

Soluble Leishmanin as an Ideal Reagent for Skin Testing in Human Leishmaniasis

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ABSTRACT

Delayed-type hypersensitivity reaction measured by skin testing, is an important tool for evaluation of cellular immune response in leishmaniasis and has been used recently as an indication of exposure to infection. Skin testing in leishmania infection needs a standard reagent with high specificity, sensitivity, and potency. In the present work, a soluble leishmanin, and three whole parasite preparations from *Leishmania major*, i.e. thimerosal, phenol, and autoclaved leishmanins, were prepared under standard conditions. These antigens were evaluated and compared in different foci of leishmaniasis. The sensitivity and potency of reagents were tested in both recovered cases and patients with active ulcer in two areas endemic to urban and rural leishmaniasis. Data obtained showed that, the sensitivity of thimerosal, phenol, and soluble leishmanins are almost equal, but are higher than autoclaved one. Moreover, the potency of soluble leishmanin was also proved to be higher than other reagents. The purity of soluble leishmanin is higher than other three particulate preparations, and it lacks the majority of membrane antigens. As a result this reagent can be used as a standard and ideal antigen for skin testing in human leishmaniasis.

Keywords: Soluble leishmanin, *Leishmania major*, Skin testing

INTRODUCTION

Leishmania parasites are pathogenic intracellular protozoa that cause a wide spectrum of infectious diseases, and their clinical outcomes, range from a self-healing cutaneous ulceration to disseminated, visceral and fatal diseases. The diversity in clinical manifestations are attributed to both species of parasite, and the ability of host to develop a specific immunity against organism[1]. It has been shown that T cell-mediated immune response plays an important role in resistance against human leishmaniasis[2, 3]. The immunity is dependent on induction of functionally distinct subsets of CD4⁺ T-cells responses. If an appropriate antigen-specific Th1 response is developed, the infection leads to recovery [4], a status usually manifested in self-healing cutaneous leishmaniasis.

In contrast, individuals cured from visceral leishmaniasis, demonstrate both Th 1 and Th2 responses [5]. Moreover, after recovery from cutaneous leishmaniasis, a prolonged immunity to

reinfection may develop in recovered individuals [6], which can be measured *in vitro* by T-cell proliferation assay and assessment of cytokines [7, 8], and *in vivo* by measurement of delayed-type hypersensitivity (DTH) response, using leishmanin skin test [9, 10].

The leishmanin skin test (LST), introduced by Montenegro in 1926 [11], has been used for years as an aid for diagnosis, and in epidemiological studies for assessing exposure to *Leishmania* [12]. At present, LST is an important tool in measurement of DTH and consequently in assessment of cell-mediated immunity. To define the immunity status of volunteers to leishmaniasis in vaccine trials, all individuals are skin tested before immunization and the positive cases are excluded from the program. Furthermore, the major role of LST appears in assessment of vaccine efficacy, and it can be used as a criterion for evaluation of the effectiveness of vaccination [13-15]. Although the use of LST in diagnosis of leishmanial infection is limited to certain situations, but LST is widely

Corresponding Author. Abbreviations: ACL, Anthroponotic cutaneous leishmaniasis; AL, Autoclaved leishmanin; CGM, Complete growth medium; CL, Cutaneous leishmaniasis; DTH, Delayed-type hypersensitivity; LST, Leishmanin skin test; PL, Phenol leishmanin; SL, Soluble leishmanin; TL, Thimerosal leishmanin; ZCL, Zoonotic cutaneous leishmaniasis

used in epidemiological studies, to define the prevalence and incidence of infection [16], scale of endemicity, and transmission risk of leishmanial infections [17, 18].

In cutaneous leishmaniasis (CL), the skin test is positive during the active and healing phases of disease and remains reactive in almost 100 percent of recovered cases [19]. Since the production of a commercially available leishmanin was stopped in the early 1980s, researchers found themselves without a reliable reagent [20]. This leads to the use of different locally produced preparations made from various *Leishmania* species with poor quality and lack of standardization, with the consequence that the results of different studies were not comparable. Therefore, the lack of a uniform reagents for vaccine trials and other epidemiological studies, was felt and production of reliable supplies was recommended by TDR, World Health Organization [20]. During the past eight years, we produced a hole parasite leishmanin from *L. major*, as a thimerosal leishmanin (TL). This reagent was evaluated in different endemic and non-endemic area. The sensitivity, and potency of different concentrations of this antigen has been analyzed and its optimum dose was selected [21]. The higher or equal reactivity of TL has been proved in comparison with available reagents such as those produced by Wellcome and Istituto Superiore di Sanita (Rome, Italy), in several labs. [22-26]. Finally, the data obtained from reactivity of this reagent, and other reagent from Italy based on *L. infantum*, in different foci in Africa and Asia, were analyzed by TDR, and proved very similar and our TL was selected by TDR for all vaccine studies and as a reference reagent [20]. At present, TL has been distributed widely for epidemiological studies and vaccine trials [27, 28].

In the present work, a soluble leishmanin (SL) was prepared from crude extract of well defined isolate of *L. major* promastigotes, under good manufacturing practice (GMP). In addition, three other whole parasite preparations including thimerosal, phenol, and autoclaved leishmanins (TL, PL, and AL), were prepared under same conditions, and the specificity, sensitivity, and potency of four preparations were carefully evaluated and compared in two known endemic areas of urban and rural cutaneous leishmaniasis (CL) in Iran, both in individuals with active lesion(s), and recovered cases from disease.

MATERIALS AND METHODS

The Parasite: The strain of *L. major* (MRHO/IR/75/ER), Lon 1, (originally isolated by Dr. A. Nadim and kindly provided by Dr. E. Javadian, School of Public Health, Tehran Medical Sciences University, Iran [29]), was used in this study. This strain was also used for preparation of killed *Leishmania* vaccine in Iran [30]. The virulence of parasites were kept by continuous passage in BALB/c mice.

Culture Media: Initially the strain was cultured on biphasic Nicole-Navy-McNeal (NNN) medium, After growth, the promastigotes were subcultured in blood free monophasic medium. This medium contains equal mixture of RPMI 1640 and Dulbecco's Modified Eagle's Medium (D-MEM), or D-MEM and Tc medium 199 (all from Sigma). These media were supplemented with 2 mM L-glutamine, 0.122 mg/ml sodium pyruvate, 2 mg/ml lactalbumin, 0.2 mg/ml yeast extract, 100 U/ml penicillin G, and 100 µg/ml streptomycin, pH 7.1. The medium was also supplemented at initial culture with 20 % fetal bovine serum (FBS) and in large scale with 10 % FBS (Gibco, from Australian origin), and is called Complete Growth Medium (CGM) hereafter.

Cultivation Procedure: One vial of stock seed, contains 5×10^6 promastigotes of *L. major*, was inoculated in CGM medium, and incubated at $25 \pm 0.1^\circ\text{C}$. After growth, the parasites were subcultured in fresh CGM into small disposable flasks (6 nil CGM), and after growth subcultured in medium flasks (50-100 ml), and then into large flasks (200-250 ml) in mass cultivation of parasite, and flasks was incubated horizontally at $25 \pm 0.1^\circ\text{C}$, until sufficient growth was obtained. Cultures were examined for possible contamination by inverted microscope, and were aerated under strict sterile conditions, every other day.

Preparation of Leishmanin Reagents: at the beginning of stationary phase, the promastigotes were harvested by centrifugation at $3400 \times g$ for 50 minutes at 5°C . Harvested parasites were washed ($3000 \times g$, 30 min) 3 times in sterile and pyrogen free PBS, pH 7.0. The sediments from different tubes were mixed, washed, and mixed with equal

volume of PBS plus 0.05% thimerosal. The killed parasites were counted and adjusted to 6×10^6 /ml, by dilution in PBS contains 0.01% (w/v) thimerosal. Phenol leishmanin was prepared by resuspending the final parasite sediment in PBS contains 0.5% (w/v) phenol. To prepare autoclaved leishmanin, the thimerosal leishmanin was autoclaved at 115°C, 15 lb. for 15 minutes. Soluble leishmanin was prepared according to the method described by Reed *et al.* [10], with a few modification. Briefly, the parasite pellet was resuspended in 5 volumes distilled water, and aliquoted in cryogenic tubes. The tubes were then immersed in liquid nitrogen, and thawed at 37°C, for 11 times. The disrupted parasites, were diluted with 10 volumes of PBS, and centrifuged at 10,000 ×g for 30 min. The supernatant was diluted with PBS containing 0.01% Thimerosal and filtered by Millipore, 0.22 µm.

Safety analysis: Sterility, toxicity, and pyrogen tests were performed according to the procedure reported previously [21].

Viability test: Viability of promastigotes were tested by cultivation of 1 ml from each lot of preparations in liquid phase of NNN medium, followed by incubation at 25°C for 30 days. The flasks were examined twice weekly for viable or mobile forms of promastigotes.

Chemical analysis: The preparations of leishmanin were tested for pH, and total proteins (Lowry method). Moreover, all four preparations were analyzed by SDS-PAGE.

Study groups and areas: To evaluate four preparations, a matched study was designed on different groups of both urban and rural endemic areas with the consent of school and health authorities. Comparative studies were performed on the same individuals, by injection of two different reagents simultaneously. Three groups were studied as follow:

a) Cured cases of anthroponotic cutaneous leishmaniasis (ACL): This study was carried out in three villages, Poonak, Hesarak, and Jannatabad, in north-west of Tehran. ACL caused by *Leishmania tropica*, is endemic in this area [31]. A total of 135 school-children, with typical atrophic scar(s), were tested. The students were of both sexes and their age ranged from 7 to 18 years, and majority had scar(s) on their hand, feet, face or

other parts of their body.

b) Recovered cases from zoonotic cutaneous leishmaniasis (ZCL): Reactivity of leishmanin preparations was studied in endemic area of Shahinshahr, which is situated 30 kilometer north of Isfahan. Isfahan is one of the most well known foci of ZCL in Iran [32]. A total of 230 schoolchildren with leishmanial scar(s) on different parts of body, from both sexes, and with age range of 6-16 years, were used for comparative skin testing.

c) Patients with ZCL: 79 patients with active lesion(s) of ZCL, from both sexes were skin tested, in an endemic suburb of Isfahan. The patients were school-children with leishmanial lesion(s) on different parts of body and their age ranged from 7 to 13 years. Moreover a total of 127 volunteers with ZCL lesion(s), from both sexes, and with an age range of 5-64 years, were tested in a newly found endemic region, south-east of Kashan. They were selected on the basis of the data from their questionnaire after an interview and physical examination. The patients or their legal guardians gave their consent to the participation in a written form. Two separate studies were carried out in this area: a single blind placebo controlled comparative study on 75 individuals, during a survey undertaken to determine the prevalence of *Leishmania* infection in a borough near the Kashan; and a double blind matched designed study on 52 individuals, in this area.

Testing and measurement of DTH reactions: In matched designed studies two different antigens were intradermally injected in the upper and lower parts of forearm 5 centimeters apart, using a 1 ml tuberculin syringe with 26-gauge needle. The diameter of induration was measured by ballpoint technique [33], using a millimeter-graduated ruler, 48 or 72 hours after injection. The mean of the two diameters was taken at 90° rotation of each reaction site. The largest measurable induration from the two readings at 48 hr and 72 hr was recorded as an indication of the DTH reactivity [34]. Induration of 5 millimeters or more in diameter was considered as a positive response.

Definitions: The sensitivity of a given antigen is defined as the percent of cured individuals or well developed cases with leishmaniasis, in whom, the antigen induced a positive leishmanin reaction. The potency of a given antigen is defined as the mean

diameter of induration, induced by the antigen in cured or cases with leishmaniasis.

Statistical analysis: For paired comparisons of means of sensitivity, the paired t-test was used. Mean diameter of induration was analyzed and compared, using Z test.

RESULTS

Safety analysis: Sterility, abnormal toxicity and pyrogen tests showed that all four preparations are sterile and non-toxic. No viable promastigote was observed in culture of leishmanins, after 30 days culture.

Chemical analysis: The range of pH for final products of all reagents were between 7.03-7.08. The amount of total protein contents for final preparations of all reagents, measured by Lowry methods, is shown in Table 1. As shown in the Figure 1, the pattern of SDS-PAGE analysis of four different preparations was identical.

Adverse Reactions: In cured cases and cases with leishmaniasis, local reactions consisted of wide erythema around the induration, in almost 100%

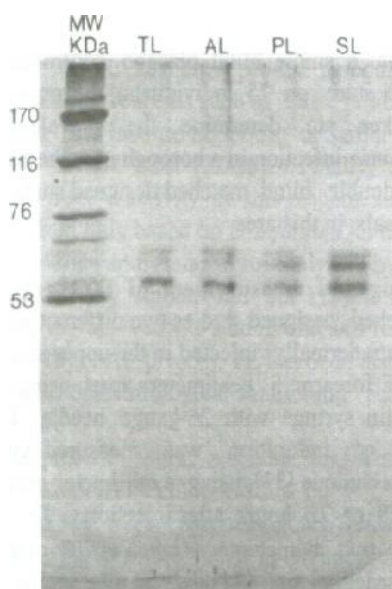


Fig. 1. Comparative analysis of soluble leishmanin and three particulate leishmanins by SDS-PAGE. 50 µg from each preparation (4.6 µg TL, 3.8 µg AL and 2.5 µg SL) was run in 10% acrylamide gel and gel was silver stained.

of cases, and occasionally pain, burning, and itching at the site of injection. All these reactions can be regarded as mild to very mild. In no case was there any severe reaction, or one that required analgesic or other treatment, and daily functions were completely normal.

Evaluation of leishmanin preparations:
Comparison of the reactivity of TL and AL: The results obtained from a matched designed study on 135 cured cases of ACL showed that TL was both more sensitive ($p < 0.05$) and more potent than AL as shown in Table 2. Similar results were obtained in a single blind study, in ZCL patients of Kashan (Table 3).

Comparing the reactivity of TL and PL: The comparison of the reactivity of TL and PL in 50 recovered cases of ZCL showed that sensitivity of both reagents are equal, but potency of TL is significantly higher than PL ($p < 0.01$). Similar results were obtained when TL and PL were compared in 52 patients with ZCL in a double blind study in Kashan, as shown in Table 4.

Comparison of the sensitivity and potency of SL & TL: When reactivity of TL was compared with four different concentrations of SL, almost equal sensitivity were observed, but the potency of all doses of SL was higher than TL, especially in higher concentrations of SL. Moreover, the results of this study showed dose-dependent potency of different concentrations of SL, as demonstrated in Table 5.

Comparing the activity of SL and PL: The reactivity of SL and PL was tested in both 74 healed cases and 79 patients with ZCL. As displayed in Table 6, the sensitivity of SL (10 mg/ml) was significantly higher than PL in both groups ($p < 0.05$), and also SL was more potent than PL in both cases ($p < 0.01$).

DISCUSSION

Although the need for a reliable and standard antigen for skin testing in leishmaniasis has almost been resolved by availability of a reference whole parasite leishmanin [20], there are still a trend towards the use of a purified, simple, and potent reagent capable to evoke a specific response in delayed type hypersensitivity reaction. Therefore, in this study, to evaluate and select the most reactive, reliable

Table 1. Total protein contents (Lowry method), number of parasite and pH of different leishmanin preparations.

| Type of reagent | TL | PL | AL | SL |
|------------------------------------|-----------------------------|-----------------------------|-----------------------------|------|
| Total protein ($\mu\text{g/ml}$) | 92 | * | 77 | 50 |
| Number of parasites | $6 \times 10^6 / \text{ml}$ | $6 \times 10^6 / \text{ml}$ | $6 \times 10^6 / \text{ml}$ | - |
| pH | 7.05 | 7.03 | 7.08 | 7.08 |

* Measurement is not possible by Lowry method.

Table 2. Comparison of the reactivity of TL and AL in recovered cases of ACL in endemic area of Tehran.

| Type of reagent | No. of cases | Age (year) | Sex | | Sensitivity | | Mean of induration (mm) |
|-----------------|--------------|------------|-----|----|----------------|-------|-------------------------|
| | | | F | M | Positive cases | % | |
| TL | 135 | 11-17 | 84 | 51 | 125 | 92.59 | 14.27 ± 6.89 |
| AL | 135 | 11-17 | 84 | 51 | 114 | 84.44 | 12.24 ± 7.39 |

Table 3. Comparison of the activity of TL and AL in patients with ZCL in endemic area of Kashan.

| Type of reagent | No. of cases | Age (year) | Sex | | Sensitivity | | Mean of induration (mm) |
|-----------------|--------------|------------|-----|----|----------------|----|-------------------------|
| | | | F | M | Positive cases | % | |
| TL | 75 | 5-64 | 43 | 32 | 69 | 92 | 10.31 ± 4.30 |
| AL | 75 | 5-64 | 43 | 32 | 54 | 72 | 8.55 ± 3.03 |

Table 4. Comparison of the reactivity of TL and PL in recovered cases of ZCL and patients with ZCL in endemic area of Isfahan and Kashan.

| Type of cases | Type of reagent | No. of cases | Age (year) | Sex | | Sensitivity | | Mean of induration (mm) |
|----------------------------------|-----------------|--------------|------------|-----|----|----------------|-------|-------------------------|
| | | | | F | M | Positive cases | % | |
| Recovered cases of ZCL (Isfahan) | TL | 50 | 8-15 | - | 50 | 49 | 98.00 | 12.89 ± 3.57 |
| | PL | 50 | 8-15 | - | 50 | 49 | 98.00 | 10.53 ± 2.65 |
| Patients with ZCL (Kashan) | TL | 52 | 2-70 | 24 | 28 | 51 | 98.76 | 13.66 ± 3.79 |
| | PL | 52 | 2-70 | 24 | 28 | 51 | 98.76 | 12.09 ± 3.99 |

Table 5. Comparison of the reactivity of TL and four concentrations of SL in recovered cases of ZCL in endemic area of Isfahan.

| Conc. of SL | Type of reagent | No. of cases | Age (year) | Sex | | Sensitivity | | Mean of induration (mm) |
|-------------|-----------------|--------------|------------|-----|----|----------------|--------|-------------------------|
| | | | | F | M | Positive cases | % | |
| 5 µg/ml | SL | 43 | 6-13 | - | 43 | 43 | 100.00 | 13.2 ± 3.63 |
| | TL | 43 | 6-13 | - | 43 | 43 | 100.00 | 12.16 ± 3.80 |
| 10 µg/ml | SL | 106 | 6-16 | 14 | 92 | 104 | 98.11 | 14.66 ± 4.93 |
| | TL | 106 | 6-16 | 14 | 92 | 105 | 99.05 | 12.96 ± 3.99 |
| 25 µg/ml | SL | 13 | 11-17 | 13 | - | 13 | 100.00 | 20.58 ± 5.32 |
| | TL | 13 | 11-17 | 13 | - | 13 | 100.00 | 14.85 ± 1.77 |
| 50 µg/ml | SL | 13 | 11-17 | 13 | - | 13 | 100.00 | 23.00 ± 5.05 |
| | TL | 13 | 11-17 | 13 | - | 13 | 100.00 | 13.73 ± 4.04 |

Table 6. Comparison of sensitivity and potency of PL with SL (101.1g/ml) in cases with CL and cured cases in endemic area of Isfahan.

| Type of leishmanin | Type of cases | No. of cases | Percent sensitivity | Mean diameter (mm) | |
|--------------------|---------------|--------------|---------------------|--------------------|--------------|
| | | | | Erythema | Induration |
| Phenol | Active Ulcer | 79 | 88.60 | 7.76 ± 5.05 | 5.25 ± 3.46 |
| Soluble | Active Ulcer | 79 | 97.46 | 15.16 ± 9.41 | 10.25 ± 5.78 |
| Phenol | Recovered | 74 | 94.59 | 14.51 ± 8.93 | 7.17 ± 3.89 |
| Soluble | Recovered | 74 | 100.00 | 23.52 ± 12.07 | 12.35 ± 4.60 |

and ideal leishmanin, we tried to focus our efforts in preparation and evaluation of a crude soluble extract of *L. major* promastigotes entitled soluble leishmanin, and three other particulate reagents.

In comparison of the three particulate preparations, autoclaved leishmanin showed lower reactivity than others. The underlying reasons for these findings are unclear, but could be due to antigenic variations caused by autoclaving. The main reason in preparation and evaluation of autoclaved leishmanin was to prepare a uniform LST antigen to be used in evaluation of the efficacy of autoclaved killed *Leishmania major* vaccine. Although both TL and PL reagents showed similar sensitivity, the potency of TL was higher than PL. This may be due to the effect of the preservative on the promastigotes, since the microscopic examination showed that in TL, promastigotes remained intact and could easily be counted under light microscope, especially during the first months of production. Whereas, in PL, the organisms seemed disrupted with obvious morphological changes, making an accurate count difficult by direct microscopy.

Thus, the results obtained from comparison of three whole parasite preparations showed that TL is both more reactive and more potent reagent between other particulate reagents for skin testing in leishmaniasis. However, in comparison with the reactivity of SL with three particulate reagents, the results clearly suggested that soluble leishmanin is more sensitive and potent than other whole parasite preparations including thimerosal leishmanin. This is similar to the results reported previously from a crude soluble promastigote extract of *L. donovani chagasi* used in skin testing of persons with past American visceral leishmaniasis [10]. Therefore, it seems that SL is a preferred reagent for skin testing in leishmaniasis.

The reason for the higher sensitivity and potency of SL is not fully understood, but probably due to disruption of promastigotes, the epitops or peptides responsible for DTH, are released in crude extract, and exert more effective immune responses in skin testing. Because, the specific antigen(s) of DTH could be easily engulfed, processed by antigen presenting cells, then, recognized by T-cells, and induce a strong hypersensitivity reaction. Another factor may be the presence of high concentration

of specific antigen in crude extract of promastigotes, which is prepared from high number of parasites. Hence, the obtained data suggested a dose-dependent potency of the soluble leishmanin, so that, by increasing the concentration of reagent, the mean diameter of induration is increased.

Considering that the sensitivity and potency of SL are higher than other particulate leishmanin, and the specificity of SL has been proved in high level, as reported elsewhere [21], the use of soluble leishmanin would be highly advisable in skin testing, in particular, in two major fields: a) for study of the efficacy of vaccine, where the LST is the only unique *in vivo* test for demonstration of the efficacy of vaccine, and because the immune response not adequately formed during the early phases of trial, there is a need for a strong reagent to reveal an early specific immune response in vaccinated individuals, b) as an aid in diagnosis, especially in the cases that parasites are rare and not easily detected in routine analysis, and clinician require to a strong antigen for differential diagnosis of disease.

In conclusion, the data presented in this paper clearly demonstrates that the effectiveness of soluble leishmanin is higher than all three whole parasite leishmanins (AL, PL and TL) for assessment of delayed-type hypersensitivity in skin testing of human leishmaniasis

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