Drug Discovery Today: Disease Models
Vol. 1, No. 1 2004

Infectious diseases

Animal models for infectious diseases caused by parasites: Leishmaniasis

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Leishmaniasis is a group of diseases caused by protozoa of the genus *Leishmania*, which affect millions of people worldwide. In this review, we focus on clinical aspects of the leishmaniasis and on currently available experimental models to study the diseases.

Section Editor:
Rudi Balling—German Research Center for Biotechnology, Braunschweig, Germany

Leishmaniasis is transmitted by sand flies. Different species can cause different clinical manifestations. In addition to this heterogeneity, the genetics of the host can markedly influence the outcome of a *Leishmania* infection. Most of what we know about the response of an individual to *Leishmania* infection stems from the study of mice. Some inbred strains of mice are very susceptible to *Leishmania* infection, others are extremely resistant. C.I. de Oliveira and colleagues review the different animal models used to study the molecular basis underlying this puzzling observation. They also make us aware of another contributory factor: components in the saliva of the sand fly can apparently modify the course of infection.

Introduction

The leishmaniases represent endemic infections that occur, predominantly, in tropical and subtropical regions. Currently, the leishmaniases are considered to be endemic in 88 countries and an estimated 12 million people are infected and 200 million people live at risk of infection (http://www.who.int/tdr). Leishmaniases are transmitted by different species of sand flies and they present a wide spectrum of clinical manifestations: tegumentary leishmaniasis, ranging from localized cutaneous and mucocutaneous leishmaniases (CL and MCL), representing the responsive pole, to diffuse cutaneous leishmaniasis (DCL) which represents the unresponsive pole and visceral leishmaniasis (VL) ranging from subclinical to fatal disease. The main clinical characteristics of the leishmaniasis are summarized in Table 1. Parasites that cause New World CL are grouped under the *Leishmania braziliensis* and *L. mexicana* complexes, whereas those that cause VL are grouped under the *Leishmania donovani* complex. *L. tropica, L. aethiopica* and *L. major* represent the etiological agents of Old World CL.

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In vivo models

Tegumentary leishmaniasis

Infection begins when an infected female sand fly takes a blood meal from a human host. Following inoculation into the skin by the sand fly bite, the flagellated promastigote penetrates into the macrophage, transforms into amastigotes and multiplies. The infected macrophage eventually bursts and the released parasites are able to infect new phagocytic cells. When the infected host is bitten by another female sand fly, parasites are ingested and the life cycle continues. Cutaneous lesions initiate at the site of parasite entrance, the incubation period ranges from two weeks to several months [1] and the most frequent aspect observed in CL cases is a single ulcer with elevated borders and a sharp crater. Diffuse CL is a rare entity in which many lesions are observed, but in

 DOI: 10.1016/j.ddmod.2004.07.005

Table 1. Clinical characteristics of human leishmaniasis

Characteristics	Mucocutaneous	Cutaneous	Visceral
Lesions	Ulcerative destruction of the nasal septum	Single (occasionally a small number) ulcerated lesion with elevated borders and necrotic center	Internal organs
Histopathology and parasite numbers	Granulomatous reactions with very few parasites	Chronic inflammatory responses with moderate number of parasites	Marked macrophagic proliferation with heavy parasitism in the hematopoietic organs
Anti-*Leishmania* antibody levels	Low	Low	High
Anti-*Leishmania* CMI (*in vitro* and *in vivo* tests)	Strongly positive	Positive	Negative

which the lesions are nodular and non-ulcerated. MCL occurs in less than 3% of patients infected with *L. braziliensis* [1], lesions occur a few weeks to many years after the onset of infection. This type of involvement usually begins in the nasal mucosa but the disease can spread and might cause extensive destruction of the nasal septum. The cutaneous lesions of Old World leishmaniasis are more benign, most lesions heal spontaneously, leaving a fibrotic scar. With few exceptions, the infection confers lifelong immunity.

Experimental models of both Old World and New World cutaneous leishmaniasis have been developed. Subcutaneous infection of mice with *L. major* is one of the best studied models [2,3]. The conventional *L. major* mouse model employs a high dose of parasites, usually stationary phase promastigotes, injected into a subcutaneous site (shaved rump or footpad). Upon experimental infection with *L. major*, distinct features of the clinical spectrum of human leishmaniasis are reproduced in inbred mice. Mice from BALB/c strains are highly susceptible: upon infection, they develop large skin ulcers, which expand and metastasize, leading to death. By contrast, C3H/He, CBA, C57BL/6, and 129Sv/Ev mice are resistant to infection with *L. major,* they develop small lesions, which cure in 10–12 weeks and are also resistant to re-infection. The relevance of the Th1/Th2 balance in the *in vivo* regulation of disease outcome has been thoroughly investigated. Resistance is linked to a Th1 response, with production of IL-12 and IFN-γ, and inhibition of Th2 cytokine production, whereas susceptibility is related to a predominant Th2 response, determined by the presence of IL-4. Th2 cytokines lead to the development of severe lesions in mice infected with *L. major*, probably by deactivating infected cells. The production of reactive nitrogen intermediates by IFN-γ-activated macrophages is inhibited by TGF-β, IL-4, IL-13, and IL-10 [4] indicating that the down-regulation of Th2 cytokines might be crucial for the development of acquired resistance. Therefore, the susceptibility or resistance of different strains of mice to infection by *L. major* depends on multiple factors.

Experimental models involving subcutaneous infection of mice with *L. braziliensis* have also been developed. Accordingly, mouse strains C3H/HeJ, C57BL/6J, and CBA/CaJ showed no evidence of infection. Mouse strains AKR/J and CBA/J showed only a slight and transient swelling of the infected tissue, when parasites were inoculated in the snout [5]. By contrast, strains such as SWR/J, C57L/J, A/J, A/HeJ, and DBA/1J developed tissue swelling or nodules, which eventually resolved. BALB/c mice were ranked as most susceptible although *L. braziliensis* does not produce severe or lasting cutaneous lesions in this mouse strain. The analysis of the immune response has shown that *L. braziliensis*-infected BALB/c mice produce less IL-4, when compared with *L. major* infected mice, and treating *L. braziliensis*-infected BALB/c mice with anti-IFN-γ significantly enhanced lesion size and prevented mice from resolving the infection [6]. These authors suggest that an IFN-γ-dependent mechanism is responsible for the killing of *L. braziliensis* in BALB/c mice and that the weak infectivity of *L. braziliensis* in this mouse strain might be due to the inability of the parasite to elicit a strong and sustained IL-4 production.

The golden hamster (*Mesocricetus auratus*) has proven particularly useful for studying cutaneous leishmaniasis caused by species of the subgenus Viannia [7]. In addition to its use as a model of disease, the hamster is the most used laboratory animal for the isolation of field strains. Hamsters are exquisitely susceptible to species of the subgenus *Viannia* (*L. braziliensis*, *L. panamensis, L. guyanensis*), which are responsible for the majority of *Leishmania*-related human pathology in the America [7]. The inoculation of 200–500 promastigotes of *L. panamensis* is able to cause overt disease, between one and two months post-inoculation. Amastigote dissemination to the draining lymph nodes occurs in less than a week when large inocula (10^6) are used, although dissemination is also observed when smaller doses are employed. [7,8].

Certain variables, such as dose, co-injection with sand fly saliva and site of inoculation, can be used to modulate disease outcome, to obtain a nodular or ulcerative lesion, fast or slow development, as seen in natural infection [9,10]. Persistence that might be responsible for recurrence in humans, can also be reproduced in hamsters: after three to nine months, some strains of *L. panamensis* and *L. guyanensis* [7,11] can produce

dermal metastasis or viceralization, as seen in *L. braziliensis* infection [12].

In summary, the hamster displays a predictable disease evolution after infection with *Leishmania* (*Viannia*) spp., characterized by fast lesion development and, under particular experimental conditions, dissemination and cutaneous metastasis. The chronic nature of leishmanial disease in this animal species allows the monitoring of immunological and therapeutical interventions over long periods of time.

Attempts to reproduce the biology of natural transmission have been made, taking into account parasite load (sand flies inoculate low numbers of parasites), the presence of saliva (parasites are injected into the host's skin in the presence of sand fly saliva) and site of inoculation (parasites are injected by the sand fly into the dermal compartments of the skin). In terms of parasite load, it has been shown that infection with 100–1000 parasites has been shown to convert the phenotype of BALB/c mice from susceptible to resistant [13,14], indicating that parasite dose can influence disease outcome. We have recently examined disease outcome in BALB/c mice inoculated in the ear dermis with *L. braziliensis:* they develop pronounced, ulcerated lesions, similar to the lesions developed upon natural infection (C.I. de Oliveira, unpublished), indicating that the site of inoculation influences the outcome of disease.

It has also been shown that the presence of sand fly saliva is able to exacerbate infection with both *L. major* [15] and *L. braziliensis* [16] in the subcutaneous route of infection. However, in the so-called natural model of cutaneous leishmaniasis, where mice are co-inoculated with 1000 metacyclic promastigotes and sand fly saliva, lesions appear earlier, are more destructive and contain a greater number of parasites, in both BALB/c and C57BL/6 mice. Moreover, co-inoculation with sand fly saliva converted C57BL/6 mice into a nonhealing phenotype [17]. Accordingly, disease exacerbation was associated with the production of Th2 cytokines. Interestingly, it was observed that the pre-exposure to sand fly saliva leads to protection against leishmaniasis [17,18]. These studies report the most comprehensive experimental model of infection, taking into account important variables in the vector–parasite–host interplay, namely: presence of saliva, parasite load and inoculation site. Accordingly, it has been used to study CL caused by other species such as *L. braziliensis* and *L. amazonensis*. Regarding *L. braziliensis*, the effect of disease exacerbation, was observed upon the co-inoculation of *L. braziliensis* and *Lu. whitmani* saliva which lead to the development of lesions that also heal spontaneously [19]. While inoculation with *L. braziliensis* alone, using the subcutaneous route of infection, produced small and rapidly regressing lesions [6]. In a recent report, it was also shown that the presence of sand fly saliva enhances *L. amazonensis* infection, because BALB/c mice infected intradermally with parasites and saliva showed an earlier onset of disease and larger lesions that contained more parasites than controls [20].

Barral *et al*. [21] have shown that BALB/c mice infected with a *Leishmania* strain isolated from a mucocutaneous leishmaniasis patient developed a rapidly progressing and widely metastic disease, resembling diffuse cutaneous leishmaniasis. C57BL/6 mice, however, initially contained parasite multiplication effectively and appeared clinically cured. By contrast, parasites were able to persist and patent disease developed later on, characterized by distinctive ulcerative metastases and destruction of the nasal region, similarly to what is observed in mucocutaneous leishmaniasis. Disease development in these mouse strains was associated with a decrease in cell-mediated immunity, as monitored by delayed type hypersensitivity and lymphoproliferative responses.

Visceral leishmaniasis

The incubation period of VL is estimated to range from two to four months. The disease can present an acute, subacute or chronic evolution, but most infected individuals remain completely asymptomatic [1]. The asymptomatic individual is characterized by positive serology to *Leishmania* and, possibly, a positive intradermal test. Infected individuals can evolve to a subclinical form of VL or directly to an overt form of disease (classical VL). The classical manifestations of VL are fever, cough, weight loss, weakness, diarrhea or dysentery, and abdominal swelling. Patients also present anemia, edema, bleeding episodes and huge hepatosplenomegaly.

Experimental models for VL have been developed in both mice and hamsters. In mice, chronic VL is successfully established after intravenous or intradermal infection. Disseminated granulomes, with parasitized macrophages, are found during infection, especially in the liver and spleen of susceptible (BALB/c, C57BL/10) and resistant (C3H.HeJ, CBA, DBA/2) mouse strains infected with *L. donovani*, *L. infantum* or *Leishmania chagasi*) [22,23]. In susceptible mice, there is an early increase of parasite burden, but the infection spontaneously declines when an anti-*Leishmania* cellular immune response, involving both CD4+ and CD8+ T cells, is mounted [24]. A high parasite load is observed in the liver and is associated with IL-10, IL-6 and no IFN-γ production. In the spleen, the parasite load is less pronounced and, accordingly, splenocytes produce IL-4, IL-6, IL-10 and IFN-γ [25,26]. Resistant mice develop self-resolving infection with high IFN-γ and TNF-α [27] production in the liver. Challenge experiments also show that although the liver is resistant to reinfection after cure [28], the spleen shows no protection against reinfection [29] characterizing murine VL, a disease model with organ-specific immune responses.

Experimental infection of the golden hamster [30] has also been evaluated as an animal model for VL. This model mimics several aspects of human disease, such as hepatosplenomegaly,

pancytopenia, progressive cachexia, hypergammaglobulinemia, and suppression of T-cell proliferative response to parasite antigens [31]. This dysfunction has been attributed to the inability of infected antigen-presenting cells (APCs) to stimulate specific T cells [32,33]. Recent data indicated that despite a strong production of Th1 cytokines (IL-2, IFN-γ, and TNF-α) in the liver, spleen, and bone marrow, there is uncontrolled parasite replication, suggesting impairment of the macrophage effector's function. Progressive disease in this model was associated to a defect in the generation of NO because inducible NO synthase was not detected in the liver or spleen during the course of infection, despite the strong IFN-γ expression [34]. In conclusion, these studies demonstrate that the golden hamster is, to our knowledge, the best experimental model to study VL, because it reproduces the clinical and pathogenesis of the disease, as seen in humans and dogs. However, the wide use of hamsters is still limited by the lack of available reagents such as antibodies to cell markers and cytokines.

In vitro models

It has been shown that an *in vitro* priming (IVP) system using lymphoid cells from naïve mice primed with *L. major* promastigotes mimics *in vivo* responses in murine leishmaniasis [35]. Using this system, it has been shown that resistant CBA mice produce more IFN-γ, IL-10, and nitric oxide, whereas cells from susceptible mice produce more IL-1α, IL-5, PGE_2, and TGF-β [36]. These data suggest that T-cell commitment is influenced by a collection of factors and this *in vitro* approach could be an alternative in elucidating the mechanisms that lead to selective priming of cells. This system has also been used with human cells to explore early events of anti-*Leishmania* responses. Most individuals develop Th1 or Th0 anti-*Leishmania* T-cell lines and infected macrophages from Th1 responders produce IL-12. By contrast, Th0 responders who produce little or no endogenous IL-12 can be converted to the Th1 phenotype by addition of IL-12 during priming [37]. We have used this system to evaluate if *in vitro* primary responses were able to predict *in vivo* anti-leishmanial responses in man. PBMC from volunteers who had negative responses against *Leishmania* were used for *in vitro* stimulation with *Leishmania* antigen and IFN-γ levels were monitored. Volunteers fell into two different types of responders: those who produced low amounts of IFN-γ and those who produced high levels of this cytokine. Low responder volunteers remained low producers of IFN-γ 40 days after vaccination, whereas high producers exhibited increased IFN-γ production after vaccination. However, six months post-vaccination, all individuals tested were producing similarly high levels of IFN-γ upon stimulation of their PBMC with *Leishmania* promastigotes, indicating that the low producers *in vitro* are slow *in vivo* responders to vaccination. High IFN-γ producers exhibited an increased frequency of activated CD8+ T lymphocytes both *in vitro* and *in vivo*, as compared to low/slow IFN-γ producers [38]. Such findings suggest that IVP response is able to, for instance, predict the rate of post vaccination response in leishmanial antigen screening to test potential vaccine candidates. Although we observed that all vaccinated individuals eventually present a potent anti-*Leishmania* cell mediated immune response, a delay in the rate of mounting the CMI response might influence resistance against leishmaniasis.

Conclusions

The use of mice and hamsters as models for leishmaniasis has pointed out important issues relating to outcome of disease and development of immune responses. These models (Tables 2 and 3) have allowed the evaluation of the cytokines as well as other immune components involved in the resolution of infection and have, therefore, allowed us to gain more insight into new therapeutic strategies and vaccine candidates. It is clear that polarization of Th responses to either Th1 or Th2 can lead to life or death outcomes from infection with *L. major* in mice; however, this is the downstream effect of the infection. The upstream events that lead to this polarization need to be explored. The models of susceptibility and resistance to *L. major* infection have been used to understand the fine balance between Th1/Th2 responses and important questions have been raised from these studies. Besides that, these studies were fundamental to figure out different mechanisms responsible for the polarization of Th1/Th2 such as cytokines, transduction signal, co-stimulatory molecules, etc.

The use of a natural model of transmission, using sand fly saliva plus low doses of parasites has shown that components present in sand fly saliva can modify the course of infection. Interestingly, new studies in this area have emphasized the possibility to use saliva components in the vaccine design. Studies performed in an endemic area for VL have shown that individuals exposed to uninfected sand flies can mount an immune response against saliva components and have shown that some of these individuals can develop a protective immune response against *Leishmania*, upon the encounter

Table 2. Doses and inoculation routes of *Leishmania* used in different studies

Animal	Parasite	Route of inoculation	Dose range
Mouse	*L. chagasi*	i.v	1×10^7–2×10^7
	L. donovani	i.v.	1×10^6–1×10^7
	Leishmania infantum	i.v	1×10^4–1×10^8
		i.d	1×10^7
		s.c	1×10^7
Hamster	*L. chagasi*	s.c	1×10^7
		i.p.	2×10^7
	L. donovani	i.c	1×10^6–2×10^7
	L. infantum	i.p	1×10^5–2×10^7
		i.c.	1×10^3–1×10^5

Abbreviations: i.v., intravenous; i.d., intradermal; s.c., subcutaneous; i.p., intraperitonial; i.c., intracardiac.

Table 3. Comparison of the available experimental models for leishmaniasis

	In vivo models	*In vitro* models
Pros	Reproduce features of clinical spectrum of disease	High frequency of given antigen specific T-cell populations
Cons	Reagents to study immunological response are not readily available for hamster models	
Best use of model	Characterization of resistant and susceptible immune responses	Study mechanisms that lead to selective activation of T cells Prediction of immune responses to selective vaccine candidates
References	[2,30,31,34]	[35–38]

with the parasite. Therefore, more studies are required to understand the parasite–vector and host relationship.

By contrast, *in vitro* studies using normal human PBMC stimulated with *Leishmania in vitro* appear to yield results that mimic infection in humans just as the murine *in vitro* system mimics infection in the mouse. Therefore, the human *in vitro* system might prove useful for dissecting the immune response of humans to *Leishmania*, especially during the first few hours and days of infection, which might prove to be crucial to understanding the immune response of humans to the parasite. This approach can be used to screen new parasite antigens as candidates for the development of a potential vaccine.

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