REVISIÓN

Development of anti-*Leishmania* vaccines: contribution of Spanish researchers

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ABSTRACT

Several *Leishmania* antigens have been characterized and assayed, as recombinant products, in vaccination trials using different experimental models of leishmaniasis. Vaccination strategies have been developed to induce adequate cellular responses against a great number of antigenic proteins. Spanish groups, working alone or, in many cases, in collaborative research projects have contributed to gather and get an important level of information about families of antigenic protein properties and procedures for immune intervention. Some of these topics are included in the present review.

Keywords: Anti-*Leishmania* vaccines; antigenic proteins; experimental models.

RESUMEN

Desarrollo de vacunas contra Leishmania: contribución de investigadores españoles

Varios antígenos de *Leishmania* han sido caracterizados y probados, como productos recombinantes, en ensayos de vacunación empleando diferentes modelos experimentales de leishmaniosis con objeto de inducir respuestas celulares adecuadas, ademas, de protección contra la infección. Diferentes grupos de investigación españoles, trabajando solos o, en muchos casos, en proyectos de investigación cooperativos, han contribuido a reunir un importante nivel de conocimiento sobre diferentes familias de proteínas antigénicas y procedimientos de intervención inmunitaria. Algunos de estos temas se discuten en la presente revisión.

Palabras clave: Vacunas contra *Leishmania*; Proteínas antigénicas; Modelos experimentales.

1. INTRODUCTION

Parasites from genus *Leishmania* have a digenetic life cycle in which parasite multiply as extracellular promastigotes in the mid-gut of their insect vectors (sand-flies from genus *Phlebotom*us in the Old World and *Lutzomyia* in the New World). Parasites are transmitted to the vertebrate host during blood meal and after infecting macrophages they are transformed in the amastigote forms that replicate in vacuoles of lysosomal origin. Infection of different vertebrate hosts with several species from genus *Leishmania* can cause a complex group of diseases globally termed as leishmaniasis. In humans, depending on the infectious species and the host immune state, the disease ranges in severity from cutaneous (CL; caused by *Leishmania major* in the Old World and *Leishmania mexicana*, *Leishmania amazonensis* and *Leishmania braziliensis*, between other species in the New World), diffuse cutaneous (DCL, caused by *Leishmania aethiopica* in the Old World and *L. mexicana* in the New World) to mucocutaneous (MCL, caused mainly by *L. braziliensis*) and visceral leishmaniasis (VL, caused by *Leishmania donovani* and *Leishmania infantum* in the Old World and by *Leishmania chagasi* (genetically identical to *L. infantum* (1)) in the New World (2). These infections are endemic in several tropical and subtropical countries around the world (3) and are responsible for the second-highest number of deaths due to a parasite infection after malaria (4). Canine viscerocutaneous leishmaniasis (VCL) is an important emerging zoonosis in Mediterranean countries, Middle East and Latin America (5). This severe form of the disease is caused by *L. infantum* and by *L. chagasi* in the Old World and in the New World, respectively. Wild canids and domestic dogs act as parasite reservoirs, playing a central role in the transmission to humans (Reviewed in (6). Different spectra of human and canine disease can be developed after infection, from subclinical infection to disseminated infection (2, 7). The outcome of infection is determined by the interactions between the host immune system and different parasite species. Generally, for all forms of leishmaniasis, except MCL, protective immunity is associated with a classical cell mediated immune response that induces macrophage activation by T cells derived cytokines, while non-healing disease is associated with the generation of humoral responses (6-8). In MCL patients an exacerbated and non-controlled inflammatory response seems to be responsible for the pathogenesis (9).

The fact that patients recovered from disease are resistant to reinfection has been taken as an indication that a vaccine is feasible. Different research strategies have been employed for the generation of vaccines against *Leishmania* although there is no vaccine against this parasite in humans. In this context, some vaccines are now at the research phase and one of them, namely Leish-110f and that is based on a three antigen fusion recombinant protein (10) is on the development phase (11). Regarding prophylaxis in dogs, there are three

commercial vaccines against canine leishmaniasis: Leishmune (based on Parasite Fucose-Manose-Ligand) (12), Leishtec, based on a recombinant amastigote antigen namely A2 protein (13) and CaniLeish, composed on promastigotes secreted-excreted factors (14, 15). In spite of the existence of these products, the search of molecules for development of *Leishmania* vaccines continues, looking for improving protection against different forms of leishmaniasis. There are recent review articles covering the progress made towards the development of *Leishmania* vaccines, including some of the most studied parasite proteins together with the effect of various adjuvants employed in experimental vaccination trials (4, 11, 16-22). There is general consensus indicating that the establishment of a protective anti-*Leishmania* response may require the induction of parasite specific long-lasting memory T cells that will expand as effector T cells for the production of IFN-gamma dependent responses specific for parasite antigens in order to activate the leishmanicidal capacities of infected macrophages. In this way, most of the recent research is focused on the identification of the *Leishmania* molecules that interacts with the host immune system and on the analysis of their prophylactic properties when immunized in experimental models of infection as second generation vaccines (based on parasite fractions or recombinant proteins) or third generation vaccines (mainly DNA-based vaccines). In this work we make a review of these studies performed with different parasite proteins, immunodominant antigens during infection, focusing on the results obtained taking into consideration the implication of different Spanish researchers, alone or in collaborative work, in order to show the important efforts made in our country during the last years, helping to mitigate the effects of this emerging disease.

2. *LEISHMANIA* SURFACE ANTIGENS

A number of surface glycoproteins are present in promastigote forms of *Leishmania* parasites. The *Leishmania* proteinase GP63, one of the most abundant surface-exposed proteins on parasite promastigotes (23, 24) has been described as an immunogenic protein during human VL (25). The GP63 is also an antigenic molecule in canine VL, as described by (26). Using a recombinant version of the *L. infantum* GP63 (LiGP63) as well as some synthetic peptides derived from its aminoacid sequence, Dr. Alonso's laboratory (Centro de Biología Molecular Severo Ochoa, CSIC-UAM) was able to demonstrate that LiGP63 was recognized by the 100% of the sera from *L. infantum* infected dogs and that the C-terminal domain was the most antigenic region of the protein. On the basis of its surface localization and its antigenicity, second generation vaccines related with GP63 and its immunodominant epitopes have been extensively studied as vaccine candidates using murine models of infection (Revised in (27)). Of special interest is the work published by Cote-Sierra and coworkers (28) with the collaboration of Dr..

Segovia's group (Facultad de Medicina, Universidad de Murcia). In this work they used a recombinant version of the C-terminal domain of the GP63 fused to an immunostimulatory molecule. The main objective was to improve protection derived from this region of the GP63, which contains the host-protective T cell epitopes (29), by its fusion with the lipoprotein OprI from *Pseudomonas aeruginosa*, an inductor of IL-12. The authors demonstrated that the fusion lipoprotein was able to induce GP63 specific Th1 and TNF-alpha mediated responses correlated to robust protection against murine CL due to *L. major* infection in the susceptible BALB/c mice (28). An additional promastigote surface glycoprotein, namely GP46, M2 or PSA has been described in different *Leishmania* species (30, 31). This protein possesses a central core composed by different repeats of leucine rich regions described as most immunodominant region recognized by human and canine VL patients (31).

Another abundant component of the promastigote surface is the Kinetoplastid Membrane Protein 11 (KMP-11) a dominant surface membrane protein associated with the promastigote lipophosphoglycan (LPG). Dr. Alonso's research group in collaboration with different laboratories has been implicated in the characterization of genes encoding the KMP-11 from *L. infantum* (32) and *L. panamensis* (33) as well as in the study of the antigenicity of this protein. The immunogenicity of the KMP-11 has been demonstrated in different hosts. Thus, the sera from human patients suffering from active VL but not individuals with subclinical *L. chagasi* infections, react with the recombinant *L. infantum* KMP-11 protein (34). In addition, patients suffering from MCL or CL showed a KMP-11 specific production of IL-10 (35). Finally, anti-KMP-11 antibodies were found in the sera from VL dogs infected with *L. infantum* (32, 36). Vaccines based on this protein have shown to be protective in different animal models. The protective capacity of the KMP-11 described in a hamster model of VL infected by both pentavalent antimonial sensitive and resistant virulent *L. donovani* strains (37) has been recently reinforced after demonstration that a DNA vaccine based on *L. infantum* KMP-11 was able to protect hamster from infection with *L. chagasi* (38) and a vaccine composition formed by the recombinant *L. infantum* KMP-11 loaded in poly(lactic-co-glycolic acid) nanoparticles was able to protect mice from CL due to *L. braziliensis* infection (39). Moreover, this last formulation stimulates macrophages for secreting pro-inflammatory cytokines and chemokines and for synthesis of superoxide resulting in intracellular *L. braziliensis* killing (40).

The antigenic nature of two different amastigote specific membrane components has been studied with the implication of different Spanish researchers. P8 antigen, a *Leishmania pifanoi* amastigote specific proteoglycolipid complex, biochemically characterized by Dr. Colmenares in Dr.. MacMahon-Pratt's laboratory (41), was able to stimulate the innate immune response of murine

macrophages in a TLR4 dependent manner (42) and also was up-regulating the expression of IFN-gamma and TNF-alpha in asymptomatic *L. infantum* infected dogs (43). The induction of $CD4^+$ and $CD8^+$ mediated responses by the immunization of the P8 complex combined with the *Propionibacterium acnes* adjuvant in C57BL/6 mice resulted in protection against *L. amazonensis* infection (44). HASPB1, an hydrophilic acylated surface protein, is another component of the amastigote membranes (45, 46). This protein is able to elicit humoral responses in humans infected by *L. donovani* (47). In a canine vaccine trial made in Madrid (Instituto de Salud Carlos III) it was shown that HASPB1 was able to induce protection against experimental infection with *L. infantum* in dogs when administered in combination with a mineral oil based adjuvant (Montanide ™ISA 720).

Globally, most of the surface components of the parasite are antigenic during infection in different hosts. Different vaccination trials were performed employing purified fractions (in some cases) and mostly recombinant versions of the antigens, combined with adjuvants that stimulate cellular responses. Depending on the model, the vaccination studies resulted in different degrees of protection, The different degrees of protection was usually correlated with the induction of cellular responses. These results can be taken as an indication that surface proteins should be taken into account for the development of anti-*Leishmania* vaccines. However, and at is indicated below, proteins with intracellular locations are also interacting with the host immune system.

3. *LEISHMANIA* INTRACELLULAR ANTIGENIC PROTEINS

Many intracellular parasite proteins interact with the host immune system after *Leishmania* infection. Most of them are members of conserved housekeeping proteins like intracellular receptors, heat shock proteins, ribosomal proteins and histones (48). In spite of their conserved nature, the humoral and cellular responses against them are specifically directed against the parasite antigens without showing cross-reactivity with the host counterparts. The specificity of the response is based on the location of their antigenic determinants in the most divergent regions of the parasite proteins (48, 49). Different Spanish scientists have been implicated in the search for this type of related proteins. Some of their results are highlighted below.

3.1. Leishmania and its antigenic histones

Leishmania histones, in spite of their nuclear location and their high degree of conservation throughout eukaryotic organisms, have been described as immunodominant antigens during *Leishmania* infection (48). The characterization

of the *L. infantum* histone H2A was made using sera from infected dogs that were recognizing this basic protein (50). The rest of the nucleosome forming histones (H3, H2B and H4) was described as antigens in serologic assays employing canine VCL sera (51, 52). Antigenicity is not only related to the VL canine infection, since the four core histones were also recognized by sera from CL and MCL human patients, being the H2A the most antigenic core histone (53). This protein is also recognized by sera from VL patients infected with *L. chagasi* (34). The antigenicity of the H1 linker histone in patients infected by *L. braziliensis* has been demonstrated by Dr. Valladares research group (Facultad de Farmacia, Universidad de La Laguna) (54, 55). Remarkably, the anti-histone humoral response elicited during infection is specific for the parasite antigens and does not show cross-reactivity with the host histone, since B cell epitopes are mainly located in the most divergent regions of the parasite histones (52, 55-57). The presence of IFN-gamma mediated specific T cell responses has been demonstrated for the H2B protein in human patients of CL and VL (49, 58) and for H2A and H3 in CL patients (59).

The prophylactic value of the *Leishmania* histones was evaluated in different experimental models with the implication of different Spanish researchers. Induction of Th1 responses against the four *L. infantum* nucleosomal histones were able to protect BALB/c mice against a virulent challenge with *L. major* (60, 61), *L. braziliensis* (62) and *L. infantum* (63). Beside data reporting the protective capacities of the H1 histone in murine (64) and monkey (65) models, a vaccine based on this protein was tested with success (62.5% of infected animals without clinical symptoms) in a vaccine trial against experimental canine VL (66).

Taking into account the high degree of immunogenicity of the parasite histones and their value as immuno-prophylactic molecules tools against leishmaniasis in different experimental models, parasite histones emerge as a powerful tool against Leishmania infection. In this sense and as it is indicated in section 4, different combination molecules designed as anti-Leishmania vaccines include Leishmania histone-genes or proteins.

3.2. Leishmania ribosomes as vaccines

Leishmania ribosomes have emerged as immunodominant particles during parasite infection. Many ribosomal proteins are recognized by the sera from VL dogs (67-70) or are antigenic in human MCL and VL patients (68, 71). *Leishmania* acidic ribosomal P proteins (namely P0, P2a and P2b) are good examples of *Leishmania* intracellular antigens. Strong humoral responses are elicited against them during infection (mainly in the VL forms of human and canine disease). Interestingly, anti-P antibodies are specifically directed against parasite P proteins without cross-reactivity with the host orthologs (reviewed in (48)) although these proteins are antigenic in patients with autoimmune diseases (72). The location of B

cell epitopes in the most variable region of the P2a, P2b and P0 proteins explains the observed specificity of the response (69, 70). The P0 protein has been employed in different vaccination assays in murine models of CL employing susceptible (BALB/c) and resistant (C57BL/6) mice. BALB/c mice immunization with a parasite P0-based DNA vaccine or with the rP0 protein combined with Th1 inducing oligonucleotides induced partial protection after challenge with *L. major.* Immunized animals showed a delay in the development of cutaneous lesions but mice ultimately developed a non-healing form of the disease (73, 74). On the other hand, the Th1 responses induced by vaccination conferred protection against CL in C57BL/6 mice (74). Since the administration of some other ribosomal constituents using immunization procedures inducing Th1 responses was related to the generation of protective responses (75, 76), vaccines based on total ribosome extract (LRP) were analyzed. In addition, a cDNA clone encoding the *L. braziliensis* ribosomal protein S4 was recognized by a T-cell clone derived from a resistant VL human donor with a positive DTH skin test (49), indicating that the recognition of some of the parasite ribosomal proteins by the host immune system is not necessarily related to disease progression. Administration of the LRP combined with Th1 inducing adjuvants prompted a ribosome-specific Th1 response in mice, correlated with protection against the development of leishmaniasis due to infective challenges with *L. major* (77), *L. amazonensis* or *L. chagasi* (78) parasites. The robust protection observed in the susceptible model BALB/c-*L. major* (detected by the absence of cutaneous lesions for long periods of time) was accompanied by the capacity to resist a secondary infection (79). Two new antigenic ribosomal molecules obtained as recombinant proteins by the expression of the *L. major* encoding LmL3 and LmL5 genes have shown immuno-prophylactic properties against infection with *L. major* and *L. braziliensis* in BALB/c mice.

3.3. Leishmania homolog of mammalian receptor for activated C kinase (LACK)

LACK protein is one of the most studied Leishmania antigens. This intracellular protein is a member of the tryptophan-aspartic acid repeat family of proteins and it has been implicated in the induction of early IL-4 responses after L. major infection (80). In this sense, BALB/c rendered tolerant to LACK, as a result of transgenic expression of this molecule in the thymus, were resistant to infection with L. major and develop a Th1 response after infection (81). Several vaccination protocols were tested in collaboration between Dr. Esteban (Centro Nacional de Biotecnología) and Dr. Larraga (Centro de Investigaciones Biológicas) research groups using different LACK preparations, based on the L. infantum LACK protein, that was characterized in Dr. Larraga's research group (82). The main strategy was the induction of robust cellular responses against LACK by the use of Th1 inducing procedures (mainly DNA vaccines (83-85)) alone or combined with recombinant Vaccinia virus expressing LACK using a prime-boost strategy (86-94). Using these

strategies involving the LACK molecule of L. infantum, cross-protective responses were found in murine models of CL due to L. major (83, 87, 88, 90, 93, 94) or L. amazonensis (84) infections. The L. infantum LACK based vaccines also protect mice against murine VL disease caused by L. infantum/L. chagasi (85, 86, 91). Recent studies have correlated the observed protection to the induction of effector memory $CD4^+$ and $CD8^+$ T cells expressing IFN-gamma and TNF-alpha in response to the LACK antigen (92). These vaccination trials were extended to the experimental model of canine leishmaniasis (89, 95). Prime-boost vaccination resulted in the induction of Th1-like specific for the LACK antigen, correlated with the induction of protective responses in the vaccinated groups: lower parasite load and humoral responses against parasite proteins, as well as less external clinical symptoms (89).

4. CONCLUDING REMARKS

Different candidates for the development of *Leishmania* vaccines have emerged from the studies described in this review. As a brief summary, vaccines against *Leishmania* may depend on the selection of the adequate parasite proteins but also on the development of immunization strategies inducing memory T cellular responses able to mount a fast but controlled Th1 response when parasite is inoculated by the insect vector. Combination of parasite surface exposed structures and intracellular antigens emerge as an interesting poly-epitope based strategy that should control de replication of different *Leishmania* species. Different poly-antigenic fusion molecules have been designed for development of *Leishmania* vaccines (10, 96). Among them, the Q protein developed and tested in collaboration between different Spanish groups, is formed by the fusion of two antigenic regions of the H2A beside the antigenic domains of the three P ribosomal antigens (P2a, P2b and P0). This protein was able to confer protection to mice (97) and dogs (98) when combined with BCG as adjuvant. In addition, the Q-protein was able to induce protection when administered in dogs without any adjuvant (99). This protection was demonstrated by Dr. Gomez-Nieto group using a model of experimental infection that reproduced the course of canine natural infection (100).

Some authors have pointed out that the induction of such complex immune responses as well as the maintenance of the effector memory T cells would require parasite chronicity (reviewed in (101, 102)). In this sense, a mutant *L. infantum* parasite strain with a limited capacity of multiplication within the vertebrate host by the deletion of part of the *hsp70* genes has been constructed in Dr. Requena's laboratory (CBMSO, UAM-CSIC). As the authors point out this mutant strain may emerge as an interesting alternative to antigen-based formulations for creating anti-*Leishmania* vaccines (103, 104)

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