MINIREVIEW

Transgenic Rodent Plasmodium berghei Parasites as Tools for Assessment of Functional Immunogenicity and Optimization of Human Malaria Vaccines

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Functional assessments of immune responses against stage-specific antigens of Plasmodium falciparum, the agent of the deadliest form of human malaria, are limited mainly to in vitro assays because P. falciparum cannot infect small animals, such as rodents. Hence, antibodies from individuals who have been naturally infected or vaccinated against malaria during clinical trials have been evaluated by enzyme-linked immunosorbent assays and, more functionally, by inhibition of liver-stage development assays (ILSDA), growth inhibition assays (GIA), or membrane feeding assays (MFA). Although the results obtained by these methods have been informative, these techniques remain in vitro assays that may not provide full explanations of the functional relevance and effectiveness of vaccine-elicited immune responses. Transgenic murine parasites expressing human malaria antigens have been developed to facilitate the optimization of the immunogenicity of vaccines and employed to directly address fundamental questions about the biological role of antibodies in vivo.

ASSESSMENT OF VACCINE-INDUCED IMMUNITY

A tremendous amount of effort has been exerted in the past two decades in identifying, characterizing, and testing various stage-specific malaria antigens as potential vaccine candidates. As a result, a number of candidate vaccines have undergone phase I and phase II clinical trials, with promising results. Some of the antigens (30) that have been well-characterized include circumsporozoite protein (CS) and thrombospondin-related anonymous protein, expressed in sporozoites; merozoite surface protein 1 (MSP-1), apical membrane antigen 1, and erythrocyte binding antigen 175, expressed on asexual blood stages; and P25, P48/45, and P230, expressed on sexual stages of the parasites. Though much is now known about the mechanisms of immunity and immune responses to some of these candidate vaccines, the main challenge that has been encountered in evaluating the functional in vivo efficacy of vaccine-induced immune responses is the lack of suitable small-animal models. By and large, most of the available assays are only in vitro surrogates, such as ILSDA (8) and hepatic invasion assays (17, 21, 31) for assessing neutralizing antisporezoite immunity, GIA (22, 24, 25, 32, 33) for assessing functional immune responses to asexual parasite stages, and MFA (3, 14, 18) for measuring immune responses against surface antigens present on gametocytes, gametes, or ookinete stages of the parasite. Nonhuman primates such as Aotus and Saimiri monkeys can be infected with adapted human malaria parasites, enabling the assessment of functional immune responses against malaria antigens in vivo; however, these animal models are not widely accessible, and the cost of maintaining primates is a limiting factor (38). The availability of suitable small-animal models for the in vivo assessment of vaccine-induced functional immune responses may play a significant role in the development and functional assessment of vaccines against human malaria. High-efficiency transfection protocols (11) have enabled the transfer of genes from human malaria parasites into rodent malaria parasites with relative ease, and here, we discuss and review the potential and feasibility of using such transgenic parasites (Table 1) in assessing antibody responses to various human malaria parasite stage-specific target antigens.

TRANSGENIC PARASITES BASED ON SPOROZOITE ANTIGEN CS

CS has been extensively characterized as a sporozoite antigen that can protect vaccinated individuals against sporozoite challenge. Various formulations of this antigen have undergone clinical trials, and in some clinical trials, the magnitude of protection was judged to be at least 50% (1, 2, 4, 35). Due to the lack of simple and convenient assays to measure the neutralizing effects of anti-CS antibodies in vivo, functional studies of anti-CS immunity have relied on in vitro assays such as ILSDA or sporozoite neutralization assays. The ability of sera from immunized animals or humans to inhibit sporozoite invasion of and development in hepatocytes has been assessed in vitro by ILSDA. In ILSDA, sporozoites are incubated with mouse or human hepatocytes in the presence or absence of test sera. After a washing step to remove excess sera or extracellular sporozoites, the infected mouse or human hepatocytes are incubated for an additional 2 or 5 days, respectively, and
TABLE 1. Transgenic parasites with potential use in assessing the functionality of human malaria immunity in vivo

<table>
<thead>
<tr>
<th>Transgenic P. berghei parasite(s)</th>
<th>Antigen</th>
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immunostained to detect the number of liver-stage exoerythrocytic schizonts (21, 31).

In an attempt to simplify the assessment of neutralizing anti-CS antibodies in vivo, Persson et al. (28) developed a P. berghei hybrid parasite expressing the repetitive region of P. falciparum CS (PfCS). In this transgenic parasite, the repetitive region of P. berghei CS containing B- and CD4+ T-cell epitopes essential for antisporeozoite protective immunity was replaced with the corresponding domains from the CS of P. falciparum. This transgenic parasite, designated CS(Pf), was employed to test whether antibodies against PfCS induced by active immunization or passively transferred would protect mice against CS(Pf) sporozoite challenge. By using this model, sterile immunity in actively immunized mice (CS7BL) was demonstrated and a significant degree of protection was achieved through passive immunization (28). The findings from this in vivo assay further substantiate the potential of CS-based target antigens to induce either sterile or protective immunity against sporozoite challenge. Other formulations of CS vaccines have also been tested and protective epitopes from linear synthetic peptides containing minimal B- and T-cell epitopes of PfCS have been examined using CS(Pf) in a murine model (6). Peptides containing CS B- and T-cell epitopes were immunogenic in mice and elicited antibodies neutralizing sporozoites of the CS(Pf) parasites (6), suggesting that preerythrocytic vaccine development can benefit from transgenic parasites in murine models. Furthermore, the neutralization potential of human sera from phase I clinical trials of a CS-based vaccine was assessed using the CS(Pf) parasite (27). The magnitude of inhibition observed in in vitro sporozoite neutralization assays (>90%) was comparable to that observed in vivo following the passive immunization of mice (28), and these results suggest that CS(Pf) can be reliably used to assess antisporeozoite immunity in vivo.

In addition to in vivo functional evaluation, the CS(Pf) parasites have been used for the development of a serum neutralization assay called a transgenic sporozoite neutralization assay (17). In this approach, the in vitro neutralization of CS(Pf) sporozoites by immune sera is assessed by a semiquantitative assay based on the estimation of the number of copies of P. berghei 18S RNA by real-time PCR (17). The transgenic sporozoite neutralization assay has been used to analyze serum samples from humans immunized with the CS-based peptide (T1B)4 MAP (17) and a different CS-formulated vaccine comprising B- and CD4+ and universal T-cell epitopes (10). Thus, CS(Pf) transgenic parasites can be applied in in vitro functional neutralization assays that may be more predictive of protection than assays such as enzyme-linked immunosorbent assays or immunofluorescence assays. Through the use of transgenic rodent parasites, it has become possible to quantify infectivity, as well as delineate humoral and cellular immune responses that are critical in anti-CS immunity (10, 17). The use of the CS(Pf) transgenic parasites has also demonstrated that linear peptides such as those described above need to be further explored for their potential as vaccines (6). Further vaccine immunogenicity and CS(Pf) sporozoite challenge studies can also help in dissecting the potential roles of various arms of cellular immune responses.

The advantages of using CS(Pf) have been further highlighted by the findings of studies demonstrating good correlation between the neutralization observed in ILSDA and sterile immunity in vivo as shown by the lack of liver exoerythrocytic forms (28). Such studies are important for vaccine development and could not otherwise be done with humans. Although the scope of this review is focused on transgenic murine parasites that express human malaria antigens for use in assessing functional immunogenicity, it should be noted that transgenic models also have applications in answering basic and fundamental biological questions that include the molecular basis for host-parasite relationships. For instance, transgenic P. berghei parasites expressing PfCS lacking the repetitive region I or region II have been used to examine the roles of these regions in sporozoite motility and the invasion of mosquito salivary glands (37).

**TRANSGENIC PARASITES BASED ON ASEXUAL BLOOD-STAGE ANTIGEN MSP-1**

The poor susceptibility of rodents to P. falciparum infection has restricted the functional assessment of antibodies against asexual stages of Plasmodium in vivo. Antibodies directed against antigens such as apical membrane antigen 1, erythrocyte binding antigen 175, and MSP-1 have been shown to interfere with parasite growth and entry into red blood cells in GIA (22, 24, 25, 32, 33). The GIA method is used to evaluate the effect of immune sera or specific antibodies on the asexual growth of parasites (19). Briefly, P. falciparum schizonts are typically cultured at 1% parasitemia and 2% hematocrit in the presence of test sera. After about 40 h of culture, infected erythrocytes are recovered and smears are prepared for microscopic examination or parasites are quantified by colorimetric assays based on the measurement of the activity of lactate dehydrogenase (16) to determine the percentage of growth inhibition.

However, in simple GIA it is not possible to appreciate the in vivo importance of other inhibitory factors, such as complement and cytokines, and other immune cells, such as monocytes. Thus, when measured by in vitro GIA, the overall in vivo potential for functional immune activity against parasite invasion and/or intracellular growth remains poorly understood at best. Limited studies have attempted to utilize an antibody-dependent cell-mediated cytotoxicity assay to overcome such a deficiency (36). Antibody-dependent cell-mediated cytotoxicity represents a humoral and cellular effector mechanism in which antigens in complexes with cytotoxic antibodies (immunoglobulin G1 [IgG1] and IgG3 in humans) trigger monocytes via the
Fc receptor to release soluble inhibitory molecules (5). These studies clearly stress the need for an in vivo model system to assess the functionality of elicited immune responses. In order to examine the in vivo biological activity of antibodies elicited by immunization against asexual-stage antigens, an allelic replacement of MSP-1, a protein essential for the invasion of red blood cells by *Plasmodium* parasites, was performed previously using *P. berghei* parasites in which native MSP-1<sub>19</sub>, the C-terminal portion of the protein which is associated with protection, was replaced by the corresponding MSP-1<sub>19</sub> region from *P. falciparum* (7). Using this chimeric *P. berghei* parasite (Pb-PfM19), de Koning-Ward et al. (7) addressed the challenging question of what protective role invasion-inhibitory antibodies play in limiting the growth of blood-stage parasites (7). Sera from mice infected with Pb-PfM19 inhibited the growth of *P. falciparum* in GIA, and when these sera were passively transferred into naive mice, they gave better protection against an infection with Pb-PfM19 parasites than immunization with *P. berghei* wild-type parasites, indicating the contribution of MSP-1<sub>19</sub> antibodies to immunity (7). Data generated by this mouse model have further established that antibodies that inhibit the entry of erythrocytes largely target the MSP-1<sub>19</sub> portion of MSP-1.

The advantages of using Pb-PfM19 transgenic parasites have been demonstrated by the importance of the type of antibodies produced against specific antigens during malaria; i.e., of all the anti-MSP-1<sub>19</sub> antibodies, it is the inhibitory antibodies that are mainly responsible for eliciting protection (7, 12, 26). These data further support the notion that a measurement of the total MSP-1<sub>19</sub> antibody titer in vaccine trials or clinical samples (9, 15, 25, 34) may not be a good predictor of protective immunity. The availability of transgenic rodent parasites has also opened numerous possibilities for studies aimed at dissecting effector mechanisms against specific human malaria antigens. In another study by McIntosh et al. (20), Pb-PfM19 parasites were used in transgenic mice expressing the human IgG1 Fc receptor to examine the role of Fcy receptor I (FcyRI) in anti-*Plasmodium* immune effector mechanisms. When passively immunized with anti-MSP-1<sub>19</sub> human IgG1, the FcyRI transgenic mice, in contrast to nontransgenic littermates, were completely protected against a lethal challenge with Pb-PfM19 parasites (20). This FcyRI transgenic mouse model, in conjunction with Pb-PfM19 parasites, revealed the importance of FcyRI-mediated pathways in parasite protective immunity (20).

**TRANSGENIC PARASITES BASED ON SEXUAL-_STAGE ANTIGENS PFS25 AND PV25**

Vaccine candidates based on sexual-stage-specific antigens such as P25, P48/45, and P230 are expected to play a critical role in reducing malaria transmission (19). However, progress in evaluating the in vivo efficacy of transmission-blocking vaccines (TBV) has been limited once again by the absence of small-animal models that can be used for *P. falciparum* and *P. vivax* challenge experiments. The evaluation of the biological activities of transmission-blocking antibodies relies on MFA (3, 14, 18). This assay involves feeding test antibodies or sera and infectious gametocytes to starved mosquitoes through a thin membrane such as parafilm by using a water-jacketed glass cylinder and then enumerating oocysts in the fed mosquitoes to evaluate the transmission-blocking effect of the test sera. Though MFA has been employed widely, the method has some drawbacks in that it is an ex vivo assay which is cumbersome and requires a reliable source of infectious gametocytes. While such gametocytes of *P. falciparum* can be obtained in culture, there is no culture system for *P. vivax* gametocytes, and blood from human volunteers or chimpanzees infected with *P. vivax* has served as the only source of gametocytes. An animal model also becomes critical especially for the development of TBV, because unlike candidate vaccines targeting other stages of the malaria parasite, functional efficacy of TBV cannot be assessed by immunizing humans, as currently there are no ethically acceptable guidelines for parasite challenge for such an approach (13).

In an attempt to assay transmission-blocking immunity in vivo, genetically modified *P. berghei* parasites expressing the zygote-ookinete antigen P25 were developed. Full-length *P. vivax* P25 (Pvs25) and truncated Pvs25 (in which the glycosylphosphatidylinositol [GPI] sequence was replaced with the GPI sequence of *P. berghei* P25 [Pb25]; the GPI moiety is important in anchoring P25 on the surfaces of zygotes and ookinetes) were used to replace endogenous Pb25 and Pb28, generating Pv25DR and Pv25DR3 transgenic parasites, respectively (29). These *P. berghei* transgenic parasites expressed Pvs25 in the zygote and ookinete stages. Subsequently, Pv25DR and Pv25DR3 were employed to test the transmission-blocking potential of human sera from a clinical trial based on Pvs25 (29). In parallel, transgenic *P. berghei* parasites expressing *P. falciparum* P25 (Pfs25) have also been developed, and these transgenic parasites, designated TrPfs25Pb, were found to be susceptible to anti-Pfs25 antibodies during mosquito-stage development (23). TrPfs25Pb parasites displayed Pfs25 on the surfaces of zygotes and ookinetes. When passively transferred into mice, immune sera from nonhuman primates (rhinos monkeys) immunized with a Pfs25-based vaccine blocked the transmission of TrPfs25Pb to *Anopheles stephensi* by a magnitude of at least 90%. Moreover, mice immunized with a Pfs25 DNA vaccine and challenged with TrPfs25Pb displayed reduced malaria transmission compared to mice immunized with a control wild-type plasmid (23). The results of these studies have established that murine malaria models can serve as an alternative to in vitro MFA for the evaluation of *P. falciparum* or *P. vivax* TBV based on the target antigen P25. Further studies to define the stage specificities of transmission-blocking antibodies were also attempted using an in vitro ookinete assay with Pv25DR, Pv25DR3, and TrPfs25Pb parasites (23, 29). In this assay, blood-stage parasites are cultured to produce ookinetes (a motile stage of the parasite formed after zygote formation) in the presence of antibodies to assess transmission-blocking activity, and the magnitude of blocking varied from 50 to about 70% (23, 29). While more work is required in refining the in vitro ookinete assay, this assay has the potential to be used as a surrogate to measure transmission-blocking immunity without the need for maintaining mosquitoes. The development of Pv25DR and Pv25DR3 transgenic *P. berghei* parasites is additionally significant because *P. vivax* gametocytes cannot be cultured in vitro, as needed for MFA. Even though erythrocytic stages of *P. falciparum* can be cultured, the maintenance of infectious gametocytes is highly sensitive to the
strain of *P. falciparum* and the quality of human serum samples, culture media, and other facilities used for the maintenance of cultures. The development of TrPfs25Pb provides a convenient and affordable alternative to expensive and specialized culture facilities needed for the routine culture of infectious *P. falciparum* gametocytes and the direct assessment of the transmission-blocking potential of vaccine-induced antibodies. The use of in vivo animal model systems is also expected to provide better correlates of immunity than in vitro/ex vivo MFA, as the latter may not represent the full potential of vaccine-induced immune sera. Transgenic parasites expressing PfS25 and Pvs25 thus provide powerful tools for assessing TBV in vivo.

**FURTHER PERSPECTIVES**

We believe that using an animal model to test immune sera against various stage-specific human malaria antigens would complement in vitro assays and provide additional valuable tools toward the development of malaria vaccines. Such animal models as those discussed in this review measure the functional activity of elicited immune mechanisms in vivo and thus take into account additional immune factors that may synergize the functional blocking activities of antibodies. The development of transgenic parasites has also provided biological information demonstrating the functional conservation of many target antigens between human and murine malaria parasites; for example, PfCS, MSP-1, PfS25, and Pvs25 successfully complement functions of their orthologues in *P. berghei* (7, 23, 28, 29). In addition to revealing this functional conservation, transgenic models have also permitted the assessment of human or nonhuman primate sera and have begun to permit the dissection of immune effector mechanisms in response to human malaria antigens, studies that could not otherwise have been feasible with humans (20). While the development of transgenic murine parasites offers opportunities for assessing the functional effectiveness of immune mechanisms in vivo, it is prudent to mention that it is possible that the allelic replacement of vaccine antigens in rodent parasites may exclude certain relevant B- and/or T-cell epitopes important for the activation of human immune cells. In other instances, although antigens may be expressed, the epitopes may be nonfunctional and may not induce a comparable functionally relevant immune response in mice.

The availability of transgenic parasites expressing representative target antigens from the three major life stages of the parasite, i.e., sporozoite, blood stage, and sexual stage (Fig. 1), presents the malaria scientific community with critical and valuable tools that permit the direct evaluation of the functionality of immunity against human malaria parasites in vivo by using murine models. These animal models should also continue to play an important role in the preclinical optimization of the various vaccine formulations under development. Even though proof of the concept that human sera can be evaluated in rodents for some of the major vaccine candidate antigens has been demonstrated previously, modifying the currently existing transgenic parasites to express multiple human malaria antigens may allow the simultaneous testing of components of cocktail vaccines targeting various stages of the parasite.

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