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Experimental models in vaccine research: malaria and leishmaniasis

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Abstract

Animal models have a long history of being useful tools, not only to test and select vaccines, but also to help understand the elaborate details of the immune response that follows infection. Different models have been extensively used to investigate putative immunological correlates of protection against parasitic diseases that are important to reach a successful vaccine. The greatest challenge has been the improvement and adaptation of these models to reflect the reality of human disease and the screening of vaccine candidates capable of overcoming the challenge of natural transmission. This review will discuss the advantages and challenges of using experimental animal models for vaccine development and how the knowledge achieved can be extrapolated to human disease by looking into two important parasitic diseases: malaria and leishmaniasis.

Key words: Human vaccines; Animal models; Malaria; Leishmaniasis

Introduction

The use and development of experimental models has been closely related to the history of vaccine research. The advantages of exploring experimental animal models include some aspects that cannot be considered in clinical trials. The animals are easily available and affordable, allowing the use of a significant number of subjects and the evaluation of numerous and diverse potential antigen candidates. Moreover, they are suitable to investigate disease progression and to analyze virulence factors of particular isolates or modified strains of parasites.

Among the different animals that have been used as models for scientific research, rodents have been used the most, especially for the numerous advantages that they offer. Concerning immunological investigations, the availability of inbred, knockout and transgenic mice that can be used to investigate specific cells and molecules of the immune system have made mice a rich source for understanding the host immune response. Mice can also be used for the evaluation of vaccine immunity and long-term immune memory, which cannot be obtained from people living in endemic areas where re-exposure or other factors are not controlled. Organs and tissues where parasites may survive or be sequestered can be easily accessed for a more accurate evaluation of the disease.

Despite the numerous advantages offered by experimental models, there are some important limitations that should always be taken into consideration. The major challenge to establish a good experimental model is the capacity of extrapolating the findings to human disease. Human populations have a diverse genetic background that has a profound influence on the immune response, while most animal models are usually based on inbred strains corresponding to a homogeneous genetic population. Data resulting from experimental models can be consistent and relevant but may not reflect how some individuals can develop disease or may remain naturally resistant.

This limitation can be solved by the use of outbreed large animal models that are more closely related to humans, like dogs and non-human primates. This characteristic makes them a useful model for investigating infectious diseases where the pathological and clinical alterations would mimic the human response. Nevertheless, in spite of the striking similarities, some differences such as the more diverse histocompatibility complex and absent HLA-A2 in chimpanzees can be sufficient to alter the disease pattern and the immune response. In addition, the cost of large animals can be high and, for some species, important immunological tools are missing, which limits the complete characterization of the host immune response (1,2).

Although there are limitations, the use of animal models, particularly regarding the relevance of the results to human trials and gaps in the knowledge of the biology

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of the host-pathogen interaction, it is important that any vaccine be tested in animal models to assess its immunogenicity, safety and efficacy. Here, we consider the knowledge acquired by using experimental models in the search for a vaccine against two major parasitic diseases: malaria and leishmaniasis.

Experimental models used to test vaccines against malaria

Malaria is a devastating disease that affects about 500 million people worldwide, mostly children under 5 years of age. It is caused by *Plasmodium* spp and is transmitted by the bite of infected *Anopheles* mosquitoes. Following inoculation of sporozoites by infected mosquitoes the parasites quickly migrate and invade hepatocytes, where they multiply and later transform into merozoites. These forms are released from the infected cells and invade erythrocytes, initiating a cycle that leads to repeated parasite multiplication causing severe anemia or cerebral malaria (3).

Malaria is one of the most devastating parasitic diseases largely due to the absence of effective vaccines and the appearance of drug-resistant strains. Protection of mice, non-human primates and humans following vaccination with irradiated sporozoites has demonstrated the feasibility of developing a universally effective, long-lasting vaccine (4-7).

Experimental models were used from the beginning in malaria studies and have provided important insights into the mechanism of *Plasmodium* spp pathogenesis (8,9). Several avian models were used in early studies to screen for new potential treatments and candidate vaccines, and to help understand parasite biology. However, avian models proved to be a poor alternative for studies with mammalian hosts. The establishment of a rodent malaria model with *P. berghei* opened a new perspective and brought new insights into malaria infection in mammals (10). Other species, like *P. chabaudi*, *P. yoelii*, and *P. vinckei*, were also shown to naturally infect mice and to provide models with different disease outcomes and levels of susceptibility in inbred mouse strains (11).

Mouse models have helped elucidate the mechanisms implicated in the protective immune response. Due to the complex life cycle of the parasite, the host immune response to infection is varied, with both humoral and cell-mediated immune responses involved in eliciting effective immunity. For instance, early reports based on murine models established that T cells are important for the immune response against the liver stage, showing that T cells are initially directed at the intrahepatic parasite. Within the T cell population, $CD8^+$ T cells play an important role in protection against rodent malaria, especially in *P. berghei* and *P. yoelii* infections, by inhibiting parasite growth in the liver. $CD4^+$ T cells also play an important role in the pre-erythrocytic stages of rodent malaria as either helper or effector cells (12-15). Although experimental models have been useful tools for dissecting the immune response, there is no concrete evidence indicating that the same mechanism takes place in human malaria.

Unfortunately, there is no reliable animal model for human malaria other than non-human primates. Non-human primates represent a valuable resource for testing vaccine candidates and drugs for human use prior to human clinical trials. The *Aotus* genus, largely used in malaria research, is not found naturally infected with human malaria parasites but represents a valuable model to test vaccines. *Aotus* can be easily infected with *P. falciparum* and *P. vivax* with different levels of susceptibility, depending on the combination of *Aotus* and parasite species. For instance, protection has been achieved in *A. nancymai* immunized with the *P. falciparum* merozoite surface protein 1 antigen against blood-stage challenge with *P. falciparum* (16). Rhesus macaques have also been used as models to assess cerebral malaria, an important clinical aspect when considering a vaccine against *P. falciparum* (17).

Although mice and non-human primates offer many advantages for the study of malaria, some limitations should be considered. The lack of immunological tools to assess the immune response of non-human primates and the poor translation of antigens that are protective in mouse models to human malaria indicate that there is an urgent need to improve the experimental models. In an attempt to circumvent these issues, researchers have engineered murine models to mimic human immune responses, such as mice that are transgenic for human molecules or receptors (18,19). Recent studies have also highlighted the importance of natural transmission by infected mosquitoes to test vaccine candidates as a more reliable form of challenge. The response of mice challenged with *P. berghei*- or *P. chabaudi*-infected mosquito bites confirmed that the natural transmission method is the most relevant challenge regarding the testing of vaccines in animal models (20,21).

Experimental models for testing vaccines in leishmaniasis

Leishmaniasis is a group of diseases caused by protozoa of the genus *Leishmania*, causing significant morbidity and mortality worldwide. The disease is endemic in 88 countries and, according to the World Health Organization, 350 million people are at risk of infection with a worldwide prevalence of 12 million cases. Different clinical manifestations (cutaneous, mucocutaneous, diffuse cutaneous, and visceral) are caused by different species of *Leishmania* and the genetic background of the host can markedly influence the outcome of infection. The infection begins when an infected female sand fly inoculates *Leishmania* parasites into the skin of a vertebrate host (22,23). The sand fly regurgitates the parasite and promastigote secretory gel (PSG) while

injecting saliva (24,25). In the skin, the parasite penetrates mainly macrophages, where they multiply transformed into amastigotes.

To date, there is no global vaccine able to prevent leishmaniasis but vaccine research has advanced in recent years. These advances are mostly due to improved mouse and large animal models (such as dogs and monkeys), which have increased the knowledge of the immunological mechanisms involving the control of infection (26,27). However, it is important to state that all the information generated using these models does not automatically extrapolate to the disease in humans (22,28). More recently, the introduction of certain elements, such as low parasite inoculum, co-injection with sand fly saliva and PSG, site of inoculation, and challenge using a live infected sand fly, have been able to improve animal models in reflecting the course of natural infection (29).

Cutaneous leishmaniasis

Cutaneous leishmaniasis (CL) is described as a localized skin lesion that develops in the same area where the infected sand fly had previously fed. Depending on various factors like parasite species, immunological status and genetic background of the host, the lesion can be self-healing or may require treatment. The infection of mice with *Leishmania major* is one of the best studied leishmaniasis models, with the mouse developing aspects similar to those of the human disease (27). The conventional *L. major* mouse model injects a high dose of parasites into a subcutaneous site. BALB/c mice are highly susceptible to *L. major* and develop uncontrolled lesions. In contrast, C57BL/6 mice are resistant to *L. major* infection, developing small lesions that heal by about 12 weeks after infection, a fact that correlates with human disease (30).

In mice, both susceptible and resistant profiles show CD4+ T cell subset differentiation *in vivo*. *L. major* infection leads to the development of a polarized Th1 or Th2 immune response that dictates resistance or susceptibility, respectively. The Th1 cytokines activate macrophages to kill the intracellular parasite, primarily through a nitric oxide-mediated mechanism (31). The importance of the Th1/Th2 balance in the regulation of disease outcome, *in vivo*, was demonstrated in several studies, which have stressed that a successful vaccine against CL must induce the differentiation and expansion of specific CD4+ Th1 lymphocytes (22,32).

In experimental sand fly transmission, the estimated number of metacyclic promastigotes inoculated by the infected sand fly into the skin revealed a wide variation in the number of parasites delivered (30). During transmission, sand fly saliva and PSG are also co-injected into the skin and were shown to exacerbate lesion development (24,25). Titus and Ribeiro (24) first demonstrated that saliva was able to enhance *Leishmania* infection. This effect was observed when different species of *Leishmania* (*L. major*, *L. braziliensis* and *L. amazonensis*) were co-injected with salivary gland sonicate (SGS) from two vector species (*Lutzomyia longipalpis* and *Phlebotomus papatasi*) (33-35). The exacerbative effect of co-injecting salivary molecules was later related to potent molecules present in the saliva that modulate the balance of Th1/Th2 to a response that is favorable to parasite establishment and survival (36-38).

Since then, research has focused on trying to adapt experimental models to incorporate sand fly saliva. Belkaid et al. (26) sought to develop an animal model of cutaneous leishmaniasis that could mimic the natural conditions of *Leishmania* infection. In their study, a small quantity of *L. major* (1000 metacyclic promastigotes) co-injected with SGS obtained from a natural vector, *P. papatasi*, was injected into the mouse ear dermis. This resulted in a dramatic exacerbative effect on lesion development in both BALB/c and C57BL/6 mice. This model was used to test a vaccine based on a single-salivary molecule, a DNA vaccine expressing a 15-kDa protein from *P. papatasi* saliva that was able to confer protection in vaccinated mice challenged with *L. major* plus SGS (39). The vaccination also provided protection in B-cell knockout mice, indicating that a delayed-type hypersensitivity (DTH) generated against sand fly saliva was responsible for most or all the protective effects of this vaccine and that molecules present in the saliva are important targets for controlling leishmaniasis. Interestingly, mice vaccinated with maxadilan, a salivary molecule from *L. longipalpis* saliva, were also protected against challenge with *L. major* co-injected with SGS (40). These results show that sand fly salivary gland components are also potent immunogenic molecules, reinforcing the importance of including sand fly saliva as a component for an anti-*Leishmania* vaccine.

Although the model of injecting saliva with the parasite into the skin brought new perspectives in conditions that mimic a natural infection, it was later adapted to a model of natural transmission with sand flies where all the elements (saliva, parasite and PSG) are present. This natural model demonstrated that it is possible to transmit *L. major* by the bite of its natural vector, *P. papatasi*, to BALB/c and C57BL/6 mice. Moreover, pre-exposure to uninfected bites resulted in a significant reduction in lesion pathology when compared with naive mice. The protection observed in mice pre-exposed to saliva, either by needle inoculation or by uninfected sand fly bites, involved a strong DTH reaction against saliva (26,41). In mice sensitized by bites, protection was associated with a strong upregulation of IFN-γ and IL-12 at the bite site, indicating the activation of macrophages to kill parasites and suggesting the acceleration of anti-*Leishmania* immunity.

The natural transmission model has been used in recent work by challenging mice with infected sand flies,

demonstrating the relevance of testing potential vaccine candidates using infected sand flies. The natural challenge model was first verified when mice vaccinated with glycoconjugates derived from PSG were protected against challenge against infected sand flies, but mice vaccinated with another antigen preparation, a *Leishmania* antigen plus IL-12 that was effective against needle challenge, had no protection against infected sand fly bites (42). In agreement with these findings, it was demonstrated that mice vaccinated with a killed vaccine comprised of an autoclaved *L. major* antigen and CpG oligodeoxynucleotides that conferred protection against needle challenge with parasites, failed to protect against infected sand fly challenge (43). More recently, it was demonstrated that two vaccine candidates, comprised of a *Leishmania* (KSAC) and a salivary gland antigen (LJM11), were able to control *Leishmania* infection following challenge with infected sand flies (32,44).

These recent findings emphasize the critical role that the sand fly plays in parasite transmission and how the adaptation of experimental models to this natural challenge should be considered in future vaccine development studies.

Visceral leishmaniasis

Visceral leishmaniasis (VL), the most severe form of leishmaniasis, is caused by parasites of the *Leishmania donovani* complex (*L. donovani* and *L. infantum*). VL severely compromises the spleen, liver and bone marrow of the host leading to fever, hepatosplenomegaly, pancytopenia, and cachexia and is fatal if not treated.

Different animal models, especially mice and hamsters, have been used and adapted to mimic human VL and to test vaccine candidates. In mice, chronic VL is successfully established after intravenous or intradermal inoculation (45). Disseminated granulomas with parasitized macrophages are found during infection, especially in the liver and spleen of susceptible (BALB/c, C57BL/6) and resistant (C3H.HeJ, CBA, DBA/2) mouse strains infected with *L. donovani* or *L. infantum* (29). Mice infected with *L. donovani* have been widely studied, but this model does not reproduce the features of active human VL (46). In mice, there is an early increase of parasite burden, but over the course of 4-8 weeks the infection spontaneously declines when an anti-*Leishmania* cellular immune response, with the participation of both $CD4^+$ and $CD8^+$ T cells, is able to control the infection. This control is mediated by IFN-γ production by splenic T cells, which are driven towards a Th1 phenotype by IL-12 (47-49). Interestingly, in endemic areas, there are significant numbers of individuals with subclinical infection associated with the development of antigen-specific T cell responses, IFN-γ production and resistance to visceral infection (50). Therefore, the *L. donovani* murine model seems to reflect the early parasite replication followed by immunological control and subclinical infection in human disease. However, there is still no murine model to study the progressive disease observed in human VL.

Vaccine studies with the murine models of VL are not as developed as the models used for CL. The levels of protection found in vaccine studies of murine VL are significantly lower when compared with murine CL models (46,51-59). It is unclear whether the low level of protection observed in VL vaccine studies is due to the higher infective dose, the routes of challenge used (intravenous versus subcutaneous or intradermal), the requirement for immunological effector control mechanisms that are not adequately induced by vaccination, or a combination of all factors (45). In order to address these variables and to improve the murine model of VL, an intradermal model of infection was explored and chronic VL was successfully established in susceptible mice. In this model, the course of infection was associated with parasite clearance in the liver and skin and persistence of *Leishmania* in the spleen and draining lymph node. Interestingly, a similar finding is also observed in subclinical canine and human VL. The site-specific parasite clearance or persistence is strongly correlated with distinct localized immune responses. Mice vaccinated with *L. infantum* D-13 (p80) antigen and challenged intradermally showed higher levels of protection when compared to a low-dose intravenous infection model (45). In contrast, the hamster model for VL mimics several aspects of human disease, such as hepatosplenomegaly, pancytopenia, progressive cachexia, hypergammaglobulinemia, and suppression of a T-cell proliferative response to parasite antigens (60-62). Hamster and human VL have been related to the inability of infected antigen-presenting cells to stimulate specific T cells (61,62). This is supported by the observation that, in spite of the production of Th1 cytokines (IL-2, IFN-γ and TNF-α) in the liver, spleen and bone marrow, the animal cannot control parasite replication, suggesting an impairment of macrophage function. Disease progression of hamster VL was associated with lack of nitric oxide (NO) due to the absence of NO synthase activity despite a strong IFN-γ production in the liver and spleen during the course of infection (63). Although the hamster VL model can be closely related to human VL in many clinical and pathological aspects, it is severely restricted by the limited availability of tools to dissect immune responses and mechanisms. Progressive disease in hamsters has been mostly achieved by intravenous, intracardiac or intraperitoneal injection of a large number of parasites (63-65). Gomes et al. (66) showed the fatal outcome of VL in 3- to 4-month-old naive hamsters after intradermal injection of *Leishmania* in the ear together with sand fly saliva. Hamsters developed classical signs of VL, culminating in a fatal outcome 5-6 months post-infection. Although saliva had no effect on the course of infection in this model, a novel 11-kDa protein (LJM19) from *L. longipalpis* saliva protected hamsters against the fatal outcome of VL.

LJM19-vaccinated hamsters maintained a low parasite load correlating with high IFN-γ, TGF-β and NO in the spleen and liver up to 5 months post-infection. Importantly, a DTH response with high expression of IFN-γ was also observed in the skin of LJM19-immunized hamsters 48 h after exposure to uninfected *L. longipalpis* bites. The induction of IFN-γ at the bite site could partly explain the protection observed in the spleen and liver of LJM19-immunized hamsters through direct parasite killing and/or priming of anti-*Leishmania* immunity (66).

The dog is considered to be the main reservoir of *L. chagasi* in Latin America. Disease control is based on culling of *Leishmania*-positive animals, which has generated ethical and social discussions. A vaccine capable of preventing canine visceral leishmaniasis (CVL) will be a good alternative for epidemiological control of all VL (67). In order to better understand leishmaniasis in dogs, several studies have investigated different inoculation routes and different parasite quantities of either amastigotes or promastigotes. Although those studies reproduced some features of naturally acquired CVL, in nature, transmission occurs after an infected sand fly takes a blood meal from the skin of the animal. Some of the naturally infected dogs will remain asymptomatic, but others will develop a progressive disease with lymphoadenopathy, weight loss, anemia, hypergammaglobulinemia and dermatitis ultimately resulting in death (68,69). Thus, a proper canine model for vaccine research should reproduce the immunopathological features of the natural disease. The dermis would be the preferential site of vaccine inoculation or infection challenge since intradermal inoculation mimics the delivery of antigen and/or infection by sand flies.

Several antigens, including live or killed parasites, purified *Leishmania* fractions and defined recombinant proteins, live recombinant bacteria expressing parasite antigens, antigen-encoding DNA plasmids and recombinant salivary proteins, have been identified and tested as potential vaccine candidates against CVL (32,66,70-73).

The use of dogs or rodents as models for a VL vaccine selection has allowed the development of two CVL vaccines that are commercially available: Leishmune and Leish-Tec. Leishmune vaccine is a prophylactic vaccine against CVL and was the first licensed second-generation vaccine against leishmaniasis (71). This vaccine consists of the fucose mannose ligand isolated from *Leishmania donovani* and saponin as an adjuvant (74). Leishmune showed efficacy in mice, hamsters and dogs and also protected dogs living in an endemic VL area in Brazil (75-77).

Leish-Tec was the second vaccine commercially developed against CVL and is composed of an amastigote-specific antigen (A2) and saponin as an adjuvant also induced protection in the field (76,77). These results support the importance of the dog as a model and target for vaccines against VL, an important approach to reducing disease incidence in dogs and ultimately reducing human infections.

Final remarks

When vaccines are being evaluated, an important point of concern is the identification of correlates of protection obtained from animal models. The selection of the right animal model, the pathogen-model combination, sometimes needs to be adapted to represent human infection. The immune response to a given pathogen may also vary from one animal strain to the other and the results of the administration of a potential vaccine candidate in an animal model may not be the same as results of human clinical and endemic area trials. Therefore, biomarkers obtained with animal models adapted for vector-borne parasitic diseases should always be carefully evaluated and validation using natural transmission of parasites is of the utmost importance since an infected bite is the final challenge that any vaccine will face in an endemic area.

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