Medical Journal of the
Islamic Republic of Iran

Volume 8
Number 4
Winter 1373
February 1995



Original Articles

# TRIAL OF A NON-LIVING CRUDE VACCINE AGAINST ZOONOTIC CUTANEOUS LEISHMANIASIS

M. MOHEBALI, E.H. JAVADIAN, R. FESHARAKI,*
M. MOHAMMADZADEH**, A. NADIM, GH. TAHVILDAR-BIDRUNI,
AND A. MESDAGHINIA

*From the School of Public Health and Institute of Public Health Research, Tehran University of Medical Sciences, *Department of Protozoology and Entomology, Razi State Serum and Vaccine Institute, Tehran, and **Amir al-Momenin Hospital, Shahid Sadooghi University of Medical Sciences, Yazd, Islamic Republic of Iran.*

## ABSTRACT

A field trial was carried out on 60 volunteers selected in Yazd province, central Iran, with a vaccine containing killed promastigotes of *L. major* prepared by the Razi Institute, Hessarak. During these phase I studies which lasted for more than two years, we examined acceptable doses of the vaccine alone or mixed with BCG. The results so far indicate that 50 to 1000 µg of the vaccine alone can be well tolerated without major side-effects. The mixture of BCG with 400 µg of the vaccine produced pain and itching in five out of six volunteers, a self-limiting lymphadenopathy in one out of six, and fever in three out of six. Further trials are planned with reduced doses of BCG. The leishmania skin test became positive in a high proportion of the treated individuals after vaccination. Circulating antibodies were detected from two weeks to one month after vaccination.

*MJIRI, Vol. 8, No. 4, 211-215, 1995.*

## INTRODUCTION

The pioneering work of Mayrink and colleagues in Brazil brought killed *L. braziliensis* vaccine to existence.[1,2] These studies showed that there are no apparent side-effects even over a long period (6-8 years) following immunization. This is in contrast to some experimental animal studies in which injection of leishmanial antigens was shown to exacerbate the subsequent challenge injection.[3,4] The results obtained by Mayrink's group were very encouraging and related skin test conversion to some degree of protection.[2] Their vaccine was a mixture of four strains of leishmania isolated from cutaneous lesions in Brazil.[1] A similar preparation from a well-characterized *L. major* strain was used in Iran with and without BCG adjuvant with the following objectives:

a) Determination of the safety and proper dosage of killed *L. major* vaccine (KLmV) ±BCG.

b) Determination of immunogenicity of KLmV as measured by the leishmania skin test.

## MATERIALS AND METHODS

*L. major* promastigotes (originally isolated by A. Nadim et al.)[5] were cultured at the Razi Institute under good

**TABLE I. The vaccination schedule**

| Group no. | Dose(μg) | PPD | BCG | Booster |
|---|---|---|---|---|
| 1 | 50 | - | - | + |
| 2 | 200 | + | - | + |
| 3 | 400 | - | - | + |
| 4 | 50 | - | + | - |
| 5 | 200 | - | + | - |
| 6 | 400 | - | + | - |
| 7 | 400(Repeated) | - | + | - |
| 8 | 600 | + | - | - |
| 9 | 800 | + | - | - |

Interval of each group was at least 2 weeks

manufacturing practice (GMP) conditions to stationary phase in culture media containing 20% fetal calf serum, washed extensively, killed with tymersol 1% (Merthiolate) and then freeze-thawed five times and stored frozen until use.[6]

In order to test the safety and immunogenicity of this KLmV, we first injected different doses of KLmV±BCG to susceptible animals at the Razi Institute. Side effects following vaccination were negligible in those animals.

Then 30 volunteers were chosen from the Taft/Yazd province, central Iran where no endogenous cases of cutaneous leishmaniasis exist. The volunteers consisted of male and female village health workers, and natives of the area, aged between 15 to 33 years. They were divided into ten groups before vaccination. Montenegro and PPD tests were performed for each individual. All were negative for Montenegro test(0-1mm induration at the site of injection), and 12 of them were PPD positive, which were selected for the group to receive vaccine without BCG.

Routine hematological (CBC,HCT), biochemical (sugar, triglycerides, uric acid, urea, bilirubin, and cholesterol) and enzymatic (SGOT, SGPT) tests were performed before the initiation of the trial and two weeks after vaccination.

The dose of the vaccine expressed as total nitrogen was different for each group, ranging from 50 to 1000μg. The vaccination schedule is shown in Table 1. The vaccines were inoculated intradermally at one or two sites of the deltoid muscle in each individual. The sites of inoculation were inspected during four weeks' time in those who received vaccine alone and in three months' time in those who received vaccine with BCG, for erythema, induration, local necrosis, lymphadenopathy, pain and itching.

The immunogenicity of the vaccine was assessed by recording change in response to the Montenegro test before and at least one month after vaccination.

### IFAT measurement of humoral responses

Sera were diluted to 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, and 1:512. IFAT and ELISA were performed as described by Hommel et al.[8]

## RESULTS

Hematological and biochemical blood tests were all normal before and after vaccination except for a very small increase in SGOT which can be expected in any inoculation damaging some cells (Tables II, III). Side effects after vaccination were negligible in groups receiving vaccine without BCG.

The volunteers receiving vaccine containing 1000μg/ total nitrogen (Group 10), developed erythema with a diameter of 35-45mm starting 5 minutes after vaccination which was reduced to 6-7mm in 72 hours after vaccination. In group 4 (50μg + BCG), erythema and induration of more than 5mm was observed after 48 hours of vaccination at the site of inoculation which gradually disappeared in aboutone month. In the fifth group (200μg + BCG), erythema and induration of more than 5mm was observed 48 hours after vaccination which gradually changed to lesions and healed, leaving a scar of approximately 5mm after one month. In

**TABLE II. Biochemical blood tests before and two weeks after vaccination**

| | Before vaccination | | | | | Two weeks after vaccination | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Dose | Sugar | Trigly-cerides | Choles-terol | Urea | Uric Acid | Sugar | Trigly-cerides | Choles-terol | Urea | Uric Acid |
| 50 μg | 89.6 | 77.6 | 147.6 | 27 | 4.6 | 84.6 | 88.6 | 111.6 | 18.6 | 3.83 |
| 200 μg | 86.3 | 129.3 | 175.3 | 14.6 | 5.56 | 70 | 90 | 162.3 | 16.6 | 4.83 |
| 400 μg | 94.3 | 99.6 | 147.6 | 19.6 | 5.46 | 84 | 115.3 | 140.6 | 15 | 5 |
| 50μg+BCG | 97.6 | 146 | 155 | 16.6 | 6.2 | 97.6 | 116 | 153.3 | 22 | 4.56 |
| 200μg+BCG | 89 | 122.66 | 138.6 | 18.6 | 4.8 | 89.3 | 122.6 | 136.6 | 19 | 4.46 |
| 400μg+BCG | 93.5 | 136.8 | 170 | 26 | 5.28 | 93.5 | 137.3 | 160.3 | 26 | 10.06 |
| 600μg | 91 | 152.6 | 160.3 | 20.6 | 5.33 | 91 | 152.66 | 160.3 | 20.6 | 5.33 |
| 800 μg | 91.6 | 139 | 158.6 | 23.6 | 5.33 | 91.6 | 139 | 158.6 | 23.6 | 5.33 |
| 1000μg | 90 | 140 | 180 | 25 | 5.60 | 92 | 130 | 185 | 23 | 5.52 |

those six individuals in groups 6 and 7 (400µg + BCG), erythema and relatively severe induration was noted after 48 hours of vaccination and even in one case an induration of about 40-45mm was seen in one of the arms (site of inoculation), which disappeared after 48 hours. Finally, three months later, only a 6×4mm scar was observed.

Two individuals were tested with BCG as a control group in whom erythema (8-10mm), induration (5-7mm), pain and itching were noted after 48 hours.

**Immunogenicity**

Before inoculation of KLmV±BCG, the Montenegro test was negative (0-1mm) in all volunteers. Table IV shows the results of Montenegro testing 30 days or more after vaccination.

The results of serological tests are summarized in Tables V and VI.

**Booster Injection**

The 3 groups (1 to 3) receiving KLmV alone at doses of 50,200 and 400µg were given a booster injection of the same dose as they had received originally on days 162, 147 and 132 after the first injection. Side effects following booster injection were negligible.

## DISCUSSION

In the process of developing a new vaccine, the first clinical step (Phase I) aims at defining the doses of vaccine which are safe and acceptable. For whole killed leishmania vaccine, although much work has been done over the past decade on the safety and immunogenicity in Latin America,[1,2] nothing was done in a systematic way to define the optimal dose and schedule of injection. In the old world, live

**TABLE III. Hematology and serum transaminases before and 2 weeks after vaccination**

| | Before vaccination | | | | | | | | | Two weeks after vaccination | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Dose | HCT | WBC% | | | | | | SGOT | SGPT | HCT | WBC% | | | | | | SGOT | SGPT |
| | | N | E | B | L | M | Total | | | | N | E | B | L | M | Total | | |
| 50 µg | 44 | 67 | 1 | - | 31 | 1 | 8850 | 12 | 4 | 43.3 | 58 | 3 | - | 38 | 1 | 7500 | 32 | 13 |
| 200µg | 46.6 | 58 | - | - | 40 | 2 | 9300 | 21.3 | 20 | 47 | 57 | 1 | - | 41 | 1 | 9000 | 37 | 17.6 |
| 400µg | 50.3 | 79 | 1 | - | 20 | - | 6500 | 17.3 | 3.66 | 47.3 | 69 | 2 | - | 29 | - | 6000 | 36 | 13.6 |
| 50µg+BCG | 47.3 | 66 | - | - | 32 | 1 | 6400 | 19.3 | 12.33 | 48 | 54 | 4 | - | 42 | - | 7800 | 32 | 13 |
| 200µg+BCG | 47 | 52 | 2 | - | 49 | - | 7000 | 33.3 | 13 | 45.6 | 50 | 2 | - | 48 | - | 6300 | 38 | 16.5 |
| 400µg+BCG | 46 | 56 | - | - | 43 | 1 | 7150 | 19.13 | 9.96 | 46 | 61 | - | - | 38 | 1 | 7000 | 20.83 | 15 |
| 600µg | 47 | 61 | - | - | 38 | 1 | 7600 | 23.63 | 20.63 | 46.6 | 60 | - | - | 45 | 1 | 7900 | 25.3 | 19.5 |
| 800µg | 48 | 62 | - | - | 37 | 1 | 6900 | 21.16 | 15.43 | 48 | 60 | 2 | - | 38 | - | 6800 | 28.7 | 23 |
| 1000µg | 49 | 67 | 2 | - | 35 | 1 | 7500 | 22 | 17 | 47 | 65 | - | - | 39 | - | 7100 | 29 | 18 |

**TABLE IV. The results of Montenegro test ≥ 22 days after vaccination.**

| Dose(µg) | Mean skin test before vaccination (mm) | No. of individuals | Skin Test positive | Skin Test negative | Day of skin test | Mean |
|---|---|---|---|---|---|---|
| 50(1) | 0 | 3 | - | 3 | 92 | $\bar{X}$=3mm |
| 200(1) | 0 | 3 | 1 | 2 | 78 | $\bar{X}$=4.8mm |
| 400(1) | 0 | 3 | 3 | - | 64 | $\bar{X}$=5.5mm |
| 50+BCG(1) | 0 | 3 | - | 3 | 50 | $\bar{X}$=2.3mm |
| 200+BCG(1) | 0 | 3 | 2 | 1 | 36 | $\bar{X}$=5mm |
| 400+BCG(1) | 0 | 6 | 5 | 1 | 22 | $\bar{X}$=4.9mm |
| 600(1) | 1 | 3 | 2 | 1 | 45 | $\bar{X}$=5.2mm |
| 800(1) | 0 | 3 | 2 | 1 | 45 | $\bar{X}$=4.7mm |
| 1000(2) | 0 | 3 | 3 | - | 45 | $\bar{X}$=6.7mm |

(1). Single injection, (2). Injection at 2 sites (0.1 ml each)
*Positive= ≥5mm

**TABLE V. ELISA leishmanial antibody level before and two weeks after vaccination in 30 volunteers**

| Dose (μg) | Vaccination | | |
|---|---|---|---|
| | before | after 2 weeks | 2 weeks after booster |
| 50 | 0 | 0 | 1:251* |
| 200 | 0 | 0 | 1:245* |
| 400 | 0 | 0 | 1:251* |
| 50+BCG | 0 | 0 | - |
| 200+BGC | 0 | 0 | - |
| 400+BCG | 0 | 1:39* | - |
| 600 | 0 | 1:199* | - |
| 800 | 0 | 1:251* | - |
| 1000 | 0 | 1:251* | - |

*Geometric mean of antibody titre

**TABLE VI. IFAT leishmanial antibody level before and two weeks after vaccination in 30 volunteers**

| Concentration (μg) | Inoculation | | |
|---|---|---|---|
| | before | after 2 weeks | 2 weeks after booster |
| 50 | 0 | 0 | 1:10* |
| 200 | 0 | 0 | 1:16* |
| 400 | 0 | 0 | 1:10* |
| 600 | 0 | 1:19* | - |
| 800 | 0 | 1:31* | - |
| 1000 | 0 | 1:32* | - |
| 50+BCG | 0 | 0 | - |
| 200+BCG | 0 | 0 | - |
| 400+BCG | 0 | 1:17* | - |

*Geometric mean of antibody titre

leishmania (leishmanization) or irradiated *L. major* has been used as the vaccine. The former is not acceptable unless in extreme conditions and the latter has never been shown to produce a protective immune response.

We used whole killed *L. major* prepared similarly to that used by Mayrink et al. in a systematic way to determine its toxicity and acceptability given as an intradermal injection.

In addition, low doses of vaccine were mixed with live BCG as an adjuvant in order to enhance the cell-mediated immune response against leishmania antigens.

To be safe, we started with a dose of 50μg. This is only 10 times more than the amount of antigen (leishmania) used in skin tests. Data from Mayrink's group indicate that 3 intramuscular injections of 360μg each, one week apart, were tolerated and not associated with measurable toxicity of short or long term. Convit et al. injected a dose well over 2 mg mixed with BCG 2-3 times with intervals of 1 month for immunotherapy. In retrospect, the 50μg dose seems too low; however, the results of this and two later injections of the same dose as the primary injection, were encouraging in that a serological secondary response was observed.

Higher doses of the vaccine, up to 1000μg (injected at 2 different sites) by itself produced no unacceptable side effects.

The major toxicity and side effects were related to the use of BCG. Depending on the source of BCG, different side effects such as lymphadenopathy, pain, ulceration and fever may be observed. We used BCG provided by the State Serum Institute, Copenhagen, Denmark which is known not to be free of side-effects. It was established that addition of 400μg KLmV to a normal dose of BCG produces reactions which would not be acceptable.

Comparison of different groups concerning immunogenicity was not possible, because the intervals between vaccination and skin testing were not uniform. The reason for this was logistic. We were about 350 km away from the site and the volunteers were students-primary health workers who spent a few months at the school but were then distributed in different villages dispersed throughout an area more than 10km distant from the school.

In addition, the injections were made sequentially with intervals of 6 weeks for KLmV alone or 3 months for KLmV+BCG, going to progressively higher doses of the vaccine.

In spite of the difficulties and the limitation of having as few as 3 volunteers per group (which was imposed by ethical considerations) these studies have shown that the KLmV can be injected up to 800 µg intradermally at one site or 500µg intradermally at two sites with acceptableside effects. Furthermore, it is not recommended to use a full dose of BCG mixed with 400µg KLmV under the conditions described.

These works have paved the way for future trials using higher amounts of KLmV combined with lower doses of BCG, if needed.

## ACKNOWLEDGEMENTS

We wish to express thanks to Dr. F. Modabber, Dr. M. Asmar, Dr. M. Assai, Dr. B. Sadrizadeh and Mr. A. Mehrabi for their collaboration.

This investigation received financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

## REFERENCES

1. Mayrink W, Da Costa CA, Magalhaes PA, et al: A field trial of vaccine against American dermal leishmaniasis. Trans Roy Soc Trop Med 73: 385, 1979.
2. Antunes CMF, Mayrink W, Magalhaes PA, et al: Controlled field trials of a vaccine against New World cutaneous leishmaniasis. Int J Epidemiol 15: 572-580, 1986.
3. Titus RG, Lima GC, Engers HD, Louis JA: Exacerbation of murinecutaneous leishmaniasis by adoptive transferof parasite-specific helper T-cell populations capable of mediating *L. major* specific delayed type hypersensitivity. J Immunol 133: 58-63, 1984.
4. Liew FY, Halec, Howard JG: Prophylactic immunization against experimental leishmaniasis; IV subcutaneous immunization prevents the induction of protective immunity against fatal *L. Major* infection. J Immunol 135: 2095-2101, 1985.
5. Nadim A, Faghih MA: The epidemiology of cutaneous leishmaniasis in the Isfahan province of Iran: I. The Reservoir. Trans Roy Soc Trop Med Hyg. 62(534-549), 1968.
6. Hashemi-Fesharki, et al: Vaccine preparation and quality control of killed *L. major* vaccine. Meeting on vaccines against leishmaniasis, Belo-Horizonte, 10-11 September, 1990.
7. Javadian E, Mohebali M, et al: Phase I studies of *L. major* vaccine ±BCG. (Abstract) Meeting on vaccines against leishmaniasis, Belo-Horizonte, 10-11 September, 1990.
8. Hommel M, Peters W, Ranque J: The micro-ELISA technique in serodiagnosis of visceral leishmaniasis. Annals of Tropical Medicine and Parasitology 72(3): 215-7, 1978.