



# Evaluation of Immunity Responses Against Excretory-Secretory and Lysate Antigens Purified from *Leishmania major*

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## Abstract

**Introduction:** Leishmaniasis is one of the 10 most important infectious diseases in the world, including the three diseases for which the development of vaccines, drugs, and insecticides is difficult to tackle. Despite over a century of efforts made in the field, no suitable vaccine, medicine, or insecticide has been introduced for the disease to date. In this survey, we intended to evaluate the immunity responses against Excretory-Secretory and Lysate Antigens purified from *Leishmania major*.

**Methods:** A sufficient amount of *L. major* [MRHO/IR/75/ER] was first cultured in Novy-MacNeal-Nicolle (NNN) and RPMI 1640 media in the *in vitro* environment. Once peripheral blood mononuclear cells (PBMCs) were prepared, they were cultured with antigens in a 5% CO<sub>2</sub> incubator, and their supernatant was then measured using the interleukin-4 (IL4) and interferon- $\gamma$  (INF- $\gamma$ ) the enzyme-linked immunosorbent assay (ELISA) kit.

**Results:** An average concentration of IL4 and INF- $\gamma$  of the cell culture containing phytohaemagglutinin showed a significant difference between the average concentration of these two cytokines in the cell culture of all antigens ( $P < 0.05$ ), but it indicated insignificant difference between the antigens ( $P > 0.05$ ).

**Conclusion:** Since the larger the immunogenic and protective components are exposed to the human immune system, the more concrete the results will be, investigations should thus be conducted at the cellular and even molecular levels. Moreover, the antigen used should be a conjugation of adjuvants, immunogens, and proliferators of macrophages to provide sufficient stimulation of the immune system against the *Leishmania* parasite.

**Keywords:** Excretory-secretory and lysate antigens, Immunity response, *Leishmania major*

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## Introduction

Leishmaniasis is a global health problem and the seventh forgotten tropical infectious parasitic disease, including a set of clinical symptoms in the cutaneous, visceral, cutaneous-mucosal, and diffuse cutaneous forms. It is caused by a flagellated protozoan that is transmitted to humans by a mosquito bite. The disease is endemic in 102 countries of the world, and 90% of its visceral form is prevalent in six countries, namely, Bangladesh, Ethiopia, Brazil, India, Sudan, and South Sudan, whereas 75% of its cutaneous form appears in 10 countries of the world, including Iran. It impinges on a total of 350 million people in the world, and its burden is reported to be 3.3 million (1-6). Drug resistance pertinent to parasitic diseases, including leishmaniasis is considered one of the most important health concerns of researchers as the management of diseases is interconnected worldwide, and it is too complex to turn the efforts for its control somehow futile. Therefore, there is a threat of worsening

the situation, which shows the need for medicine and vaccines as an urgent and first-rate necessity (7-9). Much as antimony derivatives were considered the first line of treatment for the past decades, currently, the most common drugs used against *Leishmania* are pentavalent antimony, paromomycin, liposomal amphotericin B, and oral miltefosine with many adverse effects. As an alternative to antimony, their use is also limited due to the difficulty of management as well as high cost and complications (10-13). The emergence of new resistances and failure in the treatment have caused urgency in preparing vaccines and drugs for leishmaniasis. Comprehensive studies are, therefore, being conducted in the field to prepare effective vaccines and drugs around the globe (14-16). Extensive research in vaccine design and drug development has provided new insights into *Leishmania* infection and is ongoing in all areas. This study aimed to evaluate the immunity responses against excretory-secretory and lysate antigens purified from



*Leishmania major*.

## Materials and Methods

### Parasite Culture and Preparation of Lysed Antigens (Lysate *Leishmania* Antigen)

*Leishmania major* [MRHO/IR/75/ER] parasite was propagated in Novy-MacNeal-Nicolle (NNN) medium and then transferred to enriched RPMI 1640 medium for mass propagation. At this stage, the parasite was cultured in the enriched medium, the fourth passage was stored in the stationary phase, and this continued until the preparation of five billion leptomonads of the parasite. Then, using cold phosphate-buffered saline (PBS) and pH=7.5, all the cultured parasites in one bowl were washed three times. The promastigotes were then placed in PBS containing Triton X-100 1%, and after 6 times freezing and thawing at 4 °C, they were kept at -70 °C until the next steps (17-19).

### Parasite Culture and Preparation of Excretory-Secretory Antigens (Excretory-Secretory Antigen)

To prepare excretory-secretory antigens of *L. major* parasite [MRHO/IR/75/ER], complete (enriched) RPMI 1640 single-phase medium parasites of the fourth passage were separated in the stationary phase using a centrifuge and at a speed of 3000 g for 15 minutes. The supernatant solution was discarded and the parasites (sediment) were transferred to RPMI 1640 medium without antibiotics and faecal calf serum (FCS) after washing three times (with RPMI 1640 medium without FCS and antibiotics) with the same speed and duration. This medium was used only for keeping the parasite and ESA. To isolate ESA from the above environments at different times (0, 6, 12, 24, 48, and 72 hours), sampling was done using a centrifuge with a speed of 3000 g for 15 minutes. A 0.22-micron filter was used to make the supernatant solution free from parasites, and the resulting liquid was kept at -70°C until the experiment was performed (20,21).

### Measuring the Level of Excretory-Secretory Antigen and Lysate *Leishmania* Antigen Proteins Via Bradford Method

This method is faster, more accurate, and more sensitive compared to other methods because it measures the protein in micrograms; moreover, the basis of this staining is Coomassie blue G-250 substance, which can be read with the help of a spectrophotometer and with visible light. One of the advantages of the Bradford method is that Coomassie blue binds only to the peptide bands and creates color, and its maximum light absorption changes from 465 nm before the combination to 595 nm after being combined with the protein (22).

### Isolation of Peripheral Blood Mononuclear Cells

After obtaining the necessary permission, blood samples

(peripheral) were taken from 90 people who did not show a history of AIDS, and peripheral blood mononuclear cells (PBMCs) were isolated. The blood sample was then diluted to an equal ratio with RPMI 1640 medium and carefully added with Ficoll to the centrifuge tube in a ratio of 7 to 3 mL. First, Ficoll and then blood was slowly added to it so that it did not mix, and it was centrifuged in the centrifuge at 3000 g for half an hour. The PBMC layer of all the tubes related to one sample was then collected in a centrifuge tube, and RPMI 1640 was added to it three times at 600×g for 8 minutes. For the second time, RPMI 1640 containing 10% FCS was added, and after centrifugation at 800×g for 8 minutes, the isolated PBMC was ready for performing the experiment in the next steps (23,24).

### Determining the Percentage of Live Cells

To determine the percentage of live cells, a volume of lymphocyte suspension and a volume of Trypan blue were prepared, a drop of the mixture was placed on a normal slide, and the number of stained (dead) and unstained cells was counted up to 200. This operation was performed for a maximum period of 3 minutes. The following relationship exhibits the percentage of living cells:

$$100 \times \frac{\text{the number of dead cells counted} + \text{the number of living cells counted}}{\text{the number of dead cells counted}}$$

Living cells counted = percentage of living cells

Cellular immune responses against excretory-secretory antigen (ESA) and lysate *Leishmania* antigen (LLA) of the *Leishmania* parasite purified from *L. major* species [MRHO/IR/75/ER] were evaluated in an *in vitro* environment. After the isolation of PBMCs, these cells were cultured in a Co2-containing incubator:

1. Peripheral blood mononuclear cells + phytohaemagglutinin (PBMC + PHA).
2. Peripheral blood mononuclear cells + excretion-secretion antigens (PBMC + ESA).
3. Peripheral blood mononuclear cells + lysed antigens (PBMC + LLA).
4. Peripheral blood mononuclear cells + excreted-secreted and lysed antigens (PBMC + ESA + LLA)

After incubating and establishing the culture conditions of PBMCs, each supernatant was frozen separately after centrifugation at -70 °C to be used in the next steps. In the last step, the frozen supernatant was brought to room temperature, and using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Biosource Europe S.A, Nivelles, Belgium) and according to the instructions of the kit, the concentration of Interleukin-4 (IL-4) and interferon-γ (INF-γ) in the range of 429 nm was read and recorded in pgr/mL (25,26).

**Results**

The results of the evaluation of excretory-secretory and lysed *Leishmania* parasite antigens in the *in vitro* environment are as follows:

The average concentration of IL-4 secretion in the PBMCs supernatants was stimulated with 32.12 ESA, with 42.16 LLA, with ESA + 38 LLA, and with 98.8 PHA (Table 1 and Figure 1).

The average concentration of INF-γ secretion in the supernatants of PBMCs was stimulated with 185 ± 123.8 ESA, with 132.3 ± 83.8 LLA, with 208.6 ± 93.3 ESA + LLA, and with 883.9 ± 323.1 PHA (Table 2 and Figure 2).

**Discussion**

Cutaneous leishmaniasis is an infection caused by a flagellated protozoan of the genus *Leishmania*, transmitted to humans through mosquito bite and initiates with a small nodule, the most important symptom of which is a slow-healing wound. The most common site of the lesions is in the open areas of the body, especially hands and feet. The disease is prevalent in many countries in the tropical and subtropical regions of the world in various parts of Asia such as China, Syria, Saudi Arabia, Iraq, Iran, Palestine, the Caucasus and South-Eastern Russia, Pakistan, Afghanistan, and India,

as well as in countries around the Mediterranean, African countries, and Latin America. In Iran, it is the most important common disease in humans and animals, and up to 26 000 cases of the disease are officially reported annually. The major centers of urban leishmaniasis in Iran are Tehran, Shiraz, Mashhad, Kerman, Neishabor, Isfahan, and Yazd, and the important centers of rural leishmaniasis in Iran are Sarakhs, Lotfabad, Turkman-Sahra, Esfrain in Khorasan, Khuzestan, Qom, Kashan, and Tabas provinces. Furthermore, the important centers in Isfahan are Barkhar, Mimeh, Ardestan, Natanz, Aran-Bidgol, and Kashan (27,28). Studies in the field of human leishmaniasis revealed that INF-γ and IL12 (Th1 profile) play a determining role in protecting against the disease and IL-4 and IL-10 (Th2 profile) in maintaining the disease (29,30). When anti-ESA is present in the environment, the average level of IL-4 secretion in PBMCs cultured with different *Leishmania* antigens exhibits the lowest amount of this cytokine. Furthermore, when LLA is present in the environment, the level of IL-4 secretion is low. In other words, the highest amount of this cytokine is present when both ESA and LLA antigens are present in the culture medium. At the same time, there is no significant difference in the specific and separate presence of these antigens, but all of them have a

**Table 1.** The Average Concentration of IL4 Secretion in the Supernatant of PBMCs Stimulated with ESA, LLA, ESA+LLA, and PHA Antigens

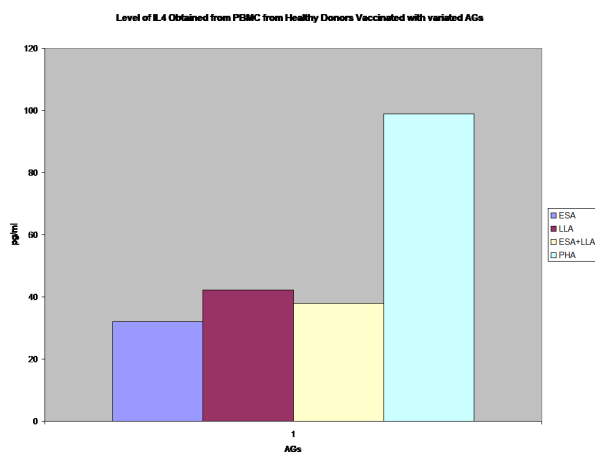
Type of Antigenic Compound	IL-4 Concentration Level (pg/mL)
ESA	32.12
LLA	42.16
ESA + LLA	38.00
PHA	98.80

Note. IL-4: Interleukin-4; PBMC: Peripheral blood mononuclear cell; ESA: Excreted-secreted antigen; LLA: Lysate leishmania antigen; PHA: Phytohaemagglutinin; P value=0.0001.

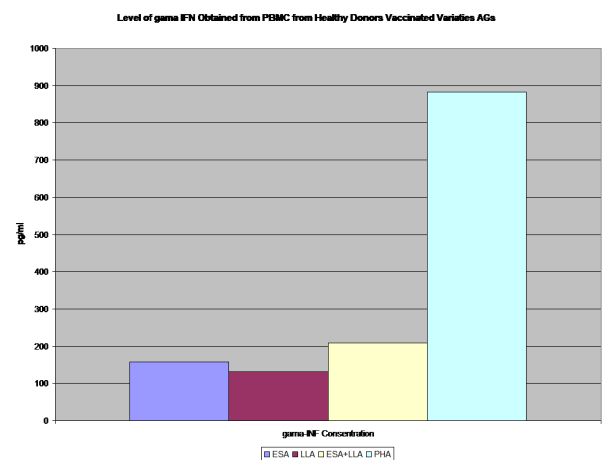
**Table 2.** The Average Concentration of INF-γ Secretion in the Supernatants of PBMC Stimulated with ESA, LLA, ESA+LLA, and PHA Antigens

Type of Antigenic Compound	INF-γ Concentration Level (pg/mL)
ESA	185.0 ± 123.8
LLA	132.3 ± 83.8
ESA + LLA	208.6 ± 93.3
PHA	883.9 ± 323.1

Note. INF-γ, Interferon-γ; PBMC, Peripheral blood mononuclear cell; ESA, Excreted-secreted antigen; LLA, Lysate leishmania antigen; PHA, Phytohaemagglutinin; P value=0.0001.



**Figure 1.** The Average Concentration of IL4 Secretion in the Supernatant of PBMCs Stimulated with ESA, LLA, ESA+LLA, and PHA Antigens. Note. IL-4: Interleukin-4; PBMC: Peripheral blood mononuclear cell; ESA: Excreted-secreted antigen; LLA: Lysate leishmania antigen; PHA: Phytohaemagglutinin



**Figure 2.** The Average Concentration of INF-γ Secretion in the Supernatants of PBMC Stimulated with ESA, LLA, ESA+LLA, and PHA Antigens. Note. INF-γ: Interferon-γ; PBMC: Peripheral blood mononuclear cell; ESA: Excreted-secreted antigen; LLA: Lysate Leishmania antigen; PHA: Phytohaemagglutinin

significant difference compared to the average level of IL4 secretion when it is in the PHA medium. In 2008, Nateghi Rostami et al also investigated the reaction of *Leishmania* to the proliferation of CD4+/CD8+ lymphocytes and obtained similar results (31). Moreover, Mahmoodi et al in 2005 demonstrated a significantly lower level of INF- $\gamma$  in PBMC of people with leishmaniasis lesions compared to people who recovered from the disease (32). Moreover, Pérez-Victoria et al investigated the production of related cytokines in PBMC cultures of people without exposure to *Leishmania* parasites and concluded that the people not being exposed to *Leishmania* parasites show fewer T cells in their immune system cycle (33). Further, in 2008, Adnan et al investigated the role of IL10 in skin lesions of leishmaniasis and concluded that the reason for this abnormality is that in some people with leishmaniasis lesions, wound expansion is seen although the wounds heal spontaneously in most people because of the presence of IL-10 (34). Moreover, in 2007, Alavi Naini investigated the initial response of T cells in the secretion of IL-4 and found that the concentration of IL-4 is low in the early stages (35-37). It is necessary to induce a complete protective response through a carrier molecule that activates the cellular immune system and to use it with a molecule that stimulates the activity of macrophages in the body (38-40).

## Conclusion

The results of research, laboratory, and clinical trials for the preparation of a vaccine against the *Leishmania* parasite over the past few decades revealed that while all types of first-to-fourth-generation vaccines being examined have been protective and immunogenic to a certain extent, none have been sufficient to provide immunity against this protozoan. Moreover, although there is a host of knowledge from research on the immunobiology of leishmaniasis, there is still a lack of high potency, immunogenic, protective, effective, and acceptable vaccine. Furthermore, it is not prepared by international standards, and this indicates our lack of complete and adequate understanding of the disease, especially the factors that control and/or regulate the development and maintenance of anti-leishmanial immunity and those associated with secondary (memory) immunity. Investigations should be conducted at the cellular, molecular, and nanotechnological levels because the greater the immunogenic and protective components are exposed to the human immune system, the more concrete the results will be. On the other hand, the utilized antigen should be a conjugate of adjuvants, immunogens, and proliferating macrophages to prepare the ground for the stimulation of the cellular immune system against the *Leishmania* parasite with comprehensive programming.

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## Authors' Contribution

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**Project administration:** Mohammad Reza Mozayan, Ali Fattahi Bafghi.

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**Supervision:** Mohammad Reza Mozayan, Ali Fattahi Bafghi.

**Validation:** Mohammad Reza Mozayan, Ali Fattahi Bafghi.

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**Writing—original draft:** Mohammad Reza Mozayan, Ali Fattahi Bafghi.

**Writing—review & editing:** Ali Fattahi Bafghi.

## Competing Interests

The authors declare no competing interests.

## Ethical Approval

Approval was obtained by the Ethical Review Board of Yazd Shahid Sadoughi University of Medical Sciences.

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