Mini-review

Challenges and perspectives in vaccination against leishmaniasis

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ABSTRACT

The leishmaniases are a group of diseases caused by protozoa of the genus Leishmania and affect millions of people worldwide. The leishmaniases are transmitted to vertebrate hosts by phlebotomine sand flies. In this review, we focus on several issues that have been poorly addressed in ongoing efforts to develop a vaccine against Leishmania, namely: vaccination with antigens present in sand fly saliva, vaccines based on intracellular Leishmania antigens, and use of recombinant BCG as a vehicle for vaccination. Additionally, we address the differences between L. major and L. braziliensis and the impact that these differences may have on strategies for immunoprophylaxis.

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Contents

Leishmaniasis is a serious and increasing public health problem. Approximately 300 million people live in or travel to tropical and subtropical risk areas. Moreover, human leishmaniasis is endemic in more than 80 countries, and its prevalence exceeds 12 million cases worldwide; 1.5–2.0 million new cases occur annually, causing a burden estimated at 2,357,000 disability-adjusted life years (DALYs) (http://www.who.int/tdr/diseases/leish/diseaseinfo.htm). Leishmania infection can be classified into three main classical syndromes: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL). Clinical manifestations of the disease depend on several factors, including the species involved, and symptoms range from self-limiting cutaneous lesions through the severe mucocutaneous form to the often fatal visceral form. In VL, which encompasses a broad range of clinical signs, infection remains asymptomatic or subclinical in many cases, while in others it follows

an acute or chronic course. Importantly, the disease does not resolve spontaneously, and the ensuing systemic infection may be fatal if left untreated [1]. In CL, lesions tend to heal spontaneously, and immunity that ensues following natural healing is lifelong. Therefore, prevention of leishmaniasis through prophylactic immunization seems feasible.

Ongoing approaches to vaccine development are largely based on identification of appropriate surface antigens of L. major. It is expected that a vaccine against leishmaniasis will likely combine more than one antigen and that antigens will preferentially be conserved among Leishmania species and present in both the amastigote and promastigote stages of the parasite. Although several current candidates fulfill such criteria, demonstration of protection by these antigens in more than one animal model is lacking. Additionally, some protective antigens are conserved among their mammalian orthologues, raising concerns over possible autoimmune reactions. This review will focus on areas poorly addressed in the ongoing efforts to develop a vaccine against Leishmania, namely, vaccination with antigens present in sand fly saliva, vaccines based on intracellular Leishmania antigens, and use of recombinant BCG as a vehicle for vaccination. An additional challenge that needs proper

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attention is the impact that recognized differences between *L. major* and *L. braziliensis* may have on strategies for immunoprophylaxis.

1. Vaccination with sand fly salivary antigens

Leishmania protozoans are transmitted to their vertebrate host by infected sand flies. While attempting to feed, these flies inject both saliva and *Leishmania* promastigotes. Sand fly saliva contains a vast repertoire of pharmacologically active molecules able to interfere with the host's hemostatic, inflammatory and immune responses. The effects of sand fly salivary products on leishmaniasis have recently been reviewed [2]. The actions of these salivary components during early interactions between *Leishmania* and the host's immune system are closely linked to disease evolution as well as to protection against the protozoan. Hence, characterization of salivary components is regarded as essential for understanding the pathogenesis of the disease as well as for providing a basis for development of novel strategies to hamper pathogen transmission. Indeed, maxadilan, a vasodilatory peptide isolated from *Lutzomyia longipalpis* saliva, decreased the secretion of IFN-γ and increased the production of IL-6 in mononuclear cells [3]. *Lu. longipalpis* saliva inhibited IL-10 and TNF-α production and enhanced IL-6, IL-8 and IL-12p40 secretion by LPS-stimulated human monocytes and dendritic cells (DCs) [4]. In parallel, reduced CD80 and increased HLA-DR expression were also observed.

These results show that sand fly salivary gland components have an immunomodulatory effect on human cells. This finding has important implications for vaccine development. Moreover, modulation of *Leishmania* infection by sand fly saliva has also been reported during experimental infection with *L. major* [5], *L. braziliensis* [6,7] and *L. amazonensis* [8]. Importantly, in a CL experimental model employing *L. major*, it was shown that prior exposure of mice to bites of uninfected sand flies conferred powerful protection against *L. major* [9] and that a DNA vaccine encoding SP15, a salivary antigen present in *Phlebotomus papatasi* saliva, provided similar protection [10]. However, when a similar approach was used in an attempt to prevent infection with *L. braziliensis*, immunization with saliva of *Lu. intermedia*, the main vector of *L. braziliensis*, was found to enhance infection [11]. Such results point to differences in the composition and immunomodulatory capacity of salivary antigens between distinct sand fly species.

With respect to the use of salivary components in the development of vaccines against VL, it has been demonstrated that immunization with a DNA plasmid coding for an 11 kDa protein from *Lu. longipalpis* saliva induced protection against intradermal co-inoculation of *L. chagasi* and salivary gland homogenate [12]. This protection was associated with the development of anti-sand fly saliva cellular immunity in the form of a DTH response and the presence of IFN-γ at the site of sand fly bites. Hence, immunity to a single salivary protein can confer protection against VL. Indeed, immunization of dogs with *Lu. longipalpis* salivary antigens led to the development of a recall response characterized by lymphocytic infiltration and expression of IFN-γ and IL-12 [13]. The recent observation that neutrophils persist at the site of sand fly bites and that this effect inhibits *L. major* elimination in mice vaccinated with *Leishmania* antigen + CpG [14] reinforces the case for development of combination vaccines, including parasite- and sand fly salivary antigens.

Sand fly salivary antigens may also serve as epidemiological tools to track vector exposure. For example, children residing in areas endemic for VL exhibit anti-*Lu. longipalpis* saliva IgG antibodies [15]. This humoral response appears simultaneously with an anti-*L. chagasi* cell-mediated immunity [16], supporting the hypothesis that induction of an immune response against saliva can facilitate induction of a protective response against leishmaniasis. Moreover, anti-sand fly saliva antibodies may be used as an important epidemiological marker of vector exposure and may even prove useful as a marker of protection. In another study, healthy volunteers exposed to laboratory-reared *Lu. longipalpis* developed anti-saliva antibodies (IgG1, IgG4 and IgE) [17]. Two major patterns of responses were observed in these volunteers: intense skin reactions with indurated nodules accompanied by delayed-type hypersensitivity (DTH)-like response with higher IgG/IgE ratio, and mild erythematous reactions with lower IgG/IgE ratio [17]. The most immunogenic proteins in individuals exposed to *Lu. longipalpis* sand flies had molecular weights of 45, 44, 43, 35, 27 and 16 kDa [16,17].

2. *Leishmania* vaccines based on intracellular antigens

Recent advances in the design of vaccines against leishmaniasis using various strains of inbred mice have shown that immunization with defined parasite antigens provides protection against challenge with several *Leishmania* species. Interestingly, many of these protective molecules have an intracellular location [18]. Some, such as A2 protein (an amastigote-specific molecule) [19] or the *Leishmania* sterol 24-c-methyltransferase (SMT) [20], are parasite-specific and are not found in mammalian cells. Other intracellular protective antigens are members of conserved protein families such as histones, the acidic ribosomal protein P0, the stress-inducible LmSTI1 protein, and LACK, the leishmanial homolog of the mammalian receptor for activated C kinase [21]. In natural infections, immune responses against these intracellular proteins are thought to result in immunopathology, since they predominantly stimulate non-protective specific humoral responses in patients with different forms of the disease [20,22–25] and in dogs with VL [26–28].

Interestingly, some of these antigenic proteins have also been implicated in the generation of protective responses. Thus, *Leishmania* histone H2B was able to stimulate the production of IFN-γ in a T cell clone established from an immune donor [29], and parasite histones H2B, H2A and H3 induced proliferation and IFN-γ production by peripheral blood mononuclear cells (PBMCs) from CL patients [30]. Similar results were observed with cells from resistant mice stimulated with A2 [31]. Generally, a high specificity was observed in the humoral and cellular responses elicited against highly conserved intracellular antigens, since T- and B-cell epitopes were restricted to the small blocks of non-conservative amino acid substitutions. Likely for that reason, neither reactive T cells [29] nor anti-intracellular protein antibodies recognized their mammalian orthologues [21]. Thus, it can be hypothesized that, depending on the immune response elicited against them, these antigens may play an important role in disease development.

The fact that the immune response to parasite antigens fails to provoke recognition of homologous regions within conserved proteins argues against nonspecific polyclonal activation as the basis of the immune response to these antigens. It therefore is expected that these antigens are exposed to the host immune system after infection. Extracellular promastigote cytolysis mediated by serum lytic factors [32] or neutrophil extracellular traps [33] may prime the immune response of the vertebrate host against intracellular antigens in the inoculation site. Later, when amastigotes replicate inside the macrophages, these antigens may be derived by spontaneous cytolysis within infected cells and become exogenously exposed after disintegration of the cells [34].

Intracellular antigens have been used for immunization in combination with Th1-inducing adjuvants or as DNA vaccines, since the development of vaccines based on these antigens is mainly focused on the generation of specific IFN-γ producing CD4+ Th1 and CD8+ T cells. Thus, immunization with *L. donovani* A2 DNA vaccines [35] or with A2 protein combined with *Propionibacterium acnes* [36] induced significant protection against VL caused by *L. donovani* in BALB/c mice. Protection was correlated with the generation of a Th1/Th2 mixed response and with A2-specific IFN-γ production. Similarly, immunization of BALB/c mice with SMT antigen plus monophosphoryl lipid A (MPL®) induced partial protection against *L. donovani* challenge and resulted in IFN-γ production upon *in vitro* stimulation with the antigen [20].

Although the generation of a vaccine-induced Th1 response is necessary for protection, it may not be sufficient. Thus, Th1 responses

elicited after LACK genetic immunization did not induce protection against *L. donovani* challenge [37]. Control of deactivating responses occurring after infection, mediated mainly by IL-10 or TGF-β [38], may improve vaccine success. In this sense, a vaccine consisting of *Leishmania* ribosomal P0 protein plus MPL® as adjuvant achieved partial protection against *L. donovani* challenge in hamsters that correlated with a decrease in IL-10 expression [39]. Partial protection against *L. infantum* challenge in BALB/c mice was observed after the adoptive transfer of bone marrow-derived DCs pulsed with four nucleosomal leishmanial histones and correlated with a decrease in CD4$^+$CD25$^+$FoxP3$^+$ T reg cells [40]. Although it has been shown that CD4$^+$CD25$^+$FoxP3$^+$ cells are involved in disease persistence in the murine model of CL [41,42], in human VL, IL-10 production is mainly associated with CD4$^+$CD25$^-$FoxP3$^-$ Tcells rather than with CD25$^+$FoxP3$^+$ cells [43].

The development of intracellular antigen-based vaccines against CL has for the most part been assayed in BALB/c mice infected by *L. major*. Th1 responses induced after immunization with LmSTI1 or LACK protein administered with IL-12 [44,45] or as DNA-based vaccines [46,47] protected BALB/c mice against *L. major* infection. Similar results were obtained in this experimental model after genetic immunization with a DNA cocktail encoding the parasite histone genes [48]. Although protection correlated with the generation of antigen-specific Th1 responses, the control of cutaneous lesion progression also depended on the suppression of IL-4 production induced by *L. major* infection. Thus, immunization of BALB/c with the parasite ribosomal protein P0 plus CpG oligodeoxynucleotides [49] or with a DNA vaccine [50] induced a Th1 response against the antigen; however, mice succumbed to progressive disease because vaccination was unable to abrogate the Th2 responses induced by *L. major* challenge. Similarly, genetic immunization with LiP2a and LiP2b acidic ribosomal proteins [51] or with HSP70 [52] also failed to inhibit the Th2 response in susceptible mice, despite production of antigen-specific IFN-γ after *L. major* challenge. Therefore, it can be concluded that protection in the BALB/c-*L. major* CL model requires not only the production of IFN-γ but also improvement of the Th1/Th2 balance in order to induce parasite elimination. Indeed, demonstration of a role for IL-10 and Treg cells in parasite persistence led to re-evaluation of vaccine-induced immunity [41,53]. In this manner, for example, low IL-10 production predicted protection upon vaccination with TryP (tryparedoxin peroxidase) antigen [54]. Challenge infection further enhanced the ratio of low IL-10/high IFN-γ upon TryP vaccination. It therefore seems that the success of a particular vaccine candidate is determined not only by its potential as an IFN-γ stimulator but also by its ability to inhibit or to reduce IL-10 production.

3. Recombinant BCG expressing *Leishmania* antigens

Several findings suggest the potential usefulness of recombinant BCG (*Mycobacterium bovis* bacillus Calmette–Guerin) in vaccination against leishmaniasis. Used as an adjuvant in *Leishmania* vaccines, BCG has shown some degree of protection; its adjuvant properties, combined with its safety and routine use worldwide, make BCG a promising candidate for expression of heterologous antigens without necessitating major alterations in public health immunization schedules [55].

BCG has been used as an adjuvant in vaccine preparations using dead *L. mexicana* or with *L. braziliensis* promastigotes. These products have been tested in humans in both prophylactic and therapeutic approaches, but with conflicting or inconclusive results [56–59]. Indeed, the therapeutic efficiency of immunotherapy (BCG plus promastigotes of *L. mexicana*) was shown to be equal to that of chemotherapy (Glucantime) and to lack the serious side effects of the drug treatment [60]. A trial in Iran showed that a single dose of autoclaved *L. major* (ALM) + BCG was safe, more immunogenic than BCG alone as measured by the leishmanin skin test, and able to confer protection against CL in boys [61]. The safety and efficacy of ALM + BCG was later tested in healthy volunteers, where it was shown that a single dose of this vaccine

was safe with no evidence of an exacerbating response following natural infection. However, ALM + BCG failed to confer significant protection when compared to BCG alone [62]. In a VL endemic setting, ALM + BCG also did not prevent development of disease when compared to individuals vaccinated with BCG alone [63]. More recently, it was shown that modification of ALM + BCG by adsorption to alum significantly increased immunogenicity. The alum-adsorbed ALM vaccine exhibited safety and immunogenicity similar to or better than multiple doses of ALM + BCG alone [64]. Overall, most studies that have employed BCG as an adjuvant reaffirmed the immunogenicity and safety of BCG.

BCG has been administered to more than three billion individuals worldwide with very few serious adverse effects, making it a suitable vehicle for the delivery of heterologous antigens. Therefore, a multivalent vaccine using BCG as a live vehicle has been advocated [65]; it is currently the only vaccine recommended for administration at birth. Thus applied, one BCG dose would be sufficient for inducing long-lasting cell-mediated immunity (CMI); it presents low production cost and is thermostable [66]. Heterologous proteins expressed in BCG induce IgG antibody production, lymphocyte proliferation and cytokine production, as well as generation of cytotoxic T lymphocytes (CTL) [67]; indeed, the first recombinant BCG was obtained for an epitope of HIV gag p17 [68]. Recombinant BCG (rBCG) vaccines have been shown to induce a protective response against *Plasmodium falciparum* [69] and *Bortedella pertussis* [70]. Promising results have also been obtained with rBCG expressing *L. major* gp63 induced CMI, which induced protection in mice [71,72]. Recombinant BCG expressing LCR1 from *L. chagasi* induced protection against homologous challenge in susceptible mice [73]. More recently, the interest in rBCG as a carrier for heterologous antigens has increased with the possibility of using new promoters to enhance antigen expression.

Recombinant BCG as an expression vehicle is under evaluation in several models ranging from expression of cytokine genes for the treatment of bladder cancer [74] to induction of protection against HIV [75], SIV [76] and respiratory syncytial virus [77]. However, despite promising initial results and recent advances in the knowledge of the molecular biology of BCG, the field is still open for development of other approaches combining different antigens.

4. Challenges of vaccination against *L. braziliensis*

In Old World CL caused by *L. major*, lesions are 2–5 cm in diameter, multiple in the majority of cases and progress to complete spontaneous healing, frequently within a few months, leaving a fibrotic scar [78]. New World CL caused by *L. braziliensis* frequently manifests as an ulcer with elevated borders and sharp crater. Lesions rapidly increase in size and show a tendency to heal slowly without treatment [78]. *L. braziliensis* can cause disseminated CL, in which hundreds of lesions erupt as a result of hematogenous spread of parasites [79], and MCL, in which parasites spread to the oral mucosa. MCL, a hallmark of *L. braziliensis* infection, leads to extensive tissue destruction as a result of the potent cell-mediated immune response triggered by the parasite [80]. Therefore, the development of an effective vaccine may contribute to the prevention of CL and MCL caused by *L. braziliensis*.

Most vaccination studies conducted thus far against CL have used genes and/or antigens isolated and characterized from *L. major*; however, *L. braziliensis* is largely divergent, genetically and biologically, from *L. major* and *L. infantum*, the two other species sequenced to date [81]. The biological diversity between *L. major* and *L. braziliensis* is observed in clinical presentations of CL caused by each [82,83], as well as in the intradermal model of experimental infection. Inoculation of *L. major* into the ear dermis of BALB/c mice leads to uncontrolled parasite proliferation [84], whereas *L. braziliensis* infection generates a cutaneous lesion that heals spontaneously [85]. In both models, control of infection was shown to be dependent on IFN-γ production [86]. BALB/c mice suffer progressive

disease after infection with *L. major*, and this outcome depends on the production of IL-4 early after infection by CD4$^+$ T cells that express V beta 4 V alpha 8 T cell receptors [87]. However, lymph node cells from BALB/c mice infected with *L. braziliensis* produced significantly less IL-4 than cells from mice infected with *L. major* [86]. This differential course of infection may also be related to the presence of *L. braziliensis* antigens that lead to strong induction of IL-12 [44]. Moreover, it was also observed that *L. braziliensis*-infected BALB/c mice showed a significantly lower expression of IL-4, IL-10 and IL-13 in cells than *L. major*-infected mice [88]. Collectively, these data reinforce the impression that the biological diversity between *L. major* and *L. braziliensis* contributes to the differences in infectiousness and pattern of disease progression induced by the two species. Importantly, of the total content of ~8300 genes in each species, only ~200 have been identified as differentially distributed between the three genomes (*L. major*, *L. braziliensis* and *L. infantum*); *L. braziliensis* possesses 47 genes that are absent from the other two species [81].

With respect to prophylactic immunization, it was observed that DNA vaccination employing *L. braziliensis* homologues of LACK, LeIF, LmSTI1 and TSA failed to confer protection against intradermal challenge with live parasites [89]. These results are in contrast with those reported for *L. major* [90]. Interestingly, *L. braziliensis* protein homologues showed a high degree of identity with those encoded by previously described genes of *L. major*, and immunization generated specific IFN-production. In contrast to observations with *L. chagasi* [12] and *L. major* [10], immunization with saliva of *Lu. intermedia*, the main vector of *L. braziliensis*, enhanced infection with *L. braziliensis* rather than inducing protection [11], as mentioned earlier. However, *Lu. intermedia* saliva produced *in vitro* responses in human monocytes similar to those observed in experiments using *Lu. longipalpis* saliva [91]. These results indicate that immunization with sand fly saliva can play an ambiguous role in leishmaniasis, depending on the vector source and the *Leishmania* species involved.

Despite the failure of some vaccines based on specific host proteins to induce immunity in experimental models, ongoing research in our laboratory has demonstrated that immunization of BALB/c mice with nucleosomal histones leads to a significant decrease in lesion development and in parasite load (M. W. Carneiro, manuscript in preparation). A similar outcome has already been observed in *L. major* [48].

Therefore, the most important factor in the development of vaccines against *L. braziliensis* may be the choice of antigen. Indeed, the problem of antigen identification represents the principal roadblock in vaccine development. Antigens are usually identified through time-consuming and labor-intensive experiments conducted both *in vitro* and *in vivo*. For example, T cell epitope prediction using a bioinformatics approach has recently been used to search for antigens in the *L. major* proteome. Using such an *in silico* approach, it was shown that a higher number of *L. major* peptides were predicted to bind to major histocompatibility (H2) molecules from BALB/c than to those from C57BL/6 background [92]. Moreover, an *in silico* search was able to identify 78 MHC class I epitopes in the *L. major* proteome. Experiments performed with this select group of 78 MHC class I epitopes narrowed the IFN-γ-inducing epitopes to eight peptides [93]. A similar approach can be employed with the *L. braziliensis* predicted proteome, also currently available for data mining. Given the striking differences in terms of disease phenotype and outcome following vaccination with different antigens, such an approach represents a valuable tool for vaccine development in the post-genomic era.

5. Concluding remarks

There are several hurdles in the path to an effective vaccine against leishmaniasis; this review has addressed only some of them. It is unlikely that an effective anti-*Leishmania* vaccine based on use of a single antigen will be achieved. A rational approach towards developing combination vaccines is the use of *Leishmania* surface antigens, a group intensively explored, along with intracellular parasite antigens and one or more salivary antigens.

Reported approaches using BCG as an adjuvant have been limited, though there are indications of its usefulness in increasing the protective potential of vaccine candidates. Of note, BCG has mainly been administered in conjunction with the candidate antigen; however, the use of rBCG expressing *Leishmania* antigens is yet to be fully explored. A point frequently overlooked in the leishmaniasis literature is the marked distinction between *L. major* and *L. braziliensis*. The New World and Old World species probably diverged 20–100 million years ago, and, indeed, these two particular species of *Leishmania* are associated with different types of disease [94]. As described above, potential candidates for vaccine development already exist. One major concern, however, is that these antigens have for the most part been selected on the basis of efficacy against *L. major* infection. Therefore, such antigens are not necessarily effective against VL or even against New World *Leishmania* species such as *L. braziliensis* and *L. amazonensis*, the species that cause debilitating MCL and DCL (diffuse cutaneous leishmaniasis). Therefore, yet another challenge for vaccine development is to obtain protection against debilitating CL or even against CL and VL where they occur in the same populations. With this in mind, several alternatives should be pursued, some of which have been addressed here.

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