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In situ cytokines (IL-4, IL-10, IL-12, IFN-γ) and chemokines (MCP-1, MIP-1α) gene expression in human *Leishmania* (*Leishmania*) *mexicana* infection

Guillermo Valencia-Pacheco [a,1], Elsy Nalleli Loría-Cervera [a,1], Erika Ivett Sosa-Bibiano [a,1], Elsy B. Canché-Pool [a,1], Alberto Vargas-Gonzalez [a,1], Peter C. Melby [b,1,2], Fernando J. Andrade-Narvaez [a,*,1]

[a] *Laboratorio de Inmunología, Centro de Investigaciones Regionales "Dr. Hideyo Noguchi", Universidad Autónoma de Yucatán, Mexico*
[b] *Department of Medicine, Microbiology and Immunology, University of Texas Health Science Center at San Antonio, San Antonio, TX, USA*

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ABSTRACT

Crucial to the defense against *Leishmania* is the ability of the host to mount a cell-mediated immune response capable of controlling and/or eliminating the parasite. The composition of the cell populations recruited in the early phase of the infection seems to be essential for defining the infection outcomes. The signals that initiate and regulate the early immune response and local accumulation of cell subsets in the skin are poorly understood. We previously studied the *in situ* expression of cytokine genes in patients with localized cutaneous leishmaniasis (LCL) caused by *Leishmania* (*Leishmania*) *mexicana*. In the present study we examined *in situ* cytokine (IL-4, IL-10, IL-12, IFN-γ) and chemokine (MCP-1, MIP-1α) gene expression in *L.* (*L.*) *mexicana* active LCL lesions, and in the delayed type hypersensitivity (DTH) skin response to *Leishmania* antigen in subjects with healed lesion and subclinical infection. Data regarding cytokines were similar to previous studies in patients with active LCL. There were no significant differences in the profile of cytokine and chemokine gene expression in DTH from subjects with healed or subclinical infection. IL-12 gene expression detected in both groups was similar. High expression of MCP-1 was detected in all patients with active LCL. There was no difference in the level of MCP-1 expression between the healed lesion and the subclinical infection groups ($p = 0.876$). IL-12 and MCP-1 in the absence of IFN-γ might be playing a crucial role in infection outcomes at skin level.

1. Introduction

The Leishmaniases are a group of vector borne diseases caused by infection with protozoan parasites of the *Leishmania* (Kinetoplastida: Trypanosomatidae) genus. They are still a major worldwide public health problem considering they are endemic in 98 countries or territories with an estimated incidence of 0.2–0.4 million of visceral leishmaniasis (VL) cases and 0.7–1.2 million of cutaneous leishmaniasis (CL) cases each year [1,2]. Nevertheless, they remain largely ignored in discussions of tropical disease priorities and they are one of the world most neglected tropical diseases [3,4]. In addition, they are not preventable and uncontrolled diseases; their epidemiological profile is shifting towards an increasing prevalence, and therefore novel instruments and approaches to reach their control are urgently necessary.

Localized cutaneous leishmaniasis (LCL) caused by *Leishmania* (*Leishmania*) *mexicana* known in the southeast of Mexico as "chiclero's ulcer" was first described by Seidelin in 1912 [5]. The sylvatic region of the Yucatan Peninsula is an endemic area of LCL [6–8]. The annual incidence of LCL reported in the State of Campeche is 5.08 × 1000 inhabitants, i.e. 0.5% [9], whereas the rate of subclinical infection is 19.8% [10].

Leishmania is transmitted to humans by sand flies displaying different infection outcomes, ranging from subclinical or asymptomatic infection to disfiguring forms of cutaneous and mucosal leishmaniasis or the potentially fatal visceral disease [11].

* Corresponding author. Address: Universidad Autónoma de Yucatán, Centro de Investigaciones Regionales "Dr. Hideyo Noguchi", Laboratorio de Inmunología, Ave. Itzáes No. 490x59-A, Mérida, Yucatán, Mexico. Tel.: +52 999 924 6412x1156; fax: +52 999 923 6120.

E-mail addresses: vpacheco@uady.mx (G. Valencia-Pacheco), nalleli.cervera@uady.mx (E.N. Loría-Cervera), erikasosa@uady.mx (E.I. Sosa-Bibiano), elsy.canche@uady.mx (E.B. Canché-Pool), gonzalez@uady.mx (A. Vargas-Gonzalez), pcmelby@utmb.edu (P.C. Melby), anarvaez@uady.mx (F.J. Andrade-Narvaez).

[1] All authors contributed equally to this work.

[2] Current address: Center for Tropical Diseases, and Departments of Internal Medicine, Microbiology and Immunology, and Pathology, University of Texas Medical Branch, Galveston, TX, USA.

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Subclinical infection and self-healing cutaneous leishmaniasis are by far the most common outcomes of *Leishmania* infection. Field studies have revealed that subclinical infection may occur in endemic areas, since positive delayed-type hypersensitivity (DTH) response to *Leishmania* antigen is detected in individuals without a clinical past history of skin ulcer suggestive of leishmaniasis [12–14]. The Montenegro skin test (MST), a test of *in vivo* parasite-specific DTH reaction, is usually used as an indicator of the prevalence of subclinical infection in endemically exposed human population.

The polymorphic outcome of *Leishmania* infection has been considered to depend on several factors such as the virulence of the infecting parasite strain, immunoregulatory effects of the sand fly saliva, host's genetic background and immune response [15–18]. It is generally accepted that the nature of the host cellular immune response largely determines the expression of disease following infection with the intracellular protozoans *Leishmania* spp.

Considering that the cellular immune response within the cutaneous lesion must be of primary importance in the outcome of infection, we previously studied the *in situ* expression of cytokine genes in patients with LCL caused by *L.* (*L.*) *mexicana.* In that study, we found that IL-lα, IL-6, IL-10, TGF-β, IFN-γ, and TNF-α mRNAs were abundant in all samples [19]. The expression of TNF-α, IL-lα, IL-10, and TGF-β were significantly increased in chronic compared to early lesions, suggesting a role of these cytokines in the immunopathogenesis of chronic disease. In contrast, we postulated that IL-12 production would be an important part of the host protective tissue response to localized *L.* (*L.*) *mexicana* infection. Also we examined the expression of IL-12 at the site of infection in human LCL to correlate its expression with its counter-regulatory cytokine, IL-10 [20]. We found that IL-10 and IL-12 mRNAs were expressed in most lesions of individuals with active LCL. The quantity of IL-12 mRNA was highly variable but correlated strongly with the level of IFN-y expression. IL-12 expression also paralleled the expression of IL-l0, a potent in vitro suppressor of IL-12 and IFN-γ production. The more chronic, non-healing lesion generally had higher levels of IL-12 mRNA indicating that the expression of this cytokine alone was not sufficient to induce healing. Although the *in situ* production of IL-10 did not appear to block IL-12 expression, IL-10 may still promote disease by direct suppression of macrophage activation.

While the cellular immune response in LCL caused by *L.* (*L.*) *mexicana* has been characterized, there are no studies focused on identifying changes in cytokine expression and their possible association with the outcome of the infection, i. e. in subjects with active lesions, as so in healed lesions, and subclinical infection. Additionally, the signals that initiate and regulate the early immune response and local accumulation of cell subsets in the skin of those patients are poorly understood. Chemokines of the CC family, such as MCP-1 (CCL2) [21] and MIP-1α (CCL3) [22], are attractive candidates for this function because they selectively attract monocytes and certain T-cell subsets.

In the present study we examined *in situ* cytokines (IL-4, IL-10, IL-12, IFN-γ) and chemokines (MCP-1, MIP-1α) gene expression in *L.* (*L.*) *mexicana* active lesions, and in the DTH skin response to *Leishmania* antigen in subjects with healed lesions and subclinical infection.

2. Materials and methods

2.1. Studied population

This study was reviewed and approved by the Bioethics Research Committee of the Universidad Autonoma de Yucatan, in agreement with international ethical guidelines for biomedical research involving human subjects. A written informed consent was obtained from each participant. A total of 26 subjects from the endemic area of LCL in the Yucatan peninsula were included as follows: Group (I) eleven patients with active lesions detected through permanent surveillance and diagnosis confirmed by *L.* (*L.*) *mexicana* identification, [23]; Group (II) nine subjects with healed lesions after receiving treatment with meglumine antimoniate [24]; and Group (III) six persons with subclinical infection (individuals with MST positive, without clinical signs and/or characteristic scars of LCL, residence in endemic area of LCL for a period $\geqslant$2 years considering seasonal transmission, and therefore exposed to the risk of be bitten by infected sand flies) selected from a database of previous epidemiological studies. The skin biopsy was taken from the MST induration site at 48 h after antigen delivery in subjects with either subclinical infection or healed lesion, and from the lesion border of patients with active LCL. The punch biopsy sample was immediately frozen in liquid nitrogen and stored until analysis.

2.2. Isolation of RNA

Each frozen tissue specimen was disrupted and homogenized with a rotorstator homogeniser in the presence of RNA lysis buffer containing guanidinium isothiocyanate. RNA isolation was performed using the RNeasy Mini Kit (QIAGEN, USA) according to the manufacturer's instructions. The RNA concentration was determined in a spectrophotometer at 260/280 nm.

2.3. RT-PCR

Reverse-transcriptase PCR was carried out following a previously described method [21,22]. Briefly, the first-strand DNA of each skin biopsy was synthesized with 0.5 μg of the RNA in Rnase-free dH_2O, 2 μl of 5× RT buffer (250 mM Tris–HCl, 375 mM KCl, 15 mM $MgCl_2$), 1 μl of DTT 0.1 M, 0.5 μl of RNAsin (20 U) (Promega, Madison, WI), 1 μl desoxynucleoside triphosphate (dNTP) mix (containing 5 mM of each of the following: dATP, dGTP, dCTP, dTTP), 1 μl of random hexadesoxynucleotide primers (0.5 μg), and 1 μl (200 U) of Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Gaithersburg, MD). The synthesis was incubated at room temperature for 10 min followed by 3 h at 37 °C and enzymes were inactivated by heating at 95 °C for 10 min. The cDNA was amplified with a PCR mixture containing 5 μl of the transcribed cDNA, 2.5 μl of 10× reaction buffer (500 mM KCl, 100 mM Tris–HCl, 1.5 mM MgCl2), 2 μl of dNTP mix (10 pmol of each dATP, dGTP, dCTP, and dTTP), 0.125 μl (2.5 U) of *Thermus aquaticus* (Taq) DNA polimerase (Boehring Mannheim), 2.5 μl of specific primer for each cytokine studied (IFNγ, Forward: CAGG TCATTCAGATGTAGCG, Reverse: ACAGTTCAGCCATCACTTG-G; IL-4: Forward: CATGTGCCGGCAACTTTGTC, Reverse: AGTGTCCTTCTC AT-GGTGGC; IL-10: Forward: GCCTAACATGCTTCGAGATC, Reverse: TGATGTCT-GGGTCTTGGTTC; IL-12: Forward: CTCCACAT TCCTAC TTCTCC, Reverse: TGG-GTCTATTCCGTTGTGTC; MCP-1: Forward: CAGCATGAAAGTCTCTGCCG, Reverse: TCAAGTCTTCGGAGTT TGGG; MIP1α: Forward: TTGCTGCTGACAC-GCCGACC, Reverse: GACCCAC TCCTCACTGGGGTC; β-actin: Forward: TGAAGTCTGACG TGGAC ATC, Reverse: ACTCGTCATACTCCTGCTTG), and sterile dH_2O free of RNAases to a total volume of 25 μl per reaction. The cDNA was amplified using a thermal cycler (Ericomp) scheduled at 95 °C (30 s) −50 °C (30 s) −72 °C (1.5 min) per cycle for a total of 35 cycles. Care was taken in the preparation of the PCR to prevent any nucleic acid contamination, and a negative control was included in each assay. The PCR product mixture was separated by agarose electrophoresis with ethidium bromide. Oligonucleotide primers for each cytokine were designed from published sequences and synthesized on a DNA synthesizer. Both 5′ primers