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Not All Antigens Are Created Equally: Progress, Challenges, and Lessons Associated with Developing a Vaccine for Leishmaniasis

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ABSTRACT From experimental models and the analyses of patients, it is well documented that antigen-specific T cells are critical for protection against *Leishmania* infection. Effective vaccines require both targeting to the pathogen and an immune stimulant to induce maturation of appropriate immune responses. While a great number of antigens have been examined as vaccine candidates against various *Leishmania* species, few have advanced to human or canine clinical trials. With emphasis on antigen expression, in this minireview we discuss some of the vaccine platforms that are currently being explored for the development of *Leishmania* vaccines. It is clear that the vaccine platform of choice can have a significant impact upon the level of protection induced by particular antigens, and we provide and highlight some examples for which the vaccine system used has impacted the protective efficacy imparted.

KEYWORDS *Leishmania*, adjuvants, protein, vaccine, vector

Hemoflagellate protozoa of the genus *Leishmania* constitute more than 20 species and subspecies of parasites that infect an estimated 12 million people from among a pool of more than 350 million people considered at risk of infection (1, 2, 3). The required interaction with infected sand flies to permit transmission renders leishmaniasis predominantly a disease of the poor, occurring mostly in remote rural villages with poor housing and little or no access to modern health care facilities. Any form of leishmaniasis therefore puts stress on the already limited financial resources of both the affected individual and the community at large (1). Vector-control programs are, however, having an impact, and on the Indian subcontinent a visceral leishmaniasis (VL) elimination target is being met in most areas of endemicity (2). With disruption in control efforts allowing new epidemics in areas of endemicity and the spread of the disease to previously free areas because of migration, tourism, and military activities, however, an effective vaccine remains highly desirable in order to retain/sustain the success of recent efforts (3).

POTENTIAL FOR VACCINES

The quality of the immune response is critical in determining if *Leishmania* infection manifests disease. The clinical symptoms are also dependent upon the particular *Leishmania* species, which vary in their geographic distributions. Manifestations range from localized, disseminated, diffuse, or recidivate cutaneous leishmaniasis (CL) or mucosal leishmaniasis (ML) to involvement of organs such as liver and spleen in cases of VL (3). CL can arise from infection with any of several *Leishmania* species (i.e., *L. major* or *L. tropica*) and can progress to the gross mucosal tissue destruction observed in ML patients. VL is the most severe form and in South Asia and Africa is caused by infection

with *L. donovani*, while in the Mediterranean, the Middle East, Latin America, and other parts of Asia, it results from infection with *L. infantum*.

Protection from, and clearance of, *Leishmania* infection is strongly associated with the generation of antigen-specific Th1 responses, knowledge that provides a clear goal for immunization. To target and appropriately skew the anti-*Leishmania* response, vaccines require both pathogen-specific antigens and immune-stimulating molecules to be rendered effective. Whole and genetically attenuated parasites, such as *L. donovani* centrin-deleted parasites (*LdCen1*$^{-/-}$), have undergone trials in both dogs and humans (4–8). Dendritic cell (DC)-based vaccines represent a more refined strategy and have been used in preclinical models. Although financial constraints and the likely difficulties in delivering such vaccines in regions of *Leishmania* endemicity seem to preclude their use in all but a very limited subset of individuals, DC-based vaccines provide further proof of concept supporting the use of more defined vaccines (9).

Our understanding of pattern recognition receptors and adjuvants that can be used in conjunction with recombinant proteins within defined subunit vaccines continues to expand (10–12). A wide variety of adjuvants have been used in *Leishmania* research programs, and the importance of adjuvants within vaccines has been the subject of numerous review articles (13–16). Simultaneous with the progress in adjuvant technologies, advances in bioinformatics and molecular techniques have expanded the number of potential *Leishmania*-specific targets for use within a vaccine (17–19). A considerable number of *Leishmania* antigens have been tested as subunit protein or DNA vaccine candidates against various *Leishmania* species, but variable results have meant that very few antigens have advanced to human or canine clinical trials (20). Factors such as the *Leishmania* source of the target gene, the *Leishmania* species involved in the challenge, the quantity of antigen/vector used, and the frequency and numbers of immunizations all impact our ability to interpret the data beyond their immediate application. In addition, once targets are selected, there are also now a plethora of strategies available with which to ready them for use within a vaccine (21). In this minireview, we discuss the current status of vaccine development for leishmaniasis with emphasis on the impact of the expression/vaccine platforms.

PURIFIED RECOMBINANT PROTEINS

Native *Leishmania* proteins have been used in either a crude or a purified manner to elicit protective immune responses. *Escherichia coli* and yeast are among the most widely used systems for the production of recombinant proteins (22–24), and bioinformatics has facilitated the production of a variety of recombinant proteins that have been investigated as *Leishmania* vaccine antigen candidates in animal models (15, 20). Key to the use of recombinant proteins is the cost-effectiveness of production, an important consideration when modeling has shown that a cost of $2 or less per dose for a preventive vaccine would be economically advantageous over the currently available leishmaniasis treatments (25). In addition, recombinant methods also provide the opportunity to manipulate and combine complementary proteins/epitopes in a single gene product.

Our first defined vaccine against leishmaniasis came with the use of *E. coli*-expressed Leish-111f (L111f; LEISH-F1) that joined the *L. major* homologue of eukaryotic thiol-specific antioxidant (TSA), the *L. major* stress-inducible protein-1 (LmSTI1) and the *L. braziliensis* elongation and initiation factor (LeIF) into a chimeric fusion protein. Leish-111f protects mice, hamsters, and rhesus macaques when appropriately formulated with adjuvant, and LEISH-F1+ monophosphoryl lipid A (MPL)-SE was the first defined *Leishmania* vaccine to enter clinical trials, where it demonstrated an excellent safety profile and induced antigen-specific responses (26–29). A second, distinct *E. coli*-derived fusion, LEISH-F3, that combines two components that can protect in animal models of VL, nucleoside hydrolase (NH) and a sterol 24-c-methyltransferase (SMT), has also progressed through phase I clinical trial (30).

The cost-effectiveness of recombinant proteins can also be enhanced by the selection of particular expression platforms. In some instances, yeast (*Saccharomyces cerevi-*
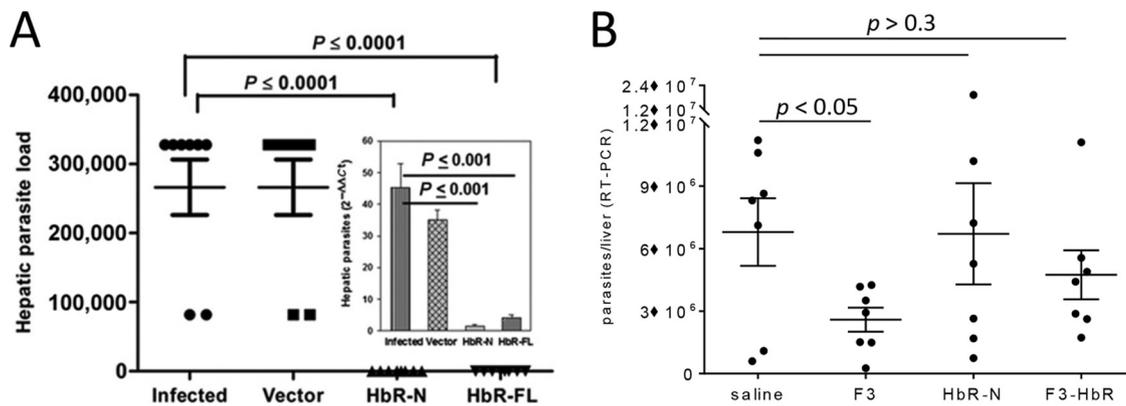
FIG 1 Influence of expression system on the protective efficacy of immunization with HbR. (A) *Leishmania* HbR was delivered as a DNA vaccine, either as full-length HbR (HbR-FL) or HbR-N coding sequence, intramuscularly into BALB/c mice. The *L. donovani* load per milligram of liver homogenate was determined 60 days after infection (inset shows real-time PCR [RT-PCR] results). Data are shown as mean and standard error of the mean (SEM), with 8 mice per group. (Republished from reference 35 with permission of the publisher.) (B) Mice were injected a total of 3 times with 5 μg *E. coli*-expressed recombinant protein formulated with GLA-SE and then 1 month after the final immunization were infected by intravenous injection of *L. donovani* promastigotes. Mice received either LEISH-F3 (30), protein derived from HbR-N, or a chimeric fusion of F3-HbR. These procedures were conducted in accordance with animal handling protocols approved by the IDRI institutional animal care and use committee. Livers were removed 1 month after parasite inoculation, and burdens were determined by quantitative PCR (qPCR). Data are shown as mean and SEM, with 7 mice per group.

siae or *Pichia pastoris*) expression systems provide benefits over *E. coli* expression systems, such as variable folding, glycosylation, or direct secretion into the fermentation media of the target protein as a soluble and stable protein (31, 32). An *E. coli*-expressed *L. donovani* nucleoside hydrolase (LdNH36) surface protein has provided partial protection against *L. donovani* infection in mice (33). Expression of LdNH36 in *P. pastoris* produced LdNH36-Y-WT that was of significantly higher molecular weight than the *E. coli* expressed protein (34). Gel analyses suggested that expression in yeast resulted in several high-mannose glycoforms, whose presence was first reduced by mutating N-linked glycosylation sites located outside the major immunogenic domain from asparagines to serines (LdNH36-dg) and then removed by mutating asparagines to glutamines. The LdNH36-dg2 product was similar in size to the nonglycosylated *E. coli* expressed protein, but the glutamine mutations resulted in increased expression levels. Mice immunized with LdNH36-dg2 did have high levels of antibodies that inhibited the hydrolase activity of the wild-type LdNH36 (34), although challenge experiments have not been reported.

DNA VACCINE CONSTRUCTS

Besides traditional recombinant expression approaches, many antigens have also been investigated in DNA vaccines (20). VL patients possess antibodies against the hemoglobin receptor (HbR) of *Leishmania*, and this protein is conserved across various *Leishmania* strains. Immunization with HbR-DNA generated multifunctional CD4 and CD8 T cells and was reported to provide complete (sterile) protection against *L. donovani* infection in both mice and hamsters (35). Given these findings, we produced recombinant HbR in *E. coli* to examine compatibility with our own vaccine candidates. Surprisingly, unlike our previous data indicating various candidates that retained their protective properties when expressed in *E. coli* (36), we did not observe protection in mice that were immunized with HbR in conjunction with the glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE) (Fig. 1). Furthermore, adding HbR as a component within fusion proteins disrupted their ability to confer protection. We still do not understand why these differences arose, but it is possible that differential processing of epitopes may occur, or some DNA vaccines may target different antigen-presenting cells than protein/adjuvant. While subtle, such changes may be enough to lead to critical variations in the protective efficacy of the respective vaccines.

Fused targets can also be created within DNA vaccines, and seven *Leishmania* genes

(H2A, H2B, H3, H4, A2, KMP11, and HSP70) are encoded within the HisAK70 DNA-vaccine (37). In the VL model, HisAK70-immunized mice exhibited many sterile hepatic granulomas associated with the resolution of hepatic parasite burdens, and in the CL model, spread of parasites to the viscera was completely inhibited in HisAK70-immunized mice. The results suggest that immunization with the HisAK70 DNA may provide a rapid, suitable, and efficient vaccination strategy to confer cross-protective immunity against VL and CL.

BACTERIAL AND VIRAL VECTORS

In terms of bacterial vectors, *Listeria monocytogenes*, *Mycobacterium bovis* BCG, and *Salmonella enterica* serovar Typhimurium have all been used as vehicles to deliver *Leishmania* targets (20). As an example of this approach, novel *Leishmania* parasite antigens were selected by an *in silico* proteomic approach and expressed in the vector *Salmonella* Typhimurium SL3261. Immunization of mice with individual *Salmonella* vaccine strains expressing the antigens LinJ08.1190 and LinJ23.0410, or a mixture of these bacilli, significantly delayed the progression of *L. major* and enhanced systemic resistance against *L. donovani* (38). These data identify *Salmonella* as another valid vaccine carrier for inducing protection against VL. Given that 2 antigens that were also selected and expressed did not protect, however, this *Salmonella* system may not be appropriate for all antigens.

With regard to viral vectors, the most advanced is a nonreplicative adenovirus vector encoding the A2 antigen (Ad5-A2) that has progressed to *L. infantum* challenge in macaques (39). Using a variety of prime boost schemes involving Ad5-A2 as well as recombinant protein and a plasmid DNA-encoding A2 gene (DNA-A2), parasite burdens in the liver were lower in immunized macaques and correlated with hepatic granuloma resolution and the reduction of clinical symptoms. Considering the importance of both CD4 and CD8 T cells in the control of *Leishmania* infection, we recently generated an adenoviral vector expressing the F3 protein (Ad5-F3) to complement the use of recombinant F3 and investigated immunization strategies with the promise of generating combined protective T-cell responses (40). Overall, a F3+GLA-SE prime/Ad5-F3 boost strategy induced the best combined CD4 and CD8 T cell memory response, although we also found that simultaneous immunization with Ad5-F3 and F3+GLA-SE in a single injection was potent enough to provide protection. As an alternative, although only limited experimental information regarding the effectiveness for use against human pathogens is available, the NYVAC poxvirus vector has also recently been explored (41). Using DNA prime/virus boost protocols, a replication-competent NYVAC expressing LACK (*Leishmania* homologue of mammalian RACKs, the receptors for activated C kinase) (NYVAC-LACK-C7L) induced more polyfunctional CD4 and CD8 T cell responses and greater protection against *L. major* than nonreplicating NYVAC-LACK.

CLOSING REMARKS

A great number of defined antigens, delivered as recombinant protein in adjuvant, as plasmid DNA, or within vector systems, have provided protection in animal models of leishmaniasis. In an attempt to consolidate information generated across many platforms, we recently selected and expressed 7 candidates as recombinant proteins in *E. coli*. Each recombinant protein was recognized by VL patients and, when formulated with the Th1-potentiating adjuvant GLA-SE, retained its protective efficacy against the challenge of mice with *L. donovani* (36). This indicates the possibility of combining candidates that have advanced in diverse vaccine platforms. It is clear, however, that the vaccine platform of choice can have a significant impact upon the level of protection induced and in some instances can negate protection altogether. Thus, without actually generating independent results, vaccine developers must be cautious when navigating through the existing data and between the platforms (Table 1). On the positive side, as is seen most clearly with viral vectors, exploring interactions between

TABLE 1 Impact of production system on protective efficacy of *Leishmania*-specific vaccine targets[a]

	Vaccine antigen system							
	recombinant		DNA		bacteria			
					Salmonella	*Listeria*	BCG	
LACK			infantum (42)					
			donovani (43)					
			chagasi (44)	chagasi (45)				
	major (46)		major (47-49)			major (50)		
	amazonensis (51)		amazonensis (51)					
	braziliensis (52)		braziliensis (52)					
gp63			infantum (53)		donovani (54)			
	major (55)	major (56)	major (57)	major (58)	major (59)	major (60)	major (61)	major (62)
			mexicana (63, 64)				mexicana (62)	mexicana (65)

protection	none	partial	strong	complete

[a]Examples of the various data generated for the *Leishmania*-specific components, LACK and gp63, across some of the vaccine systems used are shown. The protection levels are indicated by the color scheme and were obtained from the review by Costa et al. (20); the pertinent citations (42–65) are indicated in parentheses. *L. donovani*, *L. chagasi*, and *L. infantum* cause VL; *L. braziliensis*, *L. major*, and *L. mexicana*, and *L. amazonensis* cause CL or ML.

these technologies has the potential to yield regimens that augment, complement, or synergize each other to yield a robust protective regimen.

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