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Detection and chronology of parasitic kinetoplast DNA presence in hair of experimental *Leishmania major* infected BALB/c mice by Real Time PCR

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ABSTRACT

Hair can accumulate foreign chemical or biological substances. Recently, it has been reported that parasite DNA can also be detected in the hair of *Leishmania infantum* infected dogs. The aim of this work has been to find out whether parasite DNA incorporates in the hair of *Leishmania major* experimentally infected animals. For this purpose, a group of 4 BALB/c mice, intradermally inoculated in both ears with 1000 *L. major* V1 strain promastigote forms, was monitored for parameters associated to the infection during 35 days. Weekly, ear swelling was measured, and hair samples from ears and leg were collected. Blood samples were obtained before challenge and at day 35 post infection, when parasite load was measured in ear, lymph node and spleen by limit dilution. Ear swelling and other parameters observed in the infected mice were consistent with those described for this model. The presence of parasite kinetoplast DNA (kDNA) was detected by Real Time PCR in all ear and leg hair samples at the final timepoint. These data suggests that hair is a specialized tissue in the sequestration and removal of foreign DNA. Detection of DNA in hair could be, therefore, a useful tool to chronologically record the infection process during experimental mice assays.

1. Introduction

Experiments carried out in the *Leishmania major* (*L. major*) *infection*-mouse model have shown that the genetic predisposition for susceptibility or resistance to infection in different mice strains correlates with the dominance of an IL-4 driven Th2 response that causes disease or an IL-12 driven, IFN-γ dominated Th1 response that promotes healing and parasite clearance (Sacks and Noben-Trauth, 2002). Thus, the use of resistant C57BL/6 and susceptible BALB/c mice has allowed to have a deeper understanding of the immune response during *Leishmania* infection. These models have been widely employed to screen vaccine candidates against *Leishmania* (Mendez et al., 2003; Ramírez et al., 2010). The dominant Th1 response elicited in C57BL/6 mice leads to localized and benign lesions that resolve spontaneously. In contrast, the predominant Th2 response generated in BALB/c leads to a severe and non-spontaneously healing lesion, with ulceration, necrosis and extensive inflammatory reaction that produces the development of systemic disease (Sacks and Noben-Trauth, 2002), which finally drives to a visceral dissemination of the parasite to draining lymph nodes, liver and spleen (Kurey et al., 2009) that could cause even the death of the animal (Sacks and Melby, 1998).

In the mouse models, the site for injection most frequently used is the hind footpad, employing a range of 10^5–10^7 parasites (Sacks and Melby, 1998; Belkaid et al., 1998). Nevertheless, it has been reported that BALB/c mice develop cutaneous lesions in infections that mimic natural conditions using a lower number of parasites (10–1000) inoculated intradermally in the ears (Belkaid et al., 1998; Courret et al., 2003).

In most of experimental *Leishmania* infection assays monitoring is done by serological methods (Iborra et al., 2003; Iniesta et al., 2008). The presence of parasites is diagnosed by immunostaining (Cangussú et al., 2009), culture microtitration or microscopical observation of tissue samples smears (Mendez et al., 2003). Nowadays, molecular methods are becoming increasingly important,

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specially quantitative PCR. In 2002, Nicolas et al. developed a highly sensitive and specific Real time PCR method that was able to detect and quantify *Leishmania* in mouse tissues. The samples employed were obtained mainly after necropsy. Recently it has been reported that the hair may be considered as a suitable sample for the diagnosis of *Leishmania infantum* (*L. infantum*) by Real time PCR in the canine model (Belinchón-Lorenzo et al., 2013). Following this finding, the aim of this work has been to find out whether in *L. major* experimentally infected animals DNA could also be found incorporated into the hair during a follow up period of infection. For this purpose we have analyzed if parasite kinetoplast DNA (kDNA) could be detected and quantified by Real time PCR in the hair of *L. major* infected BALB/c mice. This kDNA is comprised of two units, the maxicircles (present in a number of 30–50 copies/parasite, with 20–40 kb in length) and the minicircles (present in a number of 10,000–20,000 copies/parasite with 1 kb in length), as stated by Rodgers et al. (1990). The chronology of appearance of kDNA close to the inoculation site (ear), as well as in other separate body areas (leg) has been determined, monitoring the process for five weeks.

2. Materials and methods

2.1. Mice, parasites and experimental challenge

Four-six week old female BALB/c mice ($n = 4$) were purchased from Harlan Interfauna Ibérica S.A. (Barcelona, Spain). *L. major* parasites clone V1 (MHOM/IL/80/Friedlin) were kept in a virulent state by passage in BALB/c mice. *L. major* amastigotes were obtained and transformed to promastigotes by culturing at 26 °C in Schneider's medium (Gibco, BRL, Grand Island, NY, USA) supplemented with 20% foetal calf serum. Metacyclic promastigotes of *L. major* (clone V1) were isolated from stationary cultures by negative selection as described by Sacks et al. (1985) using peanut agglutinin (Vector Laboratories, Burlingame, CA, USA). 1000 metacyclic forms were injected into the dermis (i.d.) of both ears of each mouse in a volume of 10 μl. The evolution of the ear lesion was weekly monitored measuring the diameter of the indurations with a metric calliper. The Bioethical Committees of CBMSO (ref. CEEA-CBMSO-10-044) and UAM (ref. CEI-28-713) approved all animal handling methods and the infection procedures described (Research project FIS PI11/00095).

2.2. Sample collection

A quantity of about 10–20 hair samples were weekly taken using tweezers from the region surrounding both ears and from the left leg of each animal and stored at −80 °C until DNA extraction. The sample collection in clean body areas (avoiding wounds, blood spots, . . .) assured the absence of contamination of hair with other type of cells, and the tweezers were always carefully disinfected with 1% SuredisVT® (Diversey, BCN, Spain) between samples. Blood was obtained by retroorbital bleeding just before challenge (day 0) and final timepoint (five weeks post-infection). The ears, retromandibular draining lymph nodes (DLNs) and spleen from each mouse were taken after euthanasia for parasite load determination by limiting dilution.

2.3. Serological analysis

ELISA method was used for the semi-quantitative detection of specific IgG, IgG1 and IgG2a antibodies against the total soluble antigen (SLA) of *L. major* at the two points of analysis. First, 96 wells ELISA plates were coated with 2 μg/ml of SLA antigen (obtained from *L. major* promastigotes clone V1 as described in Iborra et al. (2005). Sera were (1:100) diluted and tested in duplicate. Peroxidase-conjugated IgG (1:1000), IgG1 (1:1000) and IgG2a (1:500) purchased from Nordic Immunological Laboratories (Tilburg, The Netherlands) were used as secondary antibodies. Orto-phenylenediamine-dihydrochloride (Dako A/S, Glostrup, Denmark) was used as a peroxidase substrate. After 15 min, the reaction was stopped by the addition of 50 μl of 1 M H_2SO_4, and the absorbance was read at 450 nm. For each timepoint, results were expressed as the mean O.D ± SD of four sera assayed individually.

2.4. Parasite load determination by limiting dilution

The number of parasites was determined in ear, DLN and spleen by a limiting dilution assay (Buffet et al., 1995). Briefly, ears were recovered from infected BALB/c mice. The ventral and dorsal sheets of the infected ears were separated and deposited in Dulbecco's modified Eagle medium containing Liberase CI enzyme blend (50 μg/ml). After 2 h of incubation at 37 °C, the tissues were cut into small pieces, homogenized and filtered using a cell strainer (70 μm pore size). The homogenized tissue was serially diluted in a 96-wellflat-bottomed microtiter plate containing Schneider's medium plus 20% FCS in triplicates. The number of viable parasites was determined from the highest dilution at which promastigotes could be grown up to 7 days of incubation at 26 °C. For the determination of the number of parasites in the retromandibular DLNs and spleens, tissue samples were recovered, mechanically dissociated and then filtered and serially diluted as above. In all cases parasite load is expressed as the number of parasites in the whole organ.

2.5. DNA extraction and PCR analysis of hair samples

Each one of the hair samples were put into screw-cap sterile microtubes and incubated in 250 μl of lysis buffer (10 Mm TrisCl, 0.1 M EDTA, 0.5% SDS + 20 μg/ml pancreatic RNase – Sigma–Aldrich, St. Louis, MO, USA) for two hours at 37 °C. Proteinase K was added to a final concentration of 150 mg/ml and incubated at 56 °C overnight. Then, 200 μl of supernatant were collected and stored at −80 °C. DNA was obtained using the UltraCleanBloodSpin kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The detection and quantification of *L. major* kDNA were performed by Real time PCR, as previously described (Francino et al., 2006; Belinchón-Lorenzo et al., 2013). PCR reactions were performed in a Step One Plus Real time PCR System (Applied Biosystems Laboratories, Foster City, CA, USA) in a final volume of 25 μl (5 μl of DNA + 20 μl of Reaction Mix), containing 20 μM of each primer (Leish 1: 5′-AACTTTTCTGGTCCTCCGGGTAG-3′ and Leish2: 5′-ACCCCCAGTTTCCCGCC-3′), 10 μM of TaqMan Probe (FAM-5′-AAAAATGGGTGCAGAAAT-3′-non fluorescent quencher-MGB), and the Maxima Probe/ROX qPCR Master Mix (Fermentas GmbH Laboratories, St. Leon-Rot, Germany). The thermal cycling profile used was 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Positive (DNA extracted from 55,000 *L. infantum* promastigotes MCAN/ES/1996/BCN150, zymodeme MON-1) and negative (DNA extracted from a healthy dog blood sample) controls were included in the assay. To detect and quantify *Leishmania* kDNA in the hair, a standard curve was carried out (Rutledge and Côte, 2003). To perform this curve the DNA from six quantities of *L. infantum* parasites (MCAN/ES/1996/BCN150, zymodeme MON-1) ranging from 15,360 to 15 (dilution factor ×4) was extracted and analyzed together with the hair samples in triplicate. Samples were considered positive when the Ct of each one was lower than 35 (Applied Biosystems Application Note, 2006) and when the amplification was detected in all the replicates.

3. Results

The experimental infection led to the typical time course generation of dermal lesions detected in the ear at the inoculation

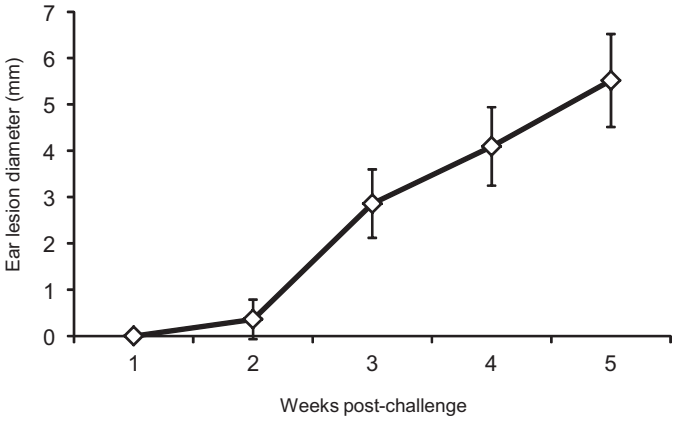

Fig. 1. Course of *Leishmania major* infection in BALB/c mice. Values represent the mean lesion diameter ± SD.

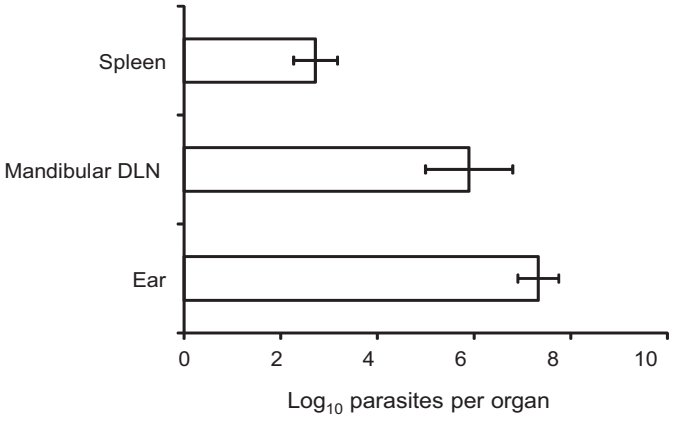

Fig. 2. Parasite burden after challenge. Five weeks after infection mice were euthanized and parasites in ear dermis, retromandibular draining lymph node (DLN) and in spleen were individually determined by a limit dilution assay. Results are expressed as the mean ± SD of eight ears, four spleens and the mandibular draining lymph nodes from four mice.

site (Fig. 1) characterized by progressive erythema, swelling and ulceration. At week 5th post challenge the lesions reached a mean diameter of about 5 mm and animals were euthanized. The isolation and quantification of the parasite load done by limiting dilution (Fig. 2) indicated that the highest amount of amastigotes was present in the skin of the ear (mean of 2×10^7 parasites per lesion). Live parasites were also detected in the retromandibular DLN and in spleen although in lower amounts (mean of 1×10^6 parasites and mean of 520 parasites per organ, respectively). Uncontrolled *L. major* replication was correlated with the induction of a non-protective Th2 response, evidenced by the presence of dominant IgG1 antibodies against parasite SLA antigen at week 5th post infection, reaching values between 2 and 3 times higher than IgG2a at the same timepoint (data not shown).

The implementation of the Real time PCR technique to hair samples in the course of the infection revealed, as reflected in Table 1, that parasitic kDNA was detected in the hair of all the infected mice. The incorporation of kDNA in the hair was progressive, being present close to the site of inoculation (ear) from the first week post challenge, and in healthy skin of a far zone (leg) starting from the third week. At the final time point (5th weeks post-infection) the kDNA was detected in the hair from both the injured ear and the healthy limbs of all the mice.

The quantification also allowed distinguishing various parasitic kDNA loads included over time in hairs from both ear and leg dermal areas (Table 1). In the ear hair a progressive increase of kDNA from the first week (mean of 0.13 estimated parasites) to the 5th week (mean of 205.77 estimated parasites) was observed. At the final timepoint all hair samples were PCR positive, being the kDNA load in ear more than 20 times higher than that found in healthy legs.

4. Discussion

It has been shown that hair is involved in the recognition, isolation and removal of foreign DNA sequences since parasite kDNA can be sequestered in the hair of *L. infantum* naturally infected dogs (Belinchón-Lorenzo et al., 2013). We have tested whether *L. major* kDNA could be also detected in the hair of experimentally infected mice. The *L. major-Mus musculus* cutaneous leishmaniasis model was used to examine, quantify and monitor the chronology of the DNA appearance in hair of both the inoculation site and in a distal body area, by Real time PCR. For this purpose, mice were intradermally inoculated in both ears with *L. major* V1 strain at a dose of 1000 metacyclic promastigote forms, that mimics a natural infection by *Phlebotomus papatasi* but without salivary gland sonicate (Belkaid et al., 1998).

The monitoring of experimental *L. major*-mice infection model is usually based on the employment of immunological techniques (Iborra et al., 2003; Iniesta et al., 2008) and also on parasite detection through microscope together with molecular techniques like conventional and Real time PCR (Buffet et al., 1995; Nicolas et al., 2002). The use of hair for PCR analyses has many advantages over other methods. It should be noticed that while the frequently blood sampling during infection could interfere with the experimental assay, the collection of hair samples is non-invasive and they are easy to store (Stolker et al., 2007).

Our results showed the presence of *L. major* kDNA in the hair close to the inoculation site as well as in body areas far from the infection site. The swelling size measured, previously described for this infection model (Ramírez et al., 2010), seems to be directly correlated to the detection of parasite DNA in ears, which progres-

Table 1
Real time PCR results of the analysis of hair from ear and leg of Balb/C mice infected by *Leishmania major*.

Timepoint post-challenge	Real time PCR results			
	Ear hair		Leg hair	
	No. positives (Ct < 35)	Mean of estimated no. of parasites[a]	No. positives (Ct < 35)	Mean of estimated no. of parasites[a]
Week 0	0	0	0	0
Week 1	1	0.13	0	0
Week 2	3	41.01	0	0
Week 3	4	102.93	3	2.04
Week 4	4	60.32	3	8.30
Week 5	4	205.77	4	8.48

[a] Average quantity of the positive samples. Each parasite contains about of 10,000–20,000 copies of the minicircle kDNA

sively increased along the experimental assay. Therefore, it is most likely that the detection and the increase in parasite DNA could be a manifestation of the infection rate in the ear's injured skin.

In view of the data obtained we propose two potential mechanisms for the origin of foreign kDNA in the hair of infected animals. One of them would be the direct incorporation of the parasite DNA among keratinocytes of skin and hair at the site of inoculation. Along with this suggestion is the observation that *L. major* amastigotes are detected in all epidermal layers and even in hair follicles of patients with cutaneous leishmaniosis (Karram et al., 2012). This phenomenon is known as "transepidermal elimination", whose mechanism is still largely unknown. Once parasite DNA is incorporated among keratinocytes it could be passed upward into the formed hair during the keratinization process. It cannot be excluded, on the other hand, that kDNA is incorporated into the hair by migration from the bloodstream, since the detection of DNA from other pathogens in blood has been reported (Corless et al., 2001; Kami et al., 2001; Kim et al., 2008; Farias et al., 2010). We think that this last mechanism would explain the presence of *L. major* kDNA in the hair of non-injured skin areas.

In summary, we consider that these data add some insights to the understanding of the physiology of hair and the involvement of this highly specialized tissue in the excretion of foreign organic toxic metabolites, and therefore in the sequestration and removal of parasite DNA. Based on the results presented we believe that hair samples could have significant scientific value for a reliable and high sensitive diagnosis of an ongoing *L. major* infection course. It provides data about the chronology of the parasite spreading at the injured and uninjured sites that can be correlated in time with the rest of analysis conventionally used for monitoring the infection and disease progression.

5. Conclusions

In conclusion, these findings, together with those previously described regarding the detection of parasitic DNA in *L. infantum* naturally infected dogs (Belinchón-Lorenzo et al., 2013) or in wild reservoirs (unpublished results), reveals a largely unknown novel mechanism of hair physiology and represents a new diagnostic method applicable to the study of different types of both natural and experimental leishmaniosis. Since the mice model is usually employed for testing new treatments as well as vaccine strategies, the detection of foreign DNA in hair could improve and facilitate the analysis of the effectiveness of anti-*Leishmania* vaccines and drugs.

In addition, we consider that the methodology described in this work may be useful to detect DNA molecules not only in cutaneous and mucocutaneous forms of leishmaniosis but also in diseases coursing with skin manifestations in which transepithelial elimination of microorganisms has been reported (Wood et al., 1976; Welykyj et al., 1991; Goette and Odom, 1986). If so, hair samples could be used to diagnose and monitor the progressive spreading of infection and the manifestations of several infectious diseases.

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