmaniasis was found during this period. Diagnosis was made on clinical presentation, Giemsa stained smear test and histopathological results. Smear test was positive in 408 cases, while 346 cases were histopathologically positive. Ultrastructural study was performed using the specimens of few cases. *Leishmania* parasites were detected in the dermal tissues as well as macrophages. All the cases were treated with the meglumine antimonate except the 8 patients who showed sensitivity to this drug, and were treated with itraconazole. Fifteen patients showed resistance to the drug. On the basis of our findings we propose that Jacobabad, Larkana and Dadu districts could be considered endemic for CL. Wet and dry type lesions indicate the presence of both the *L. (Leishmania) tropica* and *L. (L.) major* in this tropical region.

Introduction

Leishmaniasis is one of the six top most infectious diseases declared by WHO (Chance, 1981). Cutaneous leishmaniasis (CL) can be classified largely into two forms: an Old World form caused mainly by *Leishmania (Leishmania) tropica* complex (Mebrahtu *et al.*, 1992) and a New World form caused by *L. (Viannia) braziliensis* and *L. (L.) mexicana* complexes (Wirth *et al.*, 1986; Jones *et al.*, 1987). Leishmaniasis is prevalent in Pakistan as well as in its neighboring states like India (Dogra, 1992), Iran (Momeni *et al.*, 1994), Afghanistan (Hewitt *et al.*, 1998) and China (Lin *et al.*, 1986), and is a serious health problem in the region. All the three types of leishmaniasis *i.e.*, visceral, mucosal and cutaneous, have been reported from these Southeast Asian states.

Several reports have been presented regarding the prevalence of visceral leishmaniasis (VL) and CL in the northern areas of Pakistan. Ahmed *et al.* (1960) were the pioneer and reported 30 cases of visceral leishmaniasis in the northern areas from 1957 to 1960. Later, Burney *et al.* (1979) presented their study in detail. They conducted a survey on various hospital records from 1957 to 1960 from the nine villages of the northern areas and were able to find 60 cases of

VL. In 1974, they reported 20 cases of VL and CL. VL is also reported from the Balochistan province (Nagi and Nasimullah, 1993). CL is endemic in Kashmir, NWFP province, and Balochistan province and in few cities of the Punjab province. Both forms of the *Leishmania i.e., L. (L.) major* and *L. (L.) tropica* have been reported from different parts of the country. CL has never been reported endemic in the Sindh province.

The aim of this study was to present our experiences with the CL patients from the new endemic areas of 3 districts of Sindh province, which is reported for the first time.

Materials and Methods

This study was performed in the outpatient clinic of the Department of Dermatology, Chandka Medical College (CMC) Hospital, Larkana, Sindh province, Pakistan. The CMC is a well-established teaching institute attached with 1300 bed hospital located in the interior part of Sindh province. This institute has been providing medical facilities to three million population of interior Sindh, parts of Balochistan and Pujab province. There were a total of 950 cases seen in the department from 1996 to 2000. These cases were divided in two groups: 1) those who had a positive history of travel to the endemic areas of leishmaniasis before the appearance of lesion(s), 2) those who had no history of travel to the endemic areas of CL in the country. Five hundred and ten patients had a positive history of travel to the endemic areas of CL in the country. They were likely to be infected from the previously recognized endemic areas and were excluded from this study. Four hundred and forty cases never had traveled to the endemic areas before or after the appearance of lesions and they were in their residential areas during the infection. They were residing in the Jacobabad, Larkana and Dadu districts; and were included in our study. The majority of cases belonged to the various cities of Larkana district like Shahdadkot, Qambar, Mirokhan, Warah, and Larkana city. Jacobabad and Shahdadkot cities are located near the boundary of Balochistan-Sindh province; however, Larkana, Dadu, Warah, Qambar and Mirokhan cities are quite far from the inter-provincial boundary. All the cases were diagnosed on the basis of clinical findings, smear test and histopathological findings. Biopsies were taken from different sites of the lesions. One part was fixed in 10% formalin for the hematoxylin and eosin (H&E) and Giemsa stainings, and other part was fixed in ethanol for future PCR studies. Few specimens were also fixed in 2% glutaraldehyde and 2% parafarmaldehyde solution. They were post-fixed in 1% osmium tetroxide, dehydrated through a graded ethanol series, and embedded in Epon 812. The ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEM 2000 EX (Jeol Japan) electron microscope.

Results

Clinical observations

Endemic distribution of CL in Pakistan is summarized in the map of Pakistan (Fig.9.1.1). A total of 450 patients of CL who had never traveled to the endemic areas before or after the infection, were recorded during the 4-year period. Age of the patients ranged from 3 months to 60 years. Two hundred and five patients were females and 235 were males. Duration of the disease ranged from 2 months to one and half year. Most of the cases were seen during the winter season, however rare cases were seen in summer. All the patients had lesions mostly on the exposed areas of the body like face, neck, ears, hands, arms, feet and legs (Fig. 9.1.2A-D). The number of lesions in each patient was one to five. One patient had 14 dry ulcerative lesions on the abdomen, back and exposed parts of the body. Clinically, the disease was classified: a) dry papular type, 305 cases; b) dry ulcerative type, 122 cases; and wet ulcerative type, 13 cases. None of the case of mucocutaneous or visceral leishmaniasis was seen during the present study.

Light microscopic observations

Figure 9.1.1. A map of Pakistan, showing the endemic distribution of cutaneous leishmaniasis in Pakistan.

Smear test shows the evidence of numerous *Leishmania* parasites especially in the histiocytes. They were round or oval bodies with a round nucleus, and an intracellular rod-like kinetoplast was clearly seen which is the morphologic characteristic of *Leishmania.*

Histopathologically, most of the specimens showed a thin keratinized epidermis. In some specimens the epidermis was completely lost, however, parakeratosis and hyperplasia of the epidermis was also visible in many specimens. The dermis was quite thick, histiocytes were predominant in the sections, and most of them contained numerous amastigotes in large vacuoles. The tuberculoid granulomas along with the Langerhans giant cells were visible in all of the specimens, and lymphocytes, other mononuclear cells and plasma cells were also seen. We could not confirm the huge infiltration of mast cells in our human specimens naturally infected with *L. (L.) tropica* as it is previously reported in the specimens of Golden hamsters infected with an Ecuadorian isolate of *L. (L.) mexicana* (Bhutto *et al.,* 1992a).

Electron microscopically no remarkable difference was seen between the New World (Bhutto *et al.,* 1992b) and our present Old World leishmaniasis specimens. The amastigotes were confirmed in the sec-

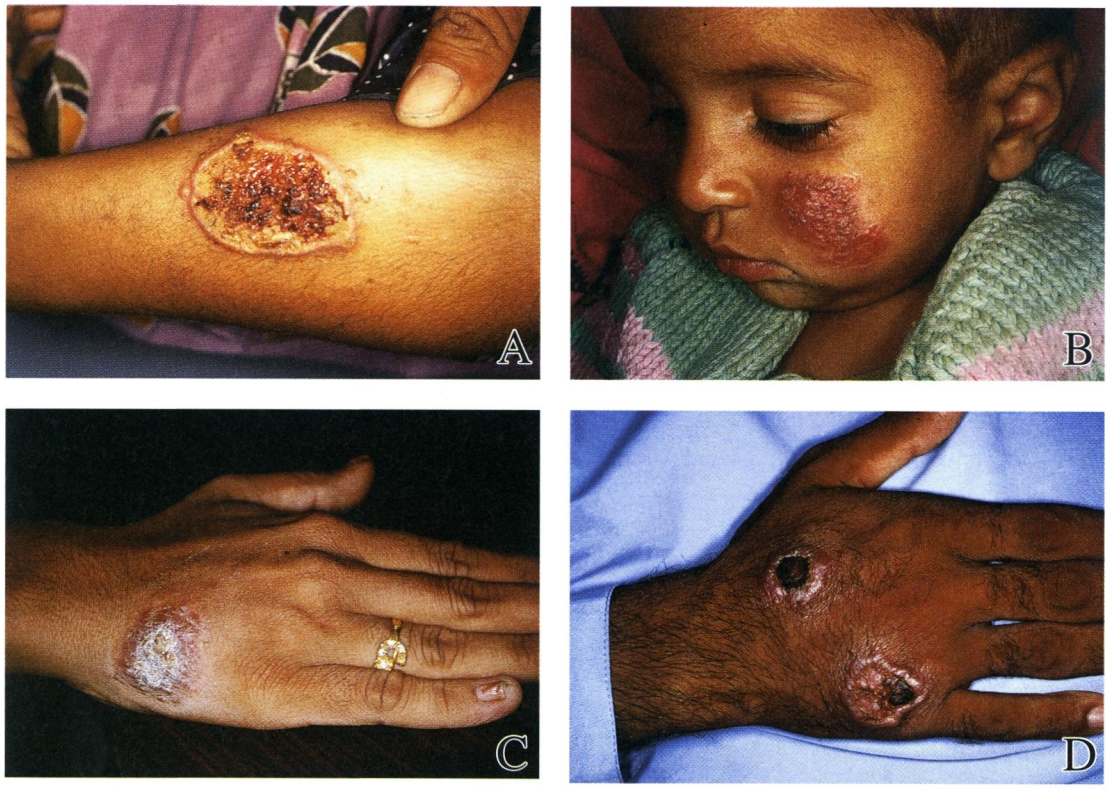

Figure 9.1.2. Clinical picture of the patients of cutaneous leishmaniasis of new endemic areas. **A.** A 10-year-old female patient has wet ulcerative lesion on the arm. **B.** One year-old girl has dry papular lesion on the left cheek. **C** and **D.** Dry ulcerative lesions on the hand of male and female patients.

tions. They were either phagocytosed by the macrophages and/or lying in the tissues (Fig. 9.1.3). The most conspicuous structures of *Leishmania* parasites in the tissues were prominent nucleus with a small nucleolus, short flagellum, kinetoplasts, vacuoles, the electron dense flagellar pockets and lysosomes.

Geographical location of Pakistan

Pakistan is situated in the north west of south Asia. On the globe it is located between the latitudes 23.45° and 36.75° North, and between longitudes 61° and 75.5° East. The border of Pakistan touches with the border of Sin Kiang province of China in the north, Afghanistan in the north-west, Iran in the west and India in the east. The total area of Pakistan is 796,096 sq. kilometers. From administrative point of view, this country is divided into four provinces namely, the North West Frontier Province (NWFP), the Punjab, Sindh and Balochistan.

Physical Features and Climate

The shape of Pakistan is evident from its physical map. In the North and the West lies a range of high mountains and plateaus, which occupy more than half of the area. Rest of the country is plain which is made of alluvium deposited by the rivers. The variety of landscape divides Pakistan into seven major regions.

1) The northern high mountainous region

This includes the Himalayas, the Karakoram, Hindukush Mountains and Ladakh range. The Himalayas are spread like an arch in the north of south Asian sub-continent. The Western parts of these ranges passing through Jammu and Kashmir enter Pakistan. The Himalayas are again sub-divided, a) the Sub-Himalayan or the Sivalik Range. The average height of this mountain ranges between 300 to 1,000 km; b) the lesser Himalayas or the Pir Punjal Range. The average height of this range is between 1800 to 4600 meters; c) central Himalayas: the highest peak of this range is Nanga Parbat, having more than 8250 meters height above the sea level; d) the Ladakh Mountain *range. These are so called low mountains where* Himalayas begin to climb down farther North.

In winters, this area is extremely cold and temperature falls below the freezing point. The winter season lasts from six to eight months.

The Karakoram Mountain is located in the north-west Himalayas in which the northern Kashmir and Gilgit are included. Its average height is 7000 meters. Its highest peak is Godwin Austin (K-2) which is 8611 meters high from the sea level. The Mountain range is situated between Pakistan and China.

The Hindukush Mountains lies to the north-west of Karakoram and are extended eastward into Afghanistan. The highest peak of these ranges is Tirich Mir, which is 7700 meters high.

2) The western low mountainous region

The western low mountainous region spread mostly in a north-south direction and is divided in five *parts.*

3) The salt range or the Potwar uplands

The average height of the salt range is about 750 to 900 meters.

4) The plateau of Balochistan

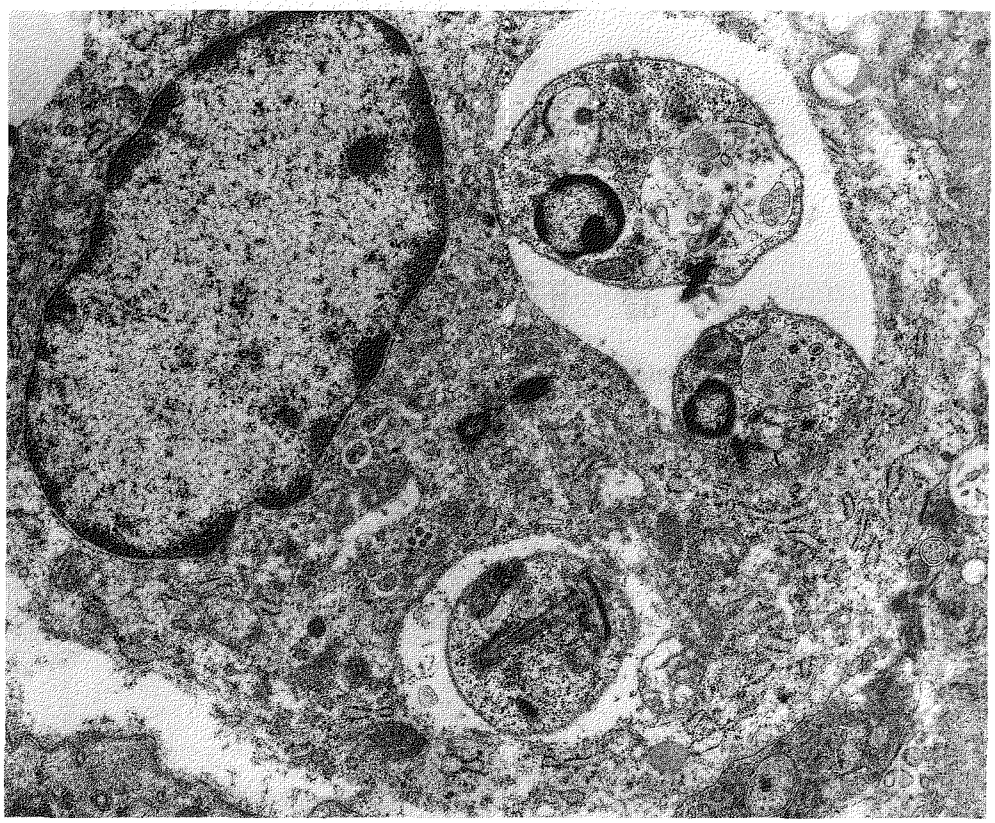

Figure 9.1.3. Electron microscopic picture showing the amastigotes phagocytosed by the macrophage.

The vast plateau of Balochistan lies in the west of Sulainman and Kirthar Mountains. This area too, like Potwar, is dry. Its average height is between 600 to 900 meters. The Balochistan plateau and the most of Thar desert area are extremely hot in the summer.

5) The upper Indus or the Punjab Plain

The plain of the upper Indus is situated in the south of Sub-Himalayas and the salt range. The Indus and its tributaries namely Jehlum, Chenab, Ravi and Sutlej irrigate it. This area is situated away from the northern mountains and the sea and is extremely hot in summer.

6) The Lower Indus or the Sindh Plain

The lower Indus plain includes the whole of the province of Sindh. It is also a flat plain, which has been formed by the Alluvium deposited by the Indus River and its tributaries. The climate is extremely hot in summer and temperature goes up to 53℃, June is the hottest month.

7) Coastal area

The total length of the coast of Pakistan is nearly 700 km, which starts from Run of Kush in Sindh and ends in the west at the Iran-Pakistan border. All the mountains, plateau, deserts, seas, plains and coastal areas form a country's natural environment. The climate of the coastal region of lower Sindh is better than that of the upper Sindh in some respect. The sea breeze does not allow the temperature of the coastal region to rise.

Discussion

Pakistan is a tropical country where leishmaniasis is highly prevalent and the disease is spreading day by day. Initial reports came from the northern areas, namely Kashmir, Gilgit, Bannu, D.I. Khan and other cities whose boundaries are in touch with the India and Afghanistan border. These reports highlighted the high incidence of CL and VL, and concluded that the said region was endemic for leishmaniasis (Burney and Lari, 1986; Burney *et al.,* 1981). Later, the studies were presented from the north-west areas of Balochistan province bordering with Afghanistan and Iran, showed the high incidence of CL and VL (Yasinzai *et al.,* 1996). The affected cities of the Balochistan province were Quetta, Sibi, Kohlu, Lehri, Lasbela and Uthal. Recently, it has been reported from the Multan city of Punjab province, a central part of the country, describing the high incidence of the disease (Mujtaba and Khalid, 1998). We explore the outbreak of CL in a couple of districts of Sindh province namely Jacobabad, Larkana and Dadu districts. During 1996, the number of CL patients was suddenly raised and the cases were frequently seen in our department, which creates our interest to conduct a careful study about the disease. We observed a total of 950 cases of CL, 450 of them were from the Jacobabad, Larkana and Dadu districts and had never traveled to the endemic areas. They had clear history of getting the infection in their residential areas. This data is allowing us to conclude that the CL is gradually spreading in the country and above-mentioned districts could be recognized as new endemic areas of CL in Pakistan.

In Pakistan, the CL is mostly prevalent in the northern mountainous areas where the winter is extremely cold and too long. Similarly the number of CL patients visited our department, was higher in winter than in summer, indicating the high prevalence rate of the disease in winter. Similar observations have been reported by the others (Mujtaba and Khalid, 1998). Further more, we could not find any case of VL in our flat tropical region. VL is frequently seen in the northern mountainous region. There are a variety of landscapes and climate in Pakistan that may affect the distribution and characteristic of leishmaniasis in the country. Geographical location has already been discussed some where. From these results, we may predict the possible relation and the effect of climate on the leishmaniasis.

It is believed that at least two *Leishmania* species cause cutaneous leishmaniasis in the Old World: a) *L. (L.) major,* usually in rural areas and clinically represents the moist or wet lesion; and b) *L. (L.) tropica,* usually in urban areas and clinically represents the dry lesion. In Pakistan, wet type lesions caused by

L. (L.) major is reported more frequently, particularly in the NWFP and Balochistan provinces (Raja *et al.,* 1998), however, Mutjaba and Khalid (1998) have detected only dry type lesions in their patients from the Multan city of Punjab province. In our patients, 427 had dry type lesions and 13 had wet type lesions on the exposed parts of the body. These results suggests the presence of both *L. (L.) tropica* and *L. (L.) major* in these new endemic areas of Sindh province.

There has been few studies regarding the reservoirs and vectors of the disease in Pakistan. Burney *et al.* (1981) captured the sandflies from the villages of northern areas of patients at night time and identified as *Phlebotomus chinensis, P. major, P. kandelakii* and *P. burneyi.* They also trapped rodents from the houses of kala-azar patients and examined, but could not find LD bodies. These results suggested that the disease may be transmitted from man to man by any of the species of sandflies, *Phlebotomus* spp. present in the areas. In Gilgit the commonest is *P. papatasi.* In later studies they proposed that the rodents are the main reservoirs of the CL (Burney and Lari, 1986), whereas *P. papatasi* and *P. sergenti* were found the main vectors (Rowland *et al.,* 1999; Nasir, 1958). At present we could not confirm the vectors of the disease. There is a need of studies to find out the reservoirs and vectors of the disease in this region. In future, we will extend our studies to perform PCR on our specimens collected.

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2. PCR, DNA Sequencing, and Monoclonal Antibody Based ELISA for the Identification of *Leishmania* Isolated from Bangladeshi Kala-azar Patients

ABSTRACT. In order to identify the causative *Leishmania* species of kala-azar (VL) in Bangladesh, we studied seven strains isolated from bone marrow or splenic aspirates of hospitalized patients. Five Old World *Leishmania* WHO reference strains were also used in this study. We developed a primer set from the internal transcribed spacer (ITS) region and amplified the *Leishmania* DNA by PCR. Subsequently, DNA sequencing of the amplified PCR products was done. All the *Leishmania* isolates of Bangladesh gave an amplified PCR product of 498 bp having sequences identical to that of *L. (L.) donovani* DD8 reference strain but different from other Old World *Leishmania* reference strains. These results suggest that this set of primers may be used for *Leishmania* species identification in the Old World especially those prevalent in Asia. All the *Leishmania* isolates from Bangladesh were also identified as *L. (L.) donovani* by monoclonal antibody based ELISA.

Introduction

Leishmania parasite typing is essential for epidemiology, clinical studies and the development of strategies to control leishmaniasis. At present, identification of clinical isolates of *Leishmania* parasites at the species level is primarily based on isozyme analysis (Kreutzer *et al.*, 1980; Evans et *al.*, 1984; Le Blank and Peter, 1986) , monoclonal antibody based ELISA (Mimori et al., 1989; Furuya *et al.*, 1998) and DNA probing (Barker *et al.*, 1986; Laskay *et al.*, 1991; Gramiccia *et al.*, 1992; Minodier *et al.*, 1997; Lambson *et al.*, 2000). In order to develop a suitable tool to identify the *Leishmania* at the species level, investigators are engaged in diagnosing the *Leishmania* species from clinical samples (Mimori et *al.*, 1998).

For typing of the *Leishmania* parasites some investigators have used nuclear or kinetoplast probes (Howard *et al.*, 1992; Guizani *et al.*, 1994). Use of several radioactive probes is often necessary for species identification. PCR tests, based either on rRNA genes or their spacers (Uliana *et al.*, 1991; Guevara *et al.*, 1992) or on the kinetoplast DNA (Rodgers *et al.*, 1990; Lopez *et al.*, 1993; Nuzum *et al.*, 1995), were developed. The kinetoplast DNA, located in minicircles, is naturally repeated up to 10000 times in the genome, but primers specific for each species must be synthesized since it has a high degree of polymorphism. The RNA genes and their spacers are repeated about 200 times in the cell, they are the interesting PCR targets because of their low level of polymorphism.

Though identification of *Leishmania* species by using probes has been increased recently, no ideal and simplified tool has been developed for wide use. In this study, we have used primers constructed from the internal transcribed spacer (ITS) of DNA and applied on different viscerotropic Old World *Leishmania* strains and strains isolated from Bangladeshi VL patients for PCR. *Leishmania* species were identified by sequencing of the amplified PCR products. We have also compared this tool with monoclonal antibody based ELISA.

Materials and Methods

Leishmania *strains studied*

Seven *Leishmnaia* strains isolated from VL patients

of Bangladesh were studied. Besides, 5 WHO reference strains of the Old World Leishmania were used in this study (Table 9.3.1.).

Collection of samples from VL patients of Bangladesh

Informed consent was obtained from each patient or from the legal guardians of children before taking samples. Bone marrow or splenic aspirate was collected from hospitalised clinically suspected kala-azar patients under aseptic condition. Few drops of the aspirate were inoculated in to modified NNN media and incubated at 22 C temperature incubator for the growth of the *Leishmania* parasite. All the *Leishmania* strains isolated from these patients were then transported to the Parasitology Department of Kochi Medical School and were maintained in USMARU medium (Evans, 1989).

Mass cultivation of the parasites

Mass cultivation of the *Leishmania* parasites was done in RPMI medium supplemented with 5% healthy human urine (Shamsuzzaman *et al.*, 1999). Stationary phase parasites were harvested, washed twice in normal saline and preserved at -30℃ until use.

Extraction of DNA

DNA was extracted following the classical protocol using phenol: chloroform:isoamyl alcohol as well as DNA extraction kit (Qiagen, Tokyo, Japan). DNA concentration was determined by spectrophotometer (ULTROSPEC 3000, UV/visible spectrophotometer, Phammacia Biotech) and preserved at 4℃ until use.

Primers used in this study

Upper primer 5' CAA CTC GGG GAG ACC TAT G 3'

Lower primer 5' AAT ATG CGC ACA ACA CAA AC 3'

PCR reactions

A 498 bp fragment of DNA was amplified. PCR amplification was carried out in a 25 µl final volume containing 2 µl DNA, 2.5 µl 1x PCR buffer, 1.5 M $MgCl_2$, 25 µM of each dNTP, 10 pmoles of each primer, and 1.25 units of Taq DNA polymerase enzyme. Samples were subjected to initial denatura-

Table 9.3.1. WHO reference strains and Leishmania isolates from VL patients of Bangladesh used in this study

Stock	Disease pattern	Species
Reference strains of the Old World:		
MHOM/IN/80/DD8	visceral	*L. (L.) donovani*
MHOM/ET/67/HU3	visceral	*L. (L.) donovani*
MHOM/TN/80/IPT-1	visceral	*L. (L.) infantum*
MHOM/SU/74/K-27	cutaneous	*L. (L.) tropica*
MHOM/SU/73/5 ASKH	cutaneous	*L. (L.) major*
Clinical isolates from Bangladesh:		
MHOM/BD/91/PG 25	visceral	*L. (L.) donovani*
MHOM/BD/91 PG 31	visceral	*L. (L.) donovani*
MHOM/BD/91 PG 34	visceral	*L. (L.) donovani*
MHOM/BD/91/ PG 38	visceral	*L. (L.) donovani*
MHOM/BD/97/ PG 192	visceral	*L. (L.) donovani*
MHOM/BD/91/Zaman-1	visceral	*L. (L.) donovani*
MHOM/BD/91/Zaman-2	visceral	*L. (L.) donovani*

tion at 94℃ for 10 min followed by 35 cycles of 94℃ for 45 sec, 55℃ for 30 sec and 72℃ for 1 min and 30 sec, followed by final extension at 72℃ for 10 min. After amplification, PCR products were separated through 1.5% agarose gel with ethidium bromide and the bands amplified were compared to the bands obtained with a positive *Leishmania* DNA control. All the chemicals without DNA sample were also run in each reaction as negative control. The bands in gel were analyzed by KODAK 1D image analysis software (Eastern Kodak Company, Scientific imaging systems, Rochester, NY, USA) using the device 'Kodak 1 DAS 290'.

DNA sequencing

Band of DNA was cut and purified in filter column (Quantum Prep™ Freeze N Squeeze DNA Gel Extraction Spin Columns, Bio-Rad Laboratories, California, USA). The purified DNA was subjected to ethanol precipitation and resuspended in 10 μl 1/10 TE buffer and aliquots were sequenced using the ABI PRISM kit. PCR primers were used as sequencing primers. The mixtures were then purified according to the manufacturer's instructions (ABI) using spin columns (CENTRI SEP, Applied Biosystems, Foster City, CA) and applied to an ABI PRISM 310 genetic analyzer. Alignment analyses were done using the program Gentyx Mac (ver. 11).

Antigen preparation for ELISA

Some of the harvested promastigotes was added to 1 ml lysis buffer (10 mM Tris-HCL, pH 7.5, 2 mM EDTA, 1.6 mM phenylmethylsulfonyl fluoride, 2 mM iodoacetamide, 100 μg/ml leupeptin). After 3 bouts of sonication for 2 min each with an interval of 1 min the homogenate was centrifuged at 20000 g for 15 min. The protein concentration of the supernatant was determined and kept at -30℃ until use.

Species identification of Leishmania by monoclonal antibodies

We used *L. (Leishmania) donovani* specific monoclonal IgG antibody (Biogenesis, England) for identification of Bangladeshi *Leishmania* strains by enzyme-linked immunosorbent assay . 96 well micro ELISA plate was coated with *Leishmania* antigen (10 μg/well), incubated at 4℃ overnight, washed, diluted monoclonal antibodies were added and incubated. After wash peroxidase conjugate was added, washed and enzyme substrate (all from Kirkegaard and Perry laboratories, Maryland, USA) was used and absorbance was measured at 405 nm.

Results

All the seven *Leishmania* strains from Bangladesh gave an amplified DNA fragment of 498 bp having the same sequences to that of *L. (L.) donovani* (DD8) WHO reference strain (Fig. 9.3.1). The other viscerotropic *Leishmania* such as *L. (L.) infantum* (MHOM /TN/80/IPT-1) and *L, (L.) donovani* (MHOM/ET/67/ HU3) also showed 493 and 495 bp PCR products using the same primer (Fig. 9.3.2). After sequencing all the *Leishmania* strains could be separated. Comparative results of DNA sequencing are shown in Fig. 9.3.3.

All the 7 *Leishmania* strains isolated from kala-azar patients of Bangladesh were characterized as *L. (L.) donovani* by zymodeme analysis (data not shown). Monoclonal antibody based ELISA could identify the *L. (L.) donovani* and could differentiate *L. (L.) donovani* from *L. (L.) major* and *L. (L.) tropica* but failed to differentiate *L. (L.) donovani* from *L. (L.) infantum.*

Discussion

About one-tenth of World population is at risk of leishmaniasis and it is a public health concern in many countries (WHO, 2000). Identification of the parasite at species level is necessary for correct diagnosis of the disease especially for cutaneous forms. Some strains such as *L. (L.) infantum* and occasionally *L. (L.) tropica,* cause both cutaneous and visceral leishmaniasis (Magill *et al.,* 1993; Sacks *et al.,* 1995). It has been reported that *L. (V.) brasiliensis,* which is strictly an agent of cutaneous or mucocutaneous leishmaniasis in the New World, causes visceral leishmaniasis in AIDS patients (Hernandez *et al.,*

1. CAACTCGGGG AGACCTATGT ATATATATAT GTAGGCCTTT
41 CCCACATACA CAGCAAAGTT TTGTACTCAA AATTTGCAGT
81 AAAAAAAGGC CGATCGACGT TATAACGCAC CGCCTATACA
121 AAAGCAAAAA TGTCCGTTTA TACAAAAAAT ATACGGCGTT
161 TCGGTTTTTG GCGGGGTGGG TGCGTGTGTG GATAACGGCT
201 CACATAACGT GTCGCGATGG ATGACTTGGC TTCCTATTTC
241 GTTGAAGAAC GCAGTAAAGT GCGATAAGTG GTATCAATTG
281 CAGAATCATT CAATTACCGA ATCTTTGAAC GCAAACGGCG
321 CATGGGAGAA GCTCTATTGT GTCATCCCCG TGCATGCCAT
361 ATTCTCAGTG TCGAACAAAA AACAACACGC CGCCTCCTCT
401 CTTCTGCACA TATATATATA TATTATACCA TACACAGTAT
441 ATATATAATT ATGTGTTGGA AGCCAAGAGG AGGCGTGTGT
481 TTGTGTTGTG CGCATATT

Figure 9.3.1. DNA sequence of the amplified region of internal transcribed spacer of Bangladesh and *L.(L.) donovani* DD8 WHO reference strain.

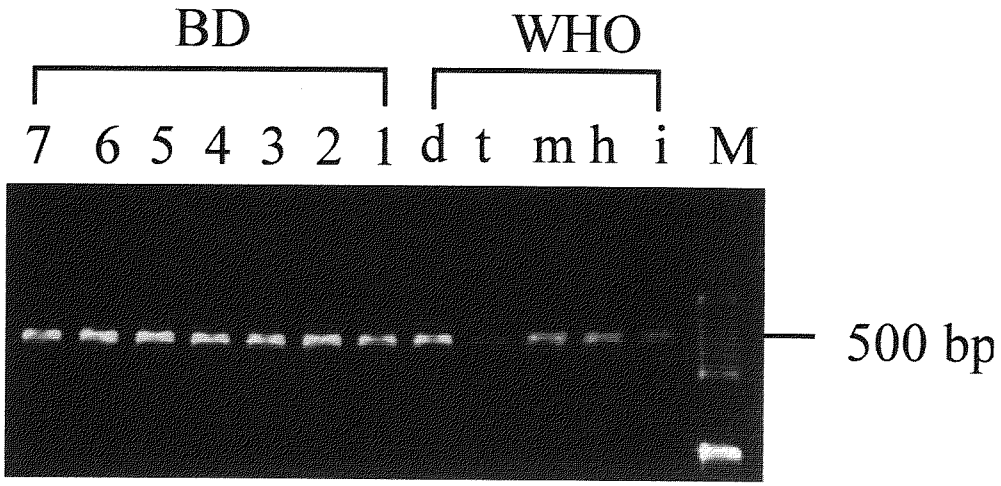

Figure 9.3.2. Amplified PCR products. M, 25 bp DNA ladder; WHO, WHO reference strains: i, *L. (L.) infantum* (1PT-1); h, *L. (L.) donovani* (HU3); m, *L. (L.) major* (5 ASKH); t, *L. (L.) tropica* (K-27); d, *L. (L.) donovani* (DD8); BD, isolates from Bangladesh: 1, PG 25; 2, PG 31;3, PG 34; 4, PG38; 5, PG 192; 6, Zaman-1; 7, Zaman-2.

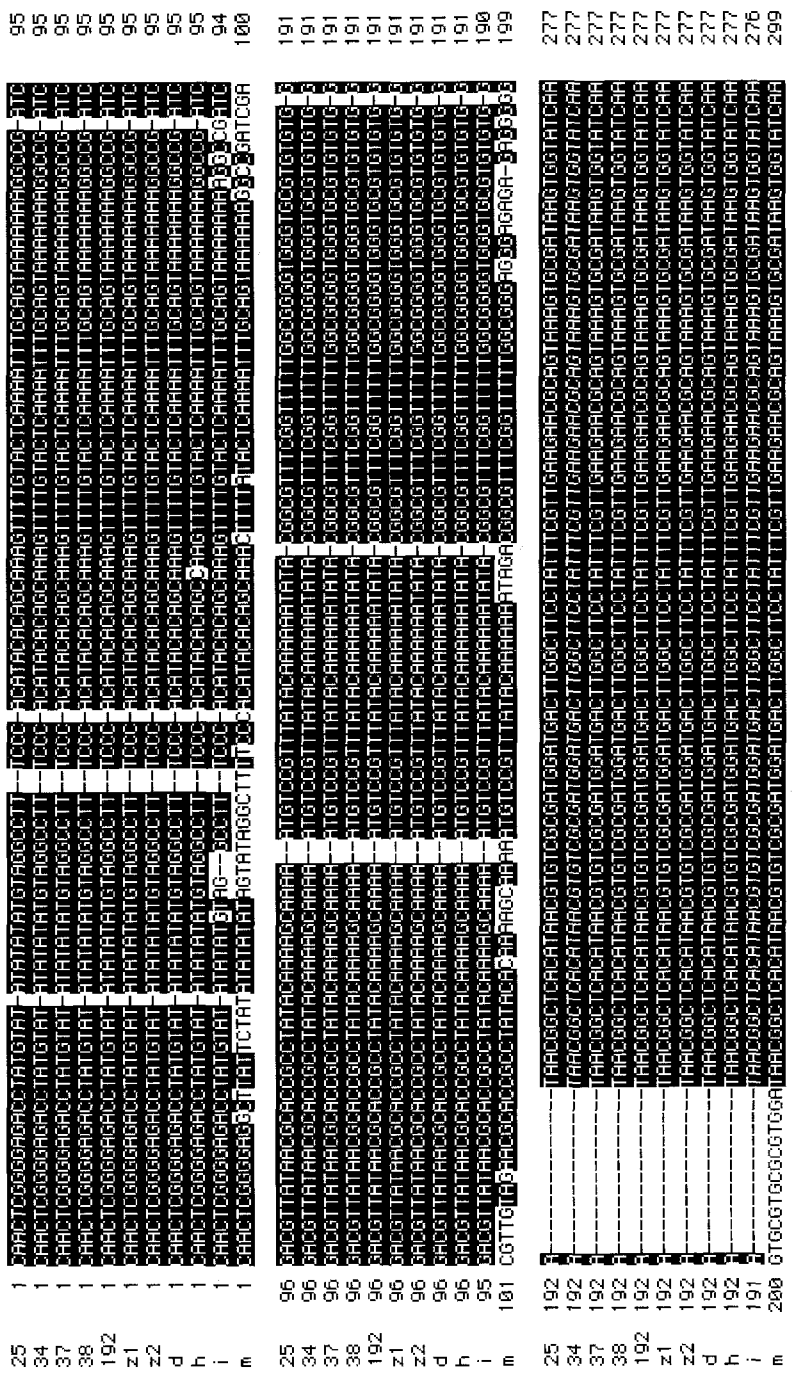

Figure 9.3.3. Multiple alignment of DNA sequences of isolates from Bangladesh and WHO reference strains: 25 PG 25; 31, PG 31; 34, PG 34; 38, PG 38; 192, PG 192; Z1, Zaman-1; Z2, Zaman-2; d, *L. (L.) donovani* (DD8) h, *L. (L.) donovani* (HU3); i, *L. (L.) infantum* (IPT-1); m, *L. (L.) major* (5 ASKH).

Figure 9.3.3. (continued).

1993). Since *Leishmania* species can not be differentiated by observing morphologies under microscope, several methods have been developed for improvement of the parasite identification including genotypic and phenotypic approaches. Isoenzyme analysis is the accurate and standard method for characterization of *Leishmania* parasite (Kreutzer *et al.,* 1980; Evans *et al.* 1984). Monoclonal antibody based ELISA had been reported to be an alternative method to identify the parasites at species level (Mimori *et al.,* 1989; Furuya *et al.,* 1998). These methods, however, need culture of the parasite which is complex and laborious, and they are usually impeded by the bacterial and/or fungal contamination and slow growth of the *Leishmania* parasite in the culture media. Moreover, the results of enzyme electrophoresis vary with electrophoresis conditions, and it is a time consuming procedure.

Now a days many investigators are interested in DNA for typing the *Leishmania* species using nuclear or kinetoplast DNA probes. The major disadvantage of this method is that use of several radioactive probes is often necessary for species identification. In the present study, we have amplified a part of ITS of DNA from most of the Old World *Leishmania* WHO reference strains using a single set of primers. By analyzing the bands of the amplified PCR products, it was not possible to distinguish the species of these reference strains. We, however, focused on direct sequencing of the amplified PCR products. By analyzing the sequences of different reference strains it was possible to identify the *Leishmania* species. In this method, the cloning step is not necessary which saves money and time and culture of parasite may also be avoided. Now we are trying to apply the same method to diagnose VL cases for detecting *Leishmania* DNA directly from clinical samples (data not shown).

In the present study, all the *Leishmania* strains isolated from VL patients of Bangladesh were identified as *L. (L.) donovani.* Sequence analysis of the isolates from Bangladeshi and WHO reference strains showed that all the isolates of Bangladesh had sequences identical to that of *L. (L.) donovani* DD8 strain. This finding corresponds to the previous reports regarding identification of *Leishmania* from Bangladesh. All the *Leishmania* strains of Bangladesh, and all but a few strains of India isolated from VL patients have been characterized as *L. (L.) donovani* by isoenzyme analysis (El-Masum and Evans, 1995; Sacks *et al.,* 1995; Chatterjee *et al.,* 1999; Shamsuzzaman *et al.,* 2000). Only 4 isolates from VL patients of India had been identified as *L. tropica* (Sacks *et al.,* 1995). In this study, not a single mutation was observed among the Isolates from Bangladesh in the amplified segment of ITS. From this result it may be said that this part of ITS region is relatively less prone to mutation. It had been speculated that ITS might identify the strains almost at species level. In this study, we observed that most *Leishmania* strains of the Old World, especially strains of Asia could be successfully identified by amplifying this segment of ITS region and sequencing analysis of the PCR products.

In conclusion, the primers set used in this study was able to amplify the DNA of most of the *Leishmania* species prevalent in the Old World. *Leishmania* species prevalent in Asia could be differentiated and identified by analyzing the sequences of the amplified PCR products. All the *Leishmania* isolates from Bangladesh could be identified as *L. (L.) donovani* having same sequences to that of *L. (L.) donovani* (DD8) reference strain. So sequencing may be an important tool to identify the *Leishmania* species. It may be extremely useful in further epidemiological studies.

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3. Diagnosis of Kala-azar in Bangladesh and Identification of *Leishmania* Species from Clinical Samples by Molecular Techniques

ABSTRACT. The general objective of the present study was to find out a suitable way to diagnose leishmaniasis from clinical samples by polymerase chain reaction (PCR). The specific objectives were to identify the *Leishmania* species directly from the clinical samples without culturing the parasite and to find out the better preservative for long term preservation. We constructed two primers, one from the internal transcribed spacer (ITS) region of the nuclear DNA and the other from kinetoplast DNA of *L. (L.) donovani* DD8 WHO reference strain, and applied on bone marrow and splenic aspirate samples collected from Bangladeshi visceral leishmaniasis (VL) patients. These primers gave positive DNA bands in 36(85.7%) clinically suspected and seropositive and/or parasite positive patients. The sensitivity of this PCR was 100% when parasite positive samples were tested. Sequencing analysis of the amplified segment of the ITS region showed that all the sequences were identical to that of *L. (L.) donovani* DD8 WHO reference strain but different from other viscerotropic *Leishmania* strains of the Old World. Samples preserved in normal saline were better than samples preserved in formalin. There was, however, no difference in the PCR results when old samples were tested for *Leishmania* DNA preserved in 70% and 100% ethanol. A search for *Leishmania* HIV/AIDS co-infection showed a negative result.

Introduction

Leishmaniasis, caused by the protozoa *Leishmania,* constitutes a diverse collection of diseases ranging from a spontaneously healing skin lesions to overwhelming visceral diseases. It is highly endemic in different parts of the New and the Old World with a 10th of the world's population at risk of infection (WHO, 2001). *L. (L.) donovani, L. (L.) infantum,* and very occasionally *L. (L.) tropica* are the causative agents of visceral leishmaniasis (VL) in the Old World with epidemics in Sudan and India, while *L. (L.) major, L. (L.) tropica* and *L. (L.) aethiopica* are the causative agents of cutaneous leishmaniasis (CL) in this area of the globe (Magil *et al.,* 1993; Sacks *et al.,* 1995). Of the 2 million new cases of VL and CL each year, Bihar state of India is the single largest focus of VL in the World producing 0.2 million of the total 0.5 million new cases of VL (WHO, 1998), and more than 15000 new cases are occurring in Bangladesh each year (Choudhury *et al.,* 1991). In southern Sudan more than 10% of the population died from VL over the past five years. On the other hand, An epidemic of CL is ongoing in Kabul, Afghanistan with an estimated 0.2 million cases (WHO, 2001)

Early diagnosis of VL is necessary to reduce the mortality. The conventional direct diagnostic methods of VL are mainly based on demonstration of parasites in biopsies or aspirates from infected spleen, bone marrow and lymph nodes (Siddig *et al.,* 188; Zijlstra *et al.,* 1992). Their sensitivities are not so high. A number of indirect methods such as ELISA, IFAT and DAT have been developed. Diagnosis of VL by detecting antibodies in sera has some disadvantages as it shows some false positive and false negative results (Hailu, 1990, Zijlstra *et al.,* 1992). Recently polymerase chain reaction (PCR) has been successfully used to diagnose VL cases from the peripheral blood, bone marrow, and lymph node aspirates (Osman *et al.,* 1997; Singh *et al.,* 1999; Hu *et al.,* 2000).

In order to make suitable plans to control the disease and to understand the epidemiology, identifica-

tion of the species is important. It is also necessary to develop diagnostic reagents and vaccine for a particular endemic area. Moreover, some *Leishmania* species which only cause CL or MCL may cause VL in HIV/AIDS patients (Roberts *et al.*, 2000). Isoenzyme analysis (Kreutzer *et al.*, 1980; Evans *et al.*, 1984; Le Blank and Peter, 1986) , monoclonal antibody based ELISA (Mimori *et al.*, 1989; Furuya *et al.*, 1998) and DNA probing (Barker *et al.*, 1986; Guizani *et al.*, 1994; Gramiccia *et al.*, 1992; Minodier *et al.*, 1997; Lambson *et al.*, 2000) are the tools used to identify species from the cultured parasites. A few reports on identification of the New World *Leishmania* species directly from the clinical samples of CL patients using PCR have been published (Mimori *et al.*, 1998, Matsumoto *et al.*, 1999). A similar method is yet to be established for the samples from VL patients. With the spread of leishmaniasis in several areas of the world, an increase number of *Leishmania* and HIV/AIDS co-infected cases are reported from 33 countries worldwide (WHO, 2001). Although a few reports on recent status of kala-azar and it's causative agents in Bangladesh have been published (El-Masum *et al.*, 1995; Shamsuzzaman *et al*, 1999, 2000), no study on systematic search for HIV/AIDS and *Leishmania* co-infections had been carried out in the country.

In this study, we have diagnosed kala-azar cases from Bangladesh by PCR and identified the causative *Leishmania* species by sequencing the amplified PCR products. We compared PCR results with direct microscopy, culture and anti-*Leishmania* antibody detection by ELISA. We also report here the comparison of formalin fixed samples with samples preserved in normal saline, and the result of a search for *Leishmania*/HIV co-infection in Bangladesh.

Materials and Methods

Patients

Forty two clinically suspected kala-azar patients who were hospitalized in Medical College Hospitals of Bangladesh, were included in this study.

Definition of kala-azar patients

Patients who were admitted to Mymensingh Medical College Hospital of Bangladesh with prolonged fever, hepatosplenomegaly, with positive aldehyde test and leucopenia with monocytosis, and were parasitologically (smear and/or culture) and /or anti-*Leishmania* antibody positive.

Definition of negative controls

Patients having fever, hepatosplenomegaly and serologically and/or parasitologically negative for leishmaniasis were recruited as negative control.

Collection of samples from VL patients of Bangladesh

Bone marrow or splenic aspirate was collected from 42 hospitalized clinically suspected kala-azar patients. Bone marrow was collected from 5 patients having chronic liver diseases, 2 patients with leukemia and 2 patients with *Plasmodium vivax* malaria under aseptic precaution as control. Giemsa stained smears were examined under microscope for detection of *Leishmania* amastigotes. The remaining portion of the aspirated material was then aliquoted in 10% formalin and normal saline in microtubes for preservation of DNA. A portion of bone marrow was inoculated into modified NNN culture medium. Five ml venous blood was collected from each patient for serological tests. All these samples were then transported to the Parasitology Department of Kochi Medical School for further study. At Kochi Medical School, bone marrow and splenic aspirates were washed in PBS and aliquoted into 70% ethanol and 100% ethanol, and some were preserved in formalin as well as normal saline. We extracted DNA every 3 months interval for up to one year and tested for *Leishmania* DNA using the same condition and primers.

*Determination of anti-*Leishmania *antibody by ELISA*

Anti-*Leishmania* IgG was detected by ELISA using crude antigen following the method described by Shamsuzzaman *et al.* (2000).

Search for Leishmania-*HIV co-infections*

Forty one sera from the suspected kala-azar

patients were screened for anti-HIV antibodies by particle agglutination test (Particle agglutination HIV-1/2, Fujirebio Co. Ltd., Tokyo, Japan) as well as by immunochromatography assay (Dina Screen HIV-1/2, Dinabot Co. Ltd., Tokyo, Japan).

Extraction of DNA

Leishmania DNA was extracted following the protocol using phenol:chloroform:isoamyl alcohol as well as DNA extraction kit (Qiagen, Tokyo, Japan). DNA concentration was determined by spectrophotometer (ULTROSPEC 3000, UV/visible spectrophotometer, Phammacia Biotech) and preserved at 4℃ until use.

Primers used in this study

The following sets of primers were used.

a. The primers constructed from kDNA of *L. (L.) donovani* DD8 strain to amplify a fragment of 354 bp in length as follows:

K upper 5' GGG ATT GGA CTT GGT GGA 3'

K lower 5' CAC AGC CCG CAG ATA CAA AT 3'b.

b. The primers constructed from the ITS region of DNA from *L. (L.) donovani* (DD8) strain to amplify a fragment of 498 bp in length as follows:

ITS upper 5' CAA CTC GGG GAG ACC TAT G 3'

ITS lower 5' AAT ATG CGC ACA ACA CAA AC 3'

Nested PCR

The nested PCR was performed using the same primers mentioned above.

PCR reactions

A 354 bp product of kDNA and a 498 bp fragment of ITS of nuclear DNA were amplified with primer sets 'a' and set 'b' respectively. PCR amplification was carried out in a 25 μl final volume containing 2 μl DNA, 2.5 μl 1x PCR buffer, 1.5 M MgCl, 25 μM of each dNTP, 10 pmoles of each primer, and 1.25 units of Taq DNA polymerase enzyme. The reaction was performed in DNA thermal cycler (Takara, Japan) with the following conditions: initial denaturation at 94℃ for 10 min followed by 35 cycles (each comprised of 94℃ for 45 sec, 55℃ for 30 sec and 72℃ for 1 min and 30 sec), followed by one cycle of final extension at 72℃ for 10 min. PCR products were then separated through 1.5% agarose gel with ethidium bromide and the bands in gel were analyzed by KODAK 1D image analysis software (Eastern Kodak Company, Scientific Imaging Systems, Rochester, NY, USA) using the device 'Kodak 1 DAS 290'. The bands amplified were compared to the bands obtained with a positive *Leishmania* DNA control. All the chemicals without DNA sample were also run in each reaction as negative control.

DNA sequencing

The 498 bp band of DNA was purified in filter column (Quantum Prep™ Freeze N Squeeze DNA Gel Extraction Spin Columns, Bio-Rad Laboratories, California, USA), ethanol precipitated and resuspended in 10 μl 1/10 TE buffer and sequenced using the ABI PRISM kit. After purifying the mixtures using spin columns (Applied Biosystems, Foster City, CA) the products were applied to an ABI Genetic analyzer. Gentyx-Mac (ver. 11.0.) software was used for alignment analyses.

Results

Of the 42 samples studied, 25 (59.5%) were amastigote positive in the Giemsa stained smears, 11 (73.3%) of the 15 samples inoculated in to culture medium were positive for *Leishmania* promastigotes (Table 9.3.1). All the control bone marrow samples were negative for amastogote. Forty sera were positive for anti-*Leishmania* antibodies. No serum was positive for anti-HIV antibodies.

Of the 42 bone marrow samples from suspected kala-azar patients 36 (85.7%) were positive by PCR by both the primers set. Four sera were antibody positive but PCR negative. Two samples were antibody negative, one of them was amastigote positive and the other was culture positive, and both were PCR positive. 29 samples which were positive in PCR showed 498 bp bands of DNA by the primers constructed from ITS region of the nuclear DNA and the remaining samples showed bands of 300-350 bp with the same

Table 9.3.1. Results of ELISA, smear examination, culture and PCR for diagnosis of kala-azar cases

Results	ELISA (n=42)	Smear (n=42)	Culture (n=15)	PCR (n=42)
Positive	40 (95.2)	24+1*(59.5)	10+1** (73.3)	34+2* (85.7)
Negative	2 (4.8)	17 (40.5)	4 (26.7)	4 (14.3)

n, total number tested; *, ELISA negative; **, smear negative, EllSA negative.

primers. All the 36 samples showed 354 bp band with the primers constructed from the *L. (L.) donovani* specific kDNA. Sequencing of the 15 PCR products which showed 498 bp bands had DNA sequences identical to that of the ITS region of the *L. (L.) donovani* DD8 reference strain (Fig.9.3.1). Bone marrow/splenic aspirates preserved in normal saline showed better results than the samples preserved in formalin. For long term preservation at -20℃, normal saline is better preservative than formalin. But there was no significant difference between the samples preserved in 70% and 100% ethanol for up to one year.

```
1.   CAACTCGGGG AGACCTATGT ATATATATAT GTAGGCCTTT
41   CCCACATACA CAGCAAAGTT TTGTACTCAA AATTTGCAGT
81   AAAAAAAGGC CGATCGACGT TATAACGCAC CGCCTATACA
121  AAAGCAAAAA TGTCCGTTTA TACAAAAAAT ATACGGCGTT
161  TCGGTTTTTG GCGGGGTGGG TGCGTGTGTG GATAACGGCT
201  CACATAACGT GTCGCGATGG ATGACTTGGC TTCCTATTTC
241  GTTGAAGAAC GCAGTAAAGT GCGATAAGTG GTATCAATTG
281  CAGAATCATT CAATTACCGA ATCTTTGAAC GCAAACGGCG
321  CATGGGAGAA GCTCTATTGT GTCATCCCCG TGCATGCCAT
361  ATTCTCAGTG TCGAACAAAA AACAACACGC CGCCTCCTCT
401  CTTCTGCACA TATATATATA TATTATACCA TACACAGTAT
441  ATATATAATT ATGTGTTGGA AGCCAAGAGG AGGCGTGTGT
481  TTGTGTTGTG CGCATATT
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Figure 9.3.1. DNA sequence of the amplified internal transcribed spacer (ITS) region of *L. (L.) donovani* DD8 WHO reference strain and 498 bp amplified PCR product.

Discussion

Mortality rate in VL cases is almost 100% in untreated group and mortality rate remains variable even in treated patients. Sometimes in endemic areas clinical features of VL are confused with other chronic febrile illnesses like chronic malaria, chronic liver disease, and some forms of leukemia. Accurate diagnosis of leishmaniasis is important to ensure the early treatment to reduce the morbidity and mortality and to know the prognosis.

In Bangladesh, VL is usually diagnosed by clinical features, supported by some nonspecific findings such as hypergammaglobulinemia estimated by aldehyde test, with leucopenia and relative monocytosis. Diagnosis is confirmed by demonstration of *Leishmania* amastigotes in the stained smears. Sometimes serological tests such as complement fixation test (CFT) for kala-azar, direct agglutination test (DAT), and occasionally ELISA and culture of *Leishmania* para-

site are done. But direct demonstration of parasite is of limited sensitivity (Zijlstra *et al.,* 1992). The conventional methods of diagnosing VL by detecting antibodies have some disadvantages. Firstly, detectable antibodies may not be found in early stages of the disease. Secondly, antibodies persist inside body several years after cure of VL, and thirdly, anti-*Leishmania* antibodies are found among residents of endemic areas who had no history of leishmaniasis. The second alternative may be antigen detection and the best method is to identify the parasite under microscope or in culture. But the sensitivity of these methods was low. So determination of *Leishmania* DNA may be the best alternative method to diagnose the cases as it is highly sensitive and specific. A series of recent studies have focused on the use of PCR amplification of kinetoplast DNA and nuclear DNA from different clinical samples (Rogers *et al.,* 1990; 1993; Rodriguez *et al.,* 1994; Singh *et al.,* 1999, Mimori *et al.,* 1999; Hu *et al.,* 2000). It has been reported that PCR was the most sensitive and specific tool among the methods available and it can detect very low quantity of DNA in a sample.

The general objective of the present study was to find out a suitable way to diagnose leishmaniasis from clinical samples by PCR. The specific objectives were to identify the *Leishmania* species directly from the clinical samples without culturing the parasite and to find out the better preservative for long term preservation of clinical samples.

In this study, PCR could diagnose VL patients and the sensitivity was 85.7% in clinically suspected patients. The sensitivity was, however, 100% when the parasite positivity was considered as gold standard since all the 25 amastgote positive and/or culture positive samples were positive by PCR. It has been reported that antibody may present in blood of the residents of endemic areas and it may persist in body up to seven years after cure from VL (Hailu, 1990). The 6 samples which were anti-*Leishmania* antibody positive but PCR negative might not be the cases of VL as all the samples were collected from the patients who were from the areas which were highly endemic for VL. On the other hand, 2 antibody negative samples were parasite positive, one was amastigoe and the other was culture positive, and both were PCR positive. These results suggest that the two seronegative cases might be the early VL cases or they had some altered immune response to *Leishmania* antigen. None of the control samples (n=9) was positive by PCR in this study.

Sequencing of the 15 PCR products which showed 498 bp amplified bands of ITS had DNA sequences identical to that of *L. (L.) donovani* DD8 reference strain. We have reported elsewhere that this segment of ITS of DNA could differentiate most of the *Leishmania* species of the Old World, especially strains from Asia. Using these primers we could diagnose the VL cases from clinical samples at the same time we could identify the causative *Leishmania* species by sequencing the amplified PCR products. It was evident from this study that bone marrow/splenic aspirates preserved in normal saline showed better results than the samples preserved in formalin. Also for long term preservation normal saline is better preservative than formalin. But there was no significant difference between the samples preserved in 70% and 100% ethanol for up to one year. A comparative study of the New World *Leishmania* diagnosis using formalin fixed and ethanol fixed samples showed that ethanol fixed samples were better than formalin fixed samples for PCR diagnosis of human CL cases (Uezato *et al.,* 1998). From the results of this study, it may be concluded that both sets of primers used in this study were efficient enough to detect *Leishmania* DNA from clinical samples of VL patients and hence these may be used in the field level. Moreover, use of primers of the ITS region of the nuclear DNA has the advantage that *Leishmania* species could be detected by sequencing the PCR products.

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Summary

The present reports dealt with the results of laboratory and field investigations conducted during the period from 1998 to 2001. The data and materials obtained were analyzed from the view of parasitological, vector entomological, pathological, electron microscopical, immunological, molecular biological and clinical points. In addition, an information on the epidemiological and clinical features of the Old World leishmaniases espeially from Pakistan and Bangladesh was also given briefly in the text. The results are summarized as follows.

The present leishmaniasis research project in Ecuador

In the current text, a brief retrospective review on the leishmaniasis research project in Ecuador was made focusing on the main topics obtained during about 18 years from 1982 to date. Causative *Leishmania* spp., vector *Lutzomyia* spp. and reservoir mammals of cutaneous leishmaniasis (CL) were incriminated at several endemic areas, performing intensive countrywide epidemiological surveys. Regarding treatment of CL, topically applicable lotions and ointments, and oral drugs, anti-malarials such as Mephaquin and Artesunate, gave good results. In a search for more simple and convenient diagnostic methods of CL, it was found that the scrape/exudate samples are suitable for polymerase chain reaction (PCR) techniques developed in this project.

A global situation of leishmaniasis in the world

By focusing on the changing patterns of transmission, clinical forms, prevalence and magnitude of leishmaniases, a global situation of the disease in the world was briefly reviewed. A special emphasis was given to *Leishmania*/HIV co-infection cases, which are increasing annually, especially in the South-western European countries such as Spain, Italy, France and Portugal.

Parasitological and vector entomological aspects

Using PCR and Southern blotting, *Endotrypanum* spp. and *L. (V.) equatorensis* were compared thoroughly. From the results obtained it was suggested that the latter species belongs to the genus *Endotrypanum.* We also designed primers specific for the detection of subgenus *Leishmania,* and the results obtained showed that the primer could be useful to detect specifically the subgenus. Isolation and characterization of *Leishmania* strains from Ecuador and Argentina were done, by using molecular tools and monoclonal antibody based ELISA. Nuclear DNA and kinetoplast DNA were amplified, and sequencing of the PCR product was done along with characterization of *Leishmania* species by serodeme analysis. In Ecuador, 8 *Leishmania* isolates from Huigra were identified as *L.(L.) mexicana,* 6 from Puerto Quito and 1 form La Mana were identified as *L.(V.) panamensis.* Two isolates from Oran, Salta, Argentina, were characterized as *L. (V.) braziliensis.* Regarding vector entomological works, natural infection rates of sandflies with *Leishmania* parasites from Andean leishmaniasis-endemic areas were examined individually by PCR; both the sensitivity and the specificity of the method employed were highly acceptable.

PCR and clinical diagnosis of CL

PCR method was compared with presently available three conventional techniques, such as smear, culture and histopathologic ones. The PCR method employed proved to be more sensitive, specific and faster in diagnosing CL cases in leishmaniasis-endemic areas of Ecuador. Differential diagnosis between the skin diseases and CL revealed that non-CL leg ulcers should be considered as a high possibility of misdiagnosis among various skin changes observed, and therefore these lesions should be properly examined.

Clinical and epidemiological aspects

Clinical survey of CL in Ecuador during 10 years between 1991 and 2000 showed that the popular types of lesions were ulcers, nodules, erythematous plaques and papules; the most frequent one was ulcer formation, showing more than 50% of the total cases exam-

ined. In the Amazonian regions of Ecuador, an active search for mucocutaneous leishmaniasis (MCL) cases was made, and 13 cases were thoroughly observed, by performing PCR, culture and histopathology. The main clinical features were erythema, ulcerations, granulomas, septal perforation, swelling of upper lip and nose, bleeding and crusts caused by the subgenus *Viannia,* especially by *L.(V.) braziliensis* (PCR identification). The mucosal tissue of nose, the oral mucous and the upper lip were the most affected. Seven anthropophilic *Lutzomyia* sandflies in the areas were identified, but no *Leishmania* parasite was found. A comparison of ultraviolet radiation energy between lowland and highland of Ecuador was done, based on the hypothesis that there might be some relationships between CL skin manifestations and ultraviolet radiation; a notable difference of CL skin lesions was observed between lowland and highland CL patients in that country.

Experimental leishmaniasis

Effects of ultraviolet A (UVA) irradiation on the mice infected with *L.(L.) amazonensis* were examined, aiming at the determination of the influence on CL pathogenesis. The results showed that both systemic and local IFN-γ cytokine responses were prominent after UVA irradiation. IFN-γ was up-regulated and IL-4 was down-regulated. This fact indicates that cytokine response shift from Th2 to Th1 pattern, which possibly protected UVA-irradiated mice from *L. (L.) amazonensis* infection. In *L.(L.) amazonensis* infected mice, the induction of delayed type hypersensitivity (DTH) reaction by DNFB (2,4-dinitrofluorobezene) significantly inhibited the development of cutaneous lesions. UVB irradiation suppressed the development of CL lesions. Pathogenesis in the mice was more effective only in the absence of DTH reaction and UV-irradiation to the control animals. A case report of the immunohistochemical investigation of the human skin lesion after sandfly bite was made. Sandfly bite induced T-lymphocytes, macrophages, mast cells and Langerhan's cells at the site of sandfly bite. The result obtained suggested that *Leishmania* infection might be easily completed through Th2 and DTH response. Pre-injection of sandly head homogenates with salivary glands enhanced significantly the infection of BALB/c mice with *Leishmania* parasites. In the lesions of pre-injected mice, electron microscopic observation revealed the presence of many *Leishmania* amastigotes outside of the macrophages, suggesting some humoral and/or cellular changes of the host immune response(s) in such treated animals.

Experimental leishmaniasis treatment

Anti-leishmanial effects of meglumine antimoniate (MA) against *Leishmania* promastigote and amastigote forms were examined. From the results obtained, it was suggested that MA inhibits directly the proliferation of promastigote, and may have inhibitory effect to interfere the entry of promastigote into macrophages. Anti-leishmanial activity of MA is probably mediated *via* promastigote proliferation, and also *via* inhibitory effects on macrophages to suppress the pathogenesis of *Leishmania* infection. A novel synthetic LPS derivative (ONO-4007) and IFN-γ were used as combination treatment of experimental leishmaniasis with MA. IFN-γ and MA completely suppressed the lesion development in the animals. Both ONO-4007 and IFN-γ exerted anti-leishmanial effect when used as combination therapy with MA.

Related papers

In Pakistan, new endemic areas of CL were detected. Among 450 cases observed, clinically the disease was classified as dry papular type, 305 cases; dry ulcerative type, 122 cases; and wet ulcerative type, 13 cases. Existence of wet and dry type of lesions indicates the presence of both *L (L.) tropica* and *L.(L.) major* in the regions detected newly. In addition, identification of parasites from Bangladeshi kala-azar patients was made by using PCR, DNA sequencing and monoclonal antibody based ELISA.

APPENDIX

Abstract of Related Papers Published

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

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

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

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

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

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

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

ABSTRACT. En el curso de nuestro estudio sobre el mecanismo de transmision de la leishmaniasis en areas endemicas del Ecuador, las primeras fases de la investigación se canalizaron hacia las busqueda de las especies de flebotominos 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 investigación se encamina a conocer el mecanismo de transmision como paso previo a la adopción de probables medi-

das 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 atencion, desde Julio a Octubre de 1983, en siete diferentes sitios del area endemica de leishmaniasis escogida por nosotros, la zona de Ocaña, Provincia del Cañar. Solo encontramos dos especies antropofilicas del genero *Lutzomyia,* en esta area de estudio; ellas fueron identificadas como *Lu. trapidoi,* y *Lu. hartmanni*, basandonos en las caracteristicas morfologicas de su espermateca y armadura cibarial. Un total de 1,452 flebotominos de ambas especies capturadas, fueron sistematicamente disecados y examinados en busqueda de la infección natural, y el resultado fue que las dos resultaron positivas con promastigotes. Los flagelados observados fueron identificados al momento como pertenecientes al complejo *Le. braziliensis,* de acuerdo a su aspecto morfologico y comportamiento en el vector, especialmente su ubicación en el tubo digestivo del huesped invertebrado. Al examinar los ejemplares recolectados a diferentes alturas sobre el nivel del mar, 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 Sud-america, 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 hrs and the second at 03:00-04:00 hrs; and that *Lu. hartmanni* bites more constantly throughout the night, with a pronounced peak between 23:00 and 24:00 hrs. 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 thespecies 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 inten sive 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 transmision 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 eclosion 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 region. The majority of leishmaniasis cases reported was from the Pacific coast, followed by the Amazon. A few cases were also observed in the Andean highland or the mid-Andes. In the text, ecological features of each region relating to the mammalian and sand fly fauna, are taken into special consideration in discussion of disease transmission.

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

Natural infections of sand flies and wild mammals : One species of *Lutzomyia, Lu. gomezi,* was added to the list of Ecuadorian leishmaniasis vectors, in addition to the two known vector species, *trapidoi* and *hartmanni.* With regard to reservoir hosts, one species, *Tamandua tetradactyla,* was newly implicated. Of these other mammal species, *Potos flavus, Sciurus vulgaris* and *Choloepus h. didactylus,* which had already been listed as leishmaniasis reservoirs, the first two mammalian species were also positive for leishmanial parasites in the current study. A search for leishmaniasis reservoir hosts was also made by the immunological method using counter immunoelectrophoresis (CIE) in this study. The CIE technique revealed that the tissue extracts (antigen) of three arboreal species, *Didelphis marsupialis, Caluromys lanatus* and *Choloepus h. didactylus,* reacted immunologically with anti-leishmanial serum, producing precipitin lines. In the first two mammalian species, no natural infections with leishmanial parasites have parasitologically been observed. It was, however, suggested that these immunologically positive mammals play an important role as reservoirs of the disease in endemic areas of Ecuador.

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

of the disease in epidemiological surveys.

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

isms might be greatly different between the 2 genera, *Leishmania* and *Trypanosoma,* and also between the 2 host animals, hamsters and mice.

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

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

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

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

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

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

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

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

Nettai, 22, 1989, 68-82

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

Yoshihisa Hashiguchi

ABSTRACT. A brief review is given of recent developments in leishmaniasis research worldwide, including details of the transmission of the three clinical forms of the disease, *viz.*, cutaneous, mucocutaneous, and visceral. 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 epdemiology 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, Psychodydae, Phlebotominae)

Alba Inchausti, Yoshihisa Hashiguchi and Antonieta de Arias

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

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

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

Yoshihisa Hashiguchi (ed.)

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

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

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

Sand fly fauna and human leishmaniasis vectors in Ecuador : In eight Departments of Ecuador where human leishmaniases are endemic, the phlebotomine sand fly was sampled. A total of 40 species was collected, of which at least 11 represented new records for Ecuador. This record increased the number of 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 activity, of the flies in the area suggesting a high transmission intensity during the rainy season. The validity of the ovarian accessory glands of seven 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* pro-

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

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

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

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

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

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

21. A Review of Leishmaniasis in Ecuador

Yoshihisa Hashiguchi and Eduardo A. Gomez L.

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

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

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

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

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

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

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

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

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

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

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

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

ABSTRACT. An epidemiological study was performed on leishmaniasis in a newly established community in south-eastern Paraguay. 149 persons, of 172 inhabitants, were thoroughly examined by clinical, parasitological and immunological (leishmanin skin test) examinations. 88 of those exam-

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

Memorias do Instituto Oswardo Cruz, 87(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 deArias, 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 leishmaniasis in the Ecuadorian Andes which has a completely different species of causative agents and vectors from those of Peruvian uta. In this text, we briefly reviewed Andean leishmaniasis including uta and revised an ecological model of the disease in the Andean plateau. Furthermore, in this text autochthonous Andean leishmaniasis cases were reported from two regions of Ecuador, Huigra (1,300 m above sea level) and Alausi (2,300 - 2,500 m a.s.l.), Department of Chimborazo. In the areas 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 leishmanias 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 evenstually 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 lst 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 Journal 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 (2,300m-1,500m above sea level), Alausi (2,300m-2,500m a.s.l.) and Huigra (1,200m-1,500m 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

Atsushi Hosokawa, Shigeo Nonaka, Miguel H. Jurado, Masato Furuya, Yuki Eshita, Tatsuyuki Mimori, Ken Katakura, Eduardo A. Gomez L., Shinzo Izumi 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 areas, 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.

Mollecular 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 ultramicroscopically 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% (EC50) varied with different *Leishmania* species, and EC50 values of the most effective lot were in a range of 20-38 μg/ml Glucantime® or 5.7-10.8 μg/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 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 (1200-1500m above sea level), Department of Chimborazo. The results obtained were compared to those in Alausi (2,300-2,500m), Department of Chimborazo and Paute (2,300-2,500m), 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 andcutaneous 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.

Japanese Journal of Tropical Medicine and Hygiene, 23(3), 1995, 151-157

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

Eduardo A. Gomez Landires, Milorad Andrial, Atsushi Hosokawa, Shigeo Nonaka and Yoshihisa Hashiguchi

ABSTRACT. The current study was designed to evaluate anti-leishmanial activity of mefloquine hydrochloride (Mephaquin®) and artesunate (Plasmotrium®) 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 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 (Fluorouracil: 5FU) was

valuated 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 7 cases, no complete cure was found within a month of treatment. It is reported that burning sensations were caused by the application of 10% paromomycin ointment. In this research, no patient with ulcerative lesions complained of burning sensation during the application of 2% 5FU ointment. It is concluded that 2% 5FU ointment may be useful for the relatively small sized shallow ulcerative lesions, but not so effective against non-ulcerative lesions (in Japanese).

Hihu-Rinsho, 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 subge, *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 casative 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, leishamaniasis 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 parasited, *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 taegeted 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 million 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.

Kochi, Japan: Research Report Series No. 5, pp. 1-207, 1997

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

Yoshihisa Hashiguchi (ed.)

ABSTRACT. 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 humdreds of HIV co-infection cases. Similar roblems 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 analysed 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 detectd 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 analysed 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 anti-

body. 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 epidemiological 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 analysed. 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 knowledge 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 anti-leishmanial activities. Almost all of cutaneous leishmniasis 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 against 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 pinon enhance the growth of the parasites during early cultivation time within 24 hrs. 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 was evaluated using *Leishmania donovani*-infected visceral leishsmaniasis 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 parassite 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.

Journal of Dermatology, 25(5), 1998, 290-298

46. A Preliminary Study Aimed at the Detection of *Leishmania* Parasites in Subjects with Cutaneous Leishmaniasis using Polymerase Chain Reaction

Hiroshi Uezato, Keisuke Hagiwara, Atsushi Hosokawa, Motoyoshi Maruno, Shigeo Nonaka, Minoru Oshiro, Masato Furuya, Eduardo A. Gomez L. and Yoshihisa Hashiguchi

ABSTRACT. As a basic study for future diagnosis of cutaneous leishmaniasis, we tried to detect *Leishmani*a parasites representing different species in the subgenera *Leishmania* and *Viannia* from subject patients with cutaneous leishmaniasis by using the polymerase chain reaction (PCR) with the subgenus *Viannia* specific primer. Four out of the 14 specimens revealed an amplified DNA of 70 bp specific for the subgenus *Viannia (L. braziliensis* complexes). No bands were detected in the rest of the specimens belonging to the subgenus *Leishmania* and unclassified groups. The base sequences of the amplified DNA corresponded with those of the *L. (V.) braziliensis* kinetoplast minicircle. We concluded that PCR using the present primer specific for the subgenus *Viannia* would be useful in detecting *Leishmania* parasites in lesions of cutaneous leishmaniasis caused by the *L. braziliensis* complex.

Igaku no ayumi, 185(7), 1998, 450-451

47. Visceral Leishmaniasis (Kala-Azar) and HIV Infection -Leishmaniasis as an Opportunistic Infection with AIDS-

Yoshihisa Hashiguchi

ABSTRACT. In the text, a brief review on the pidemiology of co-infection of visceral leishmaniasis with HIV was made, mainly based on the reports published. Such a co-infection was at first recognized in the Mediteranean countries, Spain, Italy and southern France; then the infection gradually spread to other regions, Asia, Africa, and South and Central Americas. It was emphasized that in Japan or other non-endemic countries, co-infections should be noticed as one of the important imported diseases (or travel medicines). It was also mentioned that the cases of visceral leishmaniasis co-infected with HIV are annually increasing in non-endemic British and German people who traveled to southern European and Mediteranean regions, and infected there. Moreover, clinical findings found in co-infection cases were listed, in addition to the detection sites (organs) of *Leishmania* parasites, responses to the specific medications, curations and etc. (in Japanese)

Parasitology International, 47, 1998, 121-126

48. Natural Infection of *Lutzomyia hartmanni* with *Leishmania (Viannia) equatorensis* in Ecuador

Masato Furuya, Motoyoshi Shiraishi, Yoko Akimaru, Tatsuyuki Mimori, Eduardo A. Gomez L. and Yoshihisa Hashiguchi

ABSTRACT. The sand fly vector of *Leishmania (Viannia) equatorensis* was clarified in this paper by serodeme and zymodeme analysis of three unidentified isolates from *Lutzomyia hartmanni* captured in Ocana, Department of Cañar, Ecuador. Close agreement of the electrophoretic profiles of 11 enzymes between one strain (IHAR/EC/93?OC-04) of the three isolated and two reference strains of *L. (V.) equatorensis* was observed by cellulose acetate electrophoresis. Furthermore, this strain reacted only with 7H9 monoclonal antibody of which species-specificity was shown against *L. (V.) equatorensis.* These results clearly indicate that strain OC-04 is identified as *L. (V.) equatorensis.* From these results, it appears that *Lu. hartmanni* is one of the sand fly vectors of *L. (V.) equatorensis.*

Gene, 210, 1998, 179-186

49. Rapid Identification of *Leishmania* Species from Formalin-Fixed Biopsy Samples by Polymorphism-Specific Polymerase Chain Reaction

Tatsuyuki Mimori, Ji-ichiro Sasaki, Motomi Nakata, Eduardo A. Gomez L.,Hiroshi Uezato, Shigeo Nonaka, Yoshihisa Hashiguchi, Masato Furuya and Hideyuki Saya

ABSTRACT. The precise identification and classification of *Leishmania* species is important for public health surveillance since different species cause different clinical features of the disease. A highly specific polymerase chain reaction (PCR) panel was developed to enable the identification of the five major *Leishmania* species that cause New World cuataneous leishmaniases. The primers used for this panel were designed to distinguish the polymorphism in sequences of commonly amplified DNA bands of the parasites produced by arbitrarily primed PCR. These polymorphism-specific PCR diagnoses were performed with formalin-fixed biopsy specimens of the leishmanial lesions from four patients in Ecuador and one hamster skin lesion, and these lesions were determined to be caused by *Leishmania (Viannia) panamensis, L. (Leishmania) mexicana,* and *L.(L.) amazonensis.* The PCR panel may offer an important and practical approach to the standardized identification of *Leishmania* species in field examinations.

The Journal of Dermatology, 25 (10), 1998, 623-631

50. Comparative Studies of the Detection Rates of *Leishmania* Parasites from Formalin, Ethanol-Fixed, Frozen Human Skin Specimens by Polymerase Chain Reaction and Southern Blotting

Hiroshi Uezato, Keisuke Hagiwara, Atsushi Hosokawa, Motoyoshi Maruno, Shigeo Nonaka, Minoru Oshiro, Yasutsugu Nakashima, Masato Furuya and Yoshihisa Hashiguchi

ABSTRACT. In this study, detection rates of *Leishmania* parasites from human skin were compared among three different types of specimens, formalin-fixed, ethanol-fixed, and frozen, by polymerase chain reaction (PCR) and Southern blotting. For this purpose, we used biopsy specimens colleced from 19 leishmaniasis patients and performed PCR and Southern hybridization with the probe specific for *Leishmania (Viannia) braziliensis* complex. Among these 19, 16 specimens were from cutaneous leishmaniasis (CL), one, diffuse cutaneous leishmaniasis (DCL) and 2, mucocutaneous leishmaniasis (MCL) and were formalin-fixed and paraffin-embedded. The causative agents for one case of CL and one case of DCL were already identified as *L. (Leishmania)* complex. Six specimens of CL were preserved in 100% ethanol. Two specimens of MCL were frozen tissues. PCR using the formalin-fixed

and paraffin-embedded specimens revealed positive bands at 70bp in 9 (47.4%) out of 19 specimens of CL, MCL and DCL. Southern blotting detected the signals in 12 (63.2%) out of the 19. PCR using the 100% ethanol-fixed specimens revealed positive bands in 4 (66.7%) out of 6, and Southern blotting using 2 frozen specimens of MCL were always positive (100%). Although we failed to detect significant differences by Chi-square test between the results from the formalin-fixed, paraffin-embedded specimens and those from 100% ethanol-fixed ones, we concluded that ethanol-fixed specimens, convenient for transportation and storage, would be more useful for diagnosis of leishmaniasis by PCR in a developing country.

Tokai Journal of Experimaental and Clinical Medicine, 23 (6), 1998, 393-399

51. Leishmania Mini-Exon Genes for Molecular Epidemiology of Leishmaniasis in China and Ecuador

Ken Katakura, Shin-Ichiro Kawazu, Chizu Sanjyoba, Toshimitsu Naya, Yoshitsugu Matsumoto Mamoru Ito, Koichi Nagakura, Masamichi Aikawa and Yoshihisa Hashiguchi

ABSTRACT. The mini-exon gene is unique and is tandemly repeated in the *Leishmania* genome. The transcribed region is highly conserved, but the non-transcribed spacer region is distinct in length and in sequence among different *Leishmania* species. The usefulness of PCR amplification of the *Leishmania* mini-exon gene was examined for molecular epidemiology of visceral and cutaneous leishmaniasis. We previously described a PCR method for amplification of the mini-exon gene and obtained positive amplification in bone marrow aspirates of patients with visceral leishmaniasis in China. In this study, we have cloned and sequenced two PCR products from the patients. The sequences of two products revealed 100% identity and showed more similarity to the mini-exon gene of *L. donovani* Indian strain than those of *L. donovani* complex in Africa and South America. We also applied this PCR method to the diagnosis of cutaneous leishmaniasis. We obtained positive PCR amplification in skin biopsy materials taken from patients with cutaneous leishmaniasis in Ecuador. Since this PCR amplification is simple and requires only a pair of primers to detect all *Leishmania* species distributed in Ecuador, the method may be a useful tool for the detection of parasites, not only from patients, but also from sandflies and reservoir animals in this area of endemicity.

Biophysical and Biophysical Research Communications, 255 (2), 1999, 289-294

52. Structural and Functional Analysis of the LaMDR1 Multidrug Resistance Gene in *Leishmania amazonensis*

Ken Katakura, Masaki Iwanami, Hiroshi Ohtomo, Hiroshi Fujise and Yoshihisa Hashiguchi

ABSTRACT. We determined primary sequences of the LaMDR1 gene in *Leishmania amazonensis,* a protozoan parasite that causes cutaneous leishmaniasis. The longest open reading frame encodes 1341 amino acids for a protein consisting of two similar halves, each containing six putative transmembrane domains and one ATP-binding domain. The protein has no potential N-glycosylation sites at the extracellular region. The LaMDR1 protein was 91 and 78% identical to the closely related ldmdr1 in *L. donovani* and lemdr1 in *L. enriettii,* respectively, revealing less conservation in the C-terminal than in the N-terminal transmembrane domains. Transfection of LaMDR1 conferred a multidrug resistance phenotype to wild-type promastigotes, which exhibited a significant level of resistance to vinbrastine, doxorubicin, and actinomycin D, but not to puromycin and colchicine. This drug specificity of LaMDR1 was overlapping with but distinct from that of ldmdr1, suggesting functional diversity of MDR1 proteins among different *Leishmania* species.

The Japanese Society for Systematic Parasitology, Circular 17, 1999, 1-5

53. Leishmaniasis: its Epidemiplogy and Causative Agents, with Special Reference to Ecuador -Epidemiology of Leishmaniasis-

Yoshihisa Hashiguchi

ABSTRACT. In the text, the following four points were mentioned, based on the long-term research experience of the authors in Ecuador, during about 18 years: 1) a global situation of leishmaniasis (L) in Ecuador, 2) vector sandfly species and their biting activities in areas endemic for cutaneous leishmaniasis(CL), 3) reservoir mammals of leishmaniasis and their roles as one of the typical zoonoses, 4) distributions of CL and the clinical manifestation in Ecuador. In the last session (4), clinical forms of CL divided into five types, (1) highland and lowland CL, (2) mucocutaneous L, (3) diffuse CL, (4) disseminated CL, and sporotrichoid type CL. (in Japanese)

The Japanese Society for Systematic Parasitology, Circular 17, 1999, 5-8

54. Leishmaniasis: its Epidemiology and Causative Agents, with Special Reference to Ecuador -Taxonomy of the Genus *Leishmania*-

Tatsuyuki Mimori

ABSTRACT. In the text, species status of the organisms belonging to the genus *Leishmania* was discussed briefly, following the opinions published hitherto. The author tried to review the history of parasite-isolation from the spleen of a soldier who suffered from Dam-Dam fevers and died in 1900; the case was reported in 1903 by *Leishman* as an abnormal form of typanosomes. Thus, ancient time discussions on the *Leishmania* species, causing visceral and dermal leishmaniasis were mentioned thoroughly, and then the review was made focusing on the recent methods of taxonomy of the genus *Leishmania,* employing zymodeme, serodeme, schizodeme and karyodeme analyses. Special emphasis was also given to the characterization techniques using polymerase chain reaction (PCR). Among these techniques, it was mentioned that the polymorphism specific PCR (PS-PCR) newly developed by the authors group would be very useful for future characterization of the parasite, *Leishmania* spp. and also for future diagnosis of the disease. (in Japanese)

Advances of Parasitology in Japan, 6, 1999, 527-543

55. Leishmaniasis

Yoshihisa Hashiguchi

ABSTRACT. As is well known, in Japan leishmaniasis is not prevalent and no anthropophilic sandflies, *Phlebotomus* spp. and *Lutzomyia* spp. are available. Therefore, all the cases reported in Japan were imported; the patients infected with *Leishmania* in endemic foreign countries, especially in Asia (China, India, etc.), Far East (Iran, Iraq, etc.), Africa (Kenya, Agypt, etc.), and South and Central America (Brazil, Paraguay, etc.). The author tried to review all the cases mainly reported by Japanese workers during the period from 1911 to date. In Japan, visceral leishmaniasis (kala-azar), post-kala-azar dermal leishmaniasis (PKDL), cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (MCL) were observed as clinical forms. In the text, review was made from two points of view; 1)leishmaniasis research in Japan and 2) leishmaniasis research by Japanese workers in foreign countries. In the first session, clinical cases, Kala-azar, PKDL, CL, and MCL were thoroughly described, and then researches on chemotherapy and immunology of the disease, culture in vitro, morphology, physiology, biochemistry of the *Leishmania* parasites, and experimaental studies using animal models were also reviewed. In the second session, researches mainly made by the authors group in Ecuador and Paraguay were mentioned. Main items were a) causative agents, *Leishmania* spp b) sandflies and its biting activity, c) reservoir hosts, d) epidemiology and clinical forms, and diagnosis and treatment.

Igaku no ayumi, 191(1), 1999, 29-33

56. Present and Future of the Control of Leishmaniases

Yoshihisa Hashiguchi

ABSTRACT. A brief review on the present and future of leishmaniases was made, emphasizing on the co-infection of the disease with HIV prevalent in the Mediteranean countries, and recently in Asian, African, and South and Central American countries. In the text, as the main factors of leishmaniasis spreading in the world, the following five were mentioned: 1) migration of people from urban areas to rural and/or forested areas for plantations or other purposes, 2) country- or continent-wide migration of seasonal workers, 3) ecological and geographical changes caused by land exploitation, 4) unorganized urban development, and 5) suspension of malaria control campaign. Changing patterns of *Leishmania* transmission were mentioned, citing the cases found in south-western Europe where the *Leishmania*/HIV co-infections are highly prevalent; in the area the transmission occurred directly from drug-using man to man, or from drug-using man to sandfly vecotr to man without reservoir hosts (dogs). Vector and reservoir host controls were briefly mentioned, including vaccine trials, environmental changes and a search for the suitable treatment. (in Japanese)

Annals of Tropical Medicine and Parasitology, 93(6), 1999, 613-620

57. Use of Urine Samples from Healthy Humans, Nephritis Patients or Other Animals as an Alternative to Foetal Calf Serum in the Culture of *Leishmania (L.) donovani in vitro*

S.M. Shamsuzzaman, Masato Furuya, Masataka Korenaga, Kyoko Imamura and Yoshihisa Hashiguchi

ABSTRACT. The effect of supplementing in vitro cultures of *Leishmania donovani* with urine was investigated. the parasites were isolated from Bangladeshi patients with visceral leishmaniasis. the urine samples used were collected from healthy human donors, patients with nephrotic syndrome, diabetic nephritis (DN) or diabetes mellitus, a dog and a cow. Promastigotes from blood-agar cultures were inoculated into RPMI-1640 basal medium with 10% heat-inactivated foetal calf serum (FCS) and/or 1%-20% urine. The parasites were then counted in a haemocytometer, on days 2, 4, 5, 6, 7, 8, 10, 12 and 14 post-inoculation. From day 4, the numbers of parasites/ml in cultures containing 5% healthy-human urine but no FCS were at least as high as those in cultures containing 10% FCS but no urine (P=0.191). The wet weights of parasites harvested from mass cultures of the parasites in RPMI-1640 plus 5% healthy-human urine and in RPMI-1640 plus 10% FCS were practically the same. Multiplication

of the parasites in the presence of 5% urine from a DN patient was significantly greater ($p<0.001$) than that seen with other urine samples at the same as with 5% healthy-human urine. Parasites could be maintained in RPMI-1640 plus 5% healthy-human urine for at least 40 days, sub-culturing every 4 days. Urine may be a better and much cheaper stimulant of *Leishmania* multiplication in vitro than FCS.

Japanese Journal of Tropical Medicine and Hygiene, 27, 1999, 289-294

58. Present and Future Situation of Leishmaniasis Research

Yoshihisa Hashiguchi

ABSTRACT. In order to know the global situation of leishmaniasis in the world, the transmission and clinical forms were briefly discussed, and the prevalence was also reviewed, mainly based on the reports from World Health Organization (WHO). *Leishmania*/HIV co-infection cases are increasing annually due to different factors, such as human behavioral, environmental and epidemiological changes, especially in southern Europe, Spain, Italy, France and Portugal. The co-infection cases have also been reported from other countries of different continents, Asia, Africa, and Central and South America. In such *Leishmania*/HIV co-infection cases, serological diagnosis is of little use. To overcome the diagnostic problem in HIV-infected patients, an indirect xenodiagnosis of visceral leishmaniasis using laboratory colonized sanflies was recently developed by Spanish workers; the usefulness was shortly discussed in the text as a topic.

Japanese Journal of Tropical Medicine and Hygiene, 27, 1999, 55-58

59. Leishmaniasis in Ecuador, with Special Reference to Its Andean Form

Yoshihisa Hashiguchi and Eduardo A. Gomez L.

ABSTRACT. In this text, New World leishmaniases were briefly reviewed. In addition, a history of the research on Ecuadorian leishmaniasis by the author's project from 1982 to date was also shortly given. A total of 7 species of the genus *Leishamania* as causative agents of the disease were isolated from humans, sandflies and mammals, and 4 species of *Lutzomyia* and 8 species of mammals were incriminated as probable vectors and reservoirs, respectively, in that country. In this paper, a special emphasis was given to Andean leishmaniasis which was discovered by the authors in 1986 at a

small town, Paute, located on the southern part of Ecuador, near to the Peruvian borders. The disease form is very similar to Peruvian uta especially in clinical features, but the causative agents (*Leishmania* sp.) and vector sandflies (*Lutzomyia* sp.) were completely different from Peruvian ones. Based on the results obtained from our longitudinal surveys on the epidemiology and ecology of the disease in the area, we developed a transmission model of Andean leishmaniasis. From the information collected in our studies, we recommended that measures for vector control should be applied in such an area endemic for the Andean leishmaniasis, during the dry season when the breeding site of sandflies and the transmission site were limited within and/or around rock crevices and animal burrows in the open field located at remote area from Paute town.

Japanese Journal of Tropical Medicine and Hygiene, 27, 1999, 63-65

60. Cutaneous Findings of Cutaneous Leishmaniasis and Their Differential Diagnosis in Ecuador

Atushi Hosokawa, Motoyoshi Maruno, Atsushi Takamiyagi, Shigeo Nonaka, Eduardo A. Gomez L. and Yoshihisa Hashiguchi

ABSTRACT. Ecuadorian cutaneous leishmaniasis (CL) was divided into three types, localized (LCL), generalized (GCL) and diffuse (DCL) forms. CL shows various cutaneous manifestations, such as papules, nodules, ulcers with elevated borders and erythematous plaques. In GCL, the eruptions are disseminated throughout the entire body surface. In DCL, which is associated with specific immunodeficiency against *Leishmania* antigen, different clinical manifestations such as nodules, papules and erythematous plaques are observed throughout the entire body surface, with the exception of the scalp axillary, inguinal, perineal and anal regions. Cutaneous manifestations of CL in Ecuador are very similar to those of other infectios and skin diseases. Therefore, differential diagnosis between CL and other diseases including leprosy and deep mycosis is very important, especially in countries where these diseases are relatively common. Ecuadorian LCL was clinically divided into highland type (Andean type) and lowland type. The highland type, observed in the Andes regions where the temperature and moisture is relatively low, occurs as milialy-topea-sized papules resembling insect bites and furuncles on the face and upper and lower extremities of children. The inflamation of the lesion is relatively minor compared to that of bacterial infections, although numerous Leishmania parasites are often detected within the lesions. The lowland type of CL, observed in the area with hot and humid forests, shows variable changes, including ulcer with elevated border where induration is palpable at the margin. After the infection, the lesions gradually increase in size and form relatively large and deep ulcers. A portion of each lesion heals spontaneously in about one year and leaves a relatively large scar.The inflamation of the lesion is also minor. Therefore, the clinical symptoms of the lesions such as reddness and pressure pain, are much more minor than those of bacterial infection. When the bacterial infection is coexistent at the lesion site, the ulcers tend to become large and the lesions tend to endure longer. Though various fungi have been isolated from CL ulcers, their role in the ulceration

is still obscure. During our examination in Ecuador, we saw many non-CL cases mis-diagnosed as CL and treated using antimonials for a long time. Such skin lesions required differential diagnosis between CL and other skin diseases; in the text cutaneous changes of non-CL cases mis-diagnosed as CL at health centers were listed. Based on the observations of cutaneous changes in leishmaniasis-endemic areas of Ecuador, it was suggested that special attention should be given to various infections and non-infectious diseases, including skin carcinomas, for the differential diagnosis at the examination of patients with CL. Therefore, in order to ensure the accuracy of CL diagnosis, it is important to consider the history of the present illness of the patient, and to examine the margin of ulcers by palpation; these steps are particularly important in which these diseases are endemic but parasitological and histological examinations are not available.

Japanese Journal of Dermatology, 109, 1999, 1185-1191

61. Mucocutaneous Leishmanaisis Arising in a Japanese Returnee from Paraguay

Reiko Kaneko, Toshinori Furukawa, Masataka Satoh, Keiji Iwatsuki, Fumio Kaneko, Michiko Hoshi, Ken Katakura, Hiroshi Uezato, Shigeo Nonaka, Masato Furuya and Yoshihisa Hashiguchi

ABSTRACT. We report a 17-year-old Japanese boy with mucocutaneous leishmaniasis. He was born and lived in Paraguay until the age of 9 years. At 3 years of age, he had a nodule suggestive of a primary cutaneous leishmaniasis infection on his right leg, which gradually disappeared in response to the injection of the unknown domestic medicine. At 14 years of age, he noticed a stenosis of the right nasolacrimal duct, and a small nodule in the right nasal cavity. On examination, granulomatous nodules were present on the nose and upper lip, and the nasal septum was perforated by the invasion of the lesion. *Leishmania* parasites were not detected in the infiltrates, and were negative in culture studies. The polymerase chain reaction (PCR) detected *Leishmania (Viannia) braziliensis* sequences in biopsy specimens using the subgenus *Viannia* specific primer. The patient was treated successfully with the pentavalent antimonial sodium stibogluconate at 14 mg/kg/day for three months without severe side effects. (in Japanese)

Southeast Asian Journal of Tropical Medicine and Public Health, 30(4), 1999, 682-685

62. Cost Effectiveness in the Discrimination of *Leishmania* Species Causing Anthroponotic Leishmaniases in Asia Using Selective Enzymes

S.M. Shamsuzzaman and Yoshihisa Hashiguchi

ABSTRACT. In this study, an atempt was made to evaluate the usefulness of selective enzymes in the identification of *Leishmania* spp. causing anthroponotic leishmaniasis in Asia, especially from a cost effectiveness point of view. For this purpose cellulose acetate electrophoresis was carried out to identify the *Leishmania* species of the Old World. After analyzing 11 enzymes 6PGDH was found to be the most polymorphic enzyme which could distinguish the WHO reference strains of the *Leishmania* species endemic in Asian countries like *L. (L.) donovani* (DD8), *L.(L.) infantum* (IPT-1), *L. (L.) major* (5ASKH), and *L.(L.) tropica* (K-27). Addition of another enzyme G6PDH improved the quality of diagnosis. Cost could be reduced manifold to discriminate the Asian *Leishmania* parasites by analyzing these two enzymes.

Transactions of the Royal Society of Tropical Medicine adn Hygiene, 93, 1999, 606-607

63. Comparison of PCR Results Using Scrape/Exudate, Syringe-Sucked Fluid and Biopsy Samples for Diagnosis of Cutaneous Leishmaniasis in Ecuador

Tamami Matsumoto, Yoshihisa Hashiguchi, Eduardo A. Gomez L., Manuel H. Calvopiña, Shigeo Nonaka and Hideyuki Saya

ABSTRACT. In the present study, PCR diagnosis for cutaneous leishmaniasis was performed using scrape/exudate, syringe-sucked fluid and biopsy samples from ulcerative lesions of patients in the endemic area of El Carmen, Province of Manabi, Ecuador. Of the 13 patients examined, *Leishmania* parasites were isolated from 11 by culture in blood-agar medium. However, only 7 of them were positive for parasites on microscopy examination of stained smears. Syringe-sucked samples were obtained from 10 of the 13 patients; syringe-sucked samples could not be taken from 3 patients because the method was painful for some body sites. The PCR 168-bp products of DNA were detected in 11 of the 13 patients in their scrape/exudate samples by using PCR with primer V, but no positive band was found in any of the scrape/exudate samples with primer L. Two non-leishmanial samples were negative using either of the primers. The primers used were V1 and V2 for the detection of the subgenus *Viannia,* and L1 and L2 for the subgenus *Leishmania.* Species specific primers were p1 and p2 for *L.(V.) panamensis,* b1 and b2 for *L. (V.) braziliensis,* and g1 and g2 for *L. (V.) guyanensis.* The results obtained suggested that the parasite species could be identified as *L. (V.) panamensis.* The scrape/exudate sample that was negative in PCR using primer V showed a positive reaction for primer p in polumorphism-specific PCR. The false-negative rate by PCR was 15.4% (2 negative in 13 cases) when scrape/exudate samples were used; these samples had the same sensitivity as the biopsy samples. Collection of scrape/exudate material from skin lesions was easy and painless for the patients compared with the syringe-sucked or biopsy methods. Moreover, the biopsy method of sample collection

sometimes yields undesirable results causing various secondary infections, especially in field conditions. These results suggested that scrape/exudate material taken from lesions was equally useful compared with other material for diagnosis of cutaneous leishmaniasis by PCR. We recommended scrape/exudate samples as a better alternative to biopsy samples for the diagnosis of cutaneous leishmaniasis.

Educación Medicina Contimuada, 66, 2000, 14-21

64. Leishmaniasis en el Ecuador: Diagnostico de la Leishmaniasis Cutanea con la Reaccion en Cadena de la Polimerasa (PCR) en Comparación con las Tecnicas Convencionales

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ABSTRACT. The polymerase chain reaction (PCR) technique was evaluated in the routine diagnosis of cutaneous leishmaniasis. The test was compared with standard diagnostic techniques of scraping (smear), culture and histopathology. Samples were taken from cutaneous lesions of 72 individuals from El Carmen, Province of Manabi, Ecuador, suspected of having an infection due to *Leishmania* spp. The PCR assay using specific primers for *L. (V.) braziliensis* complex, showed the highest sensitivity, 90.9%, compared to 45.5% by culture, 40.9% by scraping, and 36.4% by histopathology. According to these data, the PCR technique improves the sensitivity and speeds the diagnosis of cutaneous leishmaniasis in endemic areas of Ecuador, reducing the morbidity, costs and risks associated with inadequate treatments.

Parasitology International, 49, 2000, 139-145

65. Characterization of Bangladeshi *Leishmania* Isolated from Kala-Azar Patients by Isoenzyme Electrophoresis

S.M. Shamsuzzaman, Masato Furuya, Shamsuzzaman Choudhury, A.K.M., Masataka Korenaga and Yoshihisa Hashiguchi

ABSTRACT. To identify the prevalent *Leishmania* species in Bangladesh, a total of nine patients aged 4-35 years, were studied; six (66.7%) of them were below 20 years of age. All the patients were clinically diagnosed to have visceral leishmaniasis; their haematological profile was in accordance with

leishmaniasis and all were improved after treatment with sodium stibogluconate. All the aspirated materials (eight bone marrow and one splenic aspirate) yielded growth of *Leishmania* parasite in NNN media; *Leishmania*-Donovan bodies were found in seven (77.8%) of them in a Giemsa stained smear. Aldehyde test (AT) was positive in all the nine cases examined, whereas, complement fixation test (CFT) was positive in seven (77.8%) and indirect fluorescent antibody test (IFAT) in eight (88.9%) cases. In this study, five of the nine isolates from kala-azar patients were characterized by isoenzyme analysis comparing with five WHO reference strains, *viz., Leishmania (Leishmania) donovani* (DD8), *L. (L.) donovani* (HU3), *L. (L.) infantum* (IPT-1), *L. (L.) tropica* (K-27) and *L. (L.) major* (5-ASKH) using cellulose acetate electrophoresis. By analyzing 11 soluble isoenzymes it was found that all five WHO reference strains had distinct electrophoretic mobility of the isoenzymes studied. No interspecies difference was observed amongst the five isolates from kala-azar patients examined and their isoenzyme profiles were consistent with WHO reference strain of *L. (L.) donovani* (DD8) but different form *L. (L.) donovani* (HU3).

Journal of Dermatological Science, 26, 2001, 217-232

66. Pre-exposure with Low-dose UVA Suppresses Lesion Development and Enhances Th1 Response in BALB/c Mice Infected with *Leishmania (Leishmania) amazonensis*

Noor Mohammad Khaskhely, Motoyoshi Maruno, Atsushi Takamiyagi, Hiroshi Uezato, Khan Mohammad Abul Kasem, Atsushi Hosokawa, Ken-ichi Kariya, Yoshihisa Hashiguchi, Eduardo A. Gomez L. and Shigeo Nonaka

ABSTRACT. This study was conducted to determine whether exposing mice to ultraviolet (UV) radiation would alter the pathogenesis of infection with *Leishmania (Leishmania) amazonensis (L. amazonensis)* which causes progressive cutaneous disease in susceptible mouse strains. BALB/c mice were irradiated with 10 and 30 J/cm2 UVA on shaved skin of the back from Dermaray (M-DMR-100) for 4 consecutive days before infection with *Leishmania* promastigotes. The course of disease was recorded by measuring the size of lesions at various times after infection. Mice groups irradiated with UVA 10 and 30 J/cm^2 showed significantly suppressed lesion development compared with the non-irradiated mice. Light and electron microscopy revealed a few parasites at the site of inoculation in UVA-irradiated subjects. Sandwich enzyme-linked-immunosorbent-assay (ELISA) examination of sera showed dose dependently upregulated interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-12, and down-regulated interleukin (IL)-4 and interleukin (IL)-10 levels in UVA-irradiated as compared with the non-irradiated mice. Positive signals for IFN-γ mRNA in irradiated mice were obtained by RT-PCR, while non-irradiated mice showed negative results. None of the examined samples showed signal for IL-4 mRNA. The present study disclosed that exposure of mice to different low-doses of UVA irradiation prior to infection may interfere with immunity to *L. amazonensis* in the murine model. This indicates that the cell-mediated response switch from Th2 to Th1 pattern suppressed the cutaneous lesions of *L. amazonensis.*