Arch. Inst. RAZI (1992) **42/43**, 39-50

Vaccine Preparation and Quality Control of Killed Leishmania major

R. Hashemi-Fesharki., S. Ale-Agha., P. Ahourai., E. Djavadian* ., A. Taghavi., A. Golabi., M. Kamali., K. Esmail-Nia., H. Ghafari., F. Mofasali and R. Kheirol-Omour.

Summary: Through a series of trials, the optimum pH, suitable growing temperature, medium and a method for storing L. Major in liquid nitrogen was realized. The seed of L. major in frozen form was removed from liquid nitrogen and grown in modified NNN-medium. The culture supernatant containing promastigotes was removed and grown in RPMI 1640 containing yeast extract and lactalbumine. The harvested promastigotes were inactivated by merthiolate, at concentration of 1/5000 and then washed through centrifugation, under standard recommended method. The resulting pellet was suspended and homogenized in saline solution containing merthiolate at a concentration of 1/10000. The solution (trial vaccine)was then frozen and thawed repeatedly. The viability test was performed and 2-ml vials were filled with one ml of the trial vaccine. Three batches of this trial vaccine, were produced at three different concentration levels. The biochemical tests were performed by measuring total nitrogen, carbohydrate and lipid contents. During the production process, before and after distribution of all three batches, samples were removed and the sterility test carried out.

To perform safety, innocuity and toxicity tests, samples of the trial vaccine at different levels of total nitrogen concentration, were inoculated into laboratory animals by I. P., S. C., I. M., and I. D routes. The inoculated and the controls were kept under clinical observation. No mortality was observed among animals and no abnormality among the ones which received the trial vaccine by S.C., I.M., and I.P. routes. Howsever, the ones which had received the trial vaccine by intradermal route showed some transient skin pathological

* *University of Tehran, School of Public Health*

changes on inoculation site, which disappeared 26 days post inoculation.

The quality control test on all three batches of the trial vaccine, indicatede that it has no adverse effects. The effects of the trial vaacine on human volunteers is under study at present.

Keywords: *Leishmania / Vaccines*

Introduction

As indicated by epidemiological studies, the human leishmaniasis caused by *Lishmania tropica major,* is approximately a self healing disease, which is usually followed by a life-long immunity. In the past and to some extent at present, live-virulent *Leishmania* promastingotes, grown in culture medium and inoculated intradermally, has been used as a vaccine to prevent the disease in high risk endemic region of Iran (6,7).

As leishmanization is associated with considerable morbidity, chemotherapy with antimonial drugs is expensive, and producing side-effects associated with numerous relapses and refractory cases, therefore the need for an inactivated, safe and innocuous vaccine becomes a key objective, to encourage the Razi Institute, to accept WHO proposal for production of such a vaccine. Convit et al (1) also indicated that the immunotherapy could be an alternative way to treat the patient and reduce the prevalence of the disease. Previously, Myrink et al (3,4) had made the effort to come up with a killed vaccine against, New World cutaneous leishmaniasis, with satisfactory results.

Here, for first time, we report our studies on the production of inactivated *Leishmania* vaccine and its quality control.

Materials and Methods

a- Modified NNN meduim:

NNN medium was modified by substituting lamb blood for rabbit blood and Stocker medium (2) for saline solution. Then *Leishmania major* strain was grown in resulting medium. This strain was obtained from the School of public Health, University of Tehran, Iran, and is the one commonly used for

leishmaization in Iran (6,7).

b- Monophasic medium:

1- Eagle and TC-199 media:

Foetal calf serum, sheep, horse and calf sera either as treated with polyethylenglycol (Carbowax 6000) or as untreated alone separately and with different cocentration levels were added to the above media. Then through a number of trials, the characteristics of parasite were evaluated.

2- RPMI 1640:

The same procedure (part 1) was repeated using RPMI 1640, and this time, yeast extract and lactalbumine were also added.

c- Storing procedure:

To store the *L. major*, 2-3x10^6 promastigotes per ml of the medium were mixed with glycerol at a level of 7.5%. The mixture was then distributed in one ml aliquotes into 2-ml vials. The vials were cooled down to a temperature of 4-8 C° and frozen directly in deep freezing unit at - 70 C° and maintained there for 48 hours. These vials were then placed in liquid nitrogen and stored until usage. for viability test, every six months, samples were removed from liquid nitrogen. thawed and then evaluated.

d- Suitable temperature:

Through a number of trials, the appropriate temperature for growing *L. major* promastigotes was determined.

e- Appropriate pH:

Suitable pH was determined through a number of trials.

f- Inactivating method:

In order to inactivate the parasite, merthiolate at a concentration of 1/5000, was used.

h- Vaccine production:

1- Preparation of the trial vaccine:

The modified NNN medium was prepared and the seed of *L. major* was grown in it. A few subpassages were carried out and then culture supernatant was

grown in RPMI 1640 containing yeast extract and lactalbumine. The subpassages (not exceeding ten time) in the new medium, requiring maximum number of parasites were performed.

The parasites were harvested at stationary phase, and inactivated with merthiolate at level of 1/5000. In order to remove any trace of the medium, the harvested parasites were washed, by suspending in saline solution, and centrifuging repeatedly. The resulting pellet was homogenized in saline solution, containing merthiolate at a concentration level of 1/10000.

To make sure that the promastigotes were totaly dead, the final concentration (trial vaccine) was frozen and thawed at temperatures - 20° and 37C° respectively, for eigth times and then, viability test was performed.

To determine the number of promastigotes, they were counted after the harvest and following the concentration procedure.

2- Sterility test:

During the process of vaccine production, before and after distribution into vials, samples were removed and grown in anaerobic, aerobic and mycoplasma media to check the sterility of vaccine.

3- Safety, innocuity and toxicity tests:

To perform these tests, samples at different concentration levels were inoculated into laboratory animals (mice, guinea-pigs, rabbits and monkeys) by I.M., I.P., S.C., and I.D., routes.

The animals were kept under observation for a period of 26 days, during which the inoculation sites, general conditions and the weights of some animals were evaluated and measured (tables 1-4, figures 1-2).

4- Biochemical tests:

Samples of the trial vaccine were randomly removed from liquid nitrogen and thawed. Then through micro-Kjeldahl, ferric chloride and chlorometry methods, the total nitrogen, lipid and carbohydrate content of the sample were measured respectively.

Results and Conclusion

Our study indicated that logarithmic phase of *L. major* growth, in modified NNN medium (containing sheep blood and Stocker medium) is faster and the duration of the stationary phase is shorter than that of *L. major* in normal NNN (containing rabbit blood and saline solution). The number of

parasite increases much more in modified NNN medium as well.

The study also shows that RPMI 1640, mixed with yeast extract and lactalbumine containing 15% foetal calf serum, is a more suitable medium, to obtain the required number of promastigotes without any characteristic changes. The number of promastigotes in this monophasic particular medium, reaches more than $18x10^6$ ml. In the course of the study, it was oberved that merthiolate concentration at a level of 1/5000, is as effective as the previous concentration at level of 1/1000, and could inactivate the parasite efficiently. Since the centrifugation process takes long and the parasite could be damaged, therefore it is practical to use the 1/5000 concentation of merthiolate instead.

The method we applied, to maintaine and store the promastigotes in frozen form at-196 C° has proved to be an effective one over long periods of time without any significant losses of parasite chracteristics. As recommended by WHO, the frozen form of the seed is safer than the one removed from an infected mouse to produce the vaccine. We have therefore used this recommendation throughout our studies.

The most suitable temperature and pH in which *L. major* grows well are 25C° and 7.2 respectively.

Quality control tests of the trial vaccine, using in vitro and in vivo methods resulted in the following observations:

a- No contamination was noticed in samples taken for sterility test.

b- As shown in tabls 1-4 and figures 1-2, all animals had an overweight and their general conditions were normal as well as controls. No mortality was observed among animals and no abnormality among the ones which received the vaccine by S.C., I.M., and I.P., routes but the ones which received the vaccine by interadermal route showed some skin pathological changes such as erythema, induration and necrosis. These symptoms, however, gradually disappeared within 26 days post inoculation. Table 3 shows that a gradual increase of promastigotes number from 2×10^9 to 10×10^9/ml (containing 8000μg to 40 mg total nitrogen)is accompanied with a gradual increase of skin reactions.

The clinical skin changes are transient and gradually disappeared within 26 days post inoculation leaving slight traces on the skin.

The quality control tests of trial vaccine using in vivo and in vitro methods indicate that it is harmless and can be tested on human volunteers which is, now, under investigation. As Modabber (5) indicated a prophylactic vaccine against *Leishmania* is feasible, beneficial and cost-effective, we have also a firm opinion that the effect of the trial vaccine in volunteers will be significant.

Acknowledgment

This ivestigation received finantial support from the UNDP/World Bank/WHO Special Programme of Research and Training in Tropical Diseases.

Referenses

1 - *Convit, J., Rondon, A., Ulrich, M., Bloom., Castellanos, Castellanos., Pinardi, M.E., Castes, M., and Garcia, L., (1987): Immunotherapy Versus Chemotherapy in Localised Cutaneous Leishmaniasis., The Lancet, 401-405.*

2 - *Macpherson, I.A., and Stocker, M.G.P., (1962): Polyoma Transformation of Hamster Cell Clones-an Investigation of Genetic Factors Affecting Cell Competance.*

3 - *Mayrink, W., costa, C.A.DA., magalhaes., P.A., Melo, N.N., Dias, M., Oliveira Lima, A., Nichalick, M.A., and Williams. P., (1979): A Field Trial of a Vaccine Against American Dermal Leishmaniasis., Transactions of the Royal Society of Tropical Medicine and Hygiene, 73, No.4, 385-387.*

4 - *Mayrink, K., Williams, P., Costa, C.A., Magalhaes, P.A., Melo, M.N., Dias, M., Oliveira Lima, A., Michalick, M.S.M., Carvalho, E.F., Sessa, P.A., and Alencar, J.T.A., (1985): An Experimental Vaccine Against american Dermal Leishmaniasis: Experience in the State of Espirito Santo, Brazil., Ann. Trop. Med & Parasitology., 79, 3, 259-269.*

5 - *Modabber, F., (1989): Experiences with Vaccines Against Cutaneous Leishmaniasis of men and mice, Parasitology, 849-860.*

6 - *Nadim, A., Javadian, E., Tahvildar Bidruni, GH, Ghorbani, M., (1983): Effectivness of Leishmanization in Control of Cutaneous Leishmaniasis., Bull. Soc. Path. Ex, 76, 377-383.*

7 - *Nadim, A., & Javadian, e., (1988): Leishmanization in the Islamic Republic of Iran, Research on Control Strategies for Leishmaniases ed; Walton, PM., Wyeyartme and Modabber, F., PP. 336-9 Ottawa.*

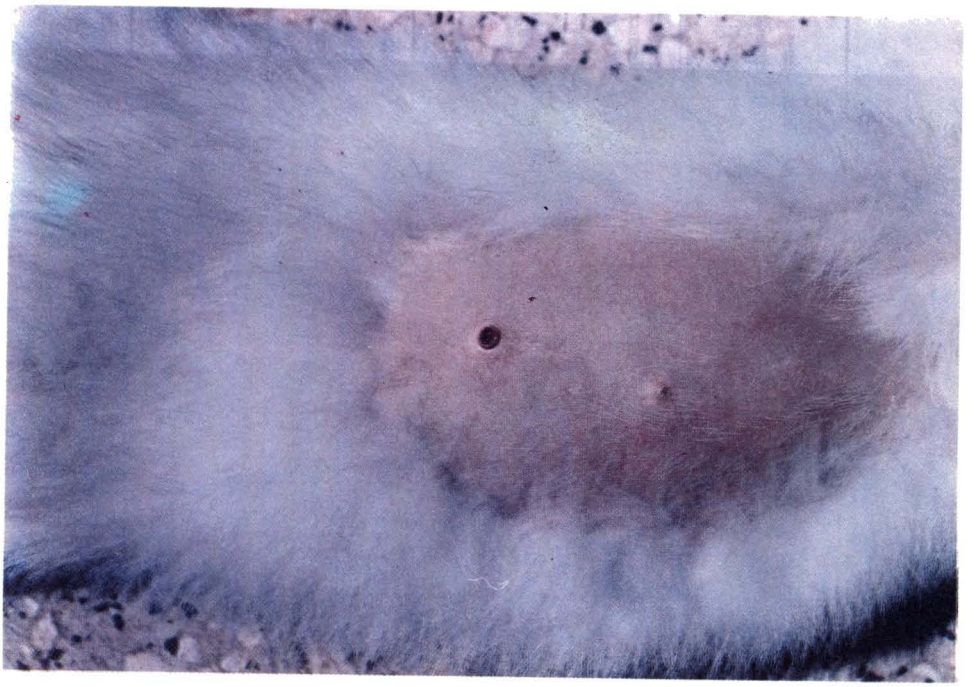

Fig 1: *Shows necrosis on the site of inoculation in Guinea pig:*

a- On the right: 0.1 ml of the vaccine, containing 400 μ g total nitrogen was inoculated

b- On the left: 0.1 ml of the vaccine, containing 200 μ g was inoculated.

Both necrosis (a&b) disappeared within 14-21 days after inoculation

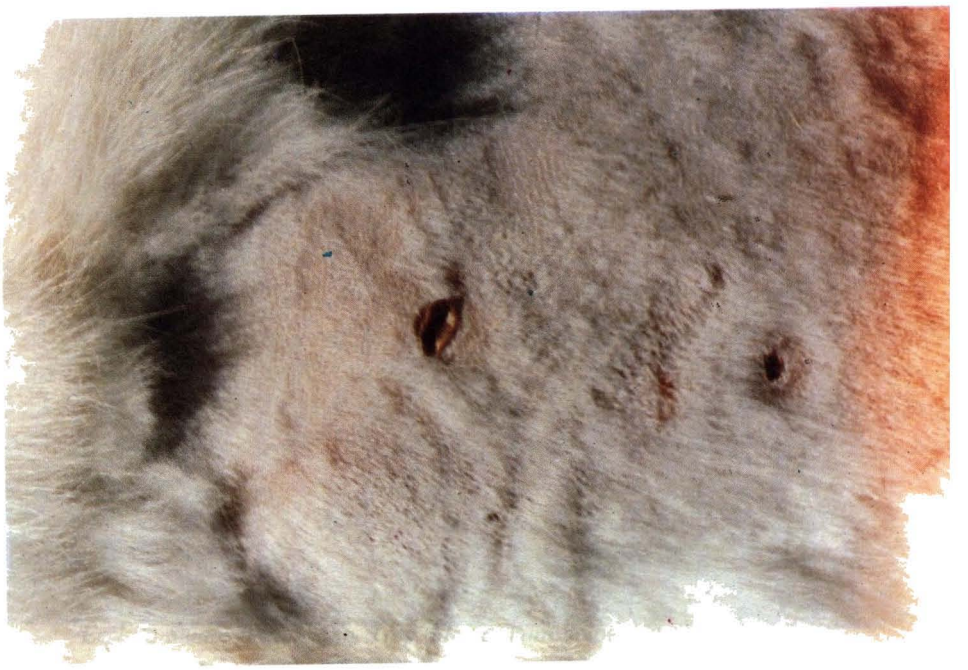

Fig 2: *Shows necrosis on the site of inoculation in rabbit.*

a- On the right: 0.3 ml of the vaccine, containing 2400 μ g total nitrogen was inoculated

b- On the left: 0.5ml of the vaccine, containing 4000 μ g total nitrogen, was inoculated

Both necrosis (a&b) disappeared within 24-26 days post inoculation.

Table No.1 - Safety, innocuity and toxicity tests of non-living L.major vaccine, containing 8mg/ml total nitrogen in mice

Group No. s	Number of animals	Inoculation route	Dosage	Total weight of animal				observations
				Before inoculation	7days after inoculation	14 days after inoculation	21 days after inoculation	
1	5	S.C.[.]	0.25ml	90gr	100gr	140gr	165gr	a-Overweight b-General condition: Normal
2	5	I.P.[..]	0.25ml	92gr	104gr	110gr	125gr	a-Overweight b-General condition: Normal
3	5	I.M.[...]	0.25ml	90gr	120gr	130gr	160gr	a-Overweight b-General condition: Normal
4	5	I.D.[....]	0.2ml	100gr	128gr	140gr	165gr	a-Over-weight b-General condition:Normal c-Erythema d-Small nodules e-Necrosis These symptoms (c,d&e) gradually disappeared 17-24 days Post-inoculation
5 Controls	8	S.C. I.P. I.M. I.D.	Received only Saline solution+1/10000 Merth.. 0.2ml/animal	160gr	180gr	196gr	220gr	a-Over-weight b-General condition: Normal c-Without any changes in inoculation sites

. = Subcutaneously
.. = Intraperitoneally
... = Intramuscularly
.... = Intradermally

Note: Average weight of each mouse ragning from 18 to 20 gr

Table No.2 - Safety, innocuity and toxicity tests of non-living L.major vaccine, containing 8mg/ml total nitrogen in guinea-pigs

Group No. s	Number of animals	Inoculation route	Dosage	Total weight of animal				observations
				Before inoculation	7days after inoculation	14 days after inoculation	21 days after inoculation	
1	5	S.C.	0.5 ml	1530 gr	1590 gr	1650gr	1800 gr	a-Over-weight b-General condition: Normal
2	5	I.P.	0.5 ml	2020 gr	2060 gr	2090 gr	2120 fgr	a-Over-weight b-General condition: Normal
3	5	I.M.	0.5 ml	1830 gr	1900 gr	1950 gr	2010 gr	a-Over-weight b-General conditoln: Normal
4	5	I.D.	0.2ml	1680gr	1800gr	1900gr	2000gr	a-Over-weight b-General condition:Normal c-Erythema d-Small nodules e-Necrosis These symptoms (c.d.& e) gradually disappeared 17-25 days after inoculation
5 Controls	8	S.C. I.P. I.M. I.D.	Received only Saline solution+1/10000 Merth.. 0.2ml/animal	2000gr	2060gr	2100gr	2200gr	a-Over-weight b-General condition: Normal c-Without any changes in inoculation sites

Note: Average weight of each guinea-pig ranging from 300-400 gr.

Table No.3 - Intradermal inoculation of non-Living L.major vaccine (Lot 6), containing 8mg/ml total nitrogen, in rabbits.

Animals No. S	Dosage Per ug total nitrogen	Dosge Per ml	Not Completely absorbed	Skin pathological changes in inoculation sites. Maximum diameter per mm			Absorbtions of the vaccine & disappearance of the symptoms after inoculation (days)
				Erythema	Induration	Necrosis	
a	800 μg	0.1	+	6	5	5	18
20 b	1600 μg	0.2	+	8	6	7.5	20
c	2400 μg	0.3	+	10.5	7.5	7.5	25
a	1600 μg	0.2	+	8	8	6.5	19
21 b	1600 μg	0.2	+	9	9	7	20
c	2000 μg	0.25	+	11	10	7	21
a	2000 μg	0.25	+	11	11	8	21
22 b	2000 μg	0.25	+	10	12	9	22
c	4000 μg	0.5	+	15	14	8.9	26
a 23 b c Controls	Received only saline solution contining 1/10000 Merthiolate (0.2 ml)	-	-	-	-	-	Without any changes in inoculated Sites.

Note: Each animal (No. S 20,21 & 22) received three different doses of the vaccine inoculated at three different spots of flank skin 3.5 centimeters aparts.

Table No.4 Intradermal inoculation of non-Living L.major vaccine (Lot 6), containing 8mg/ml total nitrogen, in cynemolgus monkeys.

Group No. S		Dosage Per total nitrogen	Dosage per ml	Skin pathological changes in inoculation sites Erythema	Induration	Necrosis	observations
29	a	1600 μg	0.2 ml	-	-	-	a-General condition: Normal b-Without any Changes in inoculation sites
	b	2400 μg	0.3 ml				
33	a	1600 μg	0.2 ml	-	-	-	a-General condition:Normal b-Without any changes in inoculation sites
	b	2800 μg	0.35 ml				
40 control		Received only saline solution containing 1/10000 Merthiolate (0.2 ml)		-	-	-	a-General condition: Normal b-Without any changes in inoculation sites

Note: Cynemolgus monkeys No.S 29& 33, each received two different doses of the vaccine inoculated at two different spots of back skin 3 centimeters aparts.