

Review Article

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Leishmaniasis vaccine candidates for development: A global overview

Ali Khamesipour, Sima Rafati*, Noushin Davoudi**, Fereidoun Maboudi** & Farrokh Modabber

Center for Research & Training in Skin Disease & Leprosy, Tehran University of Medical Sciences, Tehran
**Molecular Immunology & Vaccine Research Lab & **Biotechnology, Pasteur Institute of Iran, Iran*

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A vaccine against different forms of leishmaniasis should be feasible considering the wealth of information on genetics and biology of the parasite, clinical and experimental immunology of leishmaniasis, and the availability of vaccines that can protect experimental animals against challenge with different *Leishmania* species. However, there is no vaccine against any form of leishmaniasis for general human use. One major factor is the lack of a conceived market for human leishmaniasis vaccines. Hence pharmaceutical industries involved in vaccine development are not interested in investing millions of dollars and a decade that is required for developing a new vaccine. Besides, leishmaniasis is a local/regional problem and not a global one. According to the estimates of the World Health Organization, 90 per cent of visceral leishmaniasis occurs in five countries (Bangladesh, Brazil, India, Nepal and Sudan). Those in need are amongst the poorest people in these countries. It should therefore be the objectives of these countries to develop a vaccine. Fortunately, both Brazil and India have designated the control of visceral leishmaniasis as a top priority for their respective Ministries of Health. The purpose of this review is to present only the vaccines in use and those in development for use in dogs or humans. This is not an exhaustive review of vaccine discovery or the principles of clinical immunology underlying vaccine development.

Key words DNA vaccine - leishmaniasis - prophylactic vaccine

Development of a new vaccine must meet current requirements of Good Laboratory Practices (GLP), Good Manufacturing Practices (GMP) and Good Clinical Practices (GCP). These guidelines evolve continuously and for a prophylactic vaccine that is destined for healthy individuals - mostly children - utmost attention must be given to assure safety, reproducibility and efficacy. A candidate molecule(s) to be used in clinical trials should be developed under

these internationally accepted guidelines. All materials, their origins, sources and all procedures should be well defined/followed. Standard operating procedures (SOPs) must be developed for each step and followed meticulously to assure consistency of products at predefined high quality. Implementation of quality control (QC, of products according to predefined criteria) and quality assurance (QA, systems for equipment and personnel) for in-process

control is required to assure batch to batch reproducibility and quality of the final product. All procedures, equipment and even personnel involved would have to be validated to conform to the rules of GMP. For recombinant vaccines, the origins of genes, and all materials used in pre-industrial scale production including the gene expression system, recombinant host, and purification should be well defined and acceptable. Items required to be tested in recombinant vaccine are shown in Table I, adopted from WHO technical report series 823 and U.S. Food and Drug Administration (FDA) Code of Federal Regulations 21. In contrast to recombinant vaccines, conventional vaccines either live or killed require fewer tests on bulk intermediates and finished products. This makes the cost of production lesser than for recombinant protein production. However, cultivation of live organism and adapting a system to check the consistency in culture, and problems because of using sera in media are obstacles in conventional vaccine.

Development of vaccines can be divided into five different stages: discovery, pre-clinical development, clinical-development, registration, and post-marketing evaluation. For leishmaniasis vaccines, there has been much activity in the discovery area funded by national and international agencies, yielding many candidate second generation vaccines. These activities are carried out in research laboratories at universities and research institutes where neither the scientists nor the institutions are geared or experienced in GLP, GMP or GCP. Hence, in the absence of industrial interests, the many candidate vaccines have remained as fine publications. Only one candidate is in clinical development at this time for human use (see below under Second Generation Vaccines).

In general, the leishmaniasis vaccines in development can be divided into three categories: (i) Live *Leishmania*; including new genetically modified constructs; (ii) First generation vaccines (FGV) consisting fractions of the parasite or whole killed *Leishmania* with or without adjuvants; and (iii) Second generation vaccines (SGV) including all defined vaccines, *i.e.*, recombinant proteins, DNA vaccines and combinations thereof.

Live *Leishmania* as a prophylactic vaccine

Leishmanization (LZ)

Past experience: From ancient times it was known in western and south-western Asia, that recovery from cutaneous leishmaniasis (CL) is followed by a strong immunity to the disease¹. Hence, much like cow-pox, the exudates from active lesions were inoculated into a covered part of the body of healthy children to induce a self-healing lesion and protection against multiple lesions on the face and other exposed parts of the body. This approach later became known as “leishmanization” (LZ) and live virulent promastigotes of *Leishmania major* harvested from cell free cultures of the parasite replaced inoculation from exudates^{2,3}. Leishmanization as a prophylactic vaccine was used in Israel in 1970s and in Iran in 1980s⁴ and then in a massive programme covering over 2 million people during the Iran-Iraq war of 1982-86⁵. The programme in Israel was stopped mainly due to loss of infectivity of the parasite as a result of continuous sub-culturing. In addition they reported immunosuppression as seen by reduced responsiveness to diphtheria, pertussis, tetanus (DPT) vaccine in children following LZ. In Iran, first a pilot study was conducted, in which 250 volunteers received LZ and the rate of incidence of natural infection in “takes” vs. “non-takes” in this group was compared to unmatched control within the same geographic focus. Lesions lasted between 3.5 -13.5 months. The two year incidence of the natural infection in this hyperendemic area was 0.8 per cent for “takes” and 10.5 per cent for “non-takes”⁴.

The LZ programme was stopped in Iran after the war due to complications arising from the special war conditions under which the vaccine was produced and delivered. In addition, a few cases became chronic and did not respond to routine treatment.

Present use: At present there is only one prophylactic live vaccine in use. This is a mixture of live virulent *L. major* mixed with killed parasite registered in Uzbekistan. The parasite is isolated from an active lesion to produce the vaccine each

year to overcome the problem of loss of virulence and given a few months prior to the start of transmission cycle (February) to high risk population^{6,7}. The problems associated with this vaccine are standardization and quality control. Fortunately, there is no HIV infection at the site and amongst the target population which primarily consists of school-age children.

Future of live vaccination: Using wild type *L. major* for LZ, a lesion must develop before protection is induced. However, there are examples of protective vaccination in mice with genetically modified parasites that do not produce the pathology, yet induce protection against wild type parasites. These mutant parasites have been developed by mutagenesis and selection⁸, gene targeting methods (either to introduce foreign genes into the genome or to derive deficient strains, known as knock-out mutants) to assess the function of individual genes or gene families⁹⁻¹³. Genetic modification may consist of removal of a gene(s) as in knock-out parasites^{9-11,14-16} or by introducing several position dominant selectable markers¹⁷⁻²⁰. Another approach is to add external gene(s) "suicidal cassettes"²¹⁻²⁶ to render the parasite more responsive to drugs. In all these models, the idea is to induce protection but not the pathology associated with LZ.

Knock-out parasites: Several *Leishmania* lines have been engineered in which essential genes for survival in the host have been blocked or removed/replaced. The first construct generated by gene targeting, was a dihydrofolate reductase-thymidylate synthase (DHFR-TS) *L. major* knock-out¹⁰. This mutant was shown to survive in mice for about two months without producing a lesion. Injected mice showed significant protection (short term) against challenge with wild type^{27,28}. Although the knock-out parasite showed a type-1 cytokine response by human cells *in vitro*, further studies in monkeys were disappointing hence the DHFR-TS knock-out construct has not been further developed as a vaccine.

Using a similar technique of homologous recombination with *L. donovani*, the biopterin

transporter (BT1) was inactivated. The BT1 knock-out construct had much reduced virulence but elicited an immune response leading to a high level of resistance against challenge by the wild-type parasite³⁰. Several cysteine proteinases (CP) of *L. maxicana* knock-outs were constructed lacking CPa, CPb or both^{31,32}. These constructs had reduced pathogenicity and induced partial protection in BALB/c mice against challenge with the wild type *L. mexicana*³³.

Suicidal cassettes: Like other organisms, *Leishmania* can be genetically modified to commit suicide in response to external signals for self-destruction as a strategy for vaccination^{21,23,24,34}. By introducing genes that would produce lethal materials upon exposure to harmless substrates, toxic products are generated which are lethal to the parasite. More recently a double drug sensitive strain of *L. major* was constructed using advances in gene targeting technology by stably introducing into the chromosome modified HSV-1 thymidine kinase gene (tk), conferring increased sensitivity to ganciclovir (GCV), and a *Saccharomyces cerevisiae* cytosine deaminase gene (cd), conferring sensitivity to 5-fluorocytosine (5-FC)^{25,26}. *In vitro* studies showed that the homozygous *L. major* (tk-cd+/+) promastigotes were killed by either drug alone, and together the drugs acted synergistically²⁶. Studies conducted *in vivo* showed that progressively growing lesions in BALB/c mice, caused by *L. major* (tk-cd+/+), were completely cured by two weeks of treatment with either drug alone or in combination. Treated animals showed no signs of recurrence of infection for at least 4 months when the experiments were terminated²⁶. Further investigations, regarding the use of this interesting system in vaccination studies demonstrated that even combined therapy with GCV and 5-FC as early as day seven post infection can eliminate the parasites, yet induce a high level of IFN- γ and protection in both BALB/c C57BL/6 mice (Davoudi N, unpublished data).

None of these constructs has reached clinical development yet, however the approach provides possibilities for induction of protection with a self-limiting infection, possibly without any pathology.



Fig. 1. Appearances of lesions caused by leishmanization at the peak of lesion development. Note the different forms, (A) a small benign lesion that healed with a minimal scar; (B) satellite lesions resembling recidivans; (C) a raised lesion with strong induration at the site of infection. D1 and D2, lesions from natural infection.

Live *Leishmania* as a challenge infection to test candidate vaccines

A major time- and cost-strain for development of a vaccine is the conduct of pivotal field efficacy trials (Phase-3), with sufficient power (large sample size) needed to facilitate registration. Hence prior to embarking on costly phase-3 trial of a candidate vaccine, it would be prudent to have some indications of its efficacy in human. This is particularly important when true surrogate markers of protection are not fully identified - as is the case with leishmaniasis. Live challenge studies simulate natural infection, but can be done with a small sample size and in a short duration thereby saving a considerable amount of time and resources. To this end, live challenge has been used to measure the efficacy of candidate vaccines^{35,36}. For leishmaniasis, a seed bank and several hundred seed lots were established from the same well characterized and extensively studied parasite (*L. major*, MRHO/IR/75/ER) that was used

in the massive LZ programme at Razi Vaccine and Serum Institute in Iran³⁷. From the seed lots, several live *Leishmania* stabilates were produced for challenge studies. The autoclaved *L. major* (ALM) vaccine was also produced from the same seed lots, (see under killed vaccines). The stabilates were made from the late stationary phase of parasites in culture and were kept frozen in liquid nitrogen until used. This proved useful for maintaining parasite virulence as tested in mice. Two LZ trials were conducted in 42 inoculations in 28 male adult volunteers who were followed until complete healing of their lesions³⁸. Lesions induced by LZ were as diverse as natural infection, but much milder (Fig. 1). All volunteers who recovered from their lesions and agreed to receive a second LZ were totally protected (100% protection against second challenge 11/11). In spite of much variation and the small sample size, it was clearly demonstrated that standardized stabilates may be used to evaluate candidate vaccines in a short period (2-3 months) when 70-80 per cent of recipients

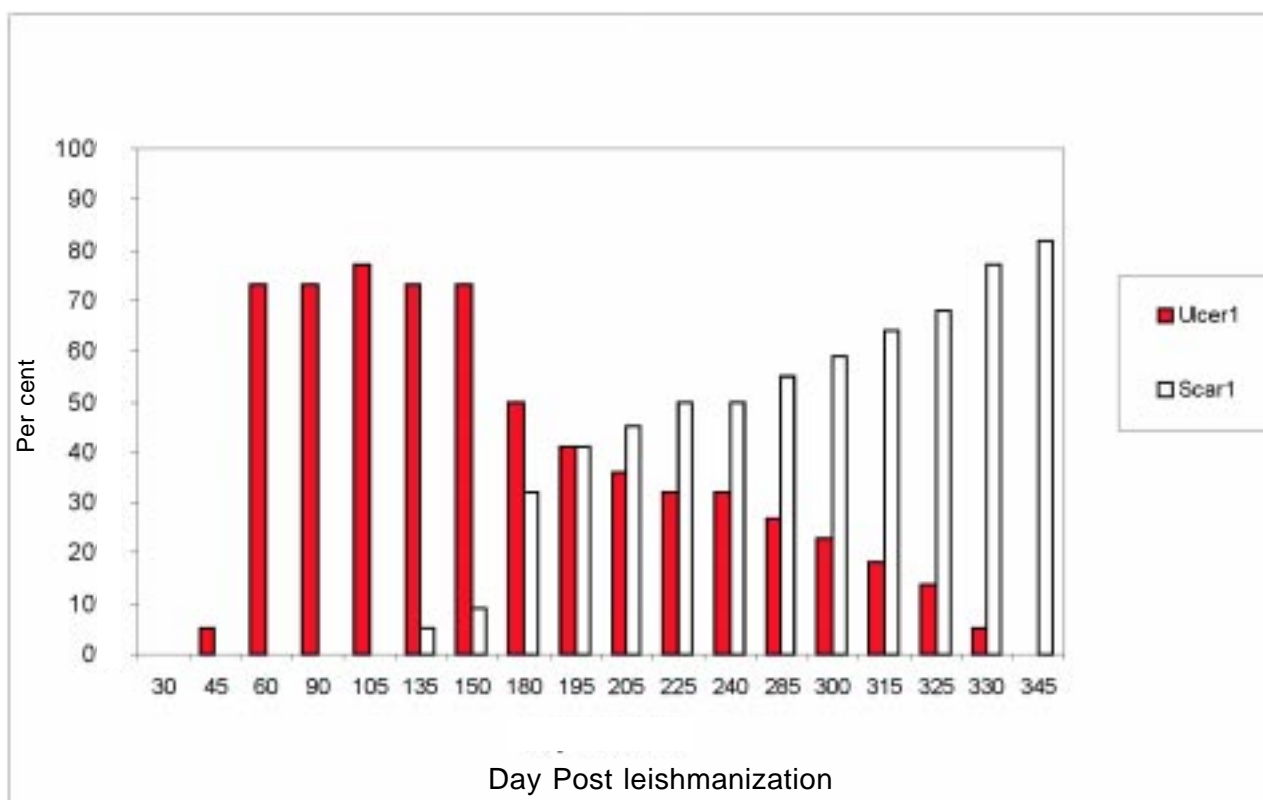


Fig.2. Ulcerated lesions development and their complete healing to scar formation in 18/23 volunteers who developed an ulcer following leishmanization. Note: Over 70 per cent had an ulcerated lesion by day 60, hence candidate vaccine efficacy can be determined in 2 months.

would develop an ulcerative lesion (Fig. 2). The stabilates proved to retain their virulence over three years and produce comparable outcomes with respect to virulence (take rates), size of lesions produced, duration to onset of ulceration and healing³⁸.

The advantages of live challenge studies over field efficacy trials against natural infection are low cost, reduced time to evaluation, all participants in a vaccine trial will end up being protected (if the candidate vaccine would not protect, then LZ would), and facility to search for surrogate markers cost-effectively. The disadvantage is a long lasting lesion (3-13 months) if untreated. In addition, *Leishmania* are believed to persist for a long time after recovery, as shown in mice^{39,40} and recurrence of leishmaniasis in immunosuppressed individuals⁴¹, hence, introduction of live *Leishmania* is not possible in an area with a risk of HIV infection. There are still certain limited isolated leishmaniasis

hyperendemic foci where HIV infection has not yet been detected.

Killed parasite (first generation) vaccines or fractions

Killed parasite - New World: The early trials with killed *Leishmania* as a vaccine were conducted in Brazil in 1940s. Later, from 1970s onwards Mayrink and his colleagues developed a killed vaccine composed of five isolates of *Leishmania* containing four different species⁴³. This was simplified to a single (*L. amazonensis*) vaccine and tested for prophylactic potential in Columbia^{48,49} and Ecuador⁴⁷ and as an adjunct to chemotherapy in Brazil⁴⁴. Convit and his group in Venezuela introduced their autoclaved *L. mexicana* + BCG for immunotherapy and/or immunochemotherapy⁴². Several prophylactic studies were done⁴³ with inconclusive results or low protection induced by the vaccine (killed *Leishmania*

Table I. Criteria for recombinant vaccine candidate (Bulk & Intermediate)

Test	Specification
Peptide map	Sample to reference peak height ratio
Methionine oxidation	< 5.5%
Carbohydrate analysis (if recombinant host is eukaryotic)	Disialo-biantennary, glycan, Trisialo-triantennary + Monosialo-biantennary glycans
N-terminal sequencing	First 10-14 amino acids
Biological potency (specific activity)	According to pre-clinical criteria
Purity	More than 96 per cent
SDS-PAGE Apparent molecular weight and impurities (Coomassie Blue stain)	Apparent molecular weight impurities bands
Size exclusion chromatography	Aggregation (both at release and expiration date).
Host cell protein	≤ 500 ng/ml
Endotoxin	≤ 30 EU/ml
DNA content	≤ 10 ng/ml
Bioburden	≤ 10 cfu/10ml

SDS-PAGE, Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Source: Adopted from WHO Expert Committee on Biological Standardisation, *Technical Report Series 760*, 1987; WHO Technical Report Series 823; WHO Expert Committee on Specifications for Pharmaceutical Preparations. Thirty- second Report, WHO, Geneva, 1992, and US Food and Drug Administration Code of Federal Regulations - Title 21 - Office of the Federal Register, National Archives and Records Administration, Washington DC, 1996

injected 3 times without any adjuvant) when given to leishmanin-negative (Montenegro skin test, MST- same as leishmanin skin test, LST-) individuals. However, a highly significant finding of this group, which has been confirmed over and over again by others, is that the incidence rate amongst the MST-converted individuals in the vaccine group was significantly lower than those in the control (unvaccinated) group or vaccinated but MST non-converted individuals. As in Venezuela with Convit's vaccine, Mayrink's vaccine was effective in reducing the dose of antimony required to achieve cure⁴⁴. Based on these trials the vaccine was registered as an adjunct to antimony therapy in Brazil but not for prophylactic use.

In Venezuela, autoclaved killed *L. mexicana* is now used to treat patients with CL. If the patient does not respond after three injections (2 months), then antimony treatment is initiated⁴⁵. In Ecuador, two doses of a vaccine composed of *L. amazonensis* and *L. mexicana* mixed with BCG induced 73 per cent protection⁴⁶. A double-blind randomized efficacy trial with Mayrink's vaccine formulated to be injected intradermal mixed with BCG could not be conducted

as planned due to flooding caused by Niño, as a results case finding was delayed and it was not possible to determine the efficacy of the vaccine, if any⁴⁷. This study unlike the claims of the authors should be considered as "inconclusive".

A comparative trial of this vaccine with and without BCG was conducted in Colombia, the formulation without BCG was chosen to proceed to efficacy trial due to lesion formation at the site of injection with BCG⁴⁸. A randomized double-blind placebo control efficacy trial against natural infection was conducted in Colombia⁴⁹. Similar to the finding of Mayrink⁴³ in Brazil, there was no significant difference in incidence rates of vaccinated vs unvaccinated controls. Unlike Mayrink's studies, there was no skin test performed after vaccination, hence the immunogenicity of the vaccine soon after immunization was not known. A low conversion rate due to whatever reason could account for lack of sufficient efficacy. Further, it is not possible to determine the incidence only in those vaccinated individuals who responded by skin test conversion, the group that in previous trials were shown to have a lower incidence rate. In general, considering all

Table II. Double-blind randomized BCG-control field efficacy trials of ALM mixed with BCG

Trial	Disease No. of volunteers No. of injection	% LST conversion in vaccinees	Incidence rate vaccines/BCG	Other outcome
1	ZCL* 2,453 One ⁵⁵	36.2 (80)	18/18.5	35% ↓ incidence in LST ⁺ $P < 0.05$ ↓ severity in vaccinated children, $P < 0.04$
2	ACL** 3,637 One ⁵⁶	16.5 (80)	2.8/3.2	Boys protected > girls $P < 0.01$
3	VL *** 2,306 Two ⁵⁷	30 (42)	11.5/12.3	43% ↓ incidence in LST converted, $P < 0.003$
4	ZCL* 2,179 Three ^a	23.3 (60)	9.8/10.3	Efficacy against severe cases 46%
5	ZCL* 2,111 3 injections a month apart ^a	Not done	14.1/14.8	Efficacy not more than single injection
6	ACL** 4,500 Three ^b	16.2 (60)	2.9/3.5	Efficacy not more than single injection, no difference between boys and girls

Causative parasite: **L. major*; ***L. tropica*; ****L. donovani*

Figures in parentheses represent time (in days after vaccination) when LST was performed

LST, Leishmania skin test; ZCL, zoonotic cutaneous leishmaniasis; ACL, anthroponotic cutaneous leishmaniasis; VL, visceral leishmaniasis

Source: Refs 56, 57 ^a, Khamesipour *et al* unpublished;^b, Sharifi *et al* unpublished

trials, based on the immunogenicity of various killed *Leishmania* preparations, it seems that a better adjuvant than BCG would be required to produce a potent vaccine.

Fractionated preparation: The fucose mannose ligand (FML) antigen is present on the surface of the parasite throughout the life cycle, and it has been shown that it is a potent immunogen in mice and rabbits and a sensitive, predictive and specific antigen in serodiagnosis of human and canine kala-azar⁵⁰. The FML saponine formulation is shown to be safe, immunogenic and protective in BALB/c, Swiss albino mice and CB hamsters⁵¹. In a Brazilian focus endemic for both human and dog visceral leishmaniasis (VL), efficacy trials using the FML vaccine in dogs induced 92 and 95 per cent protection in naturally exposed vaccinated dogs. Protection induced by the FML-*Quillaja* saponin vaccine lasted up to 3.5 yr after vaccination, therefore, it induced strong protective effect against canine kala-azar in the field⁵².

Old world killed *L. major* vaccines: In Iran, both types of zoonotic cutaneous leishmaniasis (ZCL) and anthroponotic cutaneous leishmaniasis (ACL) exist at very high incidence rates in different parts of Iran, which makes the disease one of the major health problems in this country. This has given the impetus to focus much attention and resources for developing a vaccine during the last decade.

Using the same organism that was used for LZ in Iran, a seed bank and several hundred seed lots were prepared under GMP guidelines at Razi Serum and Vaccine Research Institute, Hesarak, Iran. Several procedures were used for killing the promastigotes and finally autoclaving was selected similar to that developed in Venezuela to produce autoclaved *L. major* (ALM) as the vaccine. Stepwise phase I-II safety and dose-response trials were conducted in non endemic area^{53,54}. A dose of 1.0 mg ALM mixed with one tenth of the dose of BCG usually used for anti-tuberculosis vaccine was chosen for further development. The results showed that the mixture

was safe, acceptable and induced LST conversion in about 38 per cent and weak but measurable IFN- γ production. Efficacy trials of a single dose of 1 mg of ALM mixed with one tenth dose of BCG were conducted in a zoonotic⁵⁵ as well as anthroponotic foci of Iran⁵⁶. The immunogenicity of the vaccine in the field was much reduced and only 16.5 per cent LST conversion was seen in the anthroponotic focus of Bam, Iran (Table II). To increase immunogenicity, multiple injections were planned. Safety and immunogenicity of multiple doses were studied in non endemic foci before proceeding to field efficacy trials. Two doses of the vaccine gave a reduction of incidence of 43 per cent in LST converted volunteers in Sudan against VL involving 2306 volunteers (Table II)⁵⁷. Three injections of the vaccine did not produce any higher protection than single dose against anthroponotic or zoonotic CL in Iran (unpublished observations). It was decided that the vaccine is not immunogenic enough and another adjuvant was sought.

In order to enhance the immunogenicity of the vaccine (ALM+BCG), ALM was adsorbed to alum (aluminum hydroxide), and the resulting alum-ALM was mixed with BCG just prior to injection. Indeed, addition of alum to ALM led to enhanced immunogenicity as a single injection of killed *Leishmania* in alum plus IL-12 induced strong cellular immune responses and protection in Rhesus monkeys against cutaneous leishmaniasis⁵⁸. Likewise, alum-ALM + BCG protected Langur monkeys against visceral leishmaniasis⁵⁹. A single injection of alum-ALM mixed with BCG showed 70 per cent protection in canine leishmaniasis in Iran. The vaccine did not produce high titre of antileishmanial antibodies and the efficacy was assessed by rising serum antibodies after exposure to natural infection⁶⁰. A dose escalating trial of a single intradermal injection of alum-ALM (10, 100, 200 or 400 μ g of *Leishmania* proteins) mixed with BCG was carried out in healthy volunteers from a non endemic area of Sudan. The results showed the highest skin test conversion seen in any *Leishmania* vaccine trials so far⁶¹. With the exception of the 200 μ g arm, all volunteers developed a strong LST reaction, which remained strongly positive when tested up to 90 days post vaccination. Local side

effects were minimal and acceptable and no systemic side effect was recorded. The low immune response in the 200 μ g group was attributed to the problems with the BCG vial used on that day as BCG lesions either did not develop or developed many weeks after injection. The safety-immunogenicity trial was repeated in Sudan and essentially all participants responded strongly with a single injection⁶². Currently, an immunochemotherapy trial of 4 injections of 100 μ g of alum-ALM+BCG combined with sodium stibogluconate (Pentostam) for treatment of post-kala-azar dermal leishmaniasis (PKDL) is underway in Sudan. The initial results are highly encouraging with significant cure in the group receiving combined therapy compared to chemotherapy alone. Thus, alum precipitated ALM mixed with one tenth dose of BCG appears to constitute a safe vaccine and an appropriate candidate for further development.

Second generation vaccines

The development of a defined vaccine candidate against leishmaniasis has been made possible by our understanding of immunological mechanisms that mediate protection in animal models and to a lesser extent by supporting data from the characterization of immune responses in leishmaniasis patients. In addition, presently, genome sequencing of *L. major* is completed and one of the driving forces behind the genome project is to identify genes that are expressed in the infectious stages of the parasite and in particular, in amastigotes. Access to numerous DNA sequences will favour the development of genetic vaccines over the conventional ones, considering its simple use, low cost of production and flexibility of combining multiple genes in a single construct⁶³.

It is almost 30 yr since the first successful transfection of cells following injection of purified DNA and the expression of the encoded gene *in vivo* with production of a biologically active protein⁶⁴. This observation led to the conclusion that if a foreign protein could be expressed *in vivo* in the host, then immunity should be generated to that protein. Hence DNA vaccination was conceived which have lead to promising results in recent years. In comparison with

Table III. *Leishmania* antigens: Second generation candidate vaccines

Antigen	DNA / rProtein	Animal Model	Disease (<i>Leishmania</i> spp.)	Ref. no.
gp63	+ / +	Mice	CL (<i>L.m.</i>)	68
gp63	+ / -	Monkey	CL (<i>L.m.</i>)	70
LACK	+ / +	Mice	CL (<i>L.m.</i>)	71
LACK	+ / +	Dog	VL (<i>L.i.</i>)	72
H1	- / +	Monkey	CL (<i>L.m.</i>)	82
H2A+H2B+H3+H4	- / +	Mice	CL (<i>L.m.</i>)	81
CPB	- / +	Mice	CL (<i>L.m.</i>)	69
CPA+CPB (fused)	+ / +	Mice	CL (<i>L.m.</i>)	73
CPA+CPB	+ / +	Dog	VL (<i>L.i.</i>)	77
Leish-111f	- / +	Mice	CL (<i>L.m.</i>)	83
KMP11	- / +	Mice	CL (<i>L.m.</i>)	78
LCR1	- / +	Mice	VL (<i>L.i.</i>)	80
A2	+ / +	Mice	CL (<i>L.m.</i>)	76
HASPB1	- / +	Mice	VL (<i>L.d.</i>)	75
PapLe22	+ / -	Hamster	VL (<i>L.i.</i>)	79
PSA2	+ / +	Mice	VL (<i>L.i.</i>)	74

L.d., *L. donovani*; L.i., *L. infantum*; L.m., *L. major*

CL, Cutaneous leishmaniasis; VL, visceral leishmaniasis

the recombinant protein vaccines and attenuated organisms, DNA vaccines are relatively simple and inexpensive to produce⁶⁵. In addition, genetic vaccines effectively engage both major histocompatibility class (MHC) I and MHC II pathways, thereby allowing the induction of both CD8⁺ and CD4⁺ T cells. This feature is particularly attractive for leishmaniasis, in which both cell types are involved in protection⁶⁶. Other unique features that make DNA vaccination particularly attractive are the long lived production of the antigen, which is similar to the situation in natural *Leishmania* infection, together with improved immunological memory⁶⁷. A selected list of leishmanial antigens as second generation vaccines against leishmaniasis is shown in Table III. In this review, we concentrate on the most promising vaccine candidates and highlight new approaches for the development of new generation vaccines.

The encoding for the protein portion of the *Leishmania* surface glycoprotein (gp63) was the first *Leishmania* vaccine delivered as a plasmid⁶⁸. In this study, the level of IFN- γ but not IL-4 was high in spleen cells from immunized mice when stimulated

by freeze/thawed antigen. These mice were partially protected against challenge with infectious *L. major*. The protective efficacy of purified gp63 has been tested in several experimental models using different strains and adjuvants, giving rise to conflicting results⁸⁴. A small scale vaccine study of rgp63 against *L. major* infection was performed in vervet monkeys⁷⁰. Three doses of the recombinant antigen were administered mixed with BCG as an adjuvant. After vaccination, peripheral blood mononuclear cells (PBMC) from these animals neither proliferated nor produced IFN- γ following stimulation with antigen, and only partial protection was achieved after challenge with virulent *L. major* promastigotes. Subsequent to this work, the protective efficacy of LACK DNA was compared with that of LACK protein and IL-12⁷¹. It was shown that the LACK gene construct induced a strong protective response comparable to that achieved when LACK protein plus recombinant IL-12 was administered, and was better than protection seen with LACK protein alone. Moreover, it was demonstrated that the depletion of CD8⁺ cells at the time of vaccination or infection abolished the protective response induced by LACK DNA vaccination, suggesting a role for CD8⁺ T cells

in DNA vaccine induced protection toward *L. major*. Recently, a heterologous prime boost vaccination regime with DNA and recombinant vaccinia virus (rVV) vectors expressing LACK was shown to confer 60 per cent protection against *L. infantum* infection in dogs⁷².

The *L. major* parasite surface antigen-2 (PSA-2) is a family of glycoinositol phospholipids anchored glycoprotein expressed in both promastigote and amastigote. PSA-2 comprises three polypeptides with approximate molecular weights of 96,000, 80,000 and 50,000⁸⁵. The amastigote form expresses a distinct but closely related PSA-2 with molecular weight of 50,000 kDa. It has been shown that vaccination with native PSA-2 with *Corynebacterium parvum* as adjuvant protects mice from *Leishmania* through a Th1 mediated response, but the recombinant PSA-2 purified from *Escherichia coli* and administered in immuno stimulating complexes (ISCOMs) or mixed with *C. parvum* as an adjuvant, does not induce protective immunity despite the induction of Th1 responses. Both C3H and BALB/c mice showed good protection against *L. major* challenge when the DNA was administered as a prophylactic vaccine, but also significant healing from established *L. major* infection was seen when the plasmid was given as an immunotherapeutic agent⁷⁴.

Although vaccination studies against VL are limited in comparison to CL, there are some defined recombinant antigens with considerable level of protection. The recombinant hydrophilic acylated surface protein B1 (rHASP B1) is able to confer protection against experimental challenge with *L. donovani*. Protection induced by rHASP B1 does not require adjuvant and since vaccine induced protection correlates with the presence of rHASP B1 specific IFN- γ producing CD8⁺ T cells, it appears that the mechanism of protection is similar to DNA vaccination⁷⁵.

The A2 genes are amastigote stage specific. They form a part of a multigene family of at least 11 genes and are considered to be virulence factors that are required for the survival of *Leishmania* parasites in the mammalian host. Both A2 used as a recombinant

protein with IL-12, or as a DNA vaccine demonstrated significant protection against VL in mice challenged with *L. donovani*⁷⁶. Among the subunit vaccine candidates the kinetoplastid membrane protein-11 (KMP-11) appears highly conserved in all *Leishmania* species tested and has been described to elicit potent lymphoproliferative and antibody responses in leishmaniasis patients or experimentally infected mice^{78,86} and a significant protection in hamsters against *L. donovani* infection (S. Roy, Indian Institute of Chemical Biology, personal communications). In addition, papLe22 is another antigen which is weakly expressed and localized in the promastigotes nucleus of *L. infantum*, *L. major* and *L. guyanensis*, is highly immunogenic and recognized by sera of VL patients. The immunization of papLe22 cDNA, administered as a single intramuscular injection in golden hamsters, a highly susceptible animal, induced a marked decrease in parasite load⁷⁹.

LCR1 is another recombinant protein which shares homology with *Trypanosoma cruzi* flagellar antigen from *L. chagasi* stimulated proliferation of splenic T lymphocytes from *L. infantum* infected C3H and BALB/c mice and induced IFN- γ but not IL-4, IL-5 or IL-10 secretion. Immunization with LCR1 partially protected BALB/c mice against challenge with *L. infantum*⁸⁰. It has been shown that *Leishmania* histones are relevant immunogens for the host immune system during both *Leishmania* infection and disease. The mixture of four plasmid DNAs, encoding the *L. infantum* histones H2A, H2B, H3 and H4, was tested for protection in BALB/c mice⁸¹. It was found that the immunized animals developed a specific Th1 immune response, which was associated with an antigen specific production of IFN- γ and a limited humoral response against histones. Both CD4⁺ and CD8⁺ T cells contributed to the resistance of vaccinated mice to CL in these experiments.

Recombinant histone H1 antigen or a long synthetic peptide representing the complete *L. major* histone H1 sequence, each formulated with Montanide ISA 721 as adjuvant, did not produce significant protection in African green monkeys against CL⁸².

Some antigens expressed in amastigotes are likely to be suitable vaccine candidates. Cathepsin L-like cysteine proteinases (CPs), which are predominantly expressed and are active in the amastigote form, have received considerable attention⁸⁷. Cysteine proteinases are enzymes that belong to the papain superfamily. Three classes of CPs have been identified in *Leishmania*. Type I CP (CPB) is encoded by multicopy genes arranged in tandem arrays and is characterized by the presence of a long C-terminal extension (CTE) rich in proline, serine and/or threonine residues. Type II (CPA) and Type III (CPC) are encoded by a single copy gene. Both native and recombinant forms of the cysteine proteinases are recognized by the immune system of the individuals recovered from cutaneous leishmaniasis^{88,89}. These enzymes have also been used in immunization experiments in the mouse model. The results showed that recombinant CPB, in combination with adjuvant, induces long lasting immunity against *L. major* infection in BALB/c mice, while DNA vaccination is more efficient when a cocktail of plasmid DNAs encoding CPA and CPB is used, indicating the usefulness of the combination of these antigens⁶⁹. It has also been shown that a bacterially expressed recombinant CPA/B hybrid protein administered with poloxamer 407 as adjuvant, elicited a partial protective response against infection with *Leishmania* in BALB/c mice that was stronger than the response given by recombinant versions of CPA and CPB expressed as individual proteins⁷³. Further, the immunization of mice with either co-expressing CPA and CPB DNA plasmid or the fusion construct encoding CPA/B hybrid protein, induced partial protection against *L. major* infection⁹⁰.

The cysteine proteinases type I and II also have been used as a heterologous prime-boost regime for vaccination against experimental visceral leishmaniasis in dogs. All vaccinated dogs remained free of infection in their bone marrow. In contrast, 75 per cent in the control groups had infection in their bone marrow. Vaccinated dogs had elevated IFN- γ mRNA in their PBMC whereas control dogs had consistently increased level of IL-10. With the exception of one dog, vaccinated dogs had a stronger delayed type hypersensitivity (DTH) response than control dogs. This vaccine⁸⁹ will soon be tested

against natural infection of canine leishmaniasis in the northwest of Iran.

Recently, to overcome the genetic predisposition of the immune response amongst different individuals and population, the idea of multi-antigen vaccines using more than a single antigen has been promoted. One of the best examples of such vaccine is the tri-fusion TSA LmSTI1, and LeIF formulated in mono-phosphoryl lipid A (MPL, an amphiphilic preparation derived from the lipopolysaccharide of *Salmonella Minnesota*) in an oil/water stable emulsion (SE) using synthetic squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene, a biodegradable product). This poly protein is called Leish-111f⁸³. The antigens are present in both amastigotes and promastigotes and were selected from an expression library of *L. major* using sera from infected humans and mice. They are conserved in most *Leishmania* species that cause human disease and elicit primarily a Th1 type immune response in mice when given with appropriate adjuvants. Recombinant-LmSTI1 and r-TSA, protect mice and non human primates against *Leishmania* infection when given with IL-12 or MPL-SE. The r-LeIF antigen is therapeutically effective against leishmaniasis in mice. It has Th1 adjuvant properties, including eliciting IL-12 and IL-18 both in mouse and human cells. Leish-111f+MPL-SE produces a long term protection against *L. major* and *L. amazonensis* infection (over 14 wk) in BALB/c mice, unusual for protein antigens. No decrease in immunogenicity, diversity of epitope recognition and protection is noted with Leish-111f compared with protein mixtures⁹¹.

This is the only second generation vaccine against leishmaniasis that has reached human trials. The initial safety and dose escalating trial, conducted in volunteers in the USA, produced satisfactory results. The MPL-SE is being developed both for prophylaxis as well as therapy based on studies in mice⁹² and preliminary trials in humans on compassionate basis⁹³. There are ongoing trials in Brazil and Peru on CL and mucosal leishmaniasis (ML) patients respectively.

Discussion

There have been several initiatives in the discovery area by scientists interested in fundamental research funded by national and international agencies, yielding many candidate second generation vaccines. Many antigens with potential for protective immunization have been discovered through fundamental research rather than direct search for vaccine candidates. These activities are carried out in research laboratories at universities and research institutes where neither the scientists nor the institutions are principally involved, geared or experienced in GLP, GMP or GCP. Hence, in the absence of industrial interests, many candidate vaccines have remained as fine publications. Only one candidate is in clinical development at this time, thanks to a public (for profit)-private (not for profit) partnership, where the public partner provided the industrial expertise and the private was able to raise funds for research and development (Steve Reed, Infectious Disease Research Institute, personal communication). For neglected diseases, those with perceived lack of a profitable market, such partnership seems to be essential, without dependency on and expectation of a large return. The role of governments and industrial partners in leishmaniasis endemic countries, particularly advanced developing countries where leishmaniasis is a major public health problem, cannot be overemphasized. Fortunately, these partnerships exist or are beginning to be formed in Brazil, India and Iran where all aspects of development of new generation vaccines from basic science to registration and post market analysis are available. In addition, there are still remote pockets of foci in Iran with high incidence of CL, where no HIV infection has been detected or reported. These sites are ideal for cost-effective evaluation those candidate vaccines that have met all preclinical requirements using leishmanization.

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Reprint requests: Dr Farrokh Modabber, Center for Research and Training in Skin Disease and Leprosy
Tehran University of Medical Sciences, P.O. Box 14155-6383, Tehran 14166, Iran
e-mail: modabberf@yahoo.com