Commentary

A Robust Neutralization Test for *Plasmodium falciparum* Malaria

By Allan Saul and Louis H. Miller

From the Malaria Vaccine Development Unit, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, Maryland 20852

Malarial merozoites are released from infected erythrocytes, passing rapidly through the plasma to invade normal erythrocytes. It has long been thought that antibodies directed against merozoite surface proteins (MSPs) play an important role in natural immunity against *Plasmodium falciparum* and that these antibodies may at least in part act through blocking erythrocyte invasion by merozoites. Indeed, such a view underpins much of the search for antigens suitable for use as vaccines, targeting blood stage parasites, and has a major influence on ideas on the acquisition of active immunity and on the passive protection of infants through uptake of maternal antibodies. Although convincing evidence that antibodies from immune sera can clear an infection has been obtained by passive transfer experiments (1–4), IgG did not block invasion (3, 4). This paradox may be explained by other actions of antibody such as clearance of infected erythrocytes through recognition of *P. falciparum* erythrocyte membrane protein 1 on their surface (5) or through antibody-dependent cellular inhibition of parasite growth (6). However, data are lacking on the effect of passive transfer of IgG that has blocking antibodies.

Clearly antibodies can block invasion in vitro as has been demonstrated with monoclonal and polyclonal antibodies directed against merozoite antigens and with human antibodies affinity purified on recombinant merozoite antigens (7, 8). In keeping with the very short time that merozoites are exposed before invasion, the antibody concentrations required are high and are close to the upper limits of what is possible in immune sera (9). Without using purified and concentrated IgG, the maximum concentration of serum that can be used in in vitro invasion assays is only 20% and consequently, the antibodies are, at best, only 20% the concentration found in vivo. The assays for measuring parasite growth rates in vitro are technically difficult with relatively large errors. Thus, the levels of inhibition of parasite invasion that may be highly significant in vivo are difficult to reproducibly measure in vitro. The paper by O'Donnell et al. in this issue (10) is important because it convincingly demonstrates that most sera from two high transmission areas in Papua New Guinea are not only able to inhibit parasite invasion in vitro but that much of that inhibition can be attributed to antibodies directed against one protein, MSP-1. This is a remarkable finding since, not only is MSP-1 just one of thousands of proteins in the parasite, but this activity is directed at a small portion of MSP-1: the COOH-terminal 98 amino acids (MSP-1_{19}) of the ~1,700 amino acid–long MSP-1.

In a few special cases, it has been possible to use a naturally occurring deletion mutant or sequence variants to assess the impact of a specific antibody on parasite growth. For example, a deletion of the ring erythrocyte surface antigen gene in the FCR-3 line of *P. falciparum* was used to assess the relative importance of antibodies that reacted with both the ring erythrocyte surface antigen and Ag332 proteins in blocking invasion (11).

In the paper by O'Donnell et al. in this issue, a pair of matching parasite lines was generated by replacing the segment coding for the *P. falciparum* MSP-1_{19} portion of MSP-1 by the orthologous gene segment from a rodent malaria, *P. chabaudi*, using allelic recombination. The *P. chabaudi* MSP-1_{19} is sufficiently different to the *P. falciparum* MSP-1_{19} in that antibodies are specific for one form or the other, yet similar enough to allow the chimeric MSP-1 protein to be functional. As malaria parasites are haploid during the blood stage, this single replacement creates a parasite line genetically identical to its parent line, except for the expression of MSP-1_{19}. This allows the effect of anti–MSP-1_{19} antibodies to be more accurately measured than was previously possible. This is particularly important for assessing the anti–MSP-1_{19} activity in the complex mixture of antibodies with many different specificities to conserved and polymorphic antigens in sera from people's natural exposure to *P. falciparum*. We expect that this technique will be a valuable method for evaluating immune responses for those malarial antigens where similar replacements can be made.

Several other merozoite antigens, such as MSP-2, -3, GLURP, ABRA, the apical membrane antigen 1, erythrocyte membrane antigen 175, and the rhoptry proteins RAP-1 and RAP-2 are also under active consideration as

Address correspondence to A. Saul, MVDU/LPD/NIAID, Twinbrook I Rm. 1113, 5640 Fisher Lane, Rockville, MD 20852. Phone: 301-594-2701; Fax: 301-435-6725; E-mail: ASaul@niaid.nih.gov

targets of protective immunity (12). Presumably, other proteins, as yet undescribed, also occur on merozoites. On the assumption that different inhibitory specificities in the test sera are additive, we calculate that the anti–MSP-1_{19} activity in the sera tested by O'Donnell et al. are responsible for ~50% of the inhibition seen. This suggests that for natural immunity, antibodies to this antigen may play a major role in reducing parasite multiplication rates and, by implication, the burden of disease. If in vitro merozoite neutralization indicates protection in vivo, then these data further strengthen the belief that MSP-1_{19} is one of the top prospects for an antimalarial vaccine to reduce death in Africa.

There is already considerable evidence from studies with rodent malaria and from challenge studies with *P. falciparum* in primates that vaccines based on MSP-1_{19} and on the larger form of the antigen, MSP-1_{42}, protect against malaria (13). One of the concerns has been that protection in some of these models is only obtained at very high antibody levels and it has been unclear if these could be obtained or maintained in humans. This paper now convincingly shows that the level of antibody obtainable in natural infections is sufficient to have a significant impact on parasite growth, and presumably, the antibody is maintained by repeated boosting of natural infections. In fact, considering that the concentration of antibodies in the in vitro tests used by O'Donnell et al. is only 5% of the concentration in the circulation, one may wonder how some of these adults could be infected at all.

Using the parasites developed by O'Donnell et al., it may now be possible to follow the acquisition and loss of invasion inhibiting immunity directed to MSP-1_{19} with time. It will be interesting to reexamine the role of anti–MSP-1_{19} in the age dependence of acquisition of immunity. Early data showed that sera from adults in the Madang area have a higher level of in vitro inhibition than sera from children (14). Of practical importance, these studies may also enable the development of better in vitro correlates of protection elicited by vaccines. In particular, the ability to correlate levels of antibodies to specific MSP-1_{19} epitopes with in vitro inhibition would facilitate the development of optimum formulations and dosing regimens for human trials. Other mechanisms of action of anti–MSP-1_{19} immunity have been proposed. Now that a more defined assay is possible for measuring the role of anti–MSP-1_{19} in in vitro–growth inhibition, it may become possible to assign the relative importance of antibody-mediated inhibition or merozoites invasion and other protective mechanisms such as antibody-dependent cellular inhibition to the in vivo protection resulting from vaccination.

The observation by O'Donnell et al. that a significant part of the in vitro invasion inhibitory activity of immune human sera is directed against MSP-1_{19} provides direct evidence that this part of MSP-1 may be under immune selection pressure. Because this portion of the molecule can be replaced by the ortholog from *P. chabaudi* which differs by 57 of 98 amino acids (Fig. 1), O'Donnell et al. raise the possibility in this issue and in an earlier paper (15) that the apparent lack of strict sequence conservation required to maintain function could allow the rapid selection of mutations if this region is targeted by vaccines. Indeed some diversity is already found in this region of the *P. falciparum* sequence (16).

In our view, there are several reasons why rapid selection of escape mutants is unlikely. First, indirect evidence from genotype frequencies determined by molecular epidemiology suggests that variation in this region is primarily due to genetic drift and is not under strong immune selection for diversity ($F_{ST} > 0$; references 17 and 18). Interestingly, all monoclonal antibodies described to date that inhibit *P. falciparum* invasion in vitro do not recognize variant sequences (19, 20), although approximately half of all MSP-1_{19} monoclonal antibodies are variant specific (19). In some animals,

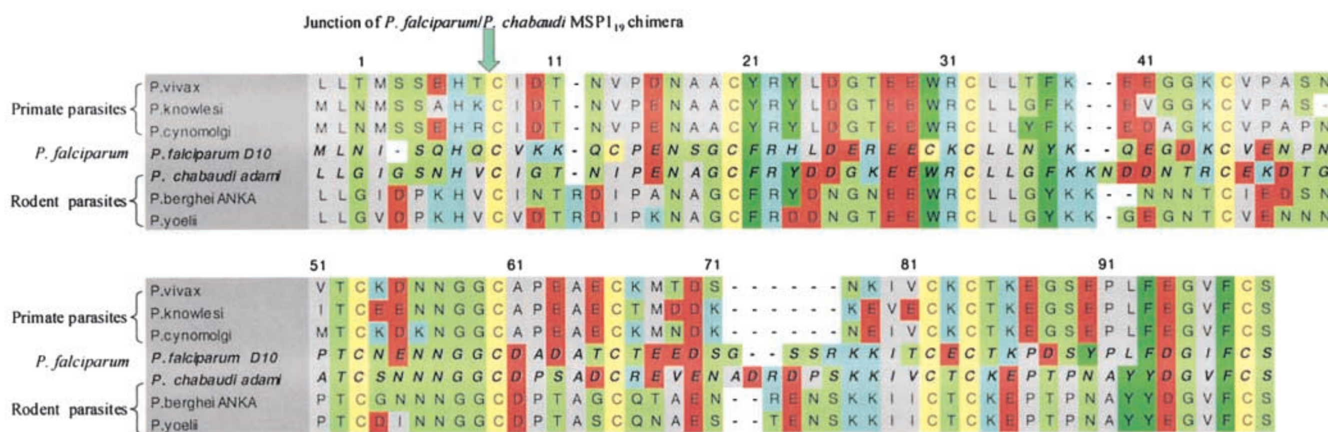

Figure 1. Alignment of the MSP-1_{19} sequences from *P. vivax* (GenBank/EMBL/DDBJ accession no. AF91157), *P. knowlesi* H strain (GenBank/EMBL/DDBJ accession no. AF298219), *P. cynomolgi* (GenBank/EMBL/DDBJ accession no. AAC46960), *P. falciparum* D10 cloned line (GenBank/EMBL/DDBJ accession no. AAA29653), *P. chabaudi adami* (GenBank/EMBL/DDBJ accession no. AF149303), *P. berghei* ANKA (GenBank/EMBL/DDBJ accession no AAF13063), and *P. yoelii* YM (GenBank/EMBL/DDBJ accession no AAA29702). Numbering commences from the start of the MSP-1_{19} fragment in *P. falciparum*. The arrow between amino acids 7 and 8 marks the junction of the *P. falciparum*–*P. chabaudi* MSP-1_{19} chimera created by O'Donnell et al. (reference 10).

recombinant MSP-1_{19} generates variant specific polyclonal antibodies (13). Thus, although variant sequences are recognized, the dominant targets of protective immunity in this molecule appear to be invariant.

Second, the constraints on the sequence may be much greater than apparent from a comparison of the *P. chabaudi* and *P. falciparum* sequences. The structure of MSP-1_{19} has been determined from *P. falciparum* (21) and the *P. cynomolgi* ortholog (22). As expected from the primary sequence, MSP-1_{19} consists of two epidermal growth factor (EGF) domains. As a result, the structure is highly constrained. MSP-1_{19} orthologs are known from seven species and, other than *P. cynomolgi*, from multiple isolates of each species. Consistent with other studies (23), these sequences fall into three clades: sequences from primate malarias (*P. vivax*, *P. knowlesi*, and *P. cynomolgi*); sequences from P. *falciparum*; and sequences from rodent malarias (*P. chabaudi*, *P. berghei*, and *P. yoelii*). A three-way comparison shows that the variation between these sequences is not random, and in many places, the three groups of sequences could be considered to be chimeras of two archetype sequences (Fig. 1). For example, in the first 35 amino acids, the primate and rodent sequences have identical or highly conserved substitutions at positions 11, 14, 18, 23, 26, 30, and 31 that differ from the *P. falciparum* sequence. On the other hand, there are many places where the *P. falciparum* and the primate malaria sequences are similar but differ from the rodent malaria sequences (e.g., amino acids 85, 86, 89, 91, 92, and 93 in the COOH-terminal portion of MSP-1_{19}). Overall, there are only 32 of 98 positions where the *P. falciparum* sequence does not follow the primate or rodent malaria sequences. As suggested by O'Donnell et al., many of the unique *P. falciparum* substitutions are apparently linked. For example, examination of the structures of *P. cynomolgi* MSP-1_{19} (22) and *P. falciparum* MSP-1_{19} (21) suggests that replacement of the disulfide bond formed from $C_{19}C_{30}$ in *P. falciparum* with $V_{14}W_{30}$ requires the replacement of S_{18} with A_{18} to create the hydrophobic pocket surrounding W_{30}. In the available structures, there are few amino acids not linked in this way. For example, K_{10} has a side chain that projects out from the first EGF domain. Even for these, their side chains may well interact with other parts of the MSP-1 molecule or other interacting proteins such as MSP-6 (24).

We come to a very different interpretation of the evolution of MSP-1_{19} to that proposed by O'Donnell et al. In our view, much of the diversity in MSP-1_{19} is very ancient, long predating the speciation that led to the current species. Therefore, it is likely that even for the potentially greater immune pressure induced by vaccination, functional constraints in the two EGF domains will substantially restrict the generation of new sequence variants.

Of all candidate vaccines for preventing disease from *P. falciparum* malaria, MSP-$1_{19/42}$–based vaccines are considered leading candidates and are to be shortly tested in the field. The discussion that we have presented argues for limited selection of mutants, even if the vaccine is effective in reducing disease. Although we cannot predict its effectiveness or the persistence of this efficacy in the coming years, the assay devised by O'Donnell et al. will greatly facilitate serologic tests for correlates of protection in the field. It will be one measure of an immune response to correlate with protection that may add power to the trial, similar to the neutralization test employed by virologists.

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