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Association of trauma with *Leishmania spp*. parasite recall. clinical and experimental evidences

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Se dan ciertas evidencias sobre el efecto que traumas o procesos inflamatorios inducidos tienen sobre la reactivación y migración de *Leishmania* en pacientes clínicamente curados, individuos con infecciones asintomáticas, o modelos experimentales. El estudio de muestras de áreas inflamadas de la piel de pacientes con las características antes mencionadas, reveló la presencia de parásitos leishmánicos o parte de su genoma mediante la utilización de métodos parasitológicos, inmunohistoquímicos (PAP) y moleculares (PCR). Asimismo, se logró la detección de ADN específico de *Leishmania* (*Viannia*) en muestras orales tomadas de pacientes con gingivitis y/o periodontitis, que habían sufrido leishmaniasis 5 a 10 años previo al presente estudio.

La dispersión observada en los casos humanos fue corroborada en un modelo animal, provocando un proceso inflamatorio artificial a hámsteres infectados con *Leishmania*. En este caso, observaciones llevadas a cabos entre 4 y 12 días mediante cultivos *in vitro*, extendidos en láminas coloreadas, o secciones histológicas, revelaron la presencia de parásitos en el sitio donde se provocó la inflamación (cavidad peritoneal) y en el hígado, el bazo, ganglio linfático, médula osea y sangre periférica circulante. Los resultados presentados apoyan el punto de vista de que en el caso de leishmaniasis, pacientes clinicamente curados con distintos esquemas terapéuticos, individuos con lesiones activas o en casos de infecciones inaparentes o asintomáticas, pueden experimentar una reactivación a distancia como consecuencia de un trauma que induzca a un proceso inflamatorio localizado.

Palabras clave: Inflammatory process, *Leishmania* reactivation.

INTRODUCTION

In the classic view of American cutaneous leishmaniasis (ACL), the parasite supposedly remains localized in the skin region around the bite of the insect vector. However, mobilization of *Leishmania* to neighboring lymph nodes, as well as to different areas of the skin different organs in experimentally infected animal models has been documented (Moreno & Scorza, 1981; Lugo *et al.*, 1993; Barral *et al.*, 1983; Hill, 1988). On the other hand, several authors have reported that in man inflammatory processes (IPs) due to different kinds of traumas provide a mileou which favors *Leishmania*-amastigote growth, triggering new lesions in naturally infected individuals who never showed apparent lesions (Walton, 1987; Long, 1973; White & Hendricks, 1982; Keane, 1994). Similar observations have been reported in experimental models exposed to artificial trauma (Bertho *et al*., 1994; Travi *et al.*, 1988; 1996).

It is known that IPs produce temporary immunosupressive stages due to the fact that the macrophages invading the inflammation site are immature and consequently unable to kill the phagocyted parasites (Ryan & Majno, 1997; Nacy *et al.*, 1981). Some authors have considered leishmaniasis as an opportunistic infection affecting individuals with other infectious diseases, such as AIDS, where the *Leishmania* parasites can spread to unusual places like the digestive

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tract and fluids such as urine, feces, sperm and pharyngeal secretion (Altes *et al*., 1991; Sedino *et al*., 1990; Fahal *et al*., 1995; Mabrahtu *et al*., 1993). In general, immunesupressed stages may increase susceptibility to infection or reactivate latent infections by *Leishmania*-parasites (Altes *et al.*, 1991). This fact was evidenced by Guevara *et al.*, (1993), who using the PCR technique demonstrated the presence of circulating *L. braziliensis* DNA in blood samples of a patient clinically cured 30 years previously while suffering a minor immunecompromise due to pregnancy. This observation also supports other workers (Saravia *et al*., 1990) describing recurrent lesions in *L.braziliensis* infected patients subjected to immunosupressive processes. The present paper deals with the use of parasitologic, immunohistochemical and molecular techniques to demonstrate the effect of trauma-induced IPs on the dispersion and/or re-activation of *Leishmania* in individuals considered to be clinically cured, and the appearance of lesions in those without apparent infection. In addition, these clinical findings are supported by experimental observations on *Leishmania*-infected hamsters in which an artificial acute IP was induced.

MATERIALS AND METHODS

Patients and sample collection: For this study we took samples from six individuals living in several villages of a CL endemic area located in Merida State in western Venezuela (71º00´-71º58´- 9º00´N). In four of these patients IPs, consisting of gingival swellings and periodontitis, were detected. The patients reported that they had suffered CL (2 with lesions on the upper members and 2 on the lower) and received antimonial therapy 5-10 years prior to examination at the out patient periodontal clinic in the Faculty of Dentistry, Universidad de Los Andes, Merida, Venezuela. 1 mm^3 biopsy samples were taken from the gingival mucosa of these patients, and processed for DNA isolation and PCR analysis as described bellow. The other two patients were diagnosed as infected with *Leishmania* at the Center for Parasitological Research, Faculty of Science, Universidad de los Andes, Mérida, Venezuela. They were: 1. A 16 year old boy who had two scars (on the right arm) from CL lesions, but was apparently cured after receiving immunotherapy. According to the clinical record the patient suffered a cut on his right elbow 10 and 20 cm away from the lesions which induced an acute IP. Using aspiration technique with a 27Gx1/2 needle and 1 ml syringe, samples were taken from the inflamed area and used for Giemsa´s stained smears, for inoculation into healthy hamsters, *in vitro* culture medium and PCR assay. 2.- A 50 year old man with no history of having suffered CL developed an ulcer as a consequence of a trauma on his righd hand. Samples consisted of aspirates taken as above for *in vitro* culture medium, Giemsa´s smears and inoculation into hamsters, and a 1 mm^3 biopsy specimen for PCR and inmmuno-histochemical methods: peroxidase anti peroxidase (PAP) and tissues indirect immuno-flurescence antibody test (IFAT) adapted from Camargo (1996).

Written consent to undergo the study protocol, was obtained from all patients. The study was approved by the Biomedical Committee of the national research council in Venezuela.

PCR detection of *Leishmania* DNA: For DNA isolation all the samples were washed in a sterile saline solution and incubated at 55ºC overnight in 1XTaq polymerase buffer without Mg++, 1% Triton X100, and proteinase K 100mg/ml. The following morning the samples were incubated at 94ºC for 15min, and cellular debris was spun down for 5 min. using an Eppendorf centrifuge.

The supernatant was kept at -20ºC until used and 2µl of this supernatant was used in the PCR reactions. The *Leishmania (Viannia)* specific PCR assay (Guevara *et al*., 1994) was driven by the forward primer 5´GCA GCA CAG GGA AAG 3´ and the reverse primer 5´ATG GAG AGA GGC ACT AGC 3´. To assess the suitability of the sample for PCR, an assay was included, targeted on human β-globine to produce 268 bp product (Saiki *et al.*, 1985), and as a negative control to check carry over contamination, all the components of the reaction mixture were used without DNA. Tissue sections of a 34 year old male who had never lived in an endemic area for leishmaniasis was used as a negative control. As positive control a blood sample from a patient with an active lesion was used. All the reactions were done simultaneously in a one-day period with the same stock of reagents and the same equipment. The PCR products (5µl samples) were electrophoresed in 2% agarose gels and stained with ethidium bromide.

Induced inflammatory process in *Leishmania*-infected hamsters: Twenty hamsters were inoculated with $2x10^5$ cultures promastigotes of *Leishmania garnhami* (MHOM/Ve/76/JAP-76), resulting in lesions on the animal´s hind feet. In ten of these animals an artificial acute intraperitoneal IP was induced by the injection of 5 ml of a 5% proteose-peptone aqueous sterile solution for two consecutive days. The rest of the infected animals were used as control. All the hamsters were kept in an animal house at 22ºC, with water and food *ad libitum* until they were sampled.

Sample Collection and Processing: A pair of infected hamsters in which an artificial acute intraperitoneal IP was induced, and their respective controls were sampled 4, 6, 8, 10 and 12 days post-inflammation. The hamsters were killed by over anesthesia with ether and samples of peritoneal liquid, the site of lesion, bone marrow, and lymph node, and biopsies from the liver and the spleen, were taken. Prior to anesthetizing the animals, a cardiopuncture was performed to take a sterile blood sample. Similarly, lh before killing the hamsters, 5 ml of Hank´s-heparine (9:1) sterile solution was injected intraperitoneally followed by gentle massage of the peritoneal cavity. Blood samples were placed in a test tube containing 0,05 ml of 15% solution of ethylene diamine tetracetic acid (EDTA), from which several micro-capillaries were filled. Both the tubes and the capillaries were spun 15 min at 1030g to separate white cells contained in the buffy coat. The samples were placed in Leighton tubes containing 1 ml of coconut water culture medium (Marquez *et al.*, 1985) and incubated at 25ºC. The peritoneal liquid was collected under sterile conditions after cutting the skin and muscle layers to introduce a sterile Pasteur pipette into the peritoneal cavity. The collected sample was placed in a centrifuge tube, spun at 114g for 10 min. The supernatant was discharged and the pellet re-suspended with 2 ml of coconut water medium to be later divided into three portions: one was placed in a Leighton tube and incubated at 25ºC, the second portion processed to estimate the number of inflammatory cells and the third smeared on a glass slide and later fixed and stained to estimate the proportional number of parasitised cells. The cultured samples of buffy coat, peritoneal liquid and the aspirated sample from the site of lesion, were observed at 24, 48, 72 and 96 h using an inverted microscope. The glass slides contained in the Leighton tubes were taken out 24 and 96 h later. They were allowed to dry, fixed with methanol and stained with May-Grümwall- Giemsa stain for lh. Biopsies from the liver, spleen, lymph node and bone marrow, were taken and divided into three portions: one was cultured in NNN culture medium, another was imbedded in paraffine was for histopathological study and the third one wax smeared on glass slides. The culture tubes were observed weekly for two months when they were eliminated if negative. The tissue sections were stained with the Giemsa-collophonium method (Bray & Garnham, 1962), and the smears stained with 10% Giemsa stain in phosphate buffer pH 7.2.

RESULTS

Detection of *Leishmania* DNA in oral samples of patients suffering gingival IP: Figure 1A shows the results of a *Leishmania Viannia* specific PCR assay carried out on the oral biopsies of patients who had suffered CL 5-10 years before examination. In all 4 cases (lanes 1 to 4) a strong amplification band of 126 bp was observed. The positive control consisting of a sample of serum from a patient with active lesion produced a similar size amplification product (lane 5), whereas a negative control consisting of a biopsy of a health y subject who never lived in endemic areas for leishmaniasis, gave no band at all (lane 7).

Reactivation of CL by Acute inflammatory processes in a cured patient and in an individual with inapparent infection: In the case of a 16 year old patient with two scars from CL infections suffered 6 months prior to the study, samples taken from an inflamed area located 10 and 20 cm far from the healed primary lesions revealed the presence of *Leishmania*-parasites (Fig. 2). Since the inflammation persisted, it was sampled again a month later. This sample revealed amastigotes in the stained smears, flagellates in a culture medium, and caused lesions in an inocultaed hamster. These results were supported by strong reaction to leishmanin-skin test (25x30 mm), high titres in IFAT and ELISA (1:1024; OD: 0,2), and a positive amplification band in PCR asays (Fig. 1B-lane 6).

The second patient, who had never showed signs of suffering CL, and who presented an IP after a trauma, developed a *Leishmania*-like lesion. Diagnosis using the leishmanin skin test showed an induration of 30x25mm at 48 h. Serological tests (IFAT and ELISA) evidenced a strong anti-*Leishmania* response judging by the high titres recorded (1:512; OD:0.2). A biopsy taken from the lesion revealed: i. specific *Leishmania* antigenic immune-fluorescent deposits detected by tissue- IFAT: ii. presence of *Leishmania*-amastigotes by immune-peroxidase technique (PAP) and iii. a positive amplification product with Leishmania (Viannia) specific PCR (Fig. 1B- lane 5).

***Leishmania* Dispersion in Experimentally Infected Hamsters after an Induced Inflammatory Process.**: The apparent mobilization of *Leishmania*-parasites induced by IPs in naturally infected patients, suggested the possibility of reproducing similar phenomenon in hamsters experimentally infected with *Leishmania*. In this case, samples taken from *Leishmania*-infected hamsters with an induced artifical acute intraperitoneal

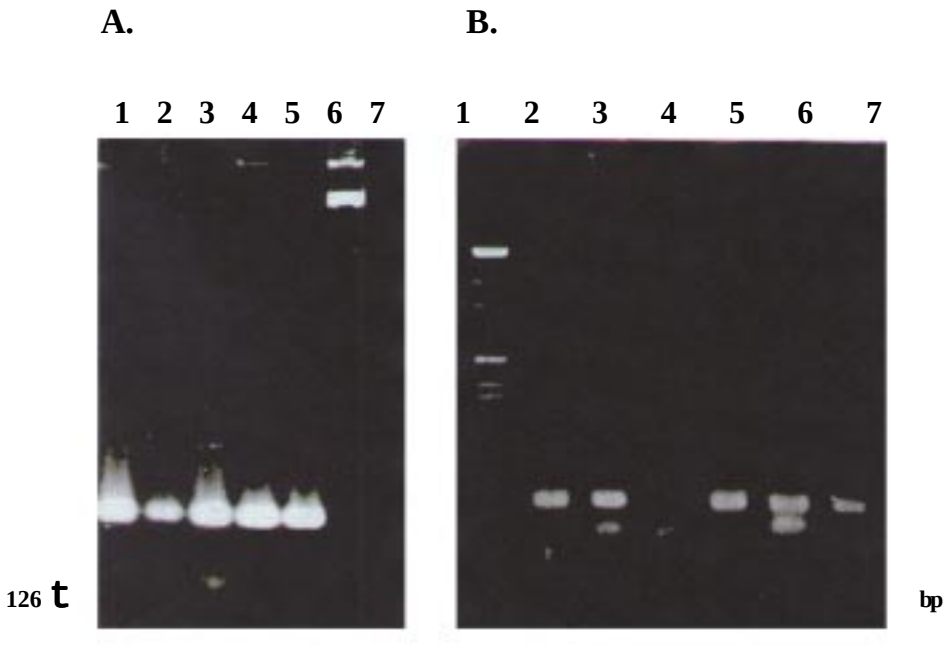

Figura 1.- Viannia subgenus specific PCR. Positive samples show a 126 bp amplification product. A.- Lanes 1-4 DNA samples from biopsies taken in patients suffering periodontitis long after healing their CL lesions. Lane 5 is positive control. Lane 6 is the λ BSTE II DNA marker. Lane 7 is a negative control.
B.- Lane 1 is the PGem® DNA marker. Lanes 2,3,7, are positive controls. Lane 5 is DNA sample of a biopsy from a patient who developed a lesion after a trauma. Lane 6 is a DNA sample from culture parasites obtained from a patient who developed an IP far from a healed primary lesion. Lane 4 is a negative control.

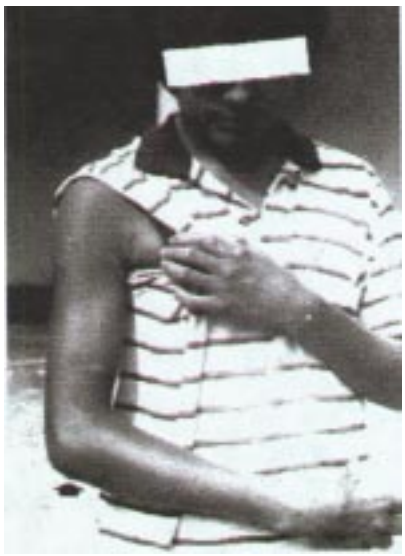

Figura 2.- Patient showing scars of heald lesions of cutaneous leishmaniasis. The arrow indicates the site of inflammatory process suffered some months after cure from which parasites were isolated.

IP showed positive results for parasites. Hemoculture analysis gave 20% of positive samples, while the examination of stained smears and *in vitro* cultures of the peritoneal liquid showed parasites in 70% of them. In addition, biopsies taken from liver, spleen, lymph node, and bone marrow revealed the presence of Leishmania-parasites in proportions from 20% to 60% with any of the methods used (seetable).

These findings differed from those observed in the infected hamsters used as control, in where the presence of *Leishmania* was restricted to the popliteum lymph node. As expected, the total number of inflammatory cells in the peritoneal cavity was higher in hamsters with induced IP (5000 cell/mm^3) than in controls (1000 cells/mm^3), with a statistically significant difference ($P \leq 0,05$). This difference was particularly marked during the 4^{th} and 6^{th} day post-inflammation. From this date onward a decrease in the number of cell per mm^3 (2000-1800 cells) was observed. The differential estimation of the type of inflammatory cells from 4 to 12 days post-inflammation made it possible to identify macrophages (70% - 95%), neutrophiles (1% - 16%) and lymphocytes (2% - 29%), the first being significantly higher than the other types of cells ($P \leq 0,05$).

DISCUSSION

In the present study it was observed that in clinically cured patients and in individuals without apparent leishmanial infection, IPs stimulated the reactivation and mobilization of Leishmania. This was evident in four patients suffering from oral IPs in which biopsy specimens from the inflamed area showed a strong signal when analyzed with a *Viannia* subgenus specific PCR assay. This fact indicates metastasis of *Leishmania* from the site of primary lesions (upper and lower members) to the gingival Ip, supporting previous clinical and experimental observation (Abbas *et al.*, 1992; Travi *et al.*, 1996), and at the same time raising concern about the oral practices in endemic areas for leishmaniasis, where dentists are in frequent contact with patients apparently cured from an episode of leishmaniasis. Similar results were obtained in two more clinical cases which showed lesions at the sites of trauma-induced IPs. In these cases, we obtained positive results for Leishmania when the inflamed areas were sampled by routine parasitological or immuno-histochemical methods or by PCR assay. This made it possible, on the one hand, to show the persistence of *Leishmania*-parasites in patients that have received a specific anti-leishmania treatment and, on the other, to reveal occult *Leishmania* in inflamed areas, which would otherwise have remained as inapparent infection. *Leishmania*- parasites isolated from the inflamed areas proved to be very active as shown by their successful establishment in culture media and/or by their ability to produce active lesions in experimentally inoculated animals. This also suggests that IP appears to act as a reactivating factor of persistent infections, and promotes the mobilization of the parasite to produce new lesions at sites far distant from the primary one.

The observed reactivation may be a consequence of the reaction of the immune-cutaneous system in which elements such as the follicular dendritic cells are responsible for the long term retention of *Leishmania*-parasites as has previously been suggested (Tapia *et al.*, 1993; Tew *et al.*, 1980). On the other hand, exacerbation of the disease may be explained by an induced increase in the number of parasites in the metastatic lesion, possibly due to a recruitment of mononuclear phagocytic cells to the site of inflammation. In this case, as it has been suggestd such cells, if not activated would act as "safe targets" for the parasite (Mirkovich *et al.*, 1986). The present observation also appears to support that of Guevara *et al.*, (1993; 1994) who demonstrated by PCR technique the presence of persistent *Leishmania* in patients with a long history of clinical cure, and corroborates the opinion of other workers on the role of parasite persistence in leishmaniasis (Saravia *et al.*, 1990; Aebischer, 1994; Ramírez & Guevara, 1997).

The presence of *Leishmania*-amastigotes in acute inflammatory *foci* of clinically cured patients may induce recurrent infective processes far from the site of primary lesions. In this case, the new lesions should be confused with re-infections. On the other hand, inflammatory *foci* may be considered as infective-sources for sand fly-vectors. This fact may provide the basis for explaining the controversial indoor transmission of leishmaniasis, and the possibility of considering man as an intradomiciliar reservoir (Rojas & Scorza, 1989). From the foregoing it is possible to suggest parasitologic examination to detect *Leishmania*-parasites in individuals that having had CL suffered an acute IP. This suggestion appears to be supported by previous clinical observations (Walton 1987) and by the increasing cases of recurrent CL due to the appearance of systemic infectious diseases such as AIDS (Badaro *et al.*, 1986; Fernández-Guerrero *et al.*, 1987; Fillola *et al.*, 1992; Martínez *et al.*, 1993; Venencie *et al.*, 1993).

Table Nº 1
Effect of an induced inflammatory process on the dispersion of *Leishmania* in experimentally infected hamsters

		Detection of *Leishmania* -Nº (%) - in					
Group	**Nº of Hamsters**	**P.M.**	**H.C.**	**Liver**	**Spleen**	**Lymph node**	**Bone marrow**
I.H.W.I.P.	10	7(70)	2(20)	2(20)	2(20)	6(60)	4(40)
C.I.H.	10	-	-	-	-	4(40)	-

I.H.W.I.P.: Infected hamsters with I.P.
C.I.H.: Control infected hamsters
P.M.: peritoneal macrophages
H.C.: Hemoculture

Similarly, the proposed examination may be useful to detect occult or inapparent infections in individuals coming from areas where CL is endemic.

As in men studied patients the effect of induced IPs on the dispersion of *Leishmania* was also demonstrated in experimentally infected hamsters. In this case, dispersion was in the induced inflamed area i.e. peritoneal cavity, which was positive for parasites in 70% of the animals sampled. These findings support the observations in human cases, showing the effect that acute IPs have on the dispersion of *Leishmania*-parasites established in primary lesions and/or any part of the body.

One more relevant aspect detected in the experimental model, was the presence of colonizacion of parasites in organs in contact with the inflamed area. This includes positive results in the liver, spleen, bone marrow and lymph node in 20%, 20%,40% and 60% respectively. This finding may be related to the ocurrence of visceralizing in individuals affected by CL.

SUMMARY

In this paper evidences are given on the effect of trauma induced and of inflammatory processes (IPs) on *Leishmania* reactivation and migration in infected patients (in cured or asymptomatic conditions), and in an experimental model. In the inflamed skin areas of the human cases we detected *Leishmania*-parasites or part of its genome by parasitologic, immuno-histochemical (PAP) and molecular (PCR) methods. We also detected *Leishmania* DNA in oral samples from patients who cured of leishmaniasis 5 to 10 years previous to the study and who were experiencing IPs due to gingival swellings and periodontitis.

The parasite migration observed in patients after suffering a trauma was corroborated in an animal model by causing an artificial intraperitoneal IP in hamsters infected with Leishmania. In this case, observations carried out from 4 to 12 days by in vitro culture medium, stained smears and/or histological sections, revealed the presence of parasites at the site of inflammation (pertoneal cavity) and in the liver, spleen, lymph nodes, bone marrow and circulating peripheral blood. These results support the view that in the case of leishmaniasis, clinically cured patients, active patients or asymptomatic individuals can experience reactivation of lesions after suffering a trauma that induce a localized IPs.

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