Randomised vaccine trial of single dose of killed *Leishmania major* plus BCG against anthroponotic cutaneous leishmaniasis in Bam, Iran

Iraj Sharifi, Ali Reza FeKri, Mohammad-Reza Aflatonian, Ali Khamesipour, Abolhassan Nadim, Mohammad-Reza Ahmadi Mousavi, Ali Z Momeni, Yahya Dowlati, Tore Godal, Fabio Zicker, Peter G Smith, Farrokh Modabber

Summary

Background A vaccine consisting of a single dose of whole-cell autoclave-killed *Leishmania major* (ALM) mixed with BCG was assessed in comparison with BCG alone against anthroponotic (human to human transmission) cutaneous leishmaniasis in a randomised double-blind trial in Bam, Iran.

Methods 3637 schoolchildren, aged 6–15 years, with no history of cutaneous leishmaniasis and no response to a leishmanin skin test, were randomly assigned to receive 1 mg ALM mixed with BCG (n=1839), or BCG alone (n=1798). Safety of the vaccine and the incidence of confirmed cases of cutaneous leishmaniasis were followed up for 2 years.

Findings Side-effects were those usually associated with BCG vaccination, but tended to persist longer in the ALM+BCG group. After exclusion of four cases occurring within 80 days of vaccination (one in the ALM+BCG group and three in the BCG group), the 2-year incidence of cutaneous leishmaniasis did not differ significantly between vaccine and BCG groups: 2·8% *vs* 3·3%, respectively (total cases 112). A sex-stratified analysis showed that in boys the vaccine conferred a protective efficacy of 18% and 78% for the first and second years, respectively—a crude 2-year overall protection of 55% (95% CI 19–75%, p<0·01). In the first 9 months after vaccination, there was a non-significant excess of cases in the ALM+BCG group (25 *vs* 16), whereas the incidence of cutaneous leishmaniasis thereafter was significantly reduced in the ALM+BCG group (27 *vs* 44, p<0·05).

Interpretation A single dose of ALM+BCG was safe and more immunogenic than BCG alone, as measured by leishmanin skin test. The exact reason for the apparent protective effect of the vaccine in boys is unknown, and may be a chance finding. However, since boys are more exposed to the infection, which is indicated by higher disease prevalence in boys in this study population, the preferential protective effect in boys may have resulted from a greater

Kerman University of Medical Sciences, Medical School, Kerman, Iran (I Sharifi PhD, A R Fekri MD, M-Z Ahmadi Mousavi MD)
Provincial Health Centre, Bam Kerman, Iran (M-R Aflatonian MPH);
Centre for Research and Training in Skin Diseases and Leprosy, Tehran University of Medical Sciences, Tehran, Iran (A Khamesipour PhD, Y Dowlati MD); **School of Public Health, Tehran University of Medical Sciences, Tehran, Iran** (Prof A Nadim MD); **Isfahan University of Medical Sciences, Isfahan, Iran** (A Z Momeni MD); **UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR), Geneva, Switzerland** (T Godal PhD, F Zicker MD, F Modabber PhD); **and London School of Hygiene and Tropical Medicine, London, UK** (Prof P G Smith DSc)

Correspondence to: Dr Farrokh Modabber, World Health Organization, CH-1211 Geneva 27, Switzerland

booster effect produced by repeated exposure to infected sandflies. Booster injections or alternative adjuvants should be tried to improve the potential efficacy of this vaccine.

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Introduction

Cutaneous leishmaniasis is endemic in more than 80 countries, notably those of south-west Asia,[1] and is an important public-health problem in many parts of Iran.[2] Vector control is difficult and complicated by the diverse ecology of many species of sandfly vectors and animal reservoirs. Existing treatments are expensive, associated with adverse effects, and of fairly low efficacy. Following recovery from leishmaniasis, a life-long immunity usually ensues. Leishmanisation (artificial inoculation of live parasites) has thus been used to induce protection. Though efficacious, this practice was abandoned because a small proportion of people developed severe side-effects, including chronic lesions.[3] A safe, efficacious, and affordable vaccine would be the most practical and cost-effective control tool in many epidemiological situations.[4]

The pioneering studies of Mayrink and colleagues in Brazil, using a killed *Leishmania* vaccine without adjuvant, showed that it was safe but gave only limited protection.[5–7] In Venezuela, Convit and colleagues successfully used killed *Leishmania* mixed with BCG for immunotherapy.[8,9] Research on a vaccine against leishmaniasis in Iran began with the preparation of a killed vaccine under good manufacturing practices at Razi Vaccine and Serum Institute. The organism used for the preparation of whole-cell autoclave-killed *Leishmania major* (ALM) was the same as that used for leishmanisation of about 2 million individuals in the 1980s in Iran. Preliminary clinical assessment of a merthiolate-killed *Leishmania* vaccine mixed with BCG was carried out in a non-endemic area—Yazd, Iran.[10] Later, phase I-II randomised double-blind controlled trials were carried out in Tehran, Iran, to compare methiolate-killed *Leishmania* vaccine with a newly produced and more stable ALM that showed a similar profile of safety and immunogenicity.[11] Those studies were designed to determine the ALM and BCG concentrations to be used in efficacy trials. The assessments were based on leishmanin skin-test conversion and γ-interferon production in the absence of any detectable antibody or interleukin-5 response.[11]

We report the result of the first population-based vaccine trial against anthroponotic cutaneous leishmaniasis. The primary objectives of the trial were to assess the efficacy of a single dose of ALM+BCG in reducing the incidence of anthroponotic cutaneous leishmaniasis among schoolchildren compared with a group receiving BCG alone, and to extend the safety assessment. The secondary objectives were to study immunogenicity (by skin-test conversion) and to assess a potential effect on reduction of disease severity.

Characteristic	ALM+BCG (n=1839)	BCG (n=1798)
Mean age (range; SD) in years	9·1 (6–15; 1·6)	9·1 (6–15; 1·5)
Female/male (% female)	865/974 (47%)	931/867 (52%)
Purified protein derivative ≥5 mm (%)	857 (46·6%)	881 (49·0%)

Table 1: **Baseline characteristics of trial participants**

Methods

Study place and population

This study was carried out from September, 1994, to February, 1997, in the city of Bam, which has a population of about 100 000 and is located along the Zagros mountains at an altitude of 1080 m in south-east Iran. Temperatures reach 48°C in summer, but winters are mild. Annual precipitation is about 58·5 mm with relative humidity 25%. The area is endemic for anthroponotic cutaneous leishmaniasis. The trial was carried out among schoolchildren, since they are the likely future target group for vaccination. Before the trial began, meetings were held with community leaders, parents, and teachers to describe the purpose, procedures, and the potential benefits and risks. The trial protocol was reviewed by the Steering Committee on Vaccines against Leishmaniasis (TDR) and approved by the ethics committee of the Ministry of Health of Iran, and the WHO Secretariat Committee on Research Involving Human Subjects.
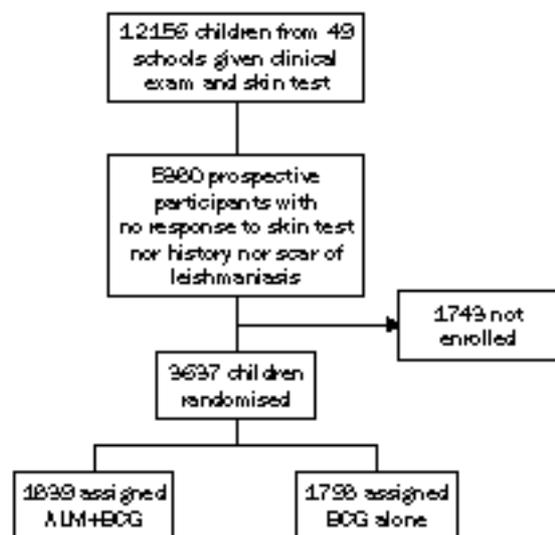
Trial design

We designed the study as a randomised, double-blind, controlled trial with 2-year follow-up after the administration of either a single dose of ALM+BCG or a single dose of BCG alone. The study was planned to include a minimum of 1430 participants in each group in order to have 80% power to detect a 50% reduction in incidence of anthroponotic cutaneous leishmaniasis at the 5% significance level assuming an annual incidence of 2% recorded in a previous study (unpublished) and a drop-out rate of 10% per year. A safety and acceptability study was carried out among a small number of children before the main trial.

Selection of participants

The trial profile is summarised in the figure. A total of 12 156 children aged 6–15 years in 49 primary schools in Bam were interviewed, clinically examined, and skin tested with leishmanin and purified protein derivative, both produced by Pasteur Institute, Tehran.[12] We measured the diameters of the indurations with the ball-point method 48–72 h after injections.[13]

Of those examined, 5380 (44%) were deemed eligible for enrolment in the trial. The main reason for exclusion was any measurable leishmania skin test response (induration >0 mm). To enter the trial, children also had to have no previous history or scar compatible with cutaneous leishmaniasis; no clinical evidence of acute or chronic disease; no history of allergic reaction; a response to purified protein derivative of less than 10 mm diameter; and no history of vaccination in the previous month.

Of those eligible for entry, 1743 (32%) were excluded for a variety of reasons, including lack of parental consent; absence on the day of vaccine administration; and the child's refusal. In addition, recruitment was slowed when it became clear that a sufficient number of children had been entered.

Vaccine administration

Sequentially numbered identical-looking vials containing 2·7 mL of either ALM or BCG diluent alone were produced by Razi Institute, Hessarak, Iran.[14] A random list of codes, balanced for every 20 vials and corresponding to each product, was prepared by the data safety and monitoring committee. The codes were kept sealed until the end of the 2-year follow-up. Just before immunisation, 0·3 mL freshly resuspended BCG was added to the next numbered vial, and children meeting the inclusion criteria were inoculated intradermally (0·1 mL) in the deltoid area. The vials were used in sequence, one at a time, and an average of roughly 25 children were inoculated from a single vial before the next sequentially numbered vial was opened. The dose of BCG received by a child corresponded to 1/10 of the standard dose. All vials and BCG were kept at 4°C and taken to schools in ice boxes. Any opened but incompletely used vials were discarded at the end of each working period (lunch break or the end of the day).

Follow-up and case-ascertainment

Following vaccination, on days 1, 7, 30, and 80, children were examined for lymphadenopathy, presence of ulcer, redness and induration at the site of injection, and asked about pain and itching. Leishmanin skin tests were done on day 80 and 1 year after vaccination. Active case-finding was implemented by clinical examination of participants in school every 2 months for a period of 2 years, except during the summer vacation when cases were reported to the trial clinic or referred by health centres. Suspected cutaneous lesions lasting for more than 4 weeks were examined parasitologically by routine methods with direct smear or culture stained by Giemsa; if positive, the child was classified as a case of cutaneous leishmaniasis.

Skin specimens were cultured in locally produced modified Nevy-Nicolle-MacNeal medium[15] at 24°C for 7 days, and transferred into Rosewell Park Memorial Institute—(RPM-1640) medium (Gibco, UK) containing 15% heat-inactivated fetal calf serum, penicillin (200 units/mL), and streptomycin (200 μg/mL). We checked cultures weekly for promastigotes for up to 4 weeks. Species identifications were made by immunofluorescence and by ELISA at Kerman University of Medical Sciences by use of specific monoclonal antibodies (provided by C Jaffe, through the Special Programme for Research and Training in Tropical Diseases), and by PCR (S Ardehali, Shiraz, Iran), as well as by isoenzyme characterisation (J P Dedet, Montpellier, France; and S Ardehali, Shiraz).

Case-management

The lesions of every confirmed case of cutaneous leishmaniasis were photographed from a fixed distance with identification number and date. We used these to verify size, and to document

Trial profile

12156 children from 49 schools given clinical exam and skin test

5380 prospective participants with no response to skin test nor history nor scar of leishmaniasis

1743 not enrolled

3637 children randomised

1839 assigned ALM+BCG

1798 assigned BCG alone

Side-effects	Day 7		Day 30	
	ALM+BCG (n=1727)	BCG (n=1713)	ALM+BCG (n=1764)	BCG (n=1726)
Redness	77·5%*	74·3%	56·8%‡	48·6%
Ulcer	19·8%‡	14·7%	48·2%†	43·0%
Lymphadenopathy	19·8%	20·0%	6·6%†	3·4%
Itch	27·0%*	23·9%	15·7%†	12·0%
Pain	15·4%	16·3%	14·0%‡	9·2%
Induration	72·4%‡	64·7%	67·2%‡	55·1%

Response to leishmanin skin test ≥5 mm	At day 80	At 1 year
ALM+BCG	16·5% (n=1807)‡	3·2% (n=1761)
BCG	11·5% (n=1681)‡	3·6% (n=1654)

*p<0·05. †p<0·01. ‡p<0·001 (comparing ALM+BCG with BCG alone).

Table 2: **Side-effects and results of leishmanin skin test following vaccination**

Vaccine group	Cases of leishmaniasis by time since vaccination								
	0	80 days	6 months (m)	9 m	12 m	15 m	18 m	21–24 m	Total
ALM+BCG (n=1839)	1	15	10	6	6	7	7	1	53
BCG (n=1798)	3	7	9	11	12	11	6	4	63

Table 3: **Cases of leishmaniasis in vaccine groups by time since vaccination**

clinical severity and evolution. Photographs were taken at first presentation with a lesion, and at each follow-up until complete scar formation occurred. Before we broke the randomisation code, we classified cases into one of five severity categories according to clinical characteristics—time to ulceration, re-epithelialisation, scar formation, and response to treatment.

Owing to known side-effects of antimonials, most patients preferred topical treatment. During the first year only, topical metronidazole cream was available; thereafter, paromomycin ointment was used.

Data processing and analysis
Special forms and database files were created on Epi Info, data entry, validation, and analyses. A plan for analyses was established by the data safety and monitoring committee before the codes were opened. Data files were reviewed by an independent monitor (L Moulton, Johns Hopkins University, School of Hygiene and Public Health, Baltimore, MD, USA) who established strict criteria for inclusion of records and locked databases before the randomisation codes were broken. We used standard statistical tests to calculate the significance of differences between proportions (χ^2) and between means (t test). Drop-out rates were very low (<10%) and similar in both groups. Calculation of leishmaniasis-incidence rates was based on the numbers randomly assigned to each group, since for cases of leishmaniasis not to have been ascertained is unlikely. We calculated vaccine efficacy as $100 \times (1\text{-RR})$, where RR is the ratio of the incidence rate in the ALM+BCG group to that in the BCG study group. Analyses that took account of the extra-binominal variation were done with GEE models in STATA (version 5.0), based on the numbers of children vaccinated from the same vaccine vial. These analyses were carried out on the basis of the numbers vaccinated from each vial, and the proportions subsequently developing disease.

Results
3637 children were randomly assigned to receive ALM+BCG (n=1839) or BCG alone (n=1798). The two groups were similar with respect to age, sex, and responses to purified protein derivative (table 1). The frequencies of postvaccination side-effects were similar to the two groups 7 days after vaccination; the side-effects seemed mostly to be those associated with BCG vaccination. However, side-effects tended to persist longer in those injected with ALM+BCG compared with those in the BCG group, and were more frequent in the former group 30 days after vaccination (table 2). No side-effects occurred that required medical attention.

On day 80, 298 (16·5%) children in the ALM+BCG group had a leishmanin skin test ≥5mm, compared with 56 (3·2%) in the BCG group. The overall mean reactivity was 2·7 (SD 1·9) mm for those who received ALM+BCG, and 0·7 (1·5) mm for those who received BCG alone

	Vaccine		Protective efficacy (95% CI)	p
	ALM+BCG	BCG		
Cases between 80 days and 2 years	52 (2·8%) (n=1838)	60 (3·3%) (n=1795)	15% (−22 to 41)	>0·05
Boys	16 (1·6%) (n=973)	32 (3·7%) (n=866)	55% (19–75)	<0·01
Girls	36 (4·2%) (n=865)	28 (3·0%) (n=929)		
Cases after 9 months	27 (1·5%) (n=1838)	44 (2·4%) (n=1795)	40% (3·7–63)	<0·05

Table 4: **Prophylactic efficacy of a single dose of ALM+BCG compared with BCG alone against cutaneous leishmaniasis**

(p<0·01). The difference in leishmanin skin test response between the two groups persisted 1 year after injection, but had declined in both groups by this time to 1·5 (1·6) mm and 0·5 (1·5) mm, respectively.

Compliance to follow-up during the eight regular follow-up examinations was high; an average of 91% of children were examined at follow-up visit. Four cases of anthroponotic cutaneous leishmaniasis occurred within 80 days of vaccination (one in the ALM+BCG group and three in the BCG group), and we decided to exclude these children from further analysis before breaking the trial codes (because the lesions may have been incubating at the time of vaccination). The cut-off of 80 days was arbitrarily chosen, since this was the day of skin testing and all participants were examined. The subsequent follow-up was 100 days later. In the interval between 80 days and 2 years postvaccination, we diagnosed 112 cases of cutaneous leishmaniasis: 52 (2·8%) in the ALM+BCG group, and 60 (3·3%) in the BCG group (table 3; vaccine efficacy=15%, 95% CI −22 to 41%). The incidence of the disease in boys was significantly reduced in the vaccine group (1·6% *vs* 3·7%, p<0·01) with an estimated protection of 18% and 78% for the first and second years, respectively, with an overall efficacy of 55% (95% CI 19% to 75%; table 4). The overall incidence in girls was higher in the ALM+BCG group (4·2%) compared with the BCG group (3·0%), though this difference was not significant.

In the period between 80 days and 9 months postvaccination, there was a non-significant excess of cases in the ALM+BCG group (25 *vs* 16), whereas the incidence of cutaneous leishmaniasis thereafter was significantly reduced in the ALM+BCG group (27 *vs* 44, p<0·05; table 3).

The anatomical distribution of lesions in boys and girls did not differ—95% of lesions were on the face and hands. The comparison of the healing processes for 59 children who recovered during the course of this study, and the degree of severity for all 112 children, revealed no pronounced difference between vaccine groups, nor between girls and boys.

Direct microscopy was positive in 109 (97·3%) cases, and culture in 92 (82·1%). Of 50 isolates characterised by various methods, 45 (90%) were *L tropica* zymodems Mon-39 and Mon-55 (20 in the ALM+BCG group and 25 in the BCG group), and five (10%) *L major* (three in the ALM+BCG group and two in the BCG group).

Discussion
Overall, the results were encouraging with respect to safety, but showed little evidence of a difference in disease incidence between those receiving ALM+BCG and those receiving BCG alone. If analyses were confined to cases of disease occurring more than 9 months after vaccination, there was a significantly lower incidence of disease in children receiving ALM+BCG compared with those receiving BCG. This 9-month period was selected on an a-priori basis, and the apparent protective effect must thus be viewed cautiously. We do not know the exact reason for this delayed protection. However, it is likely that the effect

of BCG (as an immune modifier) is short-lived compared with the effect of ALM+BCG. If any protective effect of BCG against leishmaniasis does not last as long as that of ALM+BCG, and if BCG is used for comparison, then the ALM+BCG-induced protection would become apparent later. The trial design precluded any assessment of the protective effect of BCG alone. BCG was used in the control arm of the trial to ensure that the study could be carried out with appropriate concealment of vaccine allocation, but it may not have been an ideal choice, since evidence exists that BCG alone gives some protection against experimental leishmaniasis in mice,[16,17] improves recovery in patients with American tegumentary leishmaniasis,[9] and contains antigens that are cross-reactive with *Leishmania*.[17] The true protective effect of the combined ALM+BCG vaccine may be higher than in this study if the efficacy were compared to an inert placebo group.

The immunogenicity of a single dose of ALM+BCG as measured by leishmanin skin test was low, and multiple doses might possibly have been more immunogenic and more effective in the prevention of disease. We believed it was important to gain experience with a single dose of this vaccine first, especially with regard to its safety when used in large numbers of children. This precaution is particularly important, since mice given leishmanial antigens may, under certain conditions, produce an exacerbating disease when challenged with live *L major*.[18] This effect is believed to result from stimulation of Th-2 subset of helper T cells.[19] We found no indication that ALM+BCG vaccination worsened severity of disease compared with BCG alone. In addition, ALM+BCG vaccine was not associated with any serious side-effects, and the reactions were similar to those with BCG alone and probably attributable largely to this component of the vaccine—though they tended to persist longer in those who received ALM+BCG.

Our finding of a protective effect in boys but not girls was unexpected and may result from chance. Boys generally spend more time outdoors than girls in Bam, and may therefore be at higher risk of exposure to sandfly bites. This hypothesis is consistent with the observation that more boys than girls (2·0% *vs* 1·6%, respectively, p<0·05) had active lesions when children were initially screened for entry into the trial. If repeated challenge strengthens any protection from the vaccine, this might explain the difference in findings according to sex. We would have liked to examine the correlation between skin-test reactivity (among boys and girls) and disease incidence, but the frequency and magnitude of responses to the leishmanin skin tests were too low (the mean reactions to the tests 80 days after vaccination, excluding those with no response, were 3·5 [SD 1·6] for girls and 3·3 [1·5] for boys in the ALM+BCG group; usually, reactions <5 mm diameter are deemed negative).

A vaccine made of *L tropica* may have been more effective in this area than the one used (made of *L major*). However, the various species of *Leishmania* share so many common antigens that they can not be distinguished by serology. The strain used for preparation of the vaccine was the same as that used for leishmanisation in the past.[3]

Future studies with this vaccine should be directed at improvement of the immunogenicity and protection by the administering of booster doses or the use of different adjuvants.

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