Visceral leishmaniasis: immunology and prospects for a vaccine

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Abstract

Human visceral leishmaniasis (HVL) is the most severe clinical form of a spectrum of neglected tropical diseases caused by protozoan parasites of the genus *Leishmania*. Caused mainly by *L. donovani* and *L. infantum/chagasi*, HVL accounts for more than 50,000 deaths every year. Drug therapy is available but costly, and resistance against several drug classes has evolved. Here, we review our current understanding of the immunology of HVL and approaches to and the status of vaccine development against this disease.

Keywords: Immunology, vaccine, visceral leishmaniasis

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Introduction

Proposals and broad issues regarding a research agenda for the development of vaccines against the leishmaniases have recently been discussed [1–6]. Fatal human visceral leishmaniasis (HVL) caused by *L. donovani* and *L. infantum/chagasi* in the old world and *L. infantum/chagasi* in the New World [7,8] is the focus of this review. *L. infantum/chagasi* maintains a zoonotic cycle mostly involving canine hosts, and canine visceral leishmaniasis (CVL) is a veterinary problem in its own right [9]. Reducing the incidence of CVL is a promising control strategy for zoonotic HVL [10,11]. However, *L. donovani* has an anthropoponotic cycle [12,13] (e.g. in India where HVL is known locally as kala azar). Patients treated and cured for HVL caused by *L. donovani* may subsequently develop post kala azar dermal leishmaniasis (PKDL), characterized by nodular skin lesions in which parasites can be detected [14]. Parasite persistence during PKDL is thought to be involved in igniting epidemics of HVL [15]. The epidemiological relevance of subclinical infection is only clear in dogs, where animals with subclinical infection can transmit parasites [16–18]. Our understanding of the relative contribution of subclinical vs. PKDL cases to the epidemiology of HVL has an important bearing on the design of elimination campaigns. Whereas both therapeutic and prophylactic vaccines each have their place in control programmes, prophylactic or possibly post-exposure vaccination (and vector control) may be essential if subclinical infections play a dominant role in maintaining the disease cycle.

Immunology

The immunology and immunopathology of visceral leishmaniasis in man, dog and in experimental rodent models (EVL) has been extensively studied, and in-depth reviews are
available on this topic [10,19–22]. Central to our ability to manipulate host protective immunity is an understanding of the life cycle of *Leishmania* in its mammalian host and the impact on this of various host defence mechanisms. Surprisingly, this is less complete than might be expected, with a number of questions related to vaccine success still to be answered (Fig. 1).

Although HVL is characterized by hypergammaglobulinaemia (associated with polyclonal B cell activation; [23]) and both antibody and B cells have been shown to have various regulatory roles in EVL [24,25], the precise value of high titre vaccine-induced antibodies has yet to be fully defined. Whereas opsonization of metacyclic promastigotes could reduce infectious load after sandfly transmission, there is a significant body of data derived from other models of leishmaniasis suggesting that antibodies may facilitate infection [26,27]. Unfortunately, we have little knowledge about how the infectious dose relates to the development of natural immunity (i.e. sub-clinical infection) and significantly, most experimental vaccine studies fail to measure early parasite load post-challenge, confounding the interpretation of protection measured at later time points. In HVL and CVL, immune complexes may also cause glomerulonephritis [23], though this is a factor that might only be of consideration in relation to therapeutic vaccination. What is clear is that most effective licensed human vaccines were developed for infections against which antibody-mediated protection is characteristic [28] and this fact should stimulate further research into the relationship between vaccine-induced antibody responses, infectious dose and subsequently acquired immunity.

Within hours of sandfly transmission, metacyclic promastigotes take up intracellular residence and begin conversion to intracellular amastigotes, from then on the only life cycle stage of *Leishmania* associated with human disease. Although recent evidence suggests that metacyclic forms can be found in neutrophils [29], most data indicate that long-term amastigote survival and replication occurs within mononuclear phagocytes [30]. Hence, T-cell-mediated immunity targeting either activation or killing of amastigote-infected macrophages is generally regarded as the cornerstone of host resistance and the main target for vaccine-induced responses (Fig. 1). Our knowledge of these responses derives from methodologies as diverse as serum cytokine analysis and intravital 2-photon microscopy, but rarely are multiple overlapping methodologies applied in the same study, further complicating data interpretation. In HVL, CVL and EVL, T-cell-mediated resistance is nevertheless clearly multifaceted: nitrogen and oxygen radicals serve as major leishmanicidal effectors in macrophages [31] but other pathways may also operate [32]; both Th1- and Th2-associated cytokines contribute to primary and vaccine-induced resistance [33,34]; CD4+ Foxp3+ IL-10+ Th1 cells [35,36] and/or natural CD4+ Foxp3+ IL-10+ Treg [37] may hinder macrophage activation; CD8+ T cells may provide additional beneficial cytokines and/or their cytotoxic potential may allow release of

**FIG. 1.** A vaccinologist's view of the *Leishmania* life cycle. Immune responses induced by vaccination can target metacyclic promastigotes directly, their products (e.g. promastigote secretory gel, PSG) or sandfly salivary proteins to limit initial parasite establishment. Metacyclics that survive can be targeted as amastigotes in macrophages found in skin or various systemic organs. Macrophages can be uninfected, infected or activated to kill *Leishmania* amastigotes. Parasite spread between macrophages is believed to occur through cell lysis. During subclinical infection or after clinical cure, immune responses maintain a state of persistent infection for the life of the host. Key questions pertaining to the role of Ab and CMI at each stage of the infection are listed. In most cases, the relative importance of each during vaccine-induced immunity has yet to be established. Possible transmission-blocking effects of vaccination operating in the sandfly host are not shown. Key: 4/8, naive CD4+ or CD8+ T cells; 1, 2, 10, 17, reg, activated CD4+ T cell subsets (note similar diversity may occur in activated CD8+ T cells, not shown); 4 m/8 m, memory CD4+ and CD8+ T cells; macrophages, neutrophils, dendritic cells, stromal cells, metacyclic promastigotes and amastigotes are depicted with characteristic morphology.

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amastigotes to facilitate killing by activated monocytes [38]; and effective immunity may be optimal when focused within organized tissue structures termed granuloma [22,30]. Importantly for therapeutic vaccination (and for drug action), progressive disease is associated with an increasing state of immunosuppression, attributed both to the presence of immunoregulatory cytokines, notably IL-10 and TGFβ [20], and also to destructive remodelling of lymphoid tissue [39]. Thus, interventions that alleviate the immunosuppressive state or that stimulate more potent T-cell responses have been increasingly seen as having potential for immunotherapy [40]. Nevertheless, in spite of many years of investigation, our knowledge of the mechanisms that allow life-long subclinical persistence of viscerotropic species of *Leishmania* either following natural infection or drug-induced cure are very scanty [41] and consequently there are few robust and validated correlates of protection that can be applied to studies of vaccination in man.

**Status of Vaccine Development for HVL**

In spite of an estimated 500,000 cases annually (http://www.who.int/leishmaniasis/en/), HVL has been considered an unattractive vaccine target for industry, being primarily a disease of the poor. Yet the economic impact at household and population level is substantial, even though the methodology used to quantify the problem is crude [42]. HVL ranks second only to malaria for mortality and fourth for morbidity amongst tropical parasitic diseases [43]. Even in relation to the projected most cost-effective combination therapy (estimated at ~$80 [44]), treatment and related costs (which in India surpass 10% of average household expenditure), present a financial burden that will force many into spiralling poverty [45]. A vaccine (subsidized or otherwise) will need to cost less per averted clinical case [44], and far less for a prophylactic vaccination campaign in areas where incidence rates are low. Low incidence also drives up the cost and complexity associated with prophylactic vaccine trials designed to show efficacy, leading some to conclude that testing vaccines in therapeutic trials (http://www.who.int/vaccine_research/diseases/soa_parasitic/en/index3.html) or by using leishmanization as a ‘challenge’ infection [46] is the most cost-effective way to move forward.

Although the century-old practice of leishmanization, the deliberate infection of naive people with virulent *L. major*, still provides the most compelling evidence that vaccination against leishmaniasis is feasible [46,47], the efficacy of this approach has not been shown for VL. Furthermore, whilst many whole killed parasite vaccines have been developed (http://www.upmc-biosecurity.org/website/resources/govt_docs/countermeasures/hhs/hhs_jordan_rpt_accel_dev_vac_2007.html), a recent meta-analysis of the respective prophylactic vaccine trials yielded a disillusioning summary of the lack of clinical efficacy of these first-generation vaccines [5]. In contrast, therapeutic vaccine development is supported by good clinical evidence, albeit also in the context of cutaneous or mucocutaneous leishmaniasis, because crude parasite lysates with BCG provide an effective adjunct to chemotherapy (for recent review see [48,49]) and treatment with a mixture of recombinant parasite proteins and GM-CSF combined with antimony therapy showed promise in patients that were refractory to drug-only treatment [50].

Given the complexity of the host immune response to vaccines, whole killed or lysed parasites may contain antigens or other characteristics that induce non-protective as well as protective immune responses [10,51]. Thus, second-generation vaccines, using parasite fractions or defined protein subunits, aim to reduce this complexity, and many have been tested in models of experimental leishmaniasis (though fewer in EVL; [1]), but only a few formulations have entered clinical or veterinary testing (see supplementary Table S1 for a comprehensive summary). Only a single product (Leish-111f), a fusion protein of three relatively conserved *Leishmania* proteins (thiol-specific antioxidant, stress inducible protein I and elongation initiation factor) formulated with MPL®-SE is entering phase II clinical testing in humans, including HVL as a therapeutic vaccine [6,52]. Leish-111f has been shown to have efficacy in mice [53] and under the product name rLeish-110f® has been tested as an adjunct therapy together with Glucantime® to treat a field population of dogs suffering from CVL due to *L. chagasi* infection [54]. Vaccination was safe and induced a 2–3-fold increase in antigen-specific proliferative response *in vitro* after cure, but was lacking clear clinical benefit (though the trial was not powered to reveal small effects). Leish-110f in the form of an experimental vaccine designated MML was also tested alongside recombinant *L. infantum* histone H1 and hydrophilic acylated protein B1 (HASPB1 [55]) as prophylactic vaccines against experimental CVL [56]. Dogs were vaccinated with either MML adjuvanted with MPL®-SE or H1 or HASPB1 adjuvanted with Montanide™-ISA 720 and subsequently challenged with 10⁸ *L. infantum* promastigotes. All vaccines were immunogenic, with some interesting differences in whether infection boosted these responses. For example, MML antibody responses were strongly boosted, HASPB1 responses weakly so and H1 response unaffected by infection [56]. Fewer H1 and HASPB1 immunized dogs developed CVL symptoms (37% and 50%, respectively) compared with control or MML-vaccinated dogs (71% and 75%, respectively), but larger studies
would be required to confirm these differences in efficacy and a possible correlation with antibody responses.

A very different approach has led to the development of the only currently licensed anti-Leishmania vaccine product, Leishmune® (for review see [57,58]). The vaccine is composed of a yet to be fully defined affinity purified glycoprotein fraction of *L. donovani* promastigotes, fucose-mannose-ligand (FML), formulated with a saponin adjuvant. Leishmune® is effective as a prophylactic vaccine in the field [59] and may also have an impact on human VL incidence, when combined with the cur- ing of seropositive animals [11]. Leishmune® shows therapeutic efficacy in naturally infected, outbred dogs, particularly when formulated with more saponin and in combination with otherwise poorly effective drugs [60,61]. Leishmune® seems also to block transmission. The transmission blocking activity may be a composite effect of (i) vaccinated dogs not having parasites in their skin even when infected [62] and (ii) blocking of parasite development in sandflies [63]. It is worth noting that this vaccine apart from inducing mainly IgG2 antibodies also stimulates both CD4+ and CD8+ T-cell responses that correlate with clinical cure [64,65]. Induction of CD8+ T cells may be related to adjuvant choice, as saponin is a critical component of vaccines formulated as immunostimulatory complexes (ISCOMs [66]). Recently, a dominant antigen in the FML complex, a secreted nucleoside hydrolase of 36 kDa, has been shown in recombinant form or as a DNA vaccine to reproduce some of the Leishmune® effects in mice [58], with the C-terminal domain bearing the required T-cell epitopes [67]. The rather crudely defined but nevertheless effective Leishmune® vaccine may therefore become replaced by a synthetic product comprising its active ingredients [58].

Similar to the situation in human VL patients, symptomatic disease in dogs is correlated with a ‘suppressive’ pattern of T-cell responses, with a dominant role for IL-10 in ongoing, non-protective immune responses [68,69]. In view of this similarity between HVL and CVL, the fact that Leishmune® has shown promise as a therapeutic vaccine in CVL is encouraging. The therapeutic efficacy in CVL of Leish-111f, which induces mainly B-cell and CD4+ T-cell responses, was indistinguishable from the effect of the MPL-SE adjuvants alone [54]. This may suggest that a therapeutic vaccine against HVL should, akin to CVL, aim to induce both CD4+ and CD8+ T cells. Findings in several preclinical models [70–72] support this view.

Vaccine Platforms

As mentioned, the definition of correlates of immunity to VL is still not very robust but, at the level of an individual, protection is likely to depend on antigen-specific CD4+ and CD8+ T cells able to activate or kill macrophages that are or may become infected. However, the induction of robust T-cell-mediated immunity, in particular involving CD8+ T cells, remains a general challenge for vaccine developers. To induce the latter, live vectored vaccines based on bacterial [73] and viral platforms [74] or vaccines based on recombinant DNA [75,76] are probably most promising and are in clinical development against several diseases. A series of clinical trials exploiting experimental infection of volunteers with *P. falciparum* malaria has provided much insight into the promises and challenges of these approaches [74]. Preclinical experience in other models of leishmaniasis with recombinant adenovirus [77,78], vaccinia virus [79–81], DNA [82–84] and attenuated Salmonella [85–87] suggest that these carriers should also be explored against HVL.

Ag Discovery in the Post-genomic Era (Reverse Vaccinology)

While the choice of adjuvant/carrier and formulation is critical for the performance of a vaccine, providing the key cues to instruct appropriate cell-mediated immunity, the selection of antigens is no less important. Problematically, T cells recognize protein fragments (peptide epitopes) bound to major histocompatibility (MHC) proteins that are polymorphic in a population for which the vaccine is to be developed. In the examples discussed above, two very different antigen-discovery approaches were used. Antigens contained in Leishmune® were isolated by chemical fractionation of parasite material and not by immunoscreening. In contrast, Leish-111f antigens were identified because they reacted with specific Ig in sera or T cells generated from patients or Montenegro skin-test-positive individuals in high throughput screening of gene-expression libraries [88–91]. This approach was also applied to identify B and T-cell antigens recognized during *Leishmania* infections in mice (e.g. [92,93]) and, for Leish-111f, in patients [88–91]. Other key antigens currently in late stage preclinical development have been selected based largely on serendipity, after functional testing as vaccine candidates (e.g. HASPB [55]).

With multiple genome sequences now available and high coverage proteome data available [94–97], the vaccine antigen discovery process has entered the era of reverse vaccinology [4,98]. For example, proteins featuring tandem repeats were originally identified by screening expression libraries with patient sera [89] but this approach has now been ‘reversed’ by screening protozoan genomes for genes encoding proteins with tandem repeats and subsequently...
verifying their antigenicity [99]. However, to date very few studies have used a purely reversed approach starting from genome, proteome or transcriptome data to derive and test candidate vaccine antigens.

A prerequisite of reverse vaccinology is that criteria can be identified to design an algorithm for vaccine candidate selection. Several studies [100–103] provide support for selection based on protein expression in the amastigote form, relative protein abundance and subcellular localization. It is reasonable to demand in addition that antigens show sequence conservation across parasite species and lack of homology to a vaccine recipient species (i.e. human or canine hosts). The last three criteria can be addressed using bioinformatics tools, genome information and sequence data repositories. Proteomic data are publicly available that provide a resource for the first two criteria [95–97]. Furthermore, bioinformatic analysis has verified that protein abundance is correlated with codon bias, resulting in higher translational efficiency [96,104]. Thus, even in the absence of proteomic evidence for the abundance of a particular protein (true for most membrane proteins) biased codon usage in the respective gene combined with mRNA abundance data from transcriptome analyses [105–107] can serve as a surrogate measure for relative protein abundance. Our unpublished experience with such a reverse vaccinology approach (Fig. 2) has so far identified two novel vaccine candidates from *L. donovani* that have shown promise in EVL (and CL).

The need to induce or boost specific CD8+ T cells to protect against EVL [38,70,72,108] is reflected by the inclusion of predicted CD8+ T-cell epitopes in the genomic resource TriTrypDB (Kinetoplastid Genomics Resource, http://tritrypdb.org) . Verified epitopes listed in this resource were computed by the ‘Immune Epitope Database and Analysis Resource’ (IEDB, http://www.immuneepitope.org/) , and experimentally confirmed epitopes are listed with their respective sequence, source, method of identification and reference. For non-included antigens, more than 30 programs predicting potential T-cell epitopes, mostly based on MHC-binding algorithms, are available on the Internet. A comparative study [109] assessed performance and reliability of the different algorithms and revealed that matrix-based programs (e.g. BIMAS and SYFPEITHI) were outperformed by non-linear predictors such as NetMHC that are based on artificial neural networks (ANN [110,111] http://www.cbs.dtu.dk/services/NetMHC/). To predict binding peptides, information on MHC polymorphisms is required. In dogs, these are not well characterized and hence this kind of bioinformatics may offer little to refine antigen selection approaches for CVL vaccines. However, for HVL vaccines, data on relevant human populations is available. For example, the population of India is highly diverse with several thousand endogamous groups [112] but HVL is endemic in only three states in the north of India. Focusing on Bihar, where 90% of all cases of VL are reported, eastern Uttar Pradesh and West Bengal, the composition of ethnic populations becomes less complex. The Indian Genome Variation Consortium established that the majority population of these states is Caucasian (Uttar Pradesh, Bihar and admixture in West Bengal) and Australoid (West Bengal) with mongoloid influence. Accordingly the most common HLA class I alleles

**FIG. 2.** Flow chart of a reverse vaccinology approach to identify novel antigens. Depicted is a binary decision tree and criteria to rank ORF products for candidate antigen selection starting from ‘Omics’ datasets. An exemplary analysis of CAI values for all ORFs of the *L. major* genome has been published in Paape et al. *Mol Cell Proteomics* 2008; 7: 1688–1701.
in this population are likely to be A*02, A*24, A*11 and A*33 in the HLA-A locus and B*07, B*35, B*40, B*57 and B*58 in the HLA-B locus. Thus, for the purpose of predicting vaccine antigen-derived HLA binding epitopes for people at risk in India, analysis may be limited to these most common haplotypes. HLA subtype distributions in populations at risk in other regions such as Bangladesh or Nepal will eventually also have to be considered. Of note, the normally most ubiquitous Caucasian allele A*0201 is absent and replaced with the more frequent A*0211 in the Indian population. Thus, the reverse vaccinology approach outlined above may be suited to improving the hit rate of protective antigen discovery that was comparatively low in the few studies that exploited early genomic information on the parasite [83,84,113].

**Future Directions and Vaccine Trial Strategies**

Recent reviews have highlighted the general considerations that underlie the future development of a Leishmania vaccine [1] and many of these can be applied to vaccines against HVL. However, whilst there are arguments for the development of new preclinical models of HVL, the incorporation of natural sandfly challenge [114], the identification of a greater range of antigen candidates with broad species coverage, and a greater understanding of the immunology of protective immunity, these arguments should be balanced by the need to develop a stronger base in clinical vaccinology, akin to that seen for other diseases. This end is only likely to be accomplished by an accelerated programme of well-defined clinical trials, and in this context the use of therapeutic vaccine trials as a first step has much to offer.

**Transparency Declaration**

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Human and canine VL vaccination trials assessing subunit, 2nd generation vaccines.

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