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PII: S1198-743X(16)30164-1
DOI: 10.1016/j.cmi.2016.05.021
Reference: CMI 612

To appear in: *Clinical Microbiology and Infection*

Received Date: 10 February 2016
Revised Date: 18 May 2016
Accepted Date: 22 May 2016


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IL-2 as a marker for detecting asymptomatic individuals in areas where *Leishmania infantum* is endemic

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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 IFN-γ and IL-2 become significantly elevated in samples from asymptomatic individuals (n=47) compared to those from negative controls (n=50), all them recruited from a blood bank. When compared to the reference test SLA- lymphoproliferative assay, IL-2 appears as a new, 100% sensitive and specific marker for asymptomatic individuals with positive cellular response (compared to 100% and 84.78% for IFN-γ). Further studies in other transmission areas and cohorts of exposed people need to be performed in order 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.
Key words: IL-2, marker, leishmaniasis, IGRA, asymptomatic.

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 the potential of such persons to act as disease reservoirs is to be understood.

The *Leishmania* skin test (LST) 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 LST reactivity and the results of the *in vitro* peripheral blood mononucleocyte (PBMC) 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 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 TNF-α, IL-10 and IL-2. The aim was to establish a simple method for detecting asymptomatic persons in areas where *L. infantum* is endemic.
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 subjects) between January and December 2015. All samples were subjected to CPA testing. PBMCs were isolated from whole blood and resuspended in RPMI supplemented with 10% foetal 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 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), while the other 18 were from cured VL patients who went 3 months after amphotericin B treatment to the hospital as outpatients for a medical checkup (Table 1).

Table 1. Clinical characteristics of the study population.

<table>
<thead>
<tr>
<th></th>
<th>Cured VL n=18</th>
<th>Asymptomatic individuals n=47</th>
<th>Active VL n=18</th>
<th>Negative controls n=50</th>
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<tbody>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Female</td>
<td>7</td>
<td>11</td>
<td>7</td>
<td>20</td>
</tr>
<tr>
<td>Male</td>
<td>11</td>
<td>36</td>
<td>11</td>
<td>30</td>
</tr>
<tr>
<td>Age, median (years)</td>
<td>41.5</td>
<td>42.0</td>
<td>42.0</td>
<td>42.0</td>
</tr>
<tr>
<td>CPA positive:</td>
<td>18</td>
<td>47</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CPA negative:</td>
<td>0</td>
<td>0</td>
<td>18</td>
<td>50</td>
</tr>
</tbody>
</table>

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 μl) was placed on its own in a tube (negative control), and another in a tube
containing 10 µg/ml SLA, and both were incubated at 37ºC for 24 h. They were then centrifuged, the plasma collected, and their IFN-γ, TNF-α, IL-10 and IL-2 concentrations determined using the Cytometric Bead Array Human Soluble Protein Flex Set (Becton Dickinson, BD). 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-SSUrRNA genes of was performed with DNA isolated from 2ml of blood of 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≤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% confidence interval (95%CI).

Results

Stimulation of whole blood with SLA was associated with significantly higher plasma IFN-γ concentrations in cured VL (especially) and asymptomatic participants compared to negative controls (Fig. 1A: median 343.80 pg/ml with CI95% 156.40-692.80, and 72.85 pg/ml with CI95% 45.98-110.70, respectively, vs. 0 pg/ml). Similar findings were recorded for TNF-α (Fig.1B: median 64.50 pg/ml with CI95% 34.29-247.50 and 16.75 pg/ml with CI95% 9.76-25.30 for cured VL and asymptomatic individuals, respectively, and below detectable levels for the control group). The active VL patients 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 CI95% 5.75-53.40 vs. 0 pg/ml). IL-2 was also increased in the SLA-stimulated plasma of the cured VL and (especially) asymptomatic participants compared to negative controls (Fig. 1D: median 86.55 pg/ml with CI95% 13.86-168.80 and 171.00 pg/ml with CI95% 133.40-261.40, respectively, vs. 0 pg/ml). Interestingly, no IL-2 was detected in the plasma of active VL patients. 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 (CI95% 0.98-1.00; p<0.0001), sensitivity was 100% (CI95% 0.90-1.00), specificity 84.78% (CI95% 0.71-0.94), and the cut-off 27.77 pg/ml (Fig. 1E). Importantly, the AUC for IL-2 was 1.00 (CI95% 1.00-1.00; p<0.0001), sensitivity was 100% (CI95% 0.90-1.00), specificity was 100% (CI95% 0.92-1.00), and the cut-off 50.37 pg/ml (Fig. 1F). TNF-α and IL-10 showed an AUC of 0.85 (CI95% 0.77-0.94) and 0.52 (CI95% 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 to 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, while IFN-γ detected the 83% of them. We have
also found that the increased expression of plasma IFN-\(\gamma\) 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 \(L.\) \(donovani\) is endemic [8, 11, 12]. The present results also show TNF-\(\alpha\) 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-\(\alpha\) production did not properly discriminate asymptomatic participants.

High IL-10 concentrations were also seen in the SLA-stimulated blood of some active VL patients; in these individuals the only studied Th1 cytokine not detected was IL-2. Although plasma IFN-\(\gamma\), TNF-\(\alpha\) 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-\(\gamma\) and IL-2 in individuals from the same area who had undergone solid organ transplant (and therefore took immunosuppressants) [9], but the potential of IL-2 and IFN-\(\gamma\) 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, LTBI, and healthy controls following whole blood stimulation with mycobacterial antigens [13-15].

In endemic areas of leishmaniasis, the history of active VL and treatment would be necessary to assess an asymptomatic individual, because a clear-cut for SLA-stimulated plasma IL-2 concentrations couldn’t 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, coinfected with helmints, etc) of exposed people must to be tested. An additional limitation of this study is the need of an independent validation in order 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 be not detected with this marker, and molecular tools, as PCR, should be used in combination to avoid a risk of case underestimation.

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 LST. 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 assay 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.
Funding

We gratefully acknowledge the financial support of the Drugs for Neglected Diseases Initiative (DNDi) by the Department for International Development (DFID), UK and the Swiss Agency for Development and Cooperation (SDC), Switzerland; and of the Instituto de Salud Carlos III via the Tropical Diseases Research Network (RICET RD12/0018/0003 and RD12/0018/0008) (URL) (www.ricet.es/en/) and via the ISCIII-AES project "Impact of a leishmaniasis outbreak in the southwest of Madrid in the immunosuppressed population" (PI13/00440). EC was supported by a research contract funded via VII PN I+D+I 2013-2016 and FEDER Funds (RICET RD12/0018/0003).

Support

This study was approved by the Human Research Ethics Committee of the Hospital de Fuenlabrada (APR 12-65 and APR14-64).

Acknowledgments

We thank the staff of the Blood Bank Blood and Haemotherapy Dept. of the Hospital Universitario de Fuenlabrada, Madrid, Spain, for their assistance in the collection of blood samples.

Transparency declaration

The authors have no conflicts of interest to declare.

References


Figure 1. Cytokine production in whole blood in response to SLA stimulation for 24h. (A-D) Production of IFN-\(\gamma\) (A), TNF-\(\alpha\) (B), IL-10 (C) and IL-2 (D) in plasma
following 24 h SLA stimulation of whole blood from cured 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 ≤ 0.05, **p ≤ 0.01,
***p ≤ 0.001, ****p ≤ 0.0001. (E-F) ROC analysis to determine the sensitivity and
specificity of IFN-γ and IL-2 to detect asymptomatic participants.