Genetically modified live attenuated parasites as vaccines for leishmaniasis

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Received June 27, 2005

Leishmaniasis causes significant morbidity and mortality worldwide and is an important public health problem. Even though it is endemic in developing countries in tropical regions of the world, in recent years economic globalization and increased travel has extended its reach to people in developed countries. Leishmania is usually spread by the bite of the female sandfly. In addition, naïve populations can be exposed to Leishmania infection through transfusion of blood and blood products from infected asymptomatic individuals. There are several clinical forms of leishmaniasis caused by different species of the parasite. In some cases, the only possible cure for this disease is drug treatment. However, prolonged use of such drugs has led to parasite drug resistance. At present there are no effective vaccines against Leishmania. Many vaccine strategies have been pursued, including the use of whole cell lysate, killed, avirulent or irradiated parasites. Additionally, DNA vaccines and purified or recombinant parasite antigens have also been tested. Most of these strategies have shown some degree of effectiveness in animal models but little or no protection in humans. There is now a general consensus among Leishmania vaccine researchers that parasite persistence may be important for effective protective response and could be achieved by live attenuated parasite immunization. In this article we reviewed the efforts in developing genetically defined live attenuated Leishmania parasites as vaccine candidates with the goal of achieving a low level of parasite persistence without being virulent in the host and inducing protective immunity.

Key words Animal model - Leishmania - leishmaniasis - live attenuated parasite

Trypanosomatid parasites of the genus Leishmania infect about 12 million people worldwide, with 600,000 new clinical cases reported annually and with an estimated death toll of about 50,000 persons/year. The two major clinical forms of leishmaniasis, cutaneous and visceral, are the result of infection by different species of the parasite. However, in addition to the infecting species, the clinical outcome of leishmaniasis also depends on the immune response of the host. Visceral leishmaniasis (VL), fatal if not treated, is caused by Leishmania donovani, L. infantum, and L. chagasi. More than 90 per cent of the visceral cases in the world are reported from Bangladesh, Brazil, India...
Cutaneous leishmaniasis (CL) causes lesions which are self healing and are caused by *L. major*, *L. tropica* or *L. aethiopica* in the Old World and by *L. mexicana* or *L. braziliensis* complex in the New World. Both environmental risk factors such as massive displacement of populations, urbanization, deforestation, new irrigation plans and individual risk factors such as HIV, malnutrition and genetic susceptibility make leishmaniasis an important public health problem. Though the most significant public health effects of leishmaniasis are concentrated in developing countries, occasional cases occur in developed countries as well. In the European countries around the Mediterranean basin and throughout the Middle East, as well as Latin America, there are large populations that must still consider the risk of leishmaniasis. In some of these countries, dogs represent an important reservoir for the parasite. In the US, even though leishmaniasis is not endemic, infections can be found in pockets of the country especially in the southwest. In addition, *Leishmania* infection was found in dogs in the northeastern part of the US. Increasing immigration, tourism, and military activity in *Leishmania* endemic areas, led to leishmaniasis becoming an increasing threat in non-endemic areas of the world. This was underscored by the recent military deployments to *Leishmania* endemic areas such as Iraq and Afghanistan, which have resulted in infected soldiers. In a similar way, cultured virulent promastigotes were inoculated in a procedure called leishmanization and showed protection from reinfection. Leishmanization in Uzbekistan is carried out actively in high risk populations with a mixture of live and killed *Leishmania* parasites. However, dangers of pathogenesis associated with the use of such live parasite vaccines have put a stop to this process in other areas.

**Immune response in leishmaniasis**

Development of vaccines against leishmaniasis must take into account the latest knowledge on the immune response to *Leishmania* infection. Studies in animal models, mostly the mouse, have demonstrated the requirement for a cell-mediated immune response. Working with the cutaneous disease caused by *L. major*, researchers have observed a dichotomy in the T helper (Th) lymphocyte response. Resistant mouse strains, such as C57BL/6, respond with a Th1-type response characterized by T cells that produce interferon gamma (IFN-γ). The development of the Th1 type CD4+ T cells is dependent on the cytokine IL-12.
In these mice IFN-γ activates macrophages to clear the parasites. As a source of protective INF-γ, natural killer (NK) cells have also been shown to be important. In contrast, susceptible mouse strains, such as BALB/c, respond to infection with Th2-type effector cells that secrete IL-4. In the presence of this cytokine, antibody production is favoured, IL-12 and its receptor are suppressed and the infection is not cleared. More recent work has called into question the role of IL-4 as a susceptibility factor and revealed the importance of another cytokine, IL-10, in promoting disease progression.

The immune response to Leishmania in primate models and humans does not follow the Th1/Th2 paradigm precisely. Several studies on L. major infection of primates have shown vaccine mediated protection or lack of protection that does not correlate with the INF-γ responses.

Diversity in the immune response to different species of Leishmania parasites suggests the requirements for vaccines intended to protect from cutaneous leishmaniasis may differ from those for visceral disease vaccines. Evidence shows that the immune cells that may be conferring protection after CL secrete IFN-γ, while similar cells prepared from VL patients secrete IFN-γ and IL-4. Even comparing cutaneous disease caused by L. major to that caused by the L. mexicana/L. amazonensis complex shows differences in protective or disease exacerbating responses. This rather complex picture of multiple aspects of a protective immune response that may be mounted by the host in different ways to a variety of Leishmania species clearly indicates that any vaccine must be designed to invoke such a response. Further, any vaccine candidate should be evaluated by measuring its ability to produce such responses in primates and ultimately, human trials.

Current knowledge about vaccine candidates

Early vaccine trials in human subjects utilized whole killed parasites, with variable success. The addition of adjuvants such as Bacillus Calmette-Guerin (BCG) improved the effectiveness, but still not adequately for routine clinical use. As a refinement of whole parasite lysate vaccines, fractionation led to the development of a glycoprotein enriched mixture termed the FML antigen. The FML has been reported 92 per cent effective against canine leishmaniasis, though there are no reported trials in human subjects. With the advent of molecular biological techniques, numerous Leishmania vaccines have been developed with recombinant protein antigens either mixed with appropriate adjuvants for injection or expressed by attenuated bacteria or viral vectors. Glycoprotein 63 (gp63) antigen reconstituted in liposomes was protective in animal models. However, inconsistent T cell response in humans has hindered further development of this vaccine. Another recombinant antigen, LeIF, showed promise in the BALB/c mouse model and has been added to a multi-epitope subunit vaccine. Though some recombinant antigen vaccines are still under investigation, none are ready for routine clinical use. Another application of recombinant technology is the development of DNA vaccines in which DNA sequence that encodes a Leishmania antigen is spliced into an expression vector. Immunization is achieved by injecting the expression vector into the vaccinee. DNA vaccines for Leishmania have produced an immune response in mice and in these models combinations of multiple antigen encoding plasmids have improved protection. DNA vaccine protection against visceral leishmaniasis has been more difficult to achieve though successful with the L. donovani A2 antigen. The interesting finding that a complete Leishmania cDNA expression library injected into mice was more protective than any subpools of the library plasmids reinforces the idea that the whole parasite makes the best vaccine. New discoveries of the role of sandfly saliva in facilitating the Leishmania infection have suggested an addition of saliva proteins that could improve any type of vaccine. Another new approach to the development of Leishmania vaccines is the identification of Th1-inducing adjuvants. Prime candidates include synthetic oligodeoxynucleotides containing CpG motifs, which act on professional antigen presenting cells (APC) monocytes, macrophages and dendritic cells (DC) to increase their antigen uptake and presentation as well as augment the expression of co-stimulatory molecules, and the secretion of pro-inflammatory cytokines such as IP-10, type 1 IFNs and IL-12. These cytokines, in turn, activate NK cells.
CD4⁺, CD8⁺ and gT cells enhancing their lytic activity and the secretion of high levels of IFNγ. The increased antigen presentation in a Th1 cytokine milieu supports antigen-specific humoral and cellular responses. In mice and primates, CpG DNA sequences have been shown to improve the immune response to microbial antigens including leishmanial antigen. Results from ongoing clinical studies suggest that CpG ODN are well tolerated and are effective as vaccine adjuvants.

Live attenuated vaccines

The ineffectiveness of subunit based strategies and our improved understanding of the immune response to Leishmania emphasized the need for a live attenuated vaccine that would expose the recipient to complex antigens in the right context over time without producing pathology. However, it is possible that these parasites could revert to the virulent form. Persistence of asymptomatic Leishmania infections raises the risk of subsequent reactivation, especially in AIDS where leishmaniasis is an opportunistic infection. Early attempts at development of attenuated strains, whether obtained through long term in vitro culture, γ-irradiation, selection for temperature sensitivity, or chemical mutagenesis, possess genetically undefined mutations, thus the potential for reversion cannot be predicted. Undefined attenuation can also lead to loss of effectiveness as shown by laboratory mice immunized with L. chagasi attenuated by long term in vitro culture, γ-irradiation, selection for temperature sensitivity, or chemical mutagenesis, possess genetically undefined mutations, thus the potential for reversion cannot be predicted. Undefined attenuation can also lead to loss of effectiveness as shown by laboratory mice immunized with L. chagasi attenuated by long term in vitro culture, γ-irradiation, selection for temperature sensitivity, or chemical mutagenesis, possess genetically undefined mutations, thus the potential for reversion cannot be predicted. Undefined attenuation can also lead to loss of effectiveness as shown by laboratory mice immunized with L. chagasi attenuated by long term in vitro culture, γ-irradiation, selection for temperature sensitivity, or chemical mutagenesis.

An example of an attenuated parasite generated by targeted gene deletion has been demonstrated for L. donovani in our laboratory. We have targeted the same centrin gene from L. donovani whose mutant form upon overexpression reduces the growth of the parasite in vitro as well as in the macrophage. Unlike many other eukaryotes, Leishmania is diploid throughout its life cycle. Hence it was necessary to delete both the alleles of a gene by targeting with two different selectable marker genes. We sequentially disrupted the two alleles of the centrin gene by homologous recombination with the hyg gene and the neo gene flanked at the 5' and 3' ends with the 5' and 3' untranslated regions (UTRs) of the centrin gene (Fig. 1). The centrin gene status of selected double drug resistant clones was determined by Southern blot analysis using gene specific probes for centrin, hyg and neo and further shown to be negative for RNA and protein expression of centrin. Deletion of both the alleles of centrin did not affect the growth of null mutant promastigotes (Fig. 2, left
panel). However, actively growing *LdCEN*−/− promastigotes transferred to the axenic amastigote medium, increased slightly in cell number in the first 24 h, a period required for the promastigotes to differentiate into axenic amastigotes, thereafter the *LdCEN*−/− parasites failed to grow (Fig. 2, right panel).

Electron microscopic (EM) studies showed that the centrin knockout axenic amastigotes accumulated multiple nuclei from day 1 until day 4 as opposed to the single nucleus in the axenic amastigotes of the wild type cells (Fig. 3 A & B). EM studies showing failure of basal body duplication further demonstrated the lack of centrin function in the growth defect of this parasite15.

To be a vaccine candidate, the growth defect in these parasites must be reproduced intracellularly. After *in vitro* infection of human macrophages, the *LdCEN*−/− cells became large and multinucleated (Fig. 3 D) as observed in the axenic culture. In contrast, wild type control cells continued to multiply in macrophages as uninucleated cells (Fig. 3 C) and significantly more macrophages remained infected with wild type than with *LdCEN*−/− parasites15. The parasite load at 10 days post infection was significantly greater for wild type parasites than for the mutant parasite (Fig. 3 E & F). These results suggested that the centrin deleted parasites are attenuated and unable to survive in human macrophages *in vitro*.

Thus the *LdCEN*−/− cell line can be propagated as the promastigote form for production of a potential live attenuated vaccine, yet when differentiated into the amastigote form in the mammalian host, should fail to grow thereby unable to induce pathology. The time that the differentiated cells persist in the vaccinated host may be enough to elicit a protective immune response.

In all the other gene knockout studies, the genetically altered parasite was affected equally in both the promastigote and amastigote stages. However, interestingly, *L. donovani* centrin gene disruption affects selectively only the growth of the amastigote stage of the parasite both *in vitro* and inside the human macrophages. This is the first gene knockout for a cytoskeletal structural protein in *Leishmania* that plays such an important role in the growth defect of this parasite15.
of leishmaniasis, such as cutaneous and mucocutaneous diseases, will ultimately help in developing attenuated strains for these species of *Leishmania*. Such attenuated strains could be potential vaccine candidates for these diseases. Because of the essential role of centrin in the growth of organisms, similar inactivation of the centrin gene in agents of other parasitic diseases such as malaria and Chagas also could be exploited for the development of attenuated vaccine candidates.

Similarly, other laboratories have engineered and tested genetically defined mutant parasites that could point the way toward attenuated vaccines. For example, null mutants for the glucose transporter gene family in *L. mexicana* exhibited reduced infectivity to BALB/c mouse macrophages, demonstrating that a single gene deletion can render a parasite avirulent. Additionally, although *L. major* mutants deficient for leishmanolysin genes showed normal development in macrophages, *in vitro*, they showed delayed lesion formation in susceptible BALB/c mice. *L. major* mutants that lack LPG1 (the gene encoding a galactofuranosyl transferase) showed attenuated virulence in mice. Whereas the parasites that lack LPG2 (the gene encoding a Golgi GDP-Mannose transporter) persisted indefinitely at a low level in mice without displaying disease and provided protection from virulent *L. major* challenge. Such protection from virulent challenges in mice was also achieved after gene knockout for other genes: cysteine protease in *L. mexicana*, biotin transporter in *L. donovani*, dihydrofolate reductase-thymidylate synthase (DHFR-TS) in *L. major* (Table). Though infection with DHFR-TS mutants protected mice from virulent challenge, they did not protect Rhesus monkeys.

Revealing an additional concern, follow up of lpg2 mutants, which lacked virulence, showed that after a prolonged period, these parasites are partially reverted to virulence even in the absence of LPG2-dependent glycoconjugates. Therefore, it is important to ensure lack of virulence in live attenuated parasites and to further study genetically modified parasites to understand the mechanism of pathogenesis. Such types of studies with centrin deleted parasites are underway in our laboratory.

Experiments conducted to study the survival; pathogenicity and protection from challenge by the gene knockout parasites also assessed the type of immune response that was triggered by such parasites in the host following immunization and challenge. Mice that were infected with cysteine protease deleted *L. mexicana* produced significantly less IL-4 and more IFN-γ than those infected with the wild type parasites. This displays a shift from a predominant Th2 associated immunity during wild type parasite infection to a Th1 type due to infection with mutants. In a second case, inoculation of susceptible mice with the LPG2 deficient *L. major* mutant resulted in a decreased level of IL-4 in the
host and protection from virulent challenge, although, a strong IFN-γ response was absent before challenge, describing a complex immune responses seen in mice against infection with different species/mutant strains of Leishmania.

Animal models for the evaluation and development of Leishmania vaccines

Animal models for Leishmania pathogenesis and vaccine testing have been established in the mouse and the monkey. Though visceral leishmaniasis is the greatest concern for public health, L. major, causing cutaneous disease, more closely mimics human disease in the animal model and provides a basis to demonstrate vaccine development strategies. Although most of the studies involving DNA as well as protein-based vaccines have been conducted in susceptible BALB/c mice, the healing lesions produced in C57BL/6 mice provide a more relevant model of L. major infection in natural reservoirs and in human hosts. Further, in almost every case the efficacy of Leishmania vaccines has been evaluated using a high dose of parasites (10^5–10^7) inoculated into the footpad or other subcutaneous (s.c.) sites. A natural infection model in resistant mice has been developed that takes into account three main features of natural transmission: (i) low-dose (1000) metacyclic promastigotes isolated from stationary culture by negative selection using peanut agglutinin,
combined with (ii) a salivary gland sonicate obtained from the sand fly and (iii) intradermal inoculation (the ear dermis)\(^{30}\). All of which correspond more closely to the bite of an infected sand fly. In this model, the evolution of small, healing dermal lesions exhibit features suggesting a better reproduction of the natural disease including an initial phase of parasite growth in the absence of lesion formation; an acute phase, lasting 5-10 wk, corresponding to the development and resolution of a lesion that is associated with an acute infiltration of neutrophils, macrophages, and eosinophils into the dermis, and is coincident with the onset of immunity and the killing of parasites in the site; and a chronic phase, lasting for the life of the animal, during which a low number of parasites persists in the skin in the absence of overt pathology.

Results obtained from the mouse model, though practical and informative, must be further confirmed in a primate model that more closely predicts pathogenesis and immunogenesis in humans. The rhesus macaque has demonstrated protective efficacy following infectious challenge with some *Leishmania* vaccine formulations\(^{34}\) and detailed examination of the immune response in naïve and previously infected macaques has been reported\(^{81}\).

### Challenges towards Leishmania vaccine development

As noted above, several vaccine formulations that demonstrated efficacy in the mouse models, were not protective in primates\(^{35,57}\). This underscores the need for new methods to assess the immune response of animals to vaccination that can better predict the response to a virulent organism challenge in primates and humans. Further, attenuated vaccine candidates must be evaluated for pathogenicity, which can require many months of follow up. New methods would be useful for detection of parasites as well as assessment of pathology. Effective diagnosis of the progression of infection with a candidate vaccine will require assessment of the genetic stability of the agent. An example of a tool to assess the genetic stability of the attenuated parasite vaccine on a global scale is the microarray. A microarray is a large collection of DNA sequences printed on a solid surface that can be hybridized with total RNA to measure global gene expression changes\(^{82}\) or total DNA to search for areas of deletion or amplification through comparative genome hybridization\(^{83}\). These types of approaches could be utilized to more quickly evaluate stability of attenuation or reversion.

### Conclusion

In conclusion, there is a need to develop new strategies to control this disease due to its impact on world public health. Current drug treatments alone may not successfully control this disease. Particularly since prolonged use of drugs can lead to drug resistance. The constant efforts to develop new drugs to overcome drug resistance are costly and have short-range applicability. Vaccination against leishmaniasis in humans using live parasite inoculations has been tried based on the knowledge that a cured infection protects the individuals from reinfection. Early attempts at development of avirulent parasites as vaccine candidates were made through long term culture, γ-irradiation, temperature sensitive mutations or random mutations induced by chemical agents. However, it was soon realized that these approaches also have drawbacks such as reversion to virulent forms and lack of knowledge about the type of change(s) that have occurred in such parasites. The reversion to virulent form can occur spontaneously in healthy individuals or under conditions of weak host immune system. In the last two decades, use of subunit vaccines either in the form of DNA vaccines or immunization with antigens along with adjuvants in small or large animal models has resulted in partial success in the development of *Leishmania* vaccine candidates. When this strategy was tested in humans, however, the results were not favourable. The outcome of recombinant strategies for *Leishmania* vaccine candidates led to the conclusion that a low level of parasite persistence seemed to be required to maintain the immunological memory against reinfection as was originally observed in patients who recovered from infection provided such parasites cannot revert to the virulent phenotype. These challenges can be met with newly developed live attenuated strains because they (i) possess genetically defined mutations; (ii) persist in the host without being virulent; (iii) have less chance...
of reversion to the virulent phenotype because of irreversible genetic modifications; (iv) are amenable to further genetic manipulation if a candidate vaccine causes adverse reactions; (v) can be produced in large quantities in well defined conditions suitable for human vaccination; and (vi) can be tested along with new adjuvants or parasite antigens to enhance protective immune response or in combination with antileishmanial drugs to reduce pathogenesis, if needed. Finally, in our view, to make the \textit{Leishmania} live attenuated-parasite vaccine strategy work, significant funding from governmental and non-governmental entities is needed, which can ultimately lead to eradication of leishmaniasis worldwide.

\textbf{Acknowledgment}

Authors thank Dr Daniela Verthelyi, CBER, FDA, Bethesda, MD, USA for his comments/suggestions and the support from Indo-US Vaccine Action Program grant that funded some of the work described.

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