The carboxy-terminus of merozoite surface protein 1: structure, specific antibodies and immunity to malaria

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SUMMARY

Over the last 30 years, evidence has been gathered suggesting that merozoite surface protein 1 (MSP1) is a target of protective immunity against malaria. In a variety of experimental approaches using *in vitro* methodology, animal models and sero-epidemiological techniques, the importance of antibody against MSP1 has been established but we are still finding out what are the mechanisms involved. Now that clinical trials of MSP1 vaccines are underway and the early results have been disappointing, it is increasingly clear that we need to know more about the mechanisms of immunity, because a better understanding will highlight the limitations of our current assays and identify the improvements required. Understanding the structure of MSP1 will help us design and engineer better antigens that are more effective than the first generation of vaccine candidates. This review is focused on the carboxy-terminus of MSP1.

Key words: malaria, merozoite, MSP1, structure, antibody.

INTRODUCTION

One of the best studied molecules on the surface of the asexual blood-stage malarial parasite is merozoite surface protein 1 (MSP1). This molecule was first described nearly 30 years ago, and in the intervening period much of the work on the potential of the molecule as a vaccine candidate has focused on understanding the structural diversity of the molecule and the consequences of these structural aspects for its immunogenicity and antigenicity.

In the first malaria vaccine studies with a purified protein, MSP1 was shown to confer protection against challenge infection in a rodent parasite model, *Plasmodium yoelii* in laboratory mice (Holder and Freeman, 1981). Passive immunization with certain monoclonal antibodies (mAbs) also provided protection in the same model and highlighted the importance of antibody in the protective mechanisms targeting MSP1 (Majarian *et al*. 1984; Spencer Valero *et al*. 1998). Most recently, genetic analysis of strain-specific immunity has also implicated MSP1 as a major target of strain-specific immunity in *Plasmodium chabaudi* infection of mice (Pattaradilokrat *et al*. 2007). Elucidation of the gene sequence of MSP1 from *P. falciparum* (Holder *et al*. 1985; Tanabe *et al*. 1987) and other species enabled detailed structural analysis and, in particular, identified an approximately 100 amino acid sequence at the C-terminus comprised of 2 epidermal growth factor (EGF) domains (Blackman *et al*. 1991) and called $MSP1_{19}$. This disulphide-rich structure was subsequently shown to be the target of the protective antibody used in the first passive immunization study (Burns *et al*. 1988), and other antibodies such as mAbs 12·8 and 12·10 that recognized the EGF domains in *P. falciparum* MSP1 and prevented merozoite invasion of red blood cells in culture (Blackman *et al*. 1990). The potential importance of the MSP1 EGF domains as a vaccine component was confirmed when it was shown that immunization with these domains produced in recombinant form provided protection against challenge infection with blood-stage rodent parasites (Daly and Long, 1993; Ling *et al*. 1994).

The availability of recombinant protein has allowed the numerous studies that have examined whether or not individuals naturally exposed to malaria parasites have antibodies to the protein and studies on whether or not the presence of such antibodies correlates with protective immunity. Overall the results have been contradictory, with some studies suggesting that MSP1 is important in naturally acquired immunity (Egan *et al*. 1996; Dodoo *et al*. 2008) and others suggesting that it is not (Dodoo *et al*. 1999). Similarly, the results of direct immunization studies have sometimes been contradictory. More recently transgenic methodology has allowed specific questions on the role of MSP1 and corresponding antibodies to be addressed using genetically manipulated live parasites (O'Donnell *et al*. 2001; de Koning-Ward *et al*. 2003; McIntosh *et al*. 2007). Some possible explanations for the contradictions are provided by studies on the structure of $MSP1_{19}$ and the fine specificity of the antibodies binding to it. This review will focus on some aspects of the recent work in this area.

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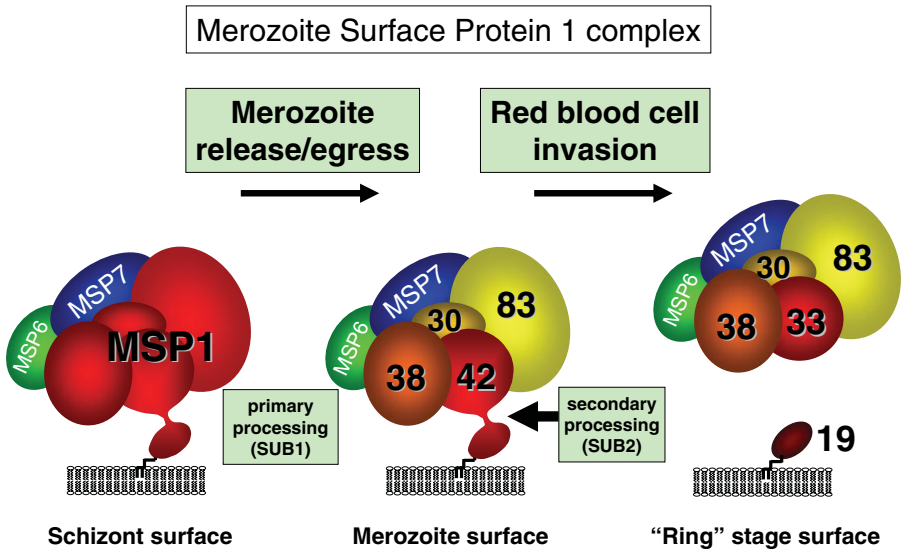

Fig. 1. The assembly and processing of the MSP1 complex. MSP1 is synthesized and associates with other proteins, particularly MSP7 and MSP6 on the surface of the developing schizont and linked to the plasma membrane by the glycosyl phosphatidyl inositol (GPI) anchor. At or just before parasite egress and merozoite release the complex is cleaved into a series of fragments by the subtilisin-like protease SUB1 (Koussis *et al*. 2009); for clarity only the processing of MSP1 is shown but both MSP6 and MSP7 are also processed. Some products of this primary processing remain associated on the merozoite surface. When the merozoite invades a red cell, secondary processing, mediated by another subtilisin, SUB2 cleaves the membrane-anchored 42 kDa MSP1 fragment into further 33- and 19 kDa fragments (Harris *et al*. 2005). The complex, including $MSP1_{33}$, is shed from the merozoite surface whilst the C-terminal $MSP1_{19}$ is carried into the newly invaded red blood cell (Blackman *et al*. 1990). Following invasion $MSP1_{19}$ is rapidly internalized into the forming nascent food vacuole, where it persists to the end of the parasite's intracellular development before being discarded in the residual body (Dluzewski *et al*. 2008)

RELATIONSHIP BETWEEN STRUCTURE, ANTIGENICITY AND IMMUNOGENICITY

In order to understand the interaction of antibodies with MSP1 it is important to understand some of the structural features of the molecule that are relevant to the biology of the parasite and how antibodies that bind to MSP1 may interfere with the processes involved.

MSP1 is synthesized from the onset of schizogony (Holder and Freeman, 1982) as a precursor that rapidly associates with MSP7 (Pachebat *et al*. 2001, 2007) and the complex is transported to the surface of the intracellular parasite where it is retained as a result of its glycosyl phosphatidyl inositol (GPI) anchor. At the end of schizogony merozoite release (or egress) from the infected red blood cell is accompanied by proteolytic processing of the complex by the protease subtilisin 1 (Sub 1) (Koussis *et al*. 2009). This so-called primary processing produces a complex of polypeptides held together by non-covalent interactions on the surface of the merozoite (Holder *et al*. 1987; Lyon *et al*. 1987; McBride and Heidrich, 1987). MSP1 gives rise to 4 fragments that have been named based on their apparent size in SDS-polyacrylamide gel electrophoresis, an N-terminal 83 kDa fragment ($MSP1_{83}$), internal 30 and 38 kDa fragments ($MSP1_{30}$ and $MSP1_{38}$) and a C-terminal 42 kDa fragment ($MSP1_{42}$). The product of a third gene (MSP6) is also part of this complex on the merozoite surface (Trucco *et al*. 2001) and both MSP7 and MSP6 are also processed. MSP1 is also dimeric (Sanders *et al*. 2007), an association in part mediated by sequences within $MSP1_{42}$ (Babon *et al*. 2007). The significance of the primary processing of the MSP1 complex is still obscure, although it is possible that it causes conformational changes leading to acquisition or change of function for the protein that is now on the surface of the free merozoite. An even more profound change to the structure of the MSP1 complex occurs at the time of merozoite invasion of red blood cells. The parasite protease subtilisin 2 (Sub2) (Barale *et al*. 1999; Hackett *et al*. 1999; Harris *et al*. 2005) cleaves the C-terminal $MSP1_{42}$ into 2 fragments: a N-terminal 33 kDa fragment ($MSP1_{33}$) and a C-terminal 19 kDa fragment ($MSP1_{19}$). As a consequence of this secondary processing $MSP1_{33}$ is shed from the surface with the rest of the MSP1 complex, and $MSP1_{19}$ is retained by its GPI anchor on the surface of the invading parasite (Blackman *et al*. 1990). This entire process is summarized in Fig. 1. Very quickly $MSP1_{19}$ is then internalized and is the first known marker of the developing food vacuole; interestingly, it remains in the food vacuole for the remainder of the parasite's intracellular development and may have a function in this location (Dluzewski *et al*. 2008).

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FUNCTION OF ANTIBODIES TO MSP1

How are antibodies binding to MSP1 likely to affect parasite growth and development in the asexual blood stage? It is clear that MSP1 is on the surface of the free merozoite and therefore specific antibodies can bind to it. However, the ways in which this binding can result in a reduction of parasite numbers are several. Largely speaking these ways are either dependent on the binding of the antibody molecule alone or mediated through additional mechanisms recruited by interactions of the constant (Fc) region with other components of the immune system. Different antibody classes and subclasses may have a different valency, shape and size as well as different Fc-based specificities, which can affect both of these mechanisms (Pleass and Holder, 2005; Shi *et al*. 2006). The concentration, avidity and fine specificity of binding of the antibodies will also have major roles, as will be outlined later. In a number of studies the binding of both mono- and polyclonal antibodies has been examined in some detail and the findings correlated with the functional properties of the antibodies. Five mechanisms can be proposed for the action of antibodies based on growth inhibition assays *in vitro* and *in vivo* studies, as follows.

Growth inhibition assays

Parasites cultured in the presence of MSP1-antibodies may show reduced growth *in vitro* suggesting that the antibodies are interfering with an essential step during merozoite invasion or subsequent development. The mechanisms are thought to depend entirely on antibody binding alone, since there are no immune cells in such assays and these antibodies have been suggested to be a major component in human plasma that inhibit erythrocyte invasion (O'Donnell *et al*. 2001). The exact mechanisms of action are unknown and may comprise several distinct activities, for which the relative importance of each is still unknown.

- Agglutination of merozoites. It has been known for some time that antibodies can agglutinate merozoites and thereby prevent their dispersal from the ruptured schizonts (Green *et al*. 1981). More recently, merozoites expressing protein on their surface from a $MSP1_{19}$ minigene construct were shown to be agglutinated by specific antibody (Gilson *et al*. 2008).
- Preventing MSP1 interaction with other parasite molecules or red cell receptor binding. Since MSP1 interacts with other molecules of both parasite and host origin it is possible that specific antibodies can interfere with both these processes. There is no evidence that host antibodies can interfere with or reverse the interaction between, for example, MSP1 and MSP7, even though a mAb designated 89·1 appears to bind to the same site on MSP1 as MSP7 (Pachebat *et al*. 2007). Several reports have suggested that MSP1 binds to the red cell surface (for example, Goel *et al*. 2003) and in some studies this attachment appears to be reversed by the binding of a specific antibody (Perkins and Rocco, 1988).
- Inhibition of the secondary processing of MSP1 by Sub2. Inhibition of secondary processing prevents release of the MSP1 complex from the merozoite surface and can be detected following antibody addition to parasite cultures or to preparations of merozoites (Blackman *et al*. 1994; Guevara Patino *et al*. 1997). Furthermore, antibodies that inhibit secondary processing of MSP1 can also be found in the sera of individuals naturally exposed to malaria (Nwuba *et al*. 2002). Thus, it is thought that these antibodies may interfere sterically with the ability of the Sub2 protease to access its cleavage site. $MSP1_{19}$-specific mAbs that inhibit secondary processing also inhibit erythrocyte invasion, whereas others that do not affect processing have no effect on invasion, suggesting that inhibition of processing may be a important antibody function (Blackman *et al*. 1994).
- Interference with parasite growth post-invasion. There have been several reports that antibodies to MSP1 can adversely affect the intracellular development of the parasite (Bergmann-Leitner *et al*. 2006; Woehlbier *et al*. 2006; Arnot *et al*. 2008). Although the mechanism of this effect is unknown, it is known that antibodies bound to $MSP1_{19}$ are carried into the erythrocyte on the surface of the newly invaded parasite and both $MSP1_{19}$ and bound antibody are rapidly transported to the food vacuole (Blackman *et al*. 1994; Dluzewski *et al*. 2008). Perhaps the antibodies interfere with a novel function of $MSP1_{19}$ within this location?

Immune mechanisms to MSP1 in vivo

In addition to the effects of MSP1-specific antibodies detected by adding them to parasites in culture, there is good evidence that the Fc portion of antibodies is important to recruit effector cells, for example to promote merozoite phagocytosis or NADPH oxidase activation and degranulation (Pleass *et al*. 2003; McIntosh *et al*. 2007).

THE IMPORTANCE OF ANTIBODY FINE SPECIFICITY

Although MSP1 is by definition on the merozoite surface, this location does not imply that all parts of the protein will be equally accessible to antibody. Some epitopes may be formed or obscured as a result of binding to other molecules or any conformation changes that result from, for example, proteolytic processing. Whilst binding to any accessible epitope

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may be sufficient for antibodies that lead to cross-linking and agglutination or Fc-mediated effects, other functions such as inhibition of processing require antibody molecules to bind to particular areas of the molecule and therefore the fine specificity of antibody binding is crucial to its function.

Studies employing the inhibition of MSP1 processing and growth inhibition assays defined 3 different classes of monoclonal and polyclonal antibody (Blackman *et al*. 1994; Guevara Patino *et al*. 1997). Inhibitory antibodies are antibodies that inhibit MSP1 secondary processing and inhibit invasion; neutral antibodies do not inhibit processing or invasion; and importantly a third class of so-called 'blocking antibodies' do not inhibit processing or invasion but facilitate invasion in the presence of inhibitory antibodies by competing with the inhibitory antibodies for binding to the antigen. Whilst all inhibitory antibodies bound to $MSP1_{19}$ some blocking antibodies are specific to epitopes formed from amino acids that are remote in the primary sequence (Guevara Patino *et al*. 1997).The presence of these different classes of antibodies in the sera of children developing immunity to malaria in a malaria-endemic area highlights the importance of understanding the fine specificity of the antibody binding (Nwuba *et al*. 2002; Corran *et al*. 2004; Okech *et al*. 2004; Omosun *et al*. 2008).

It has been proposed that the induction of blocking antibodies represents a mechanism of immune evasion, in that such antibodies will cancel out the positive effects of inhibitory antibodies (Holder *et al*. 1999). One prediction of this hypothesis is that the epitopes for blocking antibodies would be conserved since the immune selection pressure would not be to drive polymorphism but to preserve similarity in different parasite populations.

Competition between antibodies will be determined by their concentration, avidity or affinity and fine specificity, as well as the effect of overlapping epitopes and whether or not the binding of the first antibody affects the structure of the protein. The structure of the molecule on the merozoite surface is also unknown – for example it is possible that other parts of the MSP1 complex (see Fig. 1) or other surface molecules may sterically interfere with the access of an antibody. MSP1 on the parasite surface is also a dimer – and the consequences of this for antibody binding are unknown.

THE 3-DIMENSIONAL STRUCTURE OF $MSP1_{19}$, AND THE LOCATION OF EPITOPES

The 3-dimensional structure of $MSP1_{19}$ from a number of parasite species has now been solved using either crystallographic or nuclear magnetic resonance (NMR) techniques (Chitarra *et al*. 1999; Morgan *et al*. 1999; Garman *et al*. 2003; Pizarro *et al*. 2003; Babon *et al*. 2007). These data indicate that the 2 EGF domains interact closely with each other through hydrophobic interactions so that the N-terminus is close to the C-terminus (see Fig. 2). The predicted disulphide bonds are present in the structures. Although sequence alignments reveal the similarity of $MSP1_{19}$ across the species, one interesting feature is that the location and nature of charge residues differs substantially across the species. The molecule can be considered to be a relatively flat structure with 2 faces (the left and second to right panels of the displays in Fig. 2), and one face (the left hand side) is relatively hydrophobic compared with the other face. Whilst the structure is largely compact, NMR studies have shown that the large loop in the second domain is highly mobile in the solution structure (Morgan *et al*. 1999). Interestingly this loop is the location of the greatest sequence differences between the $PfMSP1_{19}$ types and of considerable heterogeneity in other species (Benjamin *et al*. 1999), but the importance of $MSP1_{19}$ sequence polymorphism in immune evasion is still unclear.

Early studies had shown that formation of disulphide bonds was important for both the binding of antibodies and the immunogenicity of the protein. For example, reduction and carboxymethylation to prevent reoxidation of the cysteines abolished the ability of the protein to provide protection in the *P. yoelii* model (Ling *et al*. 1994). Immunization with this protein induced formation of antibodies but these antibodies did not react with the native protein. The implication from these and other studies is that it is extremely important that antigen of the correct structure is used for both detecting antibodies in sero-epidemiological studies and for inducing antibodies in immunization studies.

The availability of structural information has facilitated the mapping of antibody binding sites on the molecule. This mapping has been carried out by a variety of methods including X-ray crystallography (Pizarro *et al*. 2003), NMR (Morgan *et al*. 2004, 2005), direct visualization of antigen-antibody complexes by electron microscopy (Dekker *et al*. 2004), Pepscan-based methods (Uthaipibull *et al*. 2001), and site-directed mutagenesis to produce variant proteins (Uthaipibull *et al*. 2001; Dekker *et al*. 2004; McIntosh *et al*. 2007). Competition ELISA using antibodies of known fine specificity also provides useful information on the binding sites of other antibodies (Nwuba *et al*. 2002).

The most complete set of information at the atomic level is obtained by crystallography, whereas NMR provides information on the protein in solution, predominantly from the peptide backbone. Crystallographic studies of the binding of the Fab fragment of mAb G17·12 to *P. falciparum* $MSP1_{19}$ indicate that the antigen binding site is large but restricted to the first EGF domain between residues 8 and 39 and on the outer edge (Pizarro *et al*. 2003) as shown in Fig. 2 row A; residues interacting with the antibody

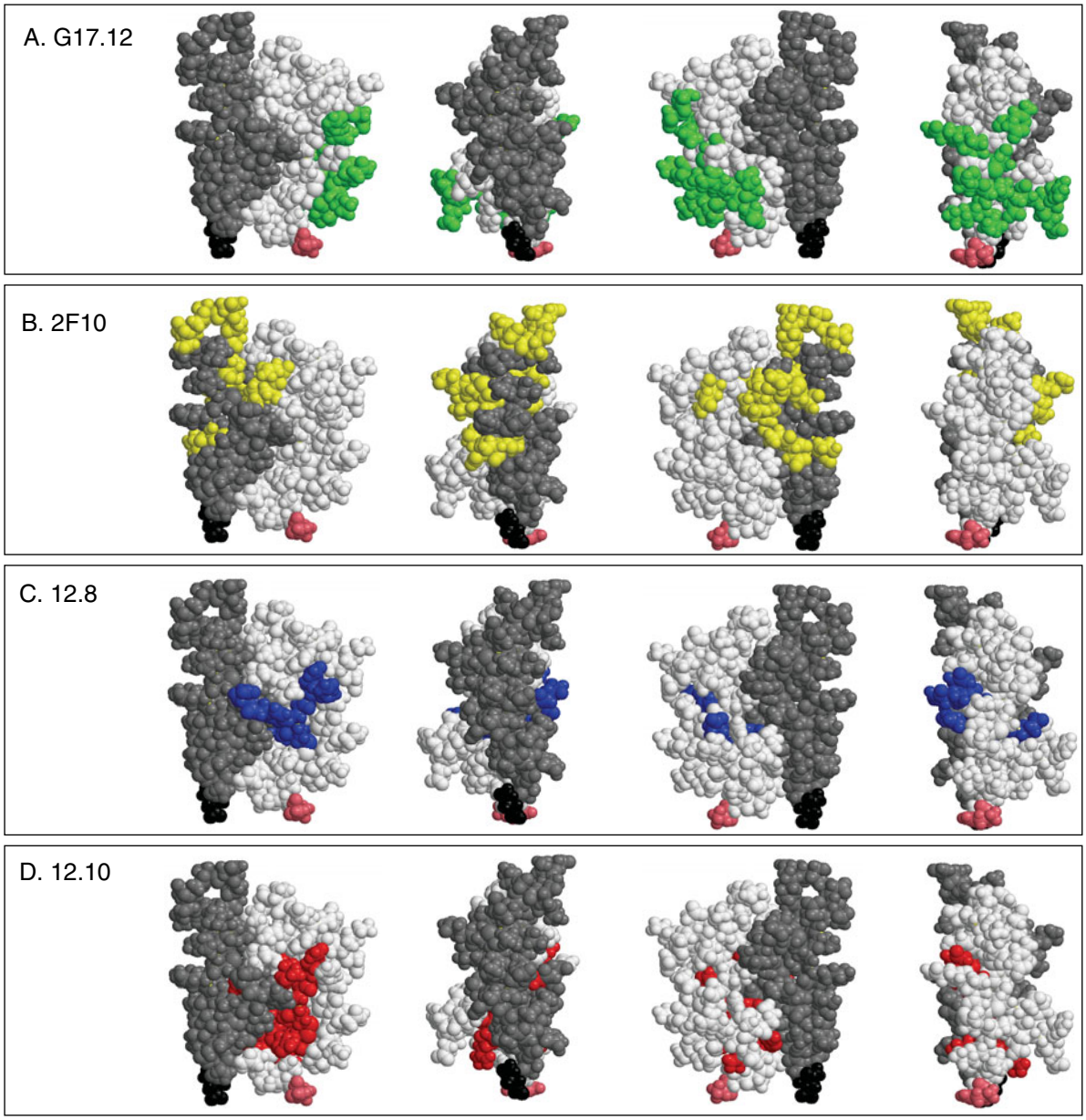

Fig. 2. The binding of monoclonal antibodies to *Plasmodium falciparum* $MSP1_{19}$. $MSP1_{19}$ is portrayed as one of the representative nmr structures (PDB: 1cej) and is orientated such that the N- and C-terminal residues (coloured pink and black, respectively) are close to the bottom; 4 views are shown rotated approximately 90° around the *y* –axis. The polypeptide consists of 2 epidermal growth factor-like (EGF) domains that interact closely with each other. In the left hand view the second EGF domain (in grey) is on the left hand side and its large flexible loop is visible at the top of the molecule, whilst the first EGF domain (in white) occupies the right side of the structure. In panel A the residues in contact with the antibody G17, identified in the crystal structure of the G17 fab-$MSP1_{19}$ are indicated in green (Pizarro *et al*. 2003). In panel B residues identified by chemical shift perturbation in the nmr analysis of mAb 2F10 fab bound to $MSP1_{19}$ are shown in yellow (Morgan *et al*. 2004). In panels C and D the residues identified by cross-saturation mapping in the nmr analysis of mAb 12·8 and 12·10 fabs bound to $MSP1_{19}$ (Morgan *et al*. 2005) are shown in blue and red respectively. The mAbs have different properties; G17 and 2F10 do not inhibit MSP1 secondary processing or affect invasion in vitro whereas 12·8 and 12·10 do. 2F10 is not a blocking antibodies but the status of G17 in this context is not known.

are shown in green. In contrast, the binding site of the Fab from mAb 2F10 as determined by chemical shift perturbation of backbone amides, a NMR-based method (Morgan *et al*. 2004), is located at the other side of the molecule, as shown in Fig. 2 row B; residues interacting with this antibody are shown in yellow, largely between residues 32 and 79, which spans the junction between the 2 EGF-domains in the linear sequence. Neither of these antibodies is inhibitory in the processing assay and mAb 2F10 is a neutral antibody in this assay; the blocking or neutral activity of G17·12 has not been determined.

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Table 1. The location of amino acid sequence changes and their effect on the binding of various monoclonal antibodies to *Plasmodium falciparum* $MSP1_{19}$

(This approach also provides data that help in the design of improved antigens to induce a more effective immune response. The antibodies are arranged into 4 groups: inhibitory, blocking and neutral antibodies as defined in the secondary processing assay (data from (Uthaipibull *et al*. 2001; Dekker *et al*. 2004)) and antibodies that mediate Fc-dependent parasite killing *in vivo* (McIntosh *et al*. 2007). Single or combinations of amino acid substitutions were made at positions throughout the $MSP1_{19}$ sequence and the identity of the changes is indicated using one-letter code for each amino acid. The data are arranged by blocks of rows: sequence changes that have no effect on the binding of any of the antibodies; changes that affect the binding of inhibitory, blocking and neutral antibodies; changes that affect the binding of blocking and neutral antibodies; changes that affect only the binding of neutral antibodies; and combinations of from 2 to 8 changes and their effect on the binding of the antibodies. Most of the substitutions were made in the first of the 2 EGF-like domains, and the nature of the new side-chains introduced may have a local effect or perturb the structure more generally, so interpretation of the link between substitution and effect on antibody binding requires caution. Antibody binding has been determined by Western blotting, and occasionally by surface plasmon resonance or ELISA. (These data are from Dekker *et al*. 2004; McIntosh *et al*. 2007; Uthaipibull *et al*. 2001 and Uthaipibull *et al*. unpublished observations).)

			Monoclonal antibody binding															
	Amino acid		Inhibitory antibodies			Blocking antibodies				Neutral antibodies							Fc-dependent antibodies	
Position	Wild type	mutant	12·8	12·10	5B1	1E1	2·2	7·5	111·4	111·2	9C8	2F10	12D11	117·2	5·2	1E8	8A12	JS1	JS2
Wild type			++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Single																			
22	L	R	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++		
32	L	R	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
35	K	I	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++		
36	Q	G	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
37	E	I	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
80	K	I	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
12	C	I	−	−	−	++	−	++	++	−	+	++	++	−	−	++	++	++	++
20	R	E	+	++	+	++	+	+	++	+	+	++	+	+	++	+	++	+	+
24	E	K	+	++	++	++	++	++	++	++	+	++	++	++	++	+	++		
26	E	I	−	++	+	++	−	++	++	++	++	++	++	++	+	+	+		
28	C	W	−	+	−	++	−	++	++	−	++	++	++	−	−	++	++	−	−
29	K	S	+	++	++	++	+	+	++	++	++	++	++	++	++	++	++	+	+
31	L	R	+	++	++	−	++	++	++	−	−	++	++	++	++	++	+		
40	K	I	+	++	+	+	++	++	+	+	+	+	+	+	++	+	++	+	+
6	Q	I	++	++	++	++	+	+	++	++	++	++	++	++	++	++	++		
14	Q	G	++	++	++	++	++	+	+	++	++	++	++	+	++	++	++		
14	Q	R	++	++	++	++	++	+	+	++	+	++	++	+	++	++	++		
15	N	R	++	++	++	++	++	−	+	++	++	++	++	++	++	++	++		
25	R	G	++	++	++	++	+	++	++	++	++	++	++	++	++	+	++		
27	E	Y	++	++	++	++	−	++	++	++	++	++	++	++	++	++	+		
34	Y	S	++	++	++	+	+	+	+	++	+	++	+	+	++	++	++		
34	Y	I	++	++	++	+	++	+	+	++	++	++	++	++	++	++	++		
39	D	T	++	++	++	++	++	++	+	++	++	++	++	++	++	++	++	+	+
43	E	L	++	++	++	+	++	+	−	++	+	++	+	+	++	++	+		

33	N	I	++	++	++	++	++	++	++	+	++	++	++	++	++	++	++	+	+
48	T	K	++	++	++	++	++	++	++	++	++	−	++	++	++	++	++		
53	N	R	++	++	++	++	++	++	++	++	++	−	−	+	++	++	++		
Combinations																			
12+28	C/C	I/W	++	++	++	++	−	++	++	+	+	++	++	+	−	++	++		
12+28	C/C	A/F	++	++	++	++	−	++	++	++	++	++	++	++	−	++	++		
34+39	Y/D	S/N	++	++	++	+	+	+	+	++	++	++	++	++	+	++	++	+	+
43+48	E/T	L/K	++	++	++	+	+	+	−	−	+	−	+	+	++	++	+	+	+
43+48	E/T	L/N	++	++	++	++	++	+	−	++	+	+	++	++	++	++	++		
47+48	P/T	S/K	+	+	+	+	+	+	+		+	−	+					+	+
27+31+43	E/L/E	Y/R/L	++	++	++	−	−	+	−	−	−	++	++	+	++	+	+		
27+31+34+43	E/L/Y/E	Y/R/S/L	++	++	++	−	−	−	−	−	−	++	++	+	++	+	++	++	++
15+27+31+43	N/E/L/E	R/Y/R/L	++	++	++	−	−	−	−	−	−	++	++	+	++	+	++	++	++
12+15+27+31+43	C/N E/L/E	I/R Y/R/L	−	−	−	−	−	−	−	−	−	++	++	−	−	−	++		
12+15+27+28+31+43	C/N E/C L/E	I/R Y/W R/L	++	+	++	+/−	−	−	−	−	−	++	++	−	−	++	++		
12+15+27+28+31+43+53	C/N E/C L/E N	I/R Y/W R/L R	+/−	−	−	−	−	−	−	−	−	−	−	−	−	+	−		
12+15+27+28+31+34+43+53	C/N E/C L/Y E/N	I/R Y/W R/S L/R	−/+	−	−	−	−	−	−	−	−	−	−	−	−	−	−		

++ =strong binding, + =binding, − =no binding

The binding sites for the inhibitory mAbs 12·8 and 12·10, as determined by chemical shift perturbation, involve residues in both EGF domains (Morgan *et al.* 2004) and by cross-saturation mapping, a measure of the proximity to the antibody of labelled side-chain nitrogen atoms, were located on the hydrophobic face of $MSP1_{19}$ and close to the interface between the 2 domains (Morgan *et al.* 2005), as shown in Fig. 2 rows C and D in red (12·10) and blue (12·8), respectively. These sites are well away from the binding sites of G17·12 and 2F10, a result consistent with the formation of complexes between $MSP1_{19}$ and both the 12·10 and 2F10 antibodies (Dekker *et al.* 2004), but include residues essentially in the first EGF domain and towards the end of the second EGF domain.

The other 2 methods used for epitope mapping of antibodies binding to $MSP1_{19}$ have some further limitations. In the Pepscan approach antibodies are allowed to bind to a series of overlapping peptides coupled at high concentration to a plastic support that creates at least part of the corresponding epitopes. In the site-directed mutagenesis approach the modification to individual amino acid side-chains needs to be sufficiently radical to affect the affinity of the antibody for the antigen and yet not have a profound effect on the overall structure of the protein. Changes of size and shape or charge may have an unpredictable consequence. For example, based on studies with *P. yoelii* $MSP1_{19}$ we know that some substitutions can have very slight and local effects, and others have very substantial consequences for the 3-D structure, as determined quite easily by comparison of 2-D HSQC spectra in NMR experiments (Curd, Birdsall, Holder and colleagues; unpublished). When the Pepscan approach was used with mAbs 12·8, 12·10 and 1E1 and 7·5 (which are both blocking antibodies) and peptides corresponding to sequences derived only from the first EGF domain, the results were largely consistent for 12·8 and 12·10 with the outputs of the direct binding methods using the full length $MSP1_{19}$. Interestingly the blocking antibodies also appeared to bind to some of the regions of sequence that comprise the epitope for the inhibitory antibodies 12·8 and 12·10. Several variants have been created by site-directed mutagenesis resulting in the replacement of one or more amino acid residue by others (see Table 1). The distribution of these changes is not uniform throughout the sequence, since the majority are located in the first EGF domain. Some of the changes are expected to have a major effect on the structure, for example, where a single cysteine is removed. As judged, for example by Western blotting, ELISA, or surface plasmon resonance, single amino acid changes were able to abolish the binding of some monoclonal antibodies but not others. Interestingly no two monoclonal antibodies have an identical pattern of reactivity with this panel of antigens. This particular analysis has been useful in determining the relationship between inhibitory, blocking and neutral antibodies, based on a panel of 3 inhibitory, 4 blocking and 8 neutral mAbs. Some changes had no effect on the binding of any antibody, some affected the binding of one or more antibodies in each of the 3 classes, some affected the blocking of only neutral antibodies and, interestingly, some affected 1 or more blocking or neutral antibodies without affecting the binding of inhibitory antibodies.

ANTIGEN DESIGN AND ENGINEERING FOR VACCINE DEVELOPMENT – CAN WE IMPROVE ON NATURE?

Despite all of the evidence to support the idea that the C-terminus of MSP1 is the target of a protective immune response, early phase IIb studies have been very disappointing in that vaccinees seemed to have boosted antibody but this had no effect on susceptibility to clinical disease (Ogutu *et al.* 2009). Does this result suggest that all of the earlier experimental studies are flawed or that immunization of humans results in largely neutral or blocking antibodies or antibodies of a non-optimal subclass? How can the information gathered on the structure and antigenicity be used in vaccine design, for example to improve the immunogenicity of the protein and the efficacy of the induced immune response? Can we modify the antigen, for example by introducing amino acid substitutions that increase its immunogenicity by allowing it to be processed and presented more rapidly by antigen presenting cells (APC), or which increase the induction of inhibitory antibodies but decrease the induction of blocking antibodies? At the same time deleterious effects due to the removal of important T-cell epitopes or unforeseen effects on the binding of antibodies that are important in one of the several functions that are known must be avoided.

In addition, it would be useful to have a panel of reagents to map the fine specificity of the antibodies induced by immunization. This panel would enable the functional attributes of antibodies induced in a vaccine trial to be classified, for example: either inhibitory, neutral or blocking. Earlier studies have suggested that measurement of total $MSP1_{19}$-specific antibodies alone is not a good predictive indicator of the function of these antibodies (Nwuba *et al.* 2002). Application of a transgenic model (McIntosh *et al.* 2007) and a variety of immunochemical approaches to define the fine specificity of antibodies in the clinical samples could provide considerable insight here.

Amino acid substitutions that affect antigen processing and presentation may be important. Studies by Hensmann and colleagues (Hensmann *et al.* 2004) showed that the wild type $MSP1_{19}$ protein from rodent parasites is a poor substrate for proteases

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important in antigen processing in the lysozome of dendritic cells, and that reduced and alkylated protein (which no longer contains any disulphide bonds) produces a faster immune response, although the response is no longer protective because the important antibodies to conformational epitopes are not produced (Ling *et al*. 1994; Hensmann *et al*. 2004). Similarly, studies with PfMSP1$_{19}$ showed the importance of disulphide bonds for both B- and T-cell epitopes (Egan *et al*. 1997). Other studies have confirmed that MSP1$_{19}$ is highly resistant to proteases, for example the protein is both present and intact in the food vacuole throughout the intracellular development (Dluzewski *et al*. 2008). Selective removal of one or more disulphide bonds to 'loosen' the structure may have the same effect as reduction and alkylation on processing but at the same time preserve the overall 3-dimensional structure necessary to get the right antibody response. This strategy has not been looked at systematically, but based on the fact that cysteines 12 and 28 of the *P. falciparum* protein are not present in MSP1$_{19}$ of other species, these two residues have been replaced. Removal of just 1 of the cysteines results in a protein that loses reactivity with mAbs 12·8/12·10 suggesting that the structure is perturbed substantially. Replacement of Cys 12 with Ile (found in the *P. yoelii* sequence) and replacement of Cys 28 with Trp, which is found at this position in all the other species (reviewed by Benjamin *et al*. 1999) has relatively little effect on the antigenicity of the protein (other than no longer binding the blocking mAb 2·2) and was carried out to see whether or not it improved the immunogenicity of the protein (see Table 1). Although the initial outcome was not very encouraging (Arnot *et al*. 2008), there is still much to do in this area. Another possible approach would be the introduction of residues that promote processing without having a substantial effect on the structure of the antigen or the presence of B and T cell epitopes; for example insertion of additional asparagines that are recognized by the asparagine endoproteinase in dendritic cell lysozomes (Hensmann *et al*. 2004) might speed up the processing of MSP1$_{19}$.

It may be possible to introduce amino acid changes that would not ablate the induction of functional antibodies, but would be advantageous, for example by reducing the binding and induction of blocking antibodies. For example, Uthaipitbull and colleagues described a number of variants that had no effect on the binding of inhibitory antibodies but did effect the binding of blocking antibodies (Uthaipibull *et al*. 2001). Several combinations of replacements have been constructed that no longer bind any of the blocking antibodies but still bind inhibitory antibodies (Table 1) and it will be interesting to see what is the outcome of immunization with these proteins. However, the situation is complex, for example, the 2 antibodies that mediate Fc-dependent parasite killing *in vivo* are blocking antibodies in the MSP1 processing assay (McIntosh *et al*. 2007).

MSP1$_{19}$ has also been used in a variety of approaches to produce chimeric antigens either with other proteins such as circumsporozoite protein (Holder *et al*. 1988; Murphy *et al*. 1990) and apical membrane antigen 1 (Faber *et al*. 2007), fused to other parts of MSP1 for use in viral vector delivery systems (Draper *et al*. 2008), or covalently coupled to proteins that might obviate the need for an adjuvant (Ogun *et al*. 2008). Another modification is removal of potential N-glycosylation sites since this modification of MSP1 does not occur in the parasite. There is still the need to construct some further variants for immunization studies, based on the previous studies reviewed here and informed by the structural information that is now available.

CONCLUDING COMMENTS

Now that clinical trials of MSP1 vaccines are underway, it is increasingly clear that we need to know more about the mechanisms of immunity, in the hope that a better understanding will highlight the limitations of our current assays and the identify the improvements required. Understanding the structure of the molecule may help us design and engineer better antigens that will be more effective than the first generation of vaccine candidates.

I would like to thank all my colleagues past and present for their tremendous contributions to the work referred to in this review and much more besides.

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