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Review

Recent updates and perspectives on leishmaniasis

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Abstract

Leishmaniasis is a neglected vector-borne tropical infection considered to be a disease of the poor. Concentrated in poverty-stricken countries within Southeast Asia, East Africa, and Latin America, it is also endemic in several Mediterranean countries. The management of the heterogeneous syndromes determined by parasites belonging to the genus *Leishmania* is particularly difficult in developed, non-endemic countries owing to the unfamiliarity of physicians with clinical symptoms, diagnostic possibilities, and available treatment options. Therefore, travelers and other people who may be exposed to sand flies in endemic areas should receive counseling regarding leishmaniasis and appropriate protective measures. Serological diagnosis is rarely used for cutaneous and mucocutaneous diseases, but it is the most commonly used technique for visceral leishmaniasis. The drugs used to treat this last disease are expensive and sometimes have toxic side effects.

This review highlights the diagnostic, chemotherapeutic, and immunizing strategies to control leishmaniasis, though no human vaccine is commercially available currently owing to the complexity of the cellular immune response to this parasite.

Key words: leishmaniasis; diagnosis; treatment; immunotherapy.

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Introduction

Leishmaniasis is a neglected vector-borne tropical infection that is considered to be a disease of the poor [1].

Concentrated in poverty-stricken countries within Southeast Asia, East Africa, and Latin America, it is also endemic in several Mediterranean countries [2]. On a global scale, ~350 million people live in areas characterized by active transmission of *Leishmania*, with 14 million people directly affected by the disease [2]. There are three main types of leishmaniasis: visceral (kala-azar), the most important disease; cutaneous, the most common; and mucocutaneous. Among parasitic diseases, mortality from leishmaniasis is second only to malaria and, in terms of disability-adjusted life years (DALYs), the third-most common cause of morbidity after malaria and schistosomiasis, with children under 15 years of age suffering most of the disease burden. International traveling has caused an increase of leishmaniasis cases in non-endemic countries, making the recognition of this parasitic infection important [4]. Moreover, the increase of immunosuppressed individuals, secondary to human immunodeficiency virus (HIV) infection and post-transplant and chemotherapeutic agents, has resulted in an increase of visceral leishmaniasis in Europe [5,6]. Kala-azar, the most deadly parasitic disease after malaria, spread by sand fly bites, afflicts hundreds of thousands of the world's poorest people in tropical countries. This vector-borne protozoan infection has clinical spectrum ranges from asymptomatic infection to fatal visceral leishmaniasis.

As reported by Alvar *et al*. [6], the number of cases of visceral leishmaniasis (VL) is calculated to be as high as 0.2–0.4 million people per year, with more than 90% of these occurring in India, Bangladesh, Sudan, Ethiopia, and Brazil, with a mortality estimated at 10%–20%, especially in poor areas. Cutaneous leishmaniasis (CL), considered by the World Health Organization (WHO) a category 1 emerging and uncontrolled disease [7], occurs mainly in the Mediterranean littoral, the Americas, and western Asia; up to 75% of these cases are found in Brazil, Syria, and Afghanistan. Approximately 35,000 cases of mucosal leishmaniasis occur annually, mainly in Brazil, Peru, and Bolivia [2]. In European countries, only two *Leishmania* species are endemic: *L. infantum*, responsible for zoonotic cutaneous and systemic disease within the Mediterranean region, with a reservoir in the domestic dog; and *L. tropica*, causing sporadic cases of anthroponotic cutaneous disease mainly in Greece. Autochthonous transmission

has been reported in Portugal, Spain, France, Italy, Greece, Malta, Cyprus, Croatia, Albania, Bulgaria, and Turkey. Most are sporadic endemic diseases, but recent outbreaks of leishmaniasis have occurred in Spain and Italy [8,9]. A recent case described by de Silva *et al*. [10] emphasizes the importance of a differential diagnosis in the presence of compatible skin lesions following foreign travel.

VL is hypoendemic in Mediterranean countries; cases in this area account for 5%–6% of the global burden. It does not necessarily lead to clinical disease; most infections remain asymptomatic, but malnutrition and immune suppression, notably HIV, predispose to clinical diseases [5]. Cases of *Leishmania*/HIV co-infection have been reported in the Mediterranean region, mainly in France, Italy, Portugal, and Spain [11]. Since highly active antiretroviral therapy was introduced in 1997, a marked decrease in the number of co-infected cases both in these regions and in India have been reported [12]. VL is a rare disease in solid-organ transplant recipients. Clemente *et al*. [13], in a multicenter study, determined the risk factors and outcomes of VL in these patients. They observed that diagnosis was frequently delayed and relapses were common after treatment.

Despite a tentative estimate of mortality of 20,000–40,000 VL deaths per year, leishmaniasis belongs to the group of neglected tropical diseases. In 2007, the World Health Assembly approved a resolution on the control of leishmaniasis, and the WHO established control programs. In 2010, an expert committee concluded that adequate worldwide leishmaniasis control is feasible but that there is a crucial lack of political commitment and international cooperation. This disease remains a major neglected tropical disease, and a strategic framework for its control in the WHO European region has been recently applied [14].

These intracellular protozoa have a complex digenetic life cycle, requiring a susceptible vertebrate host and a permissive insect vector, which allow their transmission. Diniz *et al*. [15] emphasized the importance of animal reservoirs in facilitating transmission of VL in densely populated urban areas in Brazil, and recently, Turchetti *et al*. [16] discussed the role of sexual and vertical transmission of this disease in canine populations playing an important role in the maintenance of VL.

As a result of a long host-parasite co-evolutionary process, *Leishmania* spp. has developed different immunomodulatory strategies that are essential for the establishment of infection. Understanding the mechanisms associated with immune evasion and disease progression is essential for the development of novel therapies and vaccine approaches. Cecilio *et al*. [17] recently revised how the parasite manipulates cell death and immune responses to survive. The parasite can alter the maturation process of the phagolysosome, modulate cytokine and chemokine production by host cells, impairing cell function, to enter host cells and successfully differentiate and infect.

A review concerning outbreaks in Israel over a recent 13-year period revealed an expansion of CL illness in southern, central, and northern areas of the country [18]. Therefore, studies of the responsible factors and a greater degree of public alertness are necessary. Zoonotic CL due to *L. major* is still a serious public health problem also in Tunisia. Researchers comparing the prevalence and risk factors in old and new foci using leishmanin skin test (LST) in central Tunisia observed [19] that the overall prevalence of LST positivity was 57%, and was significantly higher in the old focus than in the emerging foci. In 2012, a CL outbreak was observed among Syrian refugees in Lebanon [20] due to *L. tropica* and *L. major* infection in 85% and 15% of patients, respectively. These infections were cured in 82% of the cases by megulamine antimonite therapy for three months. An unusual presentation of imported *L. infantum* CL, diagnosed in an Australian who had traveled in Italy 19 years before, has been reported [21], supporting the importance of parasite persistence evaluation in the diagnosis of this infection.

Climate changes in Europe have increased the spread of new vectors. In northern Italy, leishmaniasis has been associated with climatic conditions that favor the development of the vector *Phlebotomus perniciosus* [22,23]. The increase of VL cases is also related to immunodepression and other variables, such as the increase of international travel, migration, and trade, which present the risk of importing parasites, vectors, and infections [24]. The main area for the acquisition of CL, especially for adventure travelers on long-term trips in highly endemic forested areas, is South America, whereas popular Mediterranean destinations are emerging as the main areas to acquire the visceral variant. The management of these infections in developed, non-endemic countries is still difficult owing to the unfamiliarity of physicians with clinical syndromes, diagnostic possibilities, and available treatment options. Therefore, travelers and other people who could be exposed to sand flies in endemic areas should receive counseling regarding leishmaniasis and appropriate protective measures.

Diagnosis

Most VL infections are diagnosed clinically with observation of irregular fever, anemia, leucopenia, and hepatosplenomegaly [2], but there are several methods of laboratory diagnosis of leishmaniasis. As recently reported [25], these include parasite detection by microscopic examination, culture and successive isoenzyme analysis for identification, or molecular biology-based assays for detecting the parasite DNA (polymerase chain reaction [PCR]). These last tests are more sensitive, but may be applied only in particular centers. Different PCR methods have been evaluated [26,27] and have been found to demonstrate a superior sensitivity compared with microscopy smear and *in vitro* culture.

Serological diagnosis, even if rarely used for CL and mucocutaneous leishmaniasis (MCL) disease, is the technique more commonly used for VL [25]. Various techniques are employed, including Immunofluorescence antibody assay (IFA), enzyme-linked immunosorbent assay (ELISA), western blot, rapid strip testing for rK39 antigen, direct agglutination technique, or detection of *Leishmania* antigen in urine by latex agglutination. High serum antibody levels are present in both asymptomatic and active VL and remain present for several years. However, the specificity of these tests in VL-endemic areas is variable, and in *Leishmania*/HIV co-infections, the serological approach for diagnosis is difficult owing to the immunosuppressive action of the virus. In fact, in these cases, additional molecular or other parasitological tests may be necessary if the results of the serological tests are negative.

Mohapatra *et al*. [28] conducted a comparative evaluation of different recombinant antigens for the serodiagnosis of Indian visceral leishmaniasis. They concluded that rK39 (a 39-aminoacid-repetitive immunodominant B-cell epitope of kinesin-related antigen from *L. chagasi*) was the most suitable antigen when compared with rK26 and rK9; this last antigen may be used as an adjunct to rK39 for accurate diagnosis of VL. The diagnostic antigens rK26 and rK18 were demonstrated to be suitable to be used as an indirect measure of parasite clearance after therapy [29]. The ELISA technique effected with a recombinant *L. infantum* heat shock protein 83 (rHsp83) represents a routine confirmatory serological assay for the diagnosis of CL, MCL, and VL [30]. Integrated diagnosis based on clinical symptoms together with an rK39 immunochromatographic rapid test is commonly used, whereas a combination of molecular and serological tests are especially useful for asymptomatic infections in endemic areas [25]. Leishmaniasis should be considered in the diagnostic assessment of patients with a compatible clinical syndrome and a history of traveling in an endemic area, even if it occurred several months or years before [21].

Treatment

The drugs employed to treat VL are expensive and sometimes have toxic side effects.

A recent review of new chemotherapeutic strategies against malaria, leishmaniasis, and trypanosomiasis [31], reports the updated literature on the challenges of drugs against leishmaniae.

Liposomal amphotericin B has the best safety profile and has been approved by the Food and Drug Administration (FDA) for the treatment of VL [32] and is the drug most used in species-directed therapy of leishmaniasis in returning travelers [33].

A multicenter, open-label study evaluated the efficacy and safety of two treatment options, single infusion of preformed amphotericin B (AmB) lipid emulsion (ABLE) and liposomal formulation (LAmB); the first type of infusion was found to be more effective and better tolerated [34]. Resveratrol, a polyphenol found in black grapes and red wine that has many biological activities, in association with amphotericin B, showed synergy against *L. amazonensis in vitro* [35].

Other drugs that have been brought onto the market more recently are paromomycin and miltefosine. The first is an aminoglycoside antibiotic, which was granted orphan drug status in 2005, and is used for the treatment of both VL and CL in parenteral and topic formulations [36]. Miltefosine was registered in India in 2002 as the first oral antileishmanial agent; its major limitations are the high cost, the need for monitoring of gastrointestinal side-effects, and occasional hepatic and renal toxicity [37]. Combining paromomycin with miltefosine, the release of nitric oxide and tumour necrosis factor by human macrophages was induced, enhancing the killing of *L. donovani* promastigotes *in vitro* [38]. Fexinidazole (formerly Hoe 239), an oral nitroimidazole, was rediscovered by the Drugs for Neglected Disease initiative (DNDi) and is in clinical trials for the treatment of African sleeping sickness; this compound or another nitroimidazole, PA-824, may be effective against leishmaniae [39].

The synthesis of polyamines and of their precursors is essential for *Leishmania* protozoa; therefore, the enzymes involved in spermidine

synthesis and utilization, in particular trypanothione synthetase and trypanothione reductase, are attractive targets for drug development [40].

Also, peptidases (proteases) are enzymes that are considered interesting targets for chemotherapy [31,41]. The only aspartic protease present in leishmaniae belongs to the family of the A2 retroviral-like aspartic proteases and is inhibited by drugs originally developed as inhibitors of HIV protease [42,43]. A recent review [42] summarizes the biology of kinetoplastid topoisomerases, which can be molecular targets in antileishmanial chemotherapy, as observed with pefloxacin in 1996 [45].

In *Leishmania*, dihydrofolate reductase (DHFR) forms a functional complex with thymidylate synthase (TS). By means of an *in silico* virtual screening technique, many DHFR-TS based drugs were screened; among these, the molecule 571633 proved promising and deserves further development [46].

High-throughput screening has also been used to identify molecules based on new molecular scaffolds. CA272 and CH872 are two molecules, based on the hydrazine and 4-hydroxyquinoline scaffolds, respectively, that are new promising antileishmanial compounds [47].

Prati *et al*. [48] investigated the feasibility of targeting *Leishmania* transporters via appropriately designed chemical probes. *L. donovani* is auxotrophic for arginine and lysine and has specific transporters (LdAAP3 and LdAAP7) to import these nutrients. Some conjugates containing cytotoxic quinone fragments (II and III) linked to amino acids (arginine and lysine) displayed toxicity against the parasites and not against mammalian THP1 cells. Recently, Nagle *et al*. [49] reported significant progress in the treatment of both leishmaniasis and African trypanosomiasis (HAT). However, new drugs for both diseases are still needed; two compounds were in clinical trials for HAT (nifurtimox and SCYX-7158) and one for VL (nifurtimox). Gupta *et al*. [50] recently identified a nitroimidazo-oxazole lead molecule for the treatment of VL. In particular, the compound DNDI-VL-2098 was characterized by a leishmanicidal activity and a capacity of inducing host-protective immune cells in hamsters. Moreover, some novel heteroretinoid-bisbenzylidine ketone hybrids have been synthesized and evaluated by Tiwari *et al*. [51] for their *in vitro* activity against *L. donovani*; a new class of compounds with antileishmanial activity was identified. Also, N-substituted β-amino alkanols were recently shown to have leishmanicidal activity, inducing severe mitochondrial swelling and vesiculation of the parasite [52].

A new delivery strategy that can increase drug distribution among different mononuclear phagocyte system tissues was studied [53]; pegylated meglumine antimoniate-containing liposomes (LMA) and their mixture with non-pegylated (conventional) LMA proved to be more effective in dogs and mice than did conventional LMA against VL. Crovirin, a cysteine-rich secretory protein (CRISP), extracted from *Crotalus viridis viridis* snake venom, used with a high-performance liquid chromatographer, had promising results against trypanosomes and leishmania [54]. This family might have potential as drugs or drug leads for the development of novel agents against these protozoa.

Different natural products were assessed. De Queiroz *et al*. [55] investigated the leishmanicidal activity of five species of plants used in folk medicine in Brazil in patients with CL (*L. amazonensis*). The plant extracts *Hyptis pectinata* (L.) Poit, *Aloe vera* (L.), *Ruta graveolens* (L.), *Pfaffia glomerata* (Spreng) Pedersen, and *Chenopodium ambrosioides* (L.) exhibited direct activity against extracellular forms of *Leishmania*. Recently, thymol derivatives were assessed in mice, and were found to demonstrate greater activity than the eugenol derivatives; benzoyl-thymol was the best inhibitor with decreased toxicity [56]. These modified compounds are promising candidates for further studies of antileishmanial drug development. The leishmanicidal effect of essential oil from *Artemisia annua* leaves against *L. donovani in vitro* and *in vivo* was also reported [57].

Nadhman *et al*. [58] described daylight responsive silver (Ag) doped semiconductor nanoparticles of zinc oxide for photodynamic therapy (PDT) against *Leishmania*. These biocompatible nanoparticles demonstrated a daylight response, causing permeability of the cell membrane and the death of parasites. Daylight-activated photodynamic therapy (DA-PDT), which abolishes the need for artificial light sources, permitting auto-treatment of the patient, was found to be effective for CL caused by *L. major* and *L. tropica* [59]. Different macrocycles, *i.e.*, cyclic macromolecules, such as sapphyrin and two related heterosapphyrins, have been investigated as potential agents for the treatment of leishmaniasis [60].

Immunity and immunotherapy

Vaccination to control leishmaniasis is promising, but at the moment, no human vaccine is commercially available due to different causes, but particularly due

to the complexity of the cellular immune response to this parasite [2]. Vaccines against canine leishmaniasis, for the reduction of the transmission of *L. infantum* to people, have been tested in Brazil and Europe with mixed results. A recent study showed that first-generation canine vaccines have inadequate ability to produce long-lasting immunity [61]. Third-generation vaccines are protective, but owing to the different nature of DNA antigens, their value is unclear. Second-generation vaccines could be one of the best choices for canine *Leishmania* vaccination. They consist of *L. donovani* glycoprotein (fucose mannose ligand) with saponin as an adjuvant. Recently, Gradoni [60] demonstrated that an effective canine *Leishmania* vaccine should induce strong and long-lasting Th1-dominated immunity to control both infection progression and parasite transmissibility via the vector. Promising antigens, including killed parasites, cell purified fractions, parasite protein components or subunits, single or multiple chimeric recombinant proteins, plasmid DNA and viral particles encoding parasite virulence factors, and adjuvant combinations have been tested on dogs. Limited or no protection was observed in phase I and II studies. The A2 antigen (a recombinant protein from the amastigote stage of different *Leishmania* species) plus saponin, which gave about 40% protection against infection, has been registered as a canine vaccine, LeishTec in Brazil and CaniLeish in Europe.

Prophylactic vaccination of both human VL carriers and dogs could generate long-term protection, reducing disease incidence. Therefore, different studies [63,64] continue to refine both antigen and adjuvant components of vaccines, as well as identify the mechanisms associated with immune evasion and disease progression to develop novel vaccine approaches. *Leishmania* parasites activate the innate and adaptive arms of the immune system, and a coordinated network of responses is required for effective immune-mediated parasite clearance. In particular, one of the reasons for anti-leishmaniasis vaccine failure is the lack of an appropriate adjuvant. Chemokine and cytokine responses are essential; however, *Leishmania* parasites have developed numerous mechanisms to prevent the development of immunity; one of these is inducing immunosuppressive cytokines. Recently, different methodologies, which include proteomics, have identified new antigens for potential polyvalent synthetic and recombinant chimeric vaccine candidates [65].

VL pathogenesis has been correlated with an overproduction of the regulatory cytokine IL-10, responsible for disease progression, whereas cytokines that selectively induce Th1 responses might be useful for VL therapy [66]. Also, dendritic cells are important in initial anti-*Leishmania* T cell responses, promoting the differentiation into memory T cells. Dendritic cell-based immunotherapy combined with antimony-based chemotherapy has been studied in mice, revealing a promising synergy. The IFNγ released by cells, particularly by CD+ T cells, from VL patients serves to limit parasite growth [67]. Arabinosylated lipoarabinomannan, a Toll-like receptor 2-ligand isolated from *Mycobacterium smegmatis*, has a strong immunomodulatory property, giving protection against *L. donovani* infection by restoring IFN-γ responsiveness important for protection against *L. donovani*-susceptible hosts [68]. The role of interleukin 17 (IL-17), an inflammatory cytokine that plays a protective role against intracellular parasites, is controversial. Nascimento *et al*. [67], evaluating the levels of IL-17A in the sera from patients with VL before and at different times after treatment with Glucantime, noted higher levels in the sera of patients with VL before treatment than in normal or endemic controls; during treatment, the levels of IL-17A decreased, but remained significantly higher than the values of normal subjects. This cytokine acts synergistically with IFN-γ, increasing nitric oxide production and leishmanicidal activity in infected macrophages. Tumor necrosis factor α (TNF-α) is another cytokine that is important in *Leishmania* infections, controlling inflammatory lesions and parasite killing. A new strategy for leishmanial treatment is based also on Toll-like receptor signal pathways [70].

Though vitamin A supplementation proved to be effective in diarrheal and respiratory diseases in several countries, few studies examined the effect in VL. Lima Maciel *et al*. [69] analyzed the effect of this vitamin on regulatory T cells and monocytes isolated from symptomatic VL and from healthy children residing in an area endemic for VL; they demonstrated that vitamin A associated to VL therapy improved recovery.

Kaur *et al*. [70] recently studied herbal drugs derived as whole-plant extracts of *Asparagus racemosus* and *Withania somnifera* to treat *L. donovani*-infected BALB/c mice. Treatment with both extracts in combination was found to be effective against parasites, generating protective Th1-type immune responses.

To identify new immunogens as vaccines in a murine model of VL, epitope-based immunogens, consisting of phage-fused peptides mimicking *L. infantum* antigens, were selected, and their affinity to antibodies from asymptomatic and symptomatic VL dogs' sera was studied [71]. Two phage clones expressing target mimotopes provided protection in mice against *L. infantum* correlated to a Th1-type immune response.

Previously, it was reported that liposomes associated with amphotericin B were used for *Leishmania* therapy [32]. Moreover, they have been found to be potent adjuvants for different antigens and are effective vehicles for peptides and proteins, increasing their immunogenicity. Liposomes carrying glycosylphosphatidylinositol-anchored proteins of *L. amazonensis* promastigotes could, in fact, induce protective immunity in mice [72].

Conclusions

Leishmaniasis is a major health problem worldwide. The spectrum of infection is subclinical (not apparent), localized (skin lesion), and disseminated (mucocutaneous and visceral); it depends on the immune status of the host, on the parasite, and on immune-inflammatory responses.

Major risk factors of *Leishmania* distribution are socioeconomic conditions, malnutrition, population mobility, and environmental and climate changes. Prevention and control are based on early diagnosis and treatment, vector control, disease surveillance, and education of the community. The European Union has been receiving, since the mid-1900s, an influx of immigrants, known to be a marginalized and vulnerable group, with an estimated 20 million migrants having arrived in the past 15 years [3]. Therefore, leishmaniasis should be considered in the diagnostic assessment of immigrants or travelers who could have been exposed to the parasite in endemic areas [24,25].

This protozoan disease is second to malaria in importance for human health; however, the options for chemotherapeutic treatment are limited owing to drug resistance and toxicity. The drug of choice is still out of reach; among the chemotherapeutic strategies, the combination of local and parenteral therapies could be more effective, shortening the duration of the treatment. A new strategy, studied *in vitro*, is based on nanoemulsions containing cholesterol and amphotericin B; the encapsulation of the drug seems to increase its efficiency [73].

To combat VL, it is important to unite groups working on drugs for the disease into a single organization, the VL Global R&D & Access Initiative [74].

For immunity to *Leishmania* antigen, persistence is necessary [64]. *L. major* vaccine consisting of needle inoculation with viable *L. major* parasites in a selected site, referred to as leishmanization, has been used in the past for people living in regions endemic for cutaneous forms of leishmaniasis. This is not a vaccine in the conventional sense, even if the cultures were less virulent than the forms inoculated by the sand-fly vector. Its use was interrupted owing to severe primary lesions. Irradiated or genetically modified organisms could be used for immunization; however, priority should be given to clinical trials of defined, subunit vaccines against this parasitic disease. Promising antigens, single or multiple chimeric recombinant proteins, plasmid DNA and viral particles encoding parasite virulence factors, and adjuvant combinations have been tested. Prophylactic and immunotherapy vaccination of both humans and dogs could generate long-term protection, reducing disease incidence responsible for protean manifestations.

Dedication

This paper is dedicated to Prof. Piero Cappuccinelli, a brilliant scientist, teacher and friend. Piero is well-known worldwide as a visiting teacher in different prestigious institutions of several countries and for various projects. He has been a curious, eclectic and inventive scientist since his training in Microbiology and Parasitology at the University of Turin. I am proud to know him not only as a scientist and colleague but also as a friend.

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