

Review Article

Indian J Med Res 123, March 2006, pp 439-454

Animal models for vaccine studies for visceral leishmaniasis

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Received April 7, 2005

Visceral leishmaniasis (VL) or kala-azar is the most dreaded and devastating amongst the various forms of leishmaniasis. The disease, though localized in certain areas only, has gained immense importance because of high mortality rate, mainly in children. The parasite is responsible for a spectrum of clinical syndromes, which can, in most extreme cases, go from an asymptomatic infection to a fatal form of VL. Chemotherapeutic measures, alone are not sufficient to control and contain the disease. As an alternate strategy, vaccination is also under experimental and clinical trials. The situation unquestionably demands the use of proper screening system, rationale chemical synthesis, vaccine development and targeted vaccine delivery. Thus, development of an acceptable vaccine is not an easy task.

While the factors, which determine clinical outcomes, are in part, a feature of the parasite, it is the nature of the host and its genetic make up and immune status that play crucial role. The prerequisite of reliable animal model is that it should have a considerably good correlation with the clinical situation and is expected to mimic the pathological features and immunological responses observed in humans when exposed to a variety of *Leishmania* spp. with different pathogenic characteristics.

Many experimental animal models like rodents, dogs and monkeys have been developed, each with specific features, but none accurately reproduces what happens in humans. In addition to the nature of the host, the major difference between natural and experimental infections is the parasite inoculum; in natural conditions, the infected sand fly vector deposits a few hundred metacyclic promastigotes into the dermis of the host, whereas experimental infections are induced by the injection (subcutaneous or intravenous) of millions of promastigotes grown in axenic cultures *in vitro* or amastigotes recovered from infected spleens.

In public health terms, VL is the disease of humans and dogs (which may be considered secondary or 'accidental' hosts in the leishmanial life cycle) who often exhibit severe clinical signs and symptoms when infected, whereas reservoir hosts generally show a few, minor or no signs. This situation makes the definition of a suitable laboratory model a difficult one since the various experimental hosts may behave either like a reservoir or an accidental host.

This review discusses the concept of animal models for VL and provides a critical evaluation of the most common experimental models and their respective advantages and disadvantages. Particular emphasis is given to the value of using mouse, hamster, dog and primate models, especially in the context of testing potential antileishmanial vaccines.

Key words Amastigotes - dog - hamster - mice - monkey - promastigotes - vaccines - visceral leishmaniasis

The leishmaniasis represent endemic infections that occur, predominantly, in tropical and subtropical regions. Currently, the leishmaniasis are considered to be endemic in 88 countries and an estimated 12 million people are infected and 350 million people live at risk of infection (<http://www.who.int/emc/disease/leish/leis.html>). Leishmaniasis 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 leishmaniasis (CL and MCL), representing the responsive pole, to diffuse cutaneous leishmaniasis (DCL) which represents the unresponsive pole and visceral leishmaniasis (VL) ranging from sub clinical to fatal disease.

Visceral leishmaniasis (VL or Kala-azar) is the most devastating type among a complex of leishmaniasis (cutaneous, mucocutaneous, and visceral) and is caused by the invasion of the reticuloendothelial system (spleen, liver and bone marrow) by the hemoflagellate protozoan parasite, *Leishmania donovani*. If left untreated the disease is almost always fatal. Humans are the main reservoir. The disease is generally restricted to the areas, which are heavily infested by the sandfly (*Phlebotomus* spp.), the vector of this disease. Although visceral leishmaniasis is widely distributed throughout the tropics, it is rampant in the Indian subcontinent and southwest Asia. Annually, about 500,000 cases of VL occur worldwide, of which 90 per cent occurs in India, Sudan, Nepal, Bangladesh and Brazil^{1,2}. The last epidemic of the disease that occurred in India (Bihar and West Bengal) in 1997-1998 caused an estimated loss of 20,000 human lives. It should thus be appreciated that this form of leishmaniasis is of great public health importance, and if proper preventive/curative measures are not adapted, the disease is likely to take a high toll of lives in endemic areas.

The symptomology of *L. donovani* infection is characteristic. The incubation period is usually from 6 wk to 6 months but can be as short as 2 wk or as long as 9 yr. The initial symptoms consist of malaise, headache, and fever occurring at irregular intervals and later becoming of daily occurrence, often accompanied by chills and sweating. Other symptoms

include cough, diarrhea, dizziness, vomiting, bleeding of gums, pains in the limbs, and weight loss. Later, the clinical picture is transformed to enlargement of spleen and liver, and anemia with leukopenia and lymphadenopathy. The skin becomes dark gray, corresponding to the name of the disease Kala-azar or black sickness.

In most acute infections death may occur within a few weeks; in sub acute cases within a year and in chronic cases within 2 to 3 yr. Since, parasites invade macrophages, which are involved in defense mechanisms, the Kala-azar patients are invariably immuno-depressed and promote speedy multiplication of parasites. The patients thus easily fall prey to secondary invaders.

Patients, who recovered from VL, usually have lifelong immunity to reinfection but occasionally relapses may occur². The incomplete treatment of the patient may lead to a condition usually referred to as post Kala-azar dermal leishmaniasis (PKDL). This condition has been witnessed very often in India and occasionally in East Africa. About 20 per cent of patients in India develop PKDL³. A year or two after antimony treatment, hypopigmented and erythematous patches are found on the face, trunk of the body, and limbs. These may develop into nodules and resemble those of lepromatous leprosy. A small number of amastigotes can be seen on the skin.

Vaccine development

There has been little advancement in the area of drug development against leishmaniasis and the age old drugs which have benefited the patient for over 40 yr are still the only hope of patients. Sodium antimony gluconate (SAG) or Sodium stibogluconate (SSG) is cardiotoxic at therapeutic doses recommended for visceral and mucocutaneous leishmaniasis. Further treatment failures reported with this drug worldwide have often been interpreted as Sb^v or SSG drug resistance². A second line drug, amphotericin B, has numerous side effects, and pentamidine is associated with disturbance of glucose metabolism and other toxicity problems.

Thus, mere chemotherapy is not sufficient to combat the disease. Hence, in addition to the therapeutic measures, vaccination is the point of serious consideration. Immunization against leishmaniasis was achieved in the past by inoculating humans with living parasites that induced localized self-healing cutaneous lesions (leishmanization). Since then, a first generation of vaccines composed of formulations including killed parasites were developed against cutaneous but not visceral leishmaniasis and were used in large clinical trials on human populations of endemic areas⁴⁻⁷. Second generation vaccines can be divided into three categories according to their composition: live mutant vaccines, defined subunits, and crude fractions. Among the recombinant and native antigens tested in murine models, the LACK protein⁸, the LPGAP peptides⁹, the LeIF protein¹⁰ and the dp72 glycoprotein of *L. donovani*¹¹ were protective immunogens for mice. Finally, protection results obtained with the third generation vaccines composed of cDNA encoding leishmanial antigens cloned into a eukaryotic expression vector are still in preliminary stage⁴. Although a great number of antigens have been tested for protection against the cutaneous disease with *in vitro* cell or mouse models, no effective vaccine against human kala-azar is yet available. Though, the solid immunity observed following cure of kala-azar has suggested that the vaccination to prevent leishmaniasis is within the reach of conventional immunization methods¹², only few reports in literature deal with vaccines *viz.*, FML, FML-QuilA Saponin *etc.*, against canine visceral leishmaniasis¹³⁻¹⁵.

To date, there are no vaccines against visceral leishmaniasis. However, there is consensus that in the long term, vaccines ought to become a major tool in the control of this group of diseases. Unfortunately, the development of vaccines has been hampered by significant antigenic diversity and the fact that the parasites have a digenetic life cycle in at least two hosts (sand fly vector and human, but there is also an animal reservoir). An equally important consideration for the design and implementation of anti parasite vaccines in general is the contribution of the genetics of the target host population and their susceptibility to infection and disease, *i.e.*, the severity of disease manifestations.

Evidence indicates that visceral leishmaniasis did not get the attention that it deserved because, until the late 1940s the disease was a local problem and the world's attention was engaged in tackling the highly fatal and epidemic bacterial and viral infections. The problem was further aggravated by lack of a proper laboratory animal model to screen potential antileishmanial compounds or vaccines, which had serious repercussions on the screening system; as such the human served both as the experimental and clinical subjects.

The development of a vaccine against leishmaniasis is a long term goal in both human and veterinary medicine. In the past decade, various subunit and DNA antigens have been identified as potential vaccine candidates in experimental animals but none have so far been approved for human use. Vaccine formulation with killed parasites is still attractive in terms of cost. To date there is no vaccine against VL in routine use anywhere in the world. Several vaccine preparations are in more or less advanced stages of testing¹⁵⁻²¹. The situation unquestionably demands the use of a proper screening system. A careful appraisal of these aspects would certainly lead to success in the development of much needed antileishmanial agents. We present here a comprehensive scenario of the laboratory models and screening systems in use.

Host immune response

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 promastigotes penetrate into the macrophage, transform into amastigotes and multiply. 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. The incubation period of VL is estimated to range from two to four months. The disease can present an acute, sub acute or chronic evolution, but most infected individuals remain completely asymptomatic. 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, diarrhoea or dysentery, and abdominal swelling. Patients also present severe cachexia, pancytopenia (anaemia, thrombocytopenia, and leukopenia, with neutropenia, marked eosinopenia, and a relative lymphocytosis and monocytosis), oedema, bleeding episodes, huge hepatosplenomegaly (splenomegaly usually predominates), and hypergammaglobulinaemia (mainly IgG from polyclonal B-cell activation) with hypoalbuminaemia.

VL is a potentially fatal disease and develops life long immunity against reinfection. Human VL caused by *L. donovani* or *L. infantum* is a severe disease with generalized spread of the parasites to reticuloendothelial system, such as spleen, liver and bone marrow. In communities exposed to infection, individuals develop a strong cellular immunity in age related fashion²². There are several studies indicating that a Th2 type response predominates during acute disease, such as suppression of T-cell reactivity to *Leishmania* antigens, predominance of endogenous interleukin-4 (IL-4) over interferon- γ (IFN- γ) production, and polyclonal B-cell activation resulting in hypergammaglobulinaemia²³. In contrast with the *L. major* mouse model, however, other studies indicate that both Th1 and Th2 like cells appear to be activated during the course of the disease, as revealed by the simultaneous high levels of IFN- γ and IL-10 detected in patients²⁴. Further, upregulation of IL-10, rather than IL-4, appears to be a constant feature in clinical disease. This cytokine has been associated to the immune suppression commonly seen in VL patients²⁵ and to the development of PKDL²⁶, a skin manifestation caused by *L. donovani* after apparent VL healing²⁷. Taken together, the above findings provide evidence for the existence of a Th1/Th2 dichotomy in the T-cell response also in human leishmaniasis. Interestingly, the outcome of the infection appears to be determined and regulated by the balance between the two parasite-specific T-cell populations²⁷. Thus, even in humans it is difficult to demarcate the responses leading either to visceral disease or to protective immunity with *L. donovani*.

Studies of tissue cytokine mRNA expression reveal a role for IL-10 in downregulating CD4⁺T-cell responses and the involvement of IL-10 in disease pathology of *L. donovani* infections²⁸. However, active VL also finds correlation with enhanced induction of IFN- γ , IL-2, IL-10 and IL-4. After cure, levels of IFN- γ , IL-4 and IL-10 persist suggesting a co-existence of Th1 and Th2 in kala-azar patients as well as in cured individuals²⁸⁻³³.

Individuals with overt visceral leishmaniasis display a negative skin test response to *Leishmania* antigens. Further, peripheral blood mononuclear cells from such individuals fail to proliferate or to produce IFN- γ when exposed to specific antigen *in vitro*³⁴. Interestingly, failure of peripheral blood mononuclear cells (PBMC) from recently infected infants to produce this cytokine is predictive of evolution of the disease towards classical, patent visceral leishmaniasis, whereas conversely, production of IFN- γ *in vitro* is indicative of maintenance of the infection at a subclinical level. These observations point to the critical importance of early T-cell activation patterns in determining the eventual outcome of the infection.

Which mechanisms underlie the poor T-cell reactivity to *Leishmania* antigens characteristic of patients with active disease? Much of this effect might be due to unbalanced production of IL-10. Indeed, elevated blood levels of IL-10 as well as high production of this cytokine (or its mRNA) by lymph node cells or PBMC from kala-azar patients appears to be a hallmark of this form of infection^{25,35,36}, whereas cell populations collected from cured individuals fail to express this immunomodulatory molecule. Of high interest, neutralizing anti-IL-10 monoclonal antibody added to PBMC from acutely infected patients markedly increased the proliferative response to a *Leishmania* lysate²⁵. In this connection, an antigen of *L. infantum* (papLe22, a 22-kDa "potentially aggravating protein" of *Leishmania*) has been described, which stimulated IL-10 production by mononuclear cells from VL patients³⁷. IL-10 therefore, appears to constitute a major regulatory cytokine whose production may critically control the outcome of infection. Failure to produce IL-12 has similarly been associated with the active form of the

Table I. Experimental models used for vaccine trials against visceral leishmaniasis (VL)

Animal/strains	Parasite	Route of inoculation	Characteristics
Mouse:			
BALB/b	<i>L. chagasi</i>	i.v	-Self curing to non healing type
BALB/c	<i>L. donovani</i>	i.d	-Th1/Th2 response
C57BL/6	<i>L. infantum</i>	s.c	-All immunological reagents are available -Good model for dissecting protective immune response
Hamster:			
Golden	<i>L. chagasi</i>	s.c	-Progressive fatal infection
Chinese	<i>L. donovani</i>	i.p	-Severe immunosuppression (Th2 response)
	<i>L. infantum</i>	i.c	-Reagents for T-cell response not available -Good experimental model for initial vaccine trial
Dog:			
Stray	<i>L. infantum</i>	i.v	-Natural reservoir (not in India)
Beagle	<i>L. chagasi</i>	i.d	-Subclinical/asymptomatic to progressive fatal infection
Mongrel			-Immunosuppression (Th2 response)-Reagents for cytokine response not available-Good secondary model for pre clinical vaccine trial
Monkey:			
Owl	<i>L. donovani</i>	i.v.	-Sub clinical to fulminating progressive fatal infection
Squirrel		i.d	-Severe immunosuppression (Th2 response)
Vervet			-All immunological reagents are available
Langurs			-Good secondary model for pre clinical vaccine trial but difficult to use due to cost, handling and immunological black boxes

i.v., intravenous; i.d., intradermal; s.c., subcutaneous; i.p., intraperitoneal; i.c., intracardial

disease³⁸. It is also being suggested that unbalanced IL-10 production might play a role in progression of the disease towards the cutaneous form of the infection known as PKDL²⁵.

Selection of models for testing of vaccine

Animal models are expected to mimic the pathological features and immunological responses observed in humans when exposed to a variety of *Leishmania* spp. with different pathogenic characteristics. Many experimental models have been developed, each with specific features, but none accurately reproduces what happens in humans. For *in vivo* testing of vaccine several animal species have served as experimental host for VL (Table I). Important among them are BALB/c mice and Syrian golden hamster (primary tests), dogs (secondary tests) and monkeys *viz.*, squirrel, vervet and Indian langur monkeys as tertiary screens.

In natural conditions, the infected sandfly deposits a few hundred metacyclic promastigotes into the

dermis of the host, whereas experimental infections are usually induced by subcutaneous (s.c.) or intravenous (i.v.) injection of millions of promastigotes grown under *in vitro* conditions or amastigotes recovered from cutaneous lesions or infected spleens. In each particular laboratory animal, the outcome of infection (*e.g.*, whether an infection remains restricted to the site of inoculation or visceralizes) will depend on a combination of factors, including the *Leishmania* species injected, virulence of the parasite isolate, nature of the inoculum and the route of inoculation. A suitable laboratory host for the target parasite (*L. donovani*) is very important from the point of view of conducting research on various aspects including host-parasite interactions, pathogenesis, biochemical changes, prophylaxis, and maintenance of parasites and above all evaluation of antileishmanial action of newer compounds for development of new drugs. For establishment of infection in animals there are few criteria *viz.*, (i) the susceptibility of the animal inoculated, (ii) the age and particular morphological phase of the parasite in culture, and (iii) the mode of infection.

The influence of the inoculum on the outcome is a particularly important consideration in experimental models and a wide variety of inoculation routes has been used. The intradermal (i.d.), subcutaneous (s.c.), intravenous (i.v.) and intraperitoneal (i.p.) routes are the most frequently used, but some experimental studies have reported unexpected results following the use of more unconventional models of inoculation. In recent years, there has been a tendency for inducing an infection similar to natural ways by using low numbers of infective parasites and the i.d. route. Of particular interest, in the context, is the improved infectivity of laboratory isolates when the inoculum contains sandfly salivary gland extracts of parasites isolated from sandflies³⁹.

Primary evaluation systems are helpful primarily in detecting leads for a vaccine development. Since the host plays an important role in the host-parasite-vaccine interaction (and we have very little knowledge about these interactions), every effort should be made to initiate evaluation of likely molecules in the target species as early as possible. This is particularly important if the translation of activity from test model to target species in the natural host is contemplated because the behaviour of the vaccine is likely to be significantly different. To substantiate the results of primary screening, the therapeutic trials of vaccines are undertaken under controlled conditions either against the target parasite in its natural host or adopted in laboratory animals. If the efficacy is established and dose relationship exists, the results of the secondary screening would help in predicting the dose against the objective parasite in man. Such an exercise may also serve as an indication of effective vaccine.

Models for primary testing: Rodents

Several attempts were made in the past to use small rodents for *L. donovani* infection. These includes hamster (European, Chinese and Syrian); mouse (BALB/c, NMRI, DBA/1, C57BL/6) rat, mastomys, squirrel, gerbil *etc*⁴⁰. Of the various animals tried, BALB/c mice and Syrian golden hamsters are the commonest and currently used animal models for drug and vaccine testing against VL.

Mice: Outbred mice are generally resistant to infection with *L. donovani*, but inbred strains display marked differences in susceptibility, which led, in the early 1970s, to the isolation of the Lsh susceptibility gene (subsequently designated *NRAMP1*)⁴¹. *NRAMP1* determines the degree of early expansion of the parasites in the liver and spleen⁴².

Studies with the mouse model also led to the characterization of the immune mechanisms important for the development of organ specific immune responses, which cause the clearance of the parasites from the liver but not the spleen⁴³. Another important contribution of the mouse model has been the discovery that chemotherapy is ineffective in the absence of intact T-cell mediated responses⁴³. These experimental studies pointed to the need to activate the immune system for successful chemotherapy and led to the successful trial in India. The mouse model has been used widely for the development of vaccines against VL^{18,44-46}. One of the difficulties with the mouse as a model for human disease is the need to inject amastigotes intravenously in order to induce a reproducible pattern of colonization of the liver and spleen of wasting, as in the human disease, and the infection is chronic but not fatal. Recently, an intradermal murine model of VL has been explored for the establishment of a chronic infection pattern in susceptible BALB/c mice resembling a course of disease similar to that of human VL⁴⁷. This model could serve as an important tool in future vaccine studies against VL.

Different *Leishmania* species cause clinically distinct diseases and the severity of the disease caused by any given parasite can vary markedly between individual hosts⁴¹. Till date, two host systems have been classified for studying *Leishmania* infection on the basis of susceptibility and resistance of the host. Murine models for experimental leishmania are well established. Parasites are injected underneath the skin of the footpad. Most of the mice strains like C57BL/6, CBA/J, C3H or BIOD2 resist the infection with clinical cure within few months⁴⁸; while BALB/c and all T-cell immunodeficient strains manifest a systemic visceral leishmania leading to death⁴⁹. Resistance and susceptibility are closely related with the development of T-cell responses of

Th1 or Th2 type, respectively. C57BL/6 mice mount early Th1 immune response and prevent the further growth of the parasite causes self-healing phenotype⁵⁰ whereas susceptible BALB/c strain mounts early Th2 response and results in non healing lesion and exaggeration of disease⁵¹. Respective resistance and susceptibility of C57BL/6 and BALB/c strains depend not only on the Th1 and Th2 type of immune response of CD4+ T cells but also on the genetic background of the host.

In general, the immune responses following infection of inbred mouse strains with viscerotropic *Leishmania* species, such as *L. donovani* or *L. infantum*, are similar to those observed in the *L. major* mouse model. However, BALB/c mice do not appear to exhibit a similarly high susceptibility to these parasites, since intravenous injection of visceral *Leishmania* results in a self-healing chronic infection. Further, cytokine phenotypes elicited by viscerotropic *Leishmania* in this mouse model are not typical of a Th2-type response⁵².

First attempts were the use of killed promastigotes together with glucan or IL-12 as an adjuvant injected subcutaneously or intravenously. The effort could impart partial resistance against challenge infection in CF1 mice⁵³. Vaccines like live attenuated promastigotes or genetically manipulated parasites, were also tested in susceptible (BALB/c) and resistant (C57BL/6) mice⁵⁴⁻⁵⁶.

This animal has also been used to assess the efficacy immunization procedures using *Leishmania* parasites of low virulence to protect against infections by the viseralizing species⁵⁷. However, it is unclear whether these very interesting results are applicable in other experimental models of visceral disease. Indeed, in a murine model, protection against visceral infection allowing intravenous injection of *L. chagasi* could be achieved only by subcutaneous inoculation of high doses of virulent promastigotes, whereas protection was not afforded by attenuated or *DHFR-TS* gene knockouts from this or other (*L. donovani*, *L. major*) species⁵⁸. Further, the bioprotein transporter (BT1) of *L. donovani* was inactivated by homologous recombination, the parasite showed a much reduced

capacity to infect BALB/c mice, yet elicited an immune response characterized by the production of IFN- γ , leading to a high level of resistance against challenge by the wild type parasite⁵⁹.

Immunization with recombinant *L. donovani* A2 protein, which is regarded as a virulence factor, led to significant protection against challenge infection by the homologous parasite, correlating with a mixed Th1/Th2 as well as a humoral response against the A2 protein⁶⁰. BALB/c mice immunized with recombinant HASPB1 were similarly protected against *L. donovani* challenge, as shown by strong reduction of spleen and liver parasite loads⁶¹. Partial protection was also achieved in BALB/c mice by subcutaneous injection of the recombinant ORFF and BTI proteins in complete, followed by incomplete Freund's adjuvant⁶², Recombinant LCR1, a protein shown to stimulate IFN- γ but not IL-4 production by T-cell enriched spleen cells from *L. chagasi* infected susceptible or resistant mice⁶³.

Though, BALB/c strain of mice infected with *L. (L.) donovani* or *L. (L.) chagasi* is the most widely studied model of VL, this is considered to be susceptible wherein the infection progresses during the first two weeks, and it is then controlled by the host immune response⁶⁴. As mentioned above, human visceral leishmaniasis presents a spectrum of clinical manifestations from a self-controlled infection to a progressive disease. The mouse model is comparable to self-controlled oligosymptomatic cases and therefore is useful for the study of the protective immune response. On the other hand, the better model to study the progressive disease is hamsters infected with *L. (L.) donovani* or *L. (L.) chagasi* that develop a disease similar to human progressive visceral leishmaniasis with hepatosplenomegaly, hypoalbuminaemia, hypergammaglobulinaemia, and pancytopenia⁶⁵. Therefore, this model is mainly used to study the mechanisms of immunosuppression.

Hamster: The Syrian golden hamster (*Mesocricetus auratus*) is uniquely susceptible to a variety of intracellular pathogens and is an excellent model for a number of human infectious diseases. The golden hamster was used as one of the early animal models for the study of visceral leishmaniasis. Infection with

L. donovani leads to visceral disease and death making it a useful tool for the characterization of molecules and mechanisms involved in pathogenesis⁴⁰. Hence, they are ideal for most of the experimental studies including vaccine testing because they almost mimic the situation in a Kala-azar patient⁶⁶. Very few inbred strains of hamsters are available from commercial sources, limiting the possibility of genetic manipulations in this model. In addition to its use as a model of disease, the hamster is also the favourite laboratory animal for the isolation and laboratory adaptation of field isolates⁴⁰. Hence, these animal models have the potential to be comparable to the clinical situation. A major disadvantage of the visceral models is that, in the prime model of visceral disease (the hamster model), only high dosages of antimony could suppress established lesions. The model has also been used for vaccination studies^{67,68} but the molecular basis for this high level of susceptibility is unknown, and immunological studies related to this model have been limited by the lack of available reagents. Interestingly, an inoculum (10^3 to 10^5) of *L. infantum* promastigotes could result in the development of both symptomatic and asymptomatic states with a spontaneous protective response⁶⁹. Heterologous protection has been observed using killed *L. major* plus BCG combination against *L. donovani* in hamsters²¹.

Melby *et al*⁷⁰ described the cloning and sequence analysis of portions of the Syrian hamster interleukin 2 (IL-2), IL-4, interferon gamma (IFN- γ), tumour necrosis factor alpha (TNF- α), IL-10, IL-12p40, and transforming growth factor beta cDNAs. In addition, they examined the cytokine response to infection with the intracellular protozoan *L. donovani* in this animal model. Sequence analysis of the hamster cytokines revealed 69 to 93 per cent homology with the corresponding mouse, rat, and human nucleotide sequences and 48 to 100 per cent homology with the deduced amino acid sequences. The hamster IFN- γ , compared with the mouse and rat homologs, had an additional 17 amino acids at the C terminus that could decrease the biological activity of this molecule and thus contribute to the extreme susceptibility of this animal to intracellular pathogens. The splenic expression of these genes in response to infection

with *L. donovani*, the cause of visceral leishmaniasis (VL), was determined by Northern blotting. VL in the hamster is a progressive, lethal disease which very closely mimics active human disease. In this model there was pronounced expression of the Th1 cytokine mRNAs, with transcripts being detected as early as 1 wk post infection. Basal expression of IL-4 in uninfected hamsters was prominent but did not increase in response to infection with *L. donovani*. IL-12 transcript expression was detected at low levels in infected animals and paralleled the expression of IFN- γ . Expression of IL-10, a potent macrophage deactivator, increased throughout the course of infection and could contribute to the progressive nature of this infection. These initial studies are the first to examine the molecular immunopathogenesis in a hamster model of VL infection and indicate that progressive disease in this model of VL is not associated with early polarization of the splenic cellular immune response toward a Th2 phenotype and away from a Th1 phenotype.

Mice control *Leishmania* infection through the generation of NO, an effector mechanism that does not have a clear role in human macrophage antimicrobial function. Remarkably, since, infection of the Syrian hamster (*M. auratus*) with *L. donovani* reproduced the clinicopathological features of human VL, investigation into the mechanisms of disease in the hamster revealed striking differences from the murine model. Uncontrolled parasite replication in the hamster liver, spleen, and bone marrow occurred despite a strong Th1-like cytokine (IL-2, IFN- γ , and TNF/lymphotoxin) response in these organs, suggesting impairment of macrophage effector function. Indeed, throughout the course of infection, inducible NO synthase (iNOS, NOS2) mRNA or enzyme activity in liver or spleen tissue was not detected. In contrast, NOS2 mRNA and enzyme activity was readily detected in the spleens of infected mice. The impaired hamster NOS2 expression could not be explained by an absence of the *NOS2* gene, overproduction of IL-4, defective TNF/lymphotoxin production (a potent second signal for *NOS2* induction), or early dominant production of the deactivating cytokines IL-10 and TGF- β . Thus, although a Th1-like cytokine response was prominent, the major antileishmanial effector

mechanism that is responsible for control of infection in mice was absent throughout the course of progressive VL in the hamster.

Recent studies have revealed that the mice model of *L. donovani* does not reproduce the features of active human VL like chronic fever, hepatosplenomegaly, pancytopenia and profound cachexia and have an ineffective antileishmanial cellular response⁶⁶. On the contrary, Syrian golden hamster model of active VL closely relates the human counterpart as shown by relentless increase in visceral parasite burden, progressive cachexia, hepatosplenomegaly, pancytopenia, hypergammaglobulinaemia and ultimately death. While the mice are either intrinsically resistant or susceptible to *Leishmania* infection and offer a well characterized genetic makeup, chiefly by the use of inbred, recombinant and naturally or experimentally mutated strains, hamsters provide an excellent model for an overtly susceptible host. Therefore, hamsters are used for histopathological and vaccine studies despite the lack of fine immunochemicals that limit the mechanistic exploration of immune responses to *Leishmania* infection⁶⁶.

Models for secondary testing: Canines

As all rodent models have the disadvantage of having different metabolisms and kinetics of the drugs, eliciting responses different from those seen in humans, the secondary testing in higher models such as dogs, cats and monkeys, which have responses close to human, would further strengthen the claim of primary screening and would help in picking the most promising molecules/epitopes, which need to be pursued in successive steps in a vaccine development programme. The dog is the major reservoir of *L. infantum* in the Middle East and the Mediterranean region and *L. chagasi* in South America. There has been no such reservoir for VL noticed in India; the disease pattern in dogs and humans is similar, with a long period of asymptomatic infection followed by wasting, anaemia, enlarged lymph nodes, and fever. As in humans, the infection remains asymptomatic in some dogs^{71,72}. One of the few differences is the presence of skin lesions in the dogs, rarely detected in

humans⁴⁰. The unpredictable nature of the infection has been a major problem in establishing experimental models for canine VL, but appears to reflect the spectrum of clinical responses seen in natural infections.

The dog may be the best animal model for VL in which relevant immunological studies and vaccine development could be performed⁷³. With the recent cloning of several dog genes encoding cytokines and immunologically important cell markers, as well as the development of monoclonal antibodies to these molecules, there is a hope for a more sustained exploitation of this excellent animal model. Dog populations are an important reservoir of visceralizing leishmania in many endemic areas, and vaccination of these animals would presumably constitute a major step towards control of the infection⁷⁴. Therefore, dogs have been proposed as a logical substitute at least for *L. infantum*, for which the dog is the natural reservoir. *L. donovani* also multiplies within the viscera of mongrel and beagle dogs. German shepherd dogs have been reported to give better results than beagles⁷⁵, whereas some researchers claimed highly successful infection rates with mixed breeds⁷¹. Studies on the prevalence of natural infections in dogs indicated that German shepherds, boxers and Dobermans exhibited VL more frequently than other breeds of dogs⁷¹.

In a pilot study by Dunan and colleagues⁷⁶, 393 seronegative dogs were inoculated with a *L. infantum* promastigote fraction (LiF2) previously shown to induce strong protection against *L. major* infection in both susceptible (BALB/c) and resistant (C57BL/6) mice. The dogs were then released and monitored for low acquisition of infection by natural routes. Surprisingly, during the first year after vaccination, the rate of infection was significantly higher in the vaccinated group relative to animals injected with adjuvant alone, a difference that disappeared during the second year. As similar immunization protocols had been used in both the mouse and the dog studies, the authors stressed the differences that may exist between infection of natural hosts and laboratory substitutes. In a recent study, dogs from an endemic area from Brazil were vaccinated subcutaneously (in the absence of adjuvant) with the fucose-mannose

ligand (FML) of *L. donovani*⁷⁷. This resulted in strong, long-lasting protection against naturally acquired infection, with only 8 per cent of the vaccinated animal developing mild disease (and no fatal infection), while 33 per cent of the control population presented with clinical or fatal disease. Recently FML vaccine and heterologous prime-boost regime using DNA and vaccinia recombinant vectors expressing LACK have been tested in canine model of VL^{13,15,78}.

Models for tertiary or pre clinical testing: Non human primates

A vaccine for man needs to be tested in primates due to their close phylogenetic relation to humans in the evolutionary tree. For VL, the availability of a non human primate model would increase the understanding of various aspects of host parasite interactions. Earlier efforts in establishing VL in New and Old World monkeys demonstrated that *Aotus trivirgatus* (owl monkeys) and *Saimiri sciureus* (Squirrel monkeys) developed fulminating but short lived infection. Antileishmanial screening was performed in owl monkey⁷⁹ and squirrel monkey⁸⁰. Old World monkeys such as *Macaca* spp. viz., *M. mulatta*, *M. fascicularis* and *M. nemestrina*, and African vervet monkeys developed low and/or inconsistent infections⁸¹. Attempts to establish VL in *Presbytis entellus* showed that this species was highly susceptible to single intravenous inoculation of hamster spleen- derived *L. donovani* amastigotes, which invariably produced consistent and progressive acute fatal infection, leading to death between 110 to 150 days post infection. The infected animals presented all the clinico-immunopathological features as observed in human kala-azar^{82,83}. The Indian langurs have also been used for preclinical evaluation of potential antileishmanial drugs⁶⁸ and vaccine^{19,20}.

Successful vaccination has been achieved against visceral leishmaniasis by intradermal inoculation of alum-precipitated autoclaved *L. major* (ALM) with BCG and autoclaved *L. donovani* (ALD) with BCG in Indian langurs. Vaccinated animals show significant lymphoproliferative response with high level of IFN- γ and IL-2^{20,21}.

Primates are less fashionable in vaccine development, because primates are not only expensive laboratory animals that are difficult to obtain and to handle, but they are also immunological black boxes.

Conclusions: Relevance of the experimental models to clinical infection and immunity

The functions of experimental animal models of VL are: (i) to provide the means for the *in vivo* maintenance of virulent strains of parasites and the production of amastigotes; (ii) to study the pathogenesis of VL and antileishmanial immunity; (iii) to test antileishmanial drugs and vaccines. Experimental infections cannot be used to predict whether an animal can or cannot be incriminated as a reservoir in the transmission cycle.

The *in vivo* maintenance of parasites may be done using a variety of animals; for *L. donovani* isolates, the hamster is usually chosen and infection is most successful when using i.p. or i.c. injection of amastigotes isolated from the spleen of a previously infected animal. The modeling of clinical features of VL is a daunting problem in view of the very different behaviour of parasites in different hosts and with different inocula. Paradoxically, any model where inoculation of parasites is not systematically followed by disease is deemed inadequate or, at best, 'capricious' (as is the case of the canine model), whereas models where inoculation is rapidly followed by a fulminating parasite proliferation and clinical VL (hamster and a majority of primate models) are considered adequate, despite failing to follow the pattern observed in humans or dogs⁴⁰.

The testing of antileishmanial vaccines presents yet a different set of constraints on models. Various models may be used to investigate new vaccination strategies, but in view of the species specificity of most antigens, it will ultimately be necessary to test a vaccine in a host permissive for the relevant human isolates. Thus, the *L. major*/mouse model is probably the best choice when investigating the fundamental immunological factors involved in vaccination against VL, but not for screening an actual vaccine against *L. donovani*^{84,40}. In as much as the function

Table II. Evaluation of different vaccines in human and experimental models for visceral leishmaniasis (VL)

Form of vaccine	Stage of parasite/ <i>Leishmania</i> strain	Evaluated in parasite-host system	References
Live vaccine	Promastigotes/ <i>L. tropica</i>	<i>L. donovani</i> /Human	85
	Promastigotes/ <i>L. donovani</i>	<i>L. donovani</i> / Human/Hamster	86, 87, 88
	10 ⁷ amastigotes/ <i>L. donovani</i>	<i>L. donovani</i> /Hamster	89
	10 ⁷ promastigotes	<i>L. chagasi</i> / Mice	58
Killed vaccine	Killed promastigote <i>L. donovani</i> + glucan	<i>L. donovani</i> /Mice	90
	Autoclaved <i>L. donovani</i> +BCG	<i>L. donovani</i> /Hamster and Langur	21
	10 ⁸ formaldehyde killed promastigotes (UR6)	<i>L. donovani</i> /Hamster/Mice	88, 91
	Autoclaved <i>L. major</i> + BCG	<i>L. donovani</i> /Hamster/Langur/Human (Clinical trial) <i>L. infantum</i> /Dog	19, 20, 7, 92
	Killed promastigote of <i>L. infantum</i> + FCA	<i>L. infantum</i> / Dog	93
	Merthiolated promastigote of <i>L. braziliensis</i> + BCG	<i>L. chagasi</i> /Dog (Phases I-III)	94
	BCG alone	<i>L. donovani</i> /Hamster	95
	fraction of <i>L. donovani</i> + β -1,3-glucan dp72 + <i>C. parvum</i>	<i>L. donovani</i> /Mice	53
	FML+ FIA/ BCG/ saponin/ IL-12/ QS 21/ Quil A/ Aluminum hydroxide/ Riedel De Haen	<i>L. donovani</i> /Mice	44
	gp36 + Reidel De Haen	<i>L. infantum</i> /Mice/Hamster/ Dog	96, 97, 14, 98, 77
Subunit vaccine	Eluted gp63 in positive liposome	<i>L. donovani</i> /Mice	99
	Q protein formulated with BCG	<i>L. donovani</i> /Mice	100
	LiF2 (<i>L. infantum</i> fraction)	<i>L. infantum</i> /Dog	101
	Glucose regulated protein-78	<i>L. infantum</i> /Dog (Phases I-III)	76
	Integral membrane protein + CFA	Mice	102
	BCG expressing flagellar antigen LCR1/recombinant LCR1	<i>L. donovani</i> /Hamster	103
	5 x10 ⁷ promastigote BT1 Null mutant	<i>L. chagasi</i> /Mice	104, 63
	Oligonucleotides primer from <i>L. donovani</i> DNA	<i>L. donovani</i> /Mice	59
	ORFF+BT1+ CFA	<i>L. donovani</i> /Mice	105
	ORFF DNA	<i>L. donovani</i> /Mice	62
	rHASPBI+ IL12	<i>L. donovani</i> /Mice	106
	15 cDNA sub libraries	<i>L. donovani</i> /Mice	61
	Only papLe 22 cDNA	<i>L. donovani</i> /Mice	107
	A2 DNA/ rA2 + heat killed <i>P. acnes</i>	<i>L. donovani</i> /Hamster	108
	IL-12 p40 -p35 fusion cDNA	<i>L. donovani</i> /Mice	109
	LACK DNA+ vaccina virus	<i>L. donovani</i> /Hamster	66
		<i>L. infantum</i> /Dog	78

FCA, Freund's complete adjuvant; FML, fucose-mannose ligand; FIA, Freund's incomplete adjuvant; CFA, Complete Freund's adjuvant

of a vaccine is to transform a susceptible animal into a resistant one, it may be logical to choose a highly susceptible animal host for initial vaccine trials (e.g., the hamster) and to follow this by trials in a host which is also a reservoir host (i.e., a host which could be used in the final stage for exposure to natural challenge). Many investigators consider the dog as the best choice for the latter, and have been working on improving protocols for obtaining more

predictable infection rates. Others have explored the possibility of using primate hosts, following the rationale that any vaccine for man needs initial trials in primates (Table II). However, in view of the fact that the pattern of leishmanial infection in primates is sub-stantially different from that observed in humans, and given the difficulty and costs of handling primates and the additional problems related with experimentation on wild-caught animals, there

does not appear to be a very strong case to support the use of primates for vaccination trials, particularly since these animals could never be used for exposure to natural challenge⁴⁰.

Further, 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 with the parasite¹¹⁰. Therefore, more studies are required to understand the parasite-vector and host relationship. By contrast, *in vitro* studies using normal human peripheral blood mononuclear cells (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 understand 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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