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THE PREPARATION AND EVALUATION OF REFERENCE LEISHMANIN FROM *LEISHMANIA MAJOR* FOR USE IN MAN FOR DIAGNOSTIC AND EXPERIMENTAL PURPOSES

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ABSTRACT

Cell-mediated immunity (CMI) plays an important role in resistance against leishmaniasis. Delayed-type hypersensitivity (DTH) reaction measured by skin testing is a practical method of evaluation of CMI and is used as an aid to diagnosis and for epidemiological assessment of exposure to leishmanial infection. Skin testing in leishmaniasis, generally known as Montenegro or leishmanin test, requires a standard antigen. At present no uniform and standard leishmanin is available for skin testing in leishmaniasis. The present work describes the preparation and testing of an antigen from *Leishmania major* using standard conditions. Three dilutions of this antigen were tested in recovered individuals in an endemic area in Iran. The results obtained showed that leishmanin preparation exhibited high specificity and sensitivity, and strong potency.
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INTRODUCTION

The leishmaniases comprise a wide spectrum of diseases of protozoan in many areas of the world.[12] Disease is caused by different species of Leishmania which are transmitted from their reservoirs to humans by bites of female sandflies of the genera Phlebotomus and Lutzomia.

In leishmaniasis, the outcome of disease depends on both the species of Leishmania and the host immune responses (Turk and Bryceson 1971). In addition, cell mediated immunity is important in the resistance against leishmanial infections. The intradermal skin test is a crucial tool for evaluation of delayed-type hypersensitivity in leishmaniases and has been used in the evaluation of leishmanization,[15] as well as killed vaccine[8] and epidemiological studies.[2]

In cutaneous leishmaniasis the skin test is positive during the active and healing phases of the disease and remains positive in almost 100 percent of cured cases.[16] Montenegro or leishmanin test could be also used as an aid for diagnosis, particularly in the cases that amastigotes are scanty and not easily detectable by routine parasitological examination. Our major difficullty has been the use of different locally produced preparations of leishmanins made

from different species by various workers. Consequently the results of various studies are not comparable. A commercially produced leishmanin was available until early 1980's which provided a common antigen in many studies of the Old World. However, after that the lack of a standard preparation was felt and production of standard leishmanins were recommended by the World Health Organization.

In the present work efforts have been made to prepare and evaluate a leishmanin produced from a well characterized *L. major* isolate under good manufacturing practices to be used as a standard reagent for diagnostic and immunological studies.

MATERIALS AND METHODS

The Parasite

The strain of *L. major* (MRHO / IR / 75 / ER) used for leishmanization in Iran during the 1980's was kindly provided by Dr. E. Javadian, School of Public Health, Tehran University, Iran, and kept virulent by continuous passage in BALB/c mice.

Leishmania enriettii was kindly provided by Dr R. Behin, WHO-IRTC, Institute of Biochemistry, University of Laussanne, Switzerland. Organisms were maintained in NNN medium or in RPMI 1640, containing 20% FCS and was kept virulent through passages in guinea pigs.

Animals

Mice of 8-10 weeks old, CBA/ca and BALB/c originally obtained from Harlan Olac Ltd, UK, were used. They were reared in the Animal Center of Pasteur Institute of Iran. Other outbred mice and guinea pigs were obtained from the Animal Labs of Pasteur Institute of Iran.

Subjects

Skin tests to leishmanin were studied in both endemic and nonendemic areas. Based on experiences of local health authorities, the hyperendemic area for *L. major* infections were chosen. This area comprised of three villages 90 kilometers east of Isfahan. Due to infection, the majority of schoolchildren in this area had scar(s) on their face, hands, feet, and other parts of their body. Reactions to leishmanial antigens were studied in 226 children with scar(s), either from infection or from leishmanization, with the consent of health and school authorities. Standard skin testing was performed by intradermal injection of 0.1 mL of undiluted and diluted antigen. Moreover, 109 healthy children from a non-endemic area of Tehran served as controls. These children applied to Pasteur Institute of Iran for BCG vaccination. As a PPD skin testing is required before BCG vaccination, the parents were asked if they would like to participate in leishmnin skin testing as well. Skin tests were performed simultaneously in all those children whose parents gave consent. 35 individuals were used for undiluted, 43 for 1/2 dilution, and 31 for 1/4 dilution tests. The age ranges in both groups were 6-14 years.

Leishmanin

Wellcome (UK) leishmanin, lot la/28 prepared from *L. major* at a concentration of 10×10^6 promastigotes/mL was kindly provided by Dr. F.Z. Modabber, TDR, WHO, Geneva, Switzerland.

Culture Media

RPMI 1640 (Gibco), supplemented with 20% fetal calf serum (FCS) (Gibco), 100 U/mL penicillin, and 100 mcg/mL streptomycin and 2 mM L-glutamine was used for parasite cultivation.

Cultivation Procedure

L. major isolated from the skin lesions of infected mice were grown in NNN medium, then were seeded into RPMI. By growing parasites passaged three times *in vitro* subsequely, a stock-seed system established, using RPMI plus 40% FCS and 10% dimethyl sulfoxide (DMSO), or 8% glycerol and aliquoted were kept in liquid nitrogen. The cultivation was performed in 25 mL universal tubes and transferred to 1 or 2 liter glass screw cap containers. The incubation temperature was 25 ± 1°C. For selection of FCS concentration in culture, different dilutions of FCS were examined using RPMI as culture medium.

Leishmanin Preparation and Control Solution

After checking for contamination, the promastigotes at the beginning of the stationary phase were treated with 0.1% filter sterilized thimerosal for 30 minutes. After killing, the parasites were harvested with centrifugation at 4000 g for 30 minutes. Harvested parasites were washed three times with filtered and autoclaved, sterilized, pyrogen-free phosphate buffered saline (PBS), pH 7.2-7.4, at 4000 g for 30 minutes. Concentrated suspension of antigens were prepared and counted with heamocytometer, using PBS containing 0.01% thimerosal, and stocks were stored at 2-8°C.

After confirmation of sterility, a suspension of the fixed and killed promastigotes were made in sterile and pyrogen free PBS, in concentration of 10^7 promastigotes per mL with addition of thimerosal in a concentration of 0.01% as preservative. The prepared leishmanin was then aliquoted into 1 mL vials and some vials were randomly chosen and sent again for sterility testing.

In addition, control solution was prepared from sterile pyrogen free PBS with 0.01% thimerosal as preservative, and was aliquoted into 1 mL, 2 mL, and 3 mL vials.

Table I. Comparison of biological activities of leishmanins obtained at log. and sta. phases of growth in exciting DTH response in CBA mice

Parasite culture time	Parasite culture phase	number of infected animals	Average of footpad swelling*
4 days	logarithmic	3	1 + 0.56 mm
10 days	stationary	3	1.9 + 0.56 mm

* 48-hr. response

Table II. Comparison of effects of the leishmanin "lot 109" with Ia/28 Wellcome leishmanin in exciting DTH responses in inbred mice

Mice strain	No. & sex of mice	No. of parasite for infection	Type of leishmanin	Dose of antigen	48 hr responses
CBA	4.F	3×10^6	Iran. 109	0.5×10^6	1.55 + 0.38 mm
CBA	4.F	3×10^6	Well. Ia/28	0.5×10^6	0.60 + 0.20 mm
BALB/c	4.F	3×10^6	Iran. 109	0.5×10^6	0.20 + 0.14 mm

Safety Analysis

Sterility testing: Sterility was assessed by cultivation of antigen and control solution in three media, tripticase soy broth, thioglycolate, and Sabouraud-dextrose broth in different volumes, for two weeks at 37°C and 25°C.

Chemical analysis: The prepared antigen was tested for pH, total proteins (Lowry method), lipids (colorimetric method), and carbohydrate contents (O-toluidine method).

Pyrogen tests: The leishmanin preparation was tested *in vitro* for endotoxin concentrations using a Limulus amoebocyte lysate (LAL) assay. This test was kindly performed at the Iranian Blood Transfusion Center, Quality Control Department. The PBS buffer used for preparation of leishmanin and control solution were also checked for pyrogen test by in vivo tests in rabbits by standard methods of the Pasteur Institute of Iran.

Abnormal toxicity: This test was carried out in five healthy mice, weighing 17 to 22 g, and two healthy guinea pigs, weighing 250 to 350 g. Each animal was injected intra-peritoneally with one human dose (0.1 mL), and after seven days the result was observed (Eur. phar., 2nd ed., 1986).

Biological Analysis

Skin test in mice: Outbred and inbred mice were injected with $2\text{-}5 \times 10^6$ infective *L. major* at the base of the tail. When ulcerous nodules appeared, antibody titration was measured by immunofluorescent method. DTH test was performed in positive and ulcerous animals by injection of infected outbred and inbred (CBA/ca) mice with leishmanin by footpad method, and BALB/c mice used as negative control, Each mouse was injected with 50 µL of undiluted antigen and control solution intradermally in right and left hindfoot pads, respectively. The increase in footpad thickness was measured after 48 hrs using a dial caliper. In these experiments, the Wellcome (UK) leishmanin was used as positive control.

Skin test in guinea pigs: Three outbred healthy guinea pigs were injected with 2×10^6 *L. enriettii* promastigotes subcutaneously in the nose. After ulceration the animals were injected intradermally with 0.1 mL of leishmanin, in a shaved area of the chest. After 48 hrs the skin induration was measured with a ruler.

Methods of Skin Testing in Man

DTH responses to leishmanin were measured by injection of 0.1 mL of each concentration of leishmanin intradermally into the inner surface of the forearm, An equivalent volume of the control solution was injected intradermally into the other forearm. The diameter of erythema and induration was measured by a millimeter-graduated ruler, 68-72 hours after injection. The mean of the two diameters was taken at 90° rotation of each reaction site. An average induration of 5 millimeters or more in diameter was considered as a positive reaction to the skin test.

RESULTS

Selection of FCS Concentration

For determination of the optimal concentration of fetal calf serum (FCS), different concentrations (10%, 15% 20%, and 30% v/v) of FCS were added to RPMI medium. The results obtained showed that the rate of growth was almost equal in four concentrations of FCS, but the parasite

Table III. Intradermal skin test with leishmanin "lot 109" in normal subjects*

leishmanin dilution	Total number of cases	age (year)	Sex M	Sex F	Negative cases	Specificity (%)	Induration of positive cases**
1	31	6-14	20	11	28	90.32	10.66 ± 0.94
1/2	35	6-14	25	10	34	97.10	11
1/4	43	6-14	35	8	43	100	---

*Individuals without leishmaniasis history and residing in a non-endemic area
** Mean diameter in millimeters

Table IV. Intradermal skin test with various dilutions of leishmanin (lot 109) in individuals recovered from *L. major* infection

Antigen dilution	Total number of cases	age (year)	Vaccinated individuals	Recovered individuals	Positive* reactions	Induration**
1	74	8-13	35	39	97.29%	15.20 ± 5.1
1/2	66	6-13	4	62	100%	17.72 ± 6.1
1/4	85	8-14	13	74	93.10%	12.28 ± 5.1

*Equal or more than 5 mm
**Mean diameter in millimeters

growth was quite satisfactory at 20% v/v FCS, therefore in the preparation of leishmanin, this concentration of FCS was used as serum supplement.

Comparison of Antigenicity of Two Stages of Parasite Growth

The leishmanin obtained from harvested promastigotes at logarithmic and stationary phases were compared for their ability to elicit effective DTH response in CBA/ca mice. It was shown that the footpad response was markedly higher when stationary phase antigen was used (Table I).

Sterility and Safety Testing

The antigen was tested for aerobic and anaerobic bacterial and fungal contamination, after preparation of antigen and distributing into vials. Neither bacterial nor fungal contamination was observed in either stage after two weeks incubation. The antigen was also cultured in NNN medium for 20 days at 25 ± 0.1°C, and no viable parasites were found in the medium.

Abnormal Toxicity: None of the animals (mice and guinea pigs) showed any signs of sickness.

Pyrogen Testing: Prepared antigen was used for endotoxin titration. The result obtained showed titer of 40.7 pg endotoxin/mL for lot 109 leishmanin. Also, all washing and dilution solutions were checked for *in vivo* pyrogen test before using preparation of leishmanin. The results obtained showed temperature increase of 0.33°C in rabbits, which was in normal and satisfactory range.

Reaction: Local reactions to leishmanin skin test such as erythema, itching and pain in healthy individuals were rare. In recovered cases, local reactions consisted of wide erythema, induration, itching and occasionally pain in the site of injection. In a few cases vesiculation occurred at the induration site.

DTH Response to Leishmanin in Mice

Mice infected with $2\text{-}5 \times 10^6$ parasites 5 1 days previously were used for skin testing with leishmanin, lot 109, and Wellcome leishmanin as a control. In CBA mice, our leishmanin showed a higher level of DTH responses (mean thickness of 1.55 mm) as compared with Wellcome leishmanin (mean thickness of 0.6 mm). BALB/c mice showed a lower response (Table II).

Evaluation of Different Concentrations of Leishmanin

Specificity of leishmanin: Specificity of prepared leishmanin was evaluated in healthy children residing in a non-endemic area (Tehran). Different dilutions (three dilutions) of leishmanin were injected intradermally and results obtained showed high specificity of antigen, i.e., 90.32%, 97.10%, and 100% for dilutions of 1, 1/2, and 1/4, respectively (Table III).

Sensitivity of leishmanin: The sensitivity of antigen was evaluated in an endemic area (Isfahan) using three dilutions of leishmanin. A high sensitivity of all concentrations of antigen was observed in recovered cases from leishmaniasis. An absolute sensitivity of 100% was demonstrated using 1/2 dilution of leishmanin. Undiluted and 1/4 dilution of antigen showed 97.29% and 93.10% sensitivity, respectively (Table IV).

Potency of antigen: Different concentrations of leishmanin showed a high reactivity in cured subjects, when the mean diameter of skin induration was measured.

The mean diameter of induration by 1/2 dilution of leishmanin was as high as 17.72 ± 16.11 mm, and mean diameters with undiluted and 1/4 diluted leishmanin were 15.20± 5.14 mm and 12.28± 5.14 mm, respectively (Table IV). A clear effect of concentration on potency could be observed in responses of skin testing, but this showed no correlation with increasing concentration of parasites.

DISCUSSION

The delayed-type hypersensitivity reaction in leishmaniasis, also known as the Montenegro leishmanin test, commonly used in epidemiological and immunological studies as well as diagnosis. In the absence of a standard and uniform leishmanin, most laboratories are required to prepare and standardize their own antigens. Thus, an overall program for preparation of reference and standard leishmanin to use for all purposes seemed useful. This was recommended and encouraged by the UNDP/World/Bank/ WHO, Special Program on Research and Training in Tropical Diseases (TDR).

Several small batches were produced under different conditions at first. Following the establishment of the best conditions, a relatively large amount (36,000 doses) of leishmanin from *L. major* was prepared and the potency, sensitivity, and specificity of the preparation was evaluated. Because of wide distribution of *L. major* infection in Iran,[15] especially after the war with Iraq, also because of a considerable cross-reactivity between *L. major* and *L. tropica* (minor) antigens,[9] the second causative agent of cutaneous leishmaniasis in Iran, and other leishmanias, this strain was chosen for preparation of leishmanin.

Safety analysis of this antigen in experimental models and subsequent biological analysis confirmed the safety of preparation and paved the way toward utilization of this leishmanin in field trials on human subjects.

The evaluation of this leishmanin (lot 109) showed that a dilution of 1/2 had the best sensitivity, as 100% of cured cases produced a positive response (mean induration 17.72 ± 6.11 mm s.d.). Undiluted leishmanin had a sensitivity of 97.29% and 1/4 dilution had a sensitivity of 93.10%. The first one represents about 5×10^5 promastigotes per injection.

A correlation between protection and positive skin test reaction (DTH) was seen in some human vaccine studies,[1] in contrast to studies in mice.[6,18] Recent studies in experimental models show that L3T4 + T-cells (CD4 + T-cells) with functional character of TH1 subset is involved in the development of both protection and specific DTH reactivity.[13,14] Also the development of resistance is associated and correlated with classical (tuberculin type) DTH.[4,7] Considering that the skin test antigens are a complex mixture of many components, and specific DTH reactivity has been often associated with acquisition of immunity to reinfection,[3] the use of skin test seems highly useful in evaluation of the immunological potential.

In contrast to other studies in the field of other leishmania species,[10,19] we found that increase in doses of antigen was not always associated with an increase in reactivity of antigen, since dilution of 1/2 demonstrated higher sensitivity, potency, and local reaction (erythema) than undiluted antigen. This was also confirmed when for comparison, we used high concentration of Roma leishmanin (5×10^6 each dose), and also Wellcome leishmanin (10^6 each dose) with our 1/2 dilution leishmanin (0.5×10^6 each dose). Our leishmanin showed higher responses than two other antigens when injected simultaneously to the same recovered individuals (unpublished data). The explanation for this finding is unclear, but probably suppressor mechanisms interfere with DTH reactivity when high doses of antigen are used. In addition, other factors including strain, virulence and culture phases, etc, may influence the property of leishmanin preparations. Our results show that in skin testing, optimal and not high dose of antigen should be used to obtain high reactivity in DTH response. Moreover, higher or lower doses of antigen would result in decreased reactivity, and therefore it is necessary to standardize the antigen to an optimal dose.

We also examined the non-specific reaction to leishmanin. The results obtained showed high specificity of three dilutions of leishmanin, which ranged between 90 to 100%, although it is possible that other conditions such as latency or cross-reactivity may cause non-specific reactions in tested individuals.

During the past three years, this leishmanin has been used and evaluated on several field trials in Iran (Shiraz, Isfahan, Tehran) and some other countries, as in Sudan, Ethiopia and Guatemala. The results obtained have been reported at satisfactory range. In a more recent study, this leishmanin has been applied as a skin test antigen for study of cutaneous leishmaniasis in south-western Ethiopia.[11]

In conclusion, the data presented here demonstrate that our leishmanin preparation exhibits high specificity, sensitivity, and potency. We therefore recommend this preparation for use in vaccine trials, as well as other seroepidemiological studies and diagnosis. The preparation can also be used as a reference leishmanin.

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REFERENCES

1. Antunes, C.M.F., Mayrink, W., Magalhaes, P.A., Costa, C.A., Melo, M.N., Dias, M., Michalick, S.M., Williams, P., Oliveira Lima, A., Vieira. J.B.F., Schettini, A.P.M: Controlled field trials of a vaccine against New World cutaneous leishmaniasis. Inter. J. Epid 15: 572-580, 1986.
2. Aston, D.L., and Thorley, A.P: Leishmaniasis in Central Brazil: results of a Montenegro skin test survey among Amerindians in Xingu National Park. Trans. Roy. Soc. Trop. Med. Hyg. 64(5): 671-678, 1970.
3. Convit, J., Pinadi, M.E., and Rondon, A.J: Diffuse cutaneous leishmaniasis: a disease due to an immunological defect of the host. Trans. Roy. Soc. Trop. Med. Hyg. 66: 603-610, 1972.
4. De-Rossell, R.A., Bray, R.S., Alexander, J: The correlation between delayed hypersensitivity, lymphocyte activation and protective immunity in experimental murine leishmaniasis. Parasite Immuunol. 9(1): 105-15, 1987.
5. European Pharmacopia. Abnormal toxicity, 2nd. ed., part II, V.2.1.5, 1986.
6. Liew, F.Y., Howard, J.G., Hale, C: Prophylactic immunization against experimental leishmaniasis. III. Protection against fatal *Leishmania tropica* infection induced by irradiated promastigotes J. Immunol. 132: 456-461, 1984.
7. Liew. F.Y., Millott, S., Lelchuk, R., Cobbold. S., Waldmann, H: Effect of CD4 monoclonal antibody *in vivo* lesion development, delayed-type hypersensitivity, and interleukin-3 production in experimental murine cutaneous leishmaniasis. Clin. Exp. Immunol. 75: 433-443, 1989.
8. Mayrink, W., Da Costa, C.A., Magalhaes, P.A., Melo, M.N., Dias, M., Olverira Lima, A., Michalick, M.S. Williams, P: A field trial o f a vaccine against American dermal leishmaniasis. Trans. Roy. Soc. Trop. Med. Hyg. 73(4): 385-387, 1979.
9. Mauel, J. and Behin, R: Immunity: clinical and experimental. In: The Leishmaniasis in Biology and Medicine. Academic Press Inc., London,. 731-791, 1987.
10. Melo, M.N., Mayrink, W., Da Costa, C.A., Magalhaes, P.A., Dias, M., Williams, P., Coelho, M.V., Batista, S.M: Stamdardization of Montenegro antigen. Rev. Inst. Med. Trop. Sao Paulo 19(3): 161-164, 1977.
11. Mengistu, G., Laskay, T., Gemetchu, T., Humber, D., Ersamo, M., Evans, D., Teferedegn, H., Phelouzat, M.A., Frommel, D: Cutaneous leishmaniasis in south-western Ethiopia: ●cholo revisited. Trans. Roy. Trop. Med. Hyg. 86: 149-153, 1992.
12. Modabber, F.Z: The leishmaniases. In: Tropical Disease Research: A Global Partnership, 8th Programme Report of the UNDP/ WORLD/ BANK/ WHO. TDR, pp. 99-112, 1987.
13. Muller, I., Louis, J.A: Immunity to experimental infection with *Leishmania major:* generation of protective L3T4 + T cell clones recognizing antigen(s) associated with live parasites. Eur. J. Immunol, 19: 865-71, 1989.
14. Moll, H., Rollinghoff, M: Resistance to murine cutaneous leishmaniasis is mediated by TH1 cells, but disease-promoting CD4+ cells are different from TH2 cells. Eur. J. Immunol. 20: 2067-2074, 1990.
15. Nadim, A., Javadian, E., Tahvildar-Bidruni, GH., Gorbani, M: Effectiveness of leishmanization in control of cutaneous leishmaniasis. Bull. Soc. Path. Ex. 76: 377-383, 1983.
16. Perston, P.M., Dumonde, D.C: Immunology of clinical and experimental leishmaniasis. In: Immunology of Parasitic Infections. Blackwell Scientific Publications. Oxford. pp. 167-202, 1976.
17. Reed, S.G., Badaro, R., Masur, H., Carvalho, E.M., Lorenco, R., Lisboa, A., Teixeira, R., Johnson, W.D., Jr., andjones, T.C: Selection of a skin test antigen for American visceral leishmaniasis. Am. J. Trop. Med. Hyg. 35(1): 79-85, 1986.
18. Titus, R.G., Lima, G.C., Enger, H.D., Louis. J.A: Exacerbation of murine cutaneous leishmaniasis by adoptive trnsfer of parasite-specific helper T-cell populations capable of mediating *Leishmania major*-specific delayed-type hypersensitivity. J. Immunol. 133: 1594-1600, 1984.
19. Weigle, K.A., Valderrama, L., Arias, A.L., Santrich, C., Saravia, N.G: Leishmanin skin test standardization and evaluation of safety, dose, storage, longevity of reaction and sensitization. Am. J. Trop. Med Hyg. 44(3): 260-271, 1991.