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Live attenuated *Leishmania* vaccines: a potential strategic alternative

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Abstract

Leishmaniasis causes significant morbidity and mortality worldwide, constituting an important public health problem. *Leishmania* infections cause a wide spectrum of diseases, ranging in severity from spontaneously healing skin lesions to fatal visceral disease. Attempts to develop an effective vaccine to control leishmaniasis have been shown to be feasible, but no vaccine is in active clinical use. The ability to create genetically modified parasites by eliminating virulence or essential genes is considered a powerful alternative in the development of an effective protective vaccine. Here, recent findings related to genetically defined live attenuated *Leishmania* parasites as promising vaccine candidates are reviewed.

Key words: *Leishmania*, leishmaniasis, live attenuated vaccines, genetically modified organisms, reverse vaccinology.

Abbreviations: *dhfr-ts* – dihydrofolate reductase-thymidylate synthase, *dhfr-ts*$^-$ – *L. major* dihydrofolate reductase-thymidylate synthase null mutant, *lpg2*$^-$ – *L. major* golgi guanosine diphosphate-mannose transporter LPG2 null mutant, PGs – phosphoglycans.

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Leishmania spp. are obligate heteroxenous kinetoplastid protozoan parasites spread by a sand fly insect vector causing a wide spectrum of diseases collectively known as leishmaniasis. Current available drugs are far from satisfactory, since the treatment requires long-term administration and is expensive and highly toxic. Also, a recent rise in the development of *Leishmania* resistance mechanisms towards first-line drugs has accentuated the problem of chemotherapy. A vaccine to prevent human leishmaniasis has been a goal for nearly a century, but there are still no effective vaccines. Extensive evidence indicates that leishmaniasis is one parasitic disease that can be controlled by vaccination [8]. The advances in parasite axenic culture and in the understanding of *Leishmania* pathogenesis coupled with the completion of the genome sequence of several *Leishmania* species and the capacity to perform genetic manipulations have opened new approaches feasible for *Leishmania* vaccine development.

An old principle of vaccination postulates that the more similar a vaccine is to the natural disease, the better is the protective immune response obtained. From a public health point of view, live attenuated vaccines are the gold standard for protection against intracellular organisms, as shown by the success of vaccines developed against smallpox, measles, mumps, and rubella, among others.

For several years now, experimentation has lead to the development of attenuated strains, in long-term *in vitro* cultures [10], selected for temperature sensitivity [6], chemical mutagenesis [9], and γ-attenuation [12] and in parasite culture under drug pressure [3]. Although these attenuated strains have been shown to yield substantial protection in murine models against challenge, a clear genetic profile and the potential for reversion cannot be predicted, making them unsuitable for use in human vaccination. Indeed, the persistence of asymptomatic *Leishmania* infections raises the risk of subsequent reactivation, especially in HIV/*Leishmania* co-infection. Moreover, undefined attenuation can lead to a loss of effectiveness for protective immunity, either because such strains fail to establish a sub-clinical infection or because they no longer express critical antigen epitopes [18]. As an alternative, a defined genetic alteration of the *Leishmania* genome can be achieved using a gene-targeted disruption strategy through homolo-

gous recombination that allows the selection of parasites lacking genes essential for long-term survival and/or virulence.

The first construct generated by gene replacement was the *L. major* dihydrofolate reductase-thymidylate synthase (*dhfr-ts*) knock-out [19]. Since *Leishmania* is diploid throughout its life cycle, a two-step process of inactivation was necessary to achieve the production of a homozygous null mutant auxotrophic for thymidine. *In vivo*, *dhfr-ts*$^-$ parasites survived but were unable to establish a persistent infection or cause disease even in the most susceptible mouse strains. Furthermore, their use as an immunizing agent induced substantial protection against a virulent *L. major* challenge. However, protective experiments in Rhesus monkeys failed [22], stopping their further development as an anti-*Leishmania* vaccine. In another approach, depleting the parasite of all surface and secreted PGs by deletion of the *L. major LPG2* gene rendered the parasites unable to cause pathology even in the absence of a host immune response, but they retained the ability to persist at a low level in mice for more than two years [16]. Hence immunizing with *lpg2*$^-$ parasites protected highly susceptible BALB/c mice against a *L. major* virulent challenge even in the absence of a strong Th1 response [20]. In contrast to *L. major* mutants, *L. mexicana lpg2*$^-$ mutants retained their virulence for macrophages and mice [7], which suggested that different *Leishmania* species possess alternative virulence repertoires to interact with the host. However, the *in vivo* follow-up of these mutants allowed the identification of a partial revertant population that regained virulence even in the absence of *LPG2*-dependent PGs [16]. Another attenuated line of *L. mexicana*, lacking cysteine proteinase genes *cpa* and *cpb*, was also successfully used to protect against homologous infection in both murine and non-murine models [1, 13]. Although *dhfr-ts*$^-$ and *lpg2*$^-$ mutants showed some inherent problems that may have excluded them as future *Leishmania* vaccines, they did open the door for live attenuated vaccination in leishmaniasis.

Recently we showed that immunizing BALB/c mice with *L. infantum* lacking one *SIR2* gene copy induced a high degree of protection against a virulent challenge [15]. The unbalanced secretion of parasite-specific IFN-γ and IL-10 was understood to allow the development of cellular and humoral anti-*Leishmania* responses that ultimately lead to an optimal elimination of the parasites. Hence it allowed the suggestion that the polarization to a high IFN-γ low IL-10 ratio after challenge would be a clear indicator of a vaccine's success. Further studies in *in vitro* and *in vivo* canine systems will help to elucidate its real capacity in protecting against canine visceral leishmaniasis. The disruption of the *SIR2* gene selectively affects the growth and development of the intracellular amastigote stage. A similar strategy was previously developed to disrupt the *L. donovani* biopterin transporter or centrin genes [11, 14]. Unfortunately, the latter is yet to be tested for protective efficacy. Therefore, targeting genes important for intracellular survival will maintain the propagation of the unaffected promastigote form for vaccine production which, when differentiated into amastigotes inside the mammalian host, will persist without any pathology, thereby ideally inducing protection.

Overall, the use of live attenuated organisms is very attractive for vaccination (Table 1). First, live attenuated vaccines, by mimicking the natural course of infection, can simultaneously deliver antigen to and enhance the innate immune activation of antigen-presenting cells, thus providing an optimal polarization of $CD4^+$ T cells [17]. Second, a complete spectrum of antigens is delivered, when compared with subunit-defined vaccines, increasing the memory repertoire of the immune system. Third, by inducing a long-lasting subclinical infection, live attenuated vaccines assure persistence of antigen that may allow the generation of antigen-specific effector and memory cells which react immediately following infection [5].

It seems now clear that antigen is not strictly required to sustain memory Th1 cells *in vivo,* since central memory T cells ($CD62L^{high}$, IL-2^{pos}, IFN-γ^{neg}), which provide an expanded pool of Ag-experienced cells, can mediate long-term protection, though of lower magnitude, even in the absence of parasites [21]. Nevertheless, the effector T cell pool that provides excellent protection is dependent upon continuous stimulation, which is only accomplished by natural infections or live attenuated vaccines. Therefore, the latter approach seems to be advantageous since it will be very difficult for a non-live vaccine to induce long-lasting protection without lifelong continuous boosting [5].

A different approach was recently proposed by Breton et al. [2], who suggested the use of a non-pathogenic *Leishmania* vector (*L. tarentolae*) as a vaccine candidate against leishmaniasis. Indeed, a single intraperitoneal immunization of *L. tarentolae* elicited a protective immune response against *L. donovani* in susceptible BALB/c mice, which was understood to be a result of an enhanced antigen presentation and potent Th1 immune response. The possibility to further refine this non-pathogenic live vaccine by genetic manipulation (generating recombinant *L. tarentolae* expressing defined *Leishmania* immunodominant epitopes) and its potential capacity to induce protection against several *Leishmania* species place this alternative strategy among the most promising live vectors towards the development of an effective and safer anti-*Leishmania* vaccine.

Although reverse vaccinology has shown encouraging advances, there is still a long road towards a live attenuated *Leishmania* vaccine. In addition, attenuated organisms might aid in the development of more potent subunit vaccines, which are useful in defining new pathways of resistance and are extremely interesting biological models to explore the correlates of protection. However, major safety constraints, such as possible reversion to virulence or reactivation in immunosuppressed individuals, and manufacturing considerations are still limiting their large-scale use. Moreover, a com-

Table 1. Live attenuated vaccines against leishmaniasis

Species	Attenuation process	Model	Outcome of immunization	References
Undefined genetic alteration				
L. major *L. tropica*	Long-term *in vitro* culture	C57BL/6 (genetically resistant mouse); BALB/c, BALB/c.H-2b and BALB/c.H-2k (genetically susceptible mouse)	Genetically susceptible vaccinated mice showed partial protection with persistent low-grade cutaneous disease for months	10
L. braziliensis	Temperature-sensitive attenuated promastigotes	BALB/c mouse	Successful immunization against infection with parental parasite clone	6
L. major	Chemical mutagenisis	BALB/c mouse	Controlled lesion size at site of challenge	9
L. major	Gamma irradiation	CBA and BALB/c mouse	Subcutaneous immunization conferred a high degree of protection against homologues and heterologous challenge	12
L. mexicana *L. major*	*In vitro* culture under drug pressure	BALB/c mouse	Attenuated lines failed to induce lesions and provide significant protection	3
Defined genetic alteration				
L. major	Dihydrofolate reductase – thymidylate synthase (*dhfr-ts*) null mutant	BALB/c mouse; primate	Do not cause disease, but protect mice against challenge; however, it failed to protect monkeys	19, 22
L. mexicana	Cysteine protease (*cpa*, *cpb*) null mutants	BALB/c and C57BL/6 mouse; hamster	Attenuated mutants do not induce lesion growth; immunization provide protection as characterized by smaller lesions and lower parasite burden	1, 13
L. donovani	Biopterin transporter (*BT1*) null mutant	BALB/c mouse	Reduced infectivity and induced protection against challenge	11
L. major	Phosphoglycans (*lpg2*) null mutant	BALB/c mouse	Persist indefinitely without causing disease and protect against challenge	20
L. infantum	Silent information regulatory 2 (*LiSIR2*) single-knockout mutant	BALB/c mouse	Attenuated mutant persists for six weeks without establishing infection, providing significant protection against challenge	15

mon additional problem in all these attenuated lines is the restraint to their use in clinical studies due to the presence of antibiotic resistance genes used as selective markers during the steps of gene deletion. The application of a versatile "hit-and-run" targeting strategy was recently proposed in which the result of gene deletion will not contain any exogenous DNA [4]. Thus, using this two-step approach (gene deletion with parasite selection and excision of the antibiotic gene cassette) it is possible to refine the generation of *Leishmania* mutants, making them suitable for use as a live vaccine.

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