



Research note

Interleukin-2 as a marker for detecting asymptomatic individuals in areas where *Leishmania infantum* is endemicA.V. Ibarra-Meneses¹, E. Carrillo^{1,*}, C. Sánchez¹, J. García-Martínez²,
D. López Lacomba², J.V. San Martín³, F. Alves⁴, J. Alvar⁴, J. Moreno¹¹ Instituto de Salud Carlos III, Centro Nacional de Microbiología, WHO Collaborating Centre for Leishmaniasis, Madrid, Spain² Hospital Universitario de Fuenlabrada, Blood Bank and Haemotherapy Department, Laboratory Medicine, Fuenlabrada, Madrid, Spain³ Hospital Universitario de Fuenlabrada, Department of Infectious Diseases, Internal Medicine, Fuenlabrada, Madrid, Spain⁴ Visceral Leishmaniasis Program, Drugs for Neglected Diseases Initiative (DNDi), Geneva, Switzerland

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ABSTRACT

No field method exists for identifying asymptomatic individuals in areas where *Leishmania infantum* is endemic. This work reports that, 24 h after stimulating whole blood with soluble *Leishmania* antigen (SLA), plasma interferon- γ (IFN- γ) and interleukin-2 (IL-2) become significantly elevated in samples from asymptomatic individuals ($n = 47$) compared with those from negative controls ($n = 50$), all of them recruited from a blood bank. When compared with the reference test SLA-lymphoproliferative assay, IL-2 appears as a new, 100% sensitive and specific marker for asymptomatic individuals with a positive cellular response (compared with 100% and 84.78%, respectively, for IFN- γ). Further studies in other transmission areas and in other cohorts of exposed people need to be performed to confirm these results. Once validated, IFN- γ and IL-2 levels in SLA-stimulated whole blood could be reliably used in the field to estimate the prevalence of those asymptomatic individuals with *Leishmania*-specific cellular immune responses. **A.V. Ibarra-Meneses, CMI 2016;22:739.e1–739.e4**

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Introduction

Asymptomatic individuals must be identified if the true prevalence of *Leishmania infantum* infection in endemic areas is to be known (different studies report 25%–80%) [1] and if the potential for such persons to act as disease reservoirs is to be understood.

The *Leishmania* skin test can be used in the field to study the prevalence of infection, but its side effects, and suspect quality in manufacturing, have seen it banned in some countries [2,3]. The good agreement between *Leishmania* skin test reactivity and the results of the *in vitro* peripheral blood mononuclear cell proliferation assay (CPA) involving soluble *Leishmania* antigen (SLA), highlights the latter's value as a detector of immune reactivity [4]. However, CPA is laborious and time-consuming; a simpler field test for identifying asymptomatic persons is required.

Commercial whole blood interferon- γ (IFN- γ) release assays (IGRA) for assessing cell-mediated immunity against cytomegalovirus and *Mycobacterium tuberculosis* may provide the basis for a field test for detecting cellular responses against *Leishmania* spp. [5,6]. The present work investigates the SLA stimulation of whole blood from cured persons, those with active disease, and asymptomatic individuals, following an outbreak of leishmaniasis caused by *L. infantum* in Fuenlabrada (Madrid, Spain). SLA-stimulated plasma IFN- γ concentrations were recorded, as were those of tumour necrosis factor- α (TNF- α), interleukin-10 (IL-10) and IL-2. The aim was to establish a simple method for detecting asymptomatic persons in areas where *L. infantum* is endemic.

Materials and methods

This study was approved by the Hospital de Fuenlabrada (Madrid) Ethics and Research Committee; all participants gave their consent to be involved. Blood was collected at the hospital blood bank (330 consecutive blood donors) and internal medicine department (36 participants) between January and December 2015.

* Corresponding author. E. Carrillo, Instituto de Salud Carlos III, Centro Nacional de Microbiología, WHO Collaborating Centre for Leishmaniasis, Ctra. Majadahonda-Pozuelo km.2, 28220 Majadahonda (Madrid), Spain.

E-mail address: ecarrillo@isciii.es (E. Carrillo).

All samples were subjected to CPA testing. Peripheral blood mononuclear cells were isolated from whole blood and resuspended in RPMI-1640 supplemented with 10% fetal calf serum, and cultivated with SLA for 5 days [7]. Of the blood bank samples, 47 were CPA-positive (stimulation index ≥ 2.27); the participants providing these samples were therefore asymptomatic and their blood was selected for analysis. Of the 283 CPA-negative samples, 50 participants were randomly selected as negative controls for analysis (simple random sample). Eighteen of the 36 samples collected at the medical department were from patients with active visceral leishmaniasis (VL), and the other 18 were from cured VL patients who attended the hospital 3 months after amphotericin B treatment as outpatients for a medical check up (Table 1).

To search for markers of asymptomatic infection, whole blood samples were stimulated with SLA as previously described [8,9]. For each sample, an aliquot of blood (500 μ L) was placed on its own in a tube (negative control), and another in a tube containing 10 mg/L SLA, and both were incubated at 37°C for 24 h. They were then centrifuged, the plasma was collected, and their IFN- γ , TNF- α , IL-10 and IL-2 concentrations were determined using the Cytometric Bead Array Human Soluble Protein Flex Set (Becton Dickinson, Franklin Lakes, NJ, USA). Results for each cytokine were expressed as the difference between the SLA-stimulated and control plasma concentrations.

A nested PCR targeting the small subunit ribosomal RNA (SSU rRNA) genes was performed with DNA isolated from 2 mL of blood from the 330 donor participants by acid guanidinium thiocyanate–phenol–chloroform extraction, as described previously [10].

Data were analysed using the Mann–Whitney *U* test. Significance was set at $p \leq 0.05$. The ability of the cytokines to detect asymptomatic participants was determined by calculating the area under the receiver operating characteristic curve (AUC) and the 95% CI.

Results

Stimulation of whole blood with SLA was associated with significantly higher plasma IFN- γ concentrations in patients with cured VL (especially) and asymptomatic participants compared with negative controls (Fig. 1a: median 343.80 pg/mL with 95% CI 156.40–692.80, and 72.85 pg/mL with 95% CI 45.98–110.70, respectively, versus 0 pg/mL). Similar findings were recorded for TNF- α (Fig. 1b: median 64.50 pg/mL with 95% CI 34.29–247.50 and 16.75 pg/mL with 95% CI 9.76–25.30 for cured VL and asymptomatic individuals, respectively, and below detectable levels for the control group). The patients with active VL returned the highest IL-10 concentrations; no IL-10 was detected in the plasma of asymptomatic participants (Fig. 1c: median 13.70 pg/mL with 95% CI 5.75–53.40 versus 0 pg/mL). Interleukin-2 was also increased in the SLA-stimulated plasma of the cured VL and (especially)

asymptomatic participants compared with negative controls (Fig. 1d: median 86.55 pg/mL with 95% CI 13.86–168.80 and 171.00 pg/mL with 95% CI 133.40–261.40, respectively, versus 0 pg/mL). Interestingly, no IL-2 was detected in the plasma of patients with active VL. No *Leishmania* DNA was detected in any blood sample from any asymptomatic or negative controls.

In the detection of asymptomatic participants, the AUC for IFN- γ was 0.99 (95% CI 0.98–1.00; $p < 0.0001$), sensitivity was 100% (95% CI 0.90–1.00), specificity was 84.78% (95% CI 0.71–0.94), and the cut-off was 27.77 pg/mL (Fig. 1e). Importantly, the AUC for IL-2 was 1.00 (95% CI 1.00–1.00; $p < 0.0001$), sensitivity was 100% (95% CI 0.90–1.00), specificity was 100% (95% CI 0.92–1.00), and the cut-off was 50.37 pg/mL (Fig. 1f). TNF- α and IL-10 showed AUC of 0.85 (95% CI 0.77–0.94) and 0.52 (95% CI 0.39–0.64), respectively.

The calculated cut-offs show that SLA-induced plasma IFN- γ identified 83% of the asymptomatic participants (39/47). However, SLA-induced plasma IL-2 detected 100% of these individuals (47/47).

Discussion

The present results show that plasma IL-2 in SLA-stimulated whole blood become significantly elevated in samples from cured VL and asymptomatic individuals compared with those from negative controls, and no IL-2 was detected in active VL. Interestingly, IL-2 was able to detect all asymptomatic individuals with *Leishmania*-specific cellular immune responses, whereas IFN- γ detected 83% of them. We have also found that the increased expression of plasma IFN- γ in SLA-stimulated whole blood distinguishes between cured VL/active VL/asymptomatic and uninfected persons living in an area where *L. infantum* is endemic. Similar results were described in the three studies that have evaluated IGRAs in areas where *Leishmania donovani* is endemic [8,11,12]. The present results also show TNF- α production to be increased in SLA-stimulated plasma from cured VL, active VL and asymptomatic individuals, a finding not seen in previous studies involving *L. donovani* asymptomatic subjects [11,12]. However, this increased TNF- α production did not properly discriminate asymptomatic participants. High IL-10 concentrations were also seen in the SLA-stimulated blood of some patients with active VL; in these individuals the only studied T helper type 1 cytokine not detected was IL-2. Although plasma IFN- γ , TNF- α and IL-2 were increased in whole blood samples from the asymptomatic individuals, IL-2 was the most sensitive and specific marker for identifying this subpopulation with positive SLA-lymphoproliferative response; this is the first time this has been described with respect to leishmaniasis. Our group recently described a strong correlation between SLA-stimulated plasma IFN- γ and IL-2 in individuals from the same area who had undergone solid organ transplant (and therefore took immunosuppressants) [9], but the potential for IL-2 and IFN- γ to detect asymptomatic individuals was not evaluated in that work. IL-2 has also been proposed as a marker to distinguish between individuals with active tuberculosis, latent tuberculosis infection and healthy controls following whole blood stimulation with mycobacterial antigens [13–15].

In areas where leishmaniasis is endemic, the history of active VL and treatment would be necessary to assess an asymptomatic individual, because a clear-cut definition for SLA-stimulated plasma IL-2 concentrations could not be established when comparing cured and asymptomatic individuals. But before considering IL-2 as an alternative or adjunct marker to identify asymptomatic individuals, other transmission areas with lower prevalence, or *L. donovani* infection, and other cohorts (malnourished, co-infected with helminths, etc.) of exposed people must be tested. An additional limitation of this study is the need for an independent

Table 1
Clinical characteristics of the study population

	Cured VL n = 18	Asymptomatic individuals n = 47	Active VL n = 18	Negative controls n = 50
Sex:				
Female	7	11	7	20
Male	11	36	11	30
Age, median (years)	41.5	42.0	42.0	42.0
CPA positive:	18	47	0	0
CPA negative:	0	0	18	50

CPA, cell proliferation assay; VL, visceral leishmaniasis.

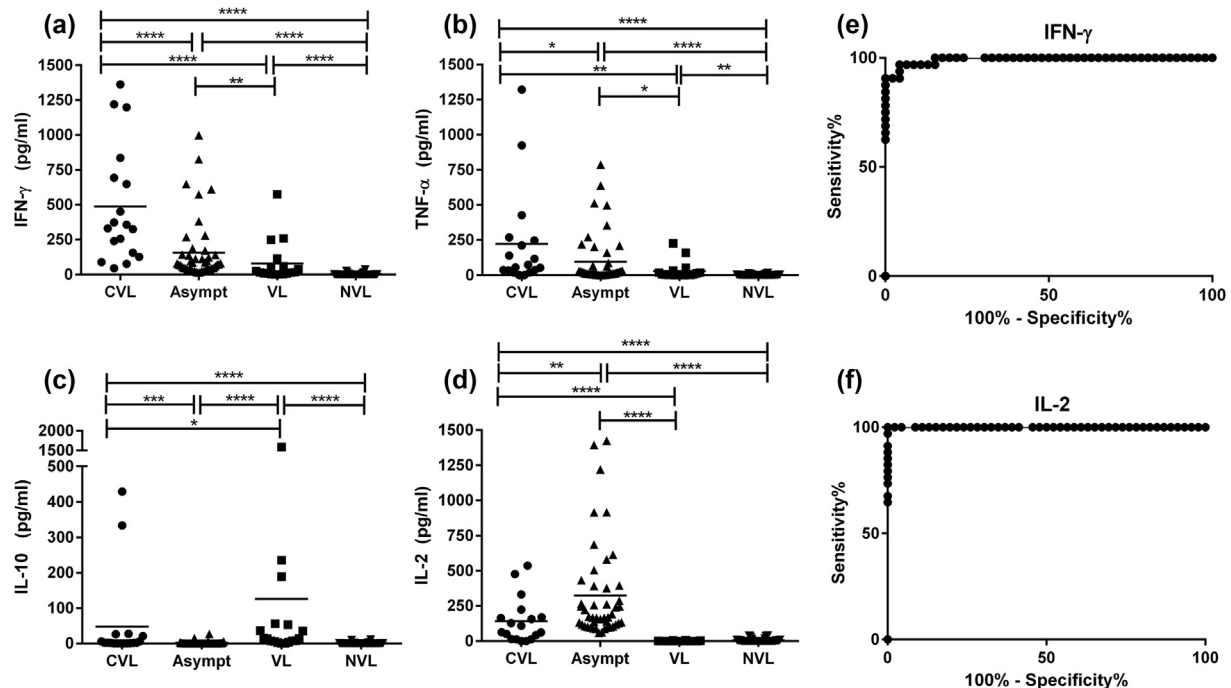


Fig. 1. Cytokine production in whole blood in response to soluble *Leishmania* antigen (SLA) stimulation for 24 h. (a–d) Production of (a) interferon-γ (IFN-γ), (b) tumour necrosis factor-α (TNF-α), (c) interleukin-10 (IL-10) and (d) IL-2 in plasma following 24 h of SLA stimulation of whole blood from patients with cured visceral leishmaniasis (VL) patients (CVL; $n = 18$), asymptomatic participants (Asympt; $n = 47$), patients with active disease (VL; $n = 18$), and negative controls (NVL; $n = 50$). Horizontal bars represent the mean concentration for each cytokine. Data were analysed using the Mann–Whitney U test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. (e,f) Receiver operating characteristic curve analysis to determine the sensitivity and specificity of IFN-γ and IL-2 to detect asymptomatic participants.

validation to confirm if its true sensitivity is between 90% and 100%, and its true specificity lies between 92% and 100%. Furthermore, it would be interesting to see long-term follow-up studies on whether a subgroup of asymptomatic individuals would develop disease and how immunological markers would evolve in such cases. Finally, it is important to highlight that those asymptomatic participants without specific cellular immune response will not be detected with this marker, and molecular tools, such as PCR, should be used in combination to avoid a risk of case under-estimation.

In conclusion, SLA-stimulated plasma IFN-γ and IL-2 concentrations would appear to be a promising new way of detecting *Leishmania* infection in individuals with specific cellular response; it may even have the potential to replace the *Leishmania* skin test. Once validated in other transmission areas and cohorts, IL-2 may be regarded as a new marker for identifying asymptomatic status with better specificity than IFN-γ. A combination of these markers and other molecular techniques like PCR might be used to better define the prevalence of the asymptomatic condition, which would be useful when taking decisions designed to control *L. infantum*. This non-invasive and non-sensitizing simple assay of blood stimulation, in which the cytokine concentration can be measured by ELISA, is easily translatable to the field. In fact, a transversal prospective study to assess the accuracy of this tool (involving a large number of samples and different transmission areas) is underway.

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Transparency declaration

The authors have no conflicts of interest to declare.

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