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Leishmanization in Small White Mice

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The main objective of this study was preparation of deep-freeze *L. major* (RHOM/IR/75/ER) Promastigotes and their ability for producing skin lesions in appropriate animal model (outbred mice). Two hundred and thirty seven out bred mice were divided into two groups. Group 1 (135) was inoculated 0.1 mL of *Leishmania* suspension subcutaneously. Group 2 (102) was injected by isotonic normal saline with the same procedure as control. The lesions were measured and parasitological examination performed too. Cutaneous lesions due to *Leishmania major* were manifested from one to six months post inoculation. Fifty eight of rest mice (63%) from interventional group were leishmanial infected six months post inoculation versus none of the mice from control group showed any lesion. In interventional group, Leishmanin Skin Test (LST) conversion after 72 h was significantly higher than the control group (54.1 vs. 4.3%, $p < 0.05$). The results of this study showed that from 5 to 25% of the deep-freeze promastigotes were alive and gradually activate within 20 min. In fact deep freezed *Leishmania major* promastigotes could be produced lesion and induced CMI in the mice.

Key words: *L. major*, Promastigote, leishmanization, Iran

INTRODUCTION

Leishmaniasis is recognized as an important public health problem after malaria in the world (Neouimine, 1996). The number of leishmaniasis cases in countries has been increased sharply in the last decade. There are several reasons behind the increased incidence of leishmaniasis in the world. The majority of them depend on human activities such as environmental modifications, resettlement of non immune populations or development of agro industrial projects, military activities and urbanization reduction in the use of residual insecticides for the control of malaria and improvements in diagnosis and reporting of positive cases (Strelkova, 1996). *Cutaneous leishmaniasis* is more prevalent than other type of *Leishmania* in Asian countries. Zoonotic Cutaneous Leishmaniasis (ZCL) and Anthroponotic Cutaneous Leishmaniasis (ACL) are found in scattered foci in various parts of Islamic Republic of Iran (Nadim and Javadian, 1998). In recent years, the incidence of both types of leishmaniasis is increasing and new areas are being invaded (Bethesda *et al.*, 2004). Still little is known the rate and efficacy of different vaccines which have been tested in last decades to prevent the infection. On the other hand, there is no effective vaccines to overcome this problem completely (CI de Oliveira *et al.*, 2003; Momeni *et al.*, 1997). Eventually physicians began taking scrapings from patients with an active Baghdad boil that were used for inoculation to create an artificial infection, called leishmanization, which occasionally resulted in secondary malaria, syphilis, or a viral infection. When *in vitro* culture became possible, various preparations were tried with mixed results. Leishmanization programmes in hyperendemic and high risk group were performed in last decades in Iran (Nadim and Javadian, 1998). When war stopped between the Islamic Republic of Iran and Iraq, Leishmanization programme was stopped too, because that programme have been accompanied with some side effects (Bora, 1996). The aim of this study was preparation of deep-freeze *L. major* (RHOM/IR/75/ER) Promastigotes and their ability for producing skin lesions in appropriate and induce CMI in appropriate animal model (outbred mice).

MATERIALS AND METHODS

Deep-frozen *Leishmania major* preparation: This was prepared by culturing of *L. major* strain (RHOM/IR/75/ER) isolated from the great gerbil (*Rhombomys opimus*) in Isfahan area in 1964 by Nadim *et al.* (1997) and maintained thereafter in the leishmaniasis laboratories of School of

Public Health, Tehran University of Medical Sciences by regular passage in out bred small white mice and cryo-conditions. Deep frozen *Leishmania major* promastigotes were prepared based on WHO protocol in 1997. Briefly, parasites were cultured from infected mice in Schnieder (Hymedia, GibCO Ltd.) and PRMI 1640 (GibCO, Ltd.) then, Sub cultured them for each 72 h interval. The Promastigotes were harvested 120 h post the fourth subculture and washed with isotonic normal saline. The parasite suspension was mixed with phosphate buffer saline (PBS, pH = 7.2) or isotonic glucose-NaCl and glycerol 10% and counted by Neobar glass (2×10^7 parasite/mL) and checked up for bacteriology and fungi contaminations. Then, gradually frozen in liquid nitrogen. Viability test showed that from 5 to 25% of promastigotes were alive after this process.

Animal inoculation: Two hundred and thirty seven mice (Out bred) were prepared from (Institute Pasture of Iran) and divided into two groups; Group I (135) was inoculated with 0.1 mL of *Leishmania* suspension subcutaneous in the base of the tail, Group II (102) was injected by isotonic normal saline as a control with the same procedure which mentioned above (Nadim *et al.*, 1997). Lesions evolution was monitored every two week until 6 months post challenge by measuring the diameter of the inoculation site with a caliper and their parasitological status determined microscopically and cultural procedures. For determining the CMI system in interventional and control groups, all the mice were tested by Leishmanin that prepared by Pasteur Institute of Iran before and 6 months post injections. The indurations measured 72-post injection. Data were analyzed by t-test and ANOVA.

RESULTS AND DISCUSSION

One hundred thirty nine laboratory mice were infected with leishmanization and were under observation within 6 months, the frequency of *Cutaneous leishmaniasis* in the mice after 6 months post inoculation was shown in Table 1.

As it could be seen 63% of cases were positive after 6 month inoculation.

Fifty eight (63%) of the test mice were infected between 1.5 to 6 months of inoculation with the lowest size 1.50 mm and the highest 11.80 mm which the LST positive after six months post leishmanization in the mice was shown in Table 2.

The skin test of cases showed that 64.1% were positive (Table 2).

Table 1: Frequency of *Cutaneous leishmaniasis* in the mice 6 months post inoculation

Group	No.	Positive		Negative	
		No.	%	No.	%
Case	92	58	63	34	37
Control	47	0	0	47	100
Total	139	58	41.7	81	58.3

Table 2: The LST positive after six months post leishmanization in the mice

Group	No.	Skin test after 6 month				Take after 6 month			
		positive		Negative		Positive		Negative	
		No.	%	No.	%	No.	%	No.	%
Case	92	59	64.1	33	35.9	58	63	81	88
Control	47	2	4.3	45	95.7	0	0	0	0
Total	139	61	43.9	78	56.1	58	41.7	81	58.3

Table 3: The LST results after 6 month post injection in the mice

Groups	%	(LST ⁻ ulcer ⁻)		(LST ⁺ ulcer ⁻)		(LST ⁻ ulcer ⁺)		(LST ⁺ ulcer ⁺)		Total
		No	%	No	%	No	%	No	%	
Case	36	39.1	23	25	21	22.8	12	13	92	100
Control	0	0	2	4.2	0	0	45	95.8	47	100

The correlation rate between lesion observations with LST results after 6 months post injection also shows in Table 3.

Currently there are no appropriate drugs or vaccines for the prevention of Leishmaniasis (CI de Oliveira *et al.*, 2003). Leishmaniasis is an intracellular infection and Live-attenuated candidates of *Leishmania* parasites have been proven to produce this kind of response, but the dangers associated with them have hindered their use (CI de Oliveira *et al.*, 2003). With the help of adjuvants and cytokines, whole-killed vaccines seem show great promise. Several research groups in the Islamic Republic of Iran, Pakistan and Sudan are at present participating in the Phase II and Phase III trials of the killed-*Leishmania major* vaccine developed by the Razi Institute, Teheran. Preliminary results indicate that leishmania vaccines will be an effective control tool in future after completion of clinical trials (Momeni *et al.*, 1997; Bora, 1996; Rab, 1996). Currently, the most successful vaccine attempts in humans has been achieved with whole-killed *Leishmania* promastigotes. Phase III clinical trials in the Middle East and South America with an autoclaved *Leishmania* and BCG have proven to be effective at reducing the incidence of *Cutaneous leishmaniasis*. Reported efficacy rates range from 18 to 78% (Sharifi *et al.*, 1998; Antunes *et al.*, 1986). However, it should be noted that leishmanization were used in different countries such as former Soviet Union and also in Islamic Republic of Iran before and after Islamic

Revolution the mass leishmanization in 1980- 1988 (during the imposed war time Iraq against Iran) were applied for Iranian soldieries (Nadim and Javadian, 1998) but nearly 2-3% side effects were seen in these group, therefore the programme was stopped in 1990 (Momeni and Aminjavaheri, 2003). Based a study which was carried out by Mohebbali and Hazrati (1990) the evaluation was carried out in 1987. Altogether, 418 leishmanized cases and 675 controls were studied. The average age in the former group was 20 years and in the latter it was 21 years. The study was carried out 15 months after leishmanization. The control group had been in the endemic area for more than 18 months. Out of the 418 men vaccinated, 237 (56.7%) were takes and 181 (43.3%) were non-takes. Almost two-thirds of the takes had been in the form of a nodule and one-third had ulcers; only four cases lasted for more than one year. It is possible that the rate of takes was higher than the figure shown because it is based on the answers of the soldiers and not direct observation. In these cases, many of them may not have noticed a nodule because it has neither pain nor pruritis. The infection rate among the vaccinated group was one-sixth that in the nonvaccinated (control) group (Mohebbali and Hazrati, 1997).

The standardization of leishmanin has been set up to overcome the possible reason in particular no vaccine or adequate preventive measures are available to reduce the risk of infection. The result of this study shows that still leishmanization could prevent the infection better than other procedures at least in Animal model (63% in case groups which was shown in Table 1 and also LST positive after six months post leishmanization were shown in Table 2 and 3 in the cases and control groups and it could be recommended when no preventive measures are available in particular in emergency situation.

In conclusion, deep frozen *Leishmania major* promastigotes that were prepared based on WHO protocol, could be produced lesion and induced CMI in the mice.

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