of fluorescent lipid analogues in the surface membrane of adult male *Schistosoma mansoni*. *Mol. Biochem. Parasitol.* 53, 233–240
58 Dixon, D.M. and Martin, R.J. (1993) **Patch clamp and chloride channels in *Ascaris suum*.** *Parasitology Today* 9, 341–344
59 Foley, M. *et al.* (1994) **Photoaffinity labelling of chloroquine-binding proteins in *Plasmodium falciparum*.** *J. Biol. Chem.* 269, 6955–6961
60 Harder, A. *et al.* (1988) **Influence of praziquantel and Ca^{2+} on the bilayer-isotropic-hexagonal transition of model membranes.** *Mol. Biochem. Parasitol.* 29, 55–60
61 Cully, D.F. *et al.* (1994) **Cloning of an avermectin-sensitive glutamate-gated chloride channel from *Caenorhabditis elegans*.** *Nature* 371, 707–711
62 McTigue, M.A. *et al.* (1995) **Crystal structure of a schistosomal drug and vaccine target: glutathione-S-transferase from *Schistosoma japonica* and its complex with the leading antischistosomal drug Praziquantel.** *J. Mol. Biol.* 246, 21–27

Plasmodium vivax: Merozoites, Invasion of Reticulocytes and Considerations for Malaria Vaccine Development

M.R. Galinski and J.W. Barnwell

Several Plasmodium vivax *merozoite proteins have been characterized over the past few years, including two that bind specifically to reticulocytes. Here, Mary Galinski and John Barnwell examine* P. vivax *merozoites and constituent molecules that are involved in host cell selection and invasion, and that also are viewed as malaria vaccine candidates. They also discuss how knowledge of the reticulocyte-binding proteins furthers the development of a conceptual framework for malaria merozoite invasion at the molecular level, not only for* P. vivax, *but for all species of the parasite.*

Plasmodium vivax and *P. falciparum* are the two most prevalent species of human malaria, together afflicting a few hundred million people annually[1]. Although the percentage of these cases caused by *P. vivax* versus *P. falciparum* is uncertain, it can be conservatively estimated that as many as 35 million cases of vivax malaria occur each year. Moreover, chloroquine-resistant strains have recently been observed[2–4], and relapse parasitemias due to primaquine-resistant liver-stage hypnozoites have become widespread and are being noted more and more frequently[5,6]. Vivax malaria is undoubtedly a major public health problem for many countries, with associated socioeconomic ramifications. Now, and probably increasingly so in the future, there is a need for alternative prophylactic and therapeutic tools for control and management of these infections.

Despite the fact that *P. vivax* is a major pathogen, basic research on molecular and cellular biological aspects of this parasite has been limited, with only a few laboratories focusing research programs on this species. One prominent reason for this lack of attention is that *P. vivax* cannot be cultured continuously *in vitro*. This contrasts with the availability of a *P. falciparum* culture system[7] that has greatly facilitated investigations of this species in many laboratories worldwide.

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An outstanding trait of *P. vivax* that has enabled it, to a large degree, to evade scientific scrutiny is that its primary host red blood cell is the reticulocyte, which normally accounts for only about 1% of the human red blood cell population. Reticulocytes are not easily obtained routinely on a daily basis in sufficient quantities to maintain laboratory cultures. This fact also hampers any systematic investigation of optimum culture conditions for *P. vivax*, which appears to be another important factor affecting the maintenance of this parasite *in vitro*. Although it is not known why *P. vivax* is restricted to this young red blood cell population, the discovery of proteins that bind specifically to reticulocytes[8] provides the beginning of an explanation for how *P. vivax* targets this host cell.

This review features the *P. vivax* reticulocyte-binding proteins[8] and also outlines work that has been accomplished in recent years to characterize *P. vivax* merozoites. We, and others, have circumvented the fact that there is no culture system for *P. vivax* by: (1) obtaining limited amounts of this parasite, as required, from Saimiri monkey adapted infections; and (2) by making use of the closely related simian malarias *P. cynomolgi* and *P. knowlesi*[9], which serve as biological model systems, and are also used to generate homologous DNA and antibody reagents/probes that crossreact with *P. vivax*. In addition, although there is no long-term culture system for *P. vivax*, short-term culture with significant reinvasion is possible, thus permitting *in vitro* investigations on merozoite invasion[10,11]. This body of *P. vivax* investigations has not only broadened our knowledge of *P. vivax* merozoite proteins, but also has provided valuable insights about functionally analogous proteins in *P. falciparum*.

Plasmodium vivax: the parasite

The distinct phylogenetic paths of *P. vivax* and *P. falciparum* probably evolved tens of millions of years ago[12]. This period of time has allowed for considerable biological differences to develop between these predominant species of malaria[13,14]. Most notably: (1) *P. vivax* can remain dormant in the liver as hypnozoites[15], which can initiate a blood-stage infection even months after an initial sporozoite infection; (2) it

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preferentially, if not exclusively, invades reticulocytes[16,17]; and (3) it develops caveolae–vesicle complexes (Schüffner's stippling) in the infected erythrocyte membrane[18,19]. In contrast: (1) *P. falciparum* does not develop a dormant liver stage; (2) it invades mature as well as immature red blood cells; (3) it produces knob protrusions in the infected erythrocyte membrane[20], as opposed to caveolae–vesicle complexes; and (4) it 'sequesters' its maturing blood-stage forms from the circulation and possible destruction in the spleen by adhering to vascular endothelium[21] (*P. vivax* does not sequester). The dramatic morphological and biological differences between *P. vivax* and *P. falciparum* have indicated that many of the proteins of these two malaria species may be quite different.

Despite the distinctive features of *P. vivax* and *P. falciparum*, however, many aspects of the biology of their blood stages are clearly similar. In particular, the merozoites of all malaria species, examined at the ultrastructural level, contain the same features and organelles typical of Apicomplexan parasites: an anterior conoid process (apical cone), paired rhoptries, micronemes and dense bodies (microspheres) (Fig. 1). These structural similarities suggest that, even though each species has different host cell requirements, the general molecular scheme of events that define invasion may be similar across species. Indeed, molecular studies have begun to denote the fine similarities, as well as differences, between the genes and proteins of these and other species of *Plasmodium*. Other recent reviews and references therein provide accounts of related studies not covered here, in particular with regard to the enzymatic and morphological events known to occur as merozoites enter erythrocytes[22,23].

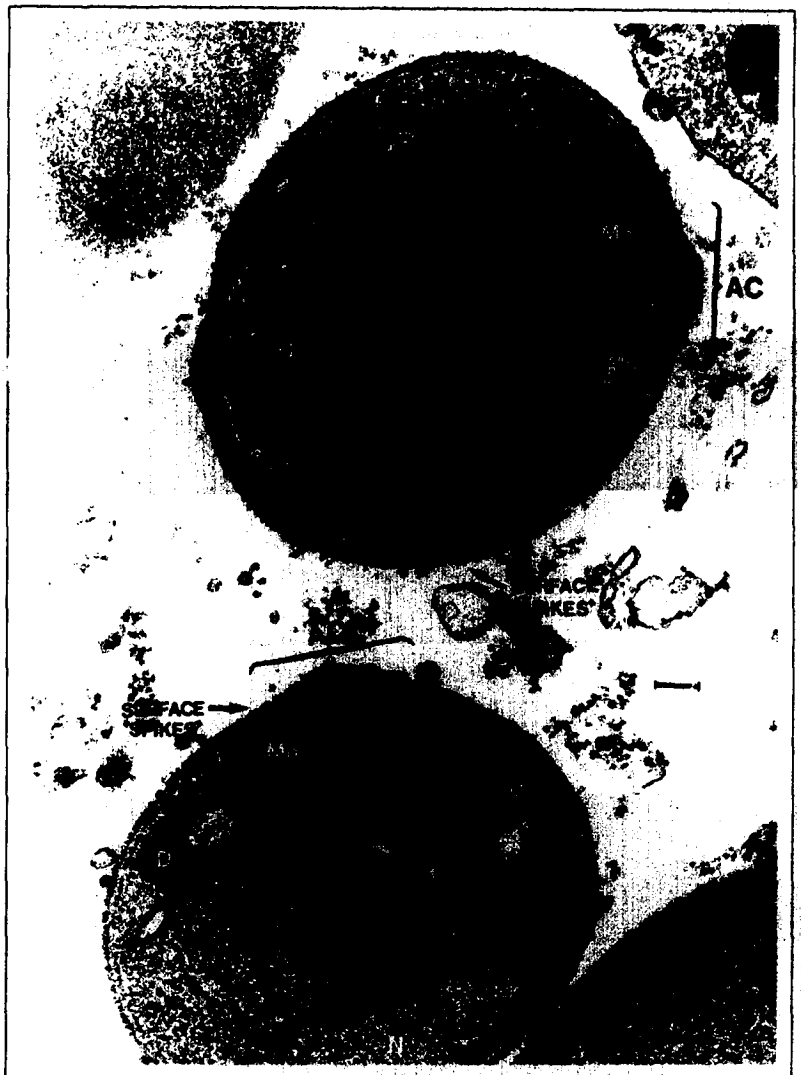

Fig. 1. Transmission electron micrograph of two *Plasmodium vivax* merozoites after *in vitro* maturation from trophozoite stage to differentiated segmented schizonts (merozoites) and mechanical release by small bore needle (25G) passage. The organized 'spiked' nature of the merozoite surface coat (20–25 nm projections) is evident in several areas, as noted. Other structures are labeled including the apical cone (AC), rhoptries (only one of each rhoptry pair is visible in each section) (Rh), micronemes (Mn), dense bodies (D) and nucleus (N). Fixation in 2% tannic acid and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer. Scale bar = 200 nm. (This image was kindly produced by Michael J. Stewart.)

The merozoite 'surface coat'

Plasmodium vivax merozoites have a 'surface coat' that appears to be composed of a highly structured array of regularly spaced protruding macromolecular complexes (Fig. 1). Although sometimes appearing as a 'fuzzy' or amorphous surface, the clear structural arrangement that we have observed on freshly released, rapidly fixed *P. vivax* merozoites has also been observed previously for other species of malaria. Bannister and colleagues have reviewed in detail the ultrastructure of the merozoite surface, emphasizing the visual complexity of the surface components (mostly for *P. knowlesi*), and discussing how this surface may participate in and facilitate the process of merozoite entry into red blood cells[24,25].

The first *Plasmodium* merozoite surface protein to be identified was the major merozoite surface protein (MSP-1) of *P. yoelii*[26], soon followed by identification of the homologous protein in *P. falciparum*[27] and *P. knowlesi*[28]. Merozoite surface protein-1 genes have since been characterized in *P. falciparum*[29,30], *P. vivax*[31,32] and several rodent malaria species[33–35]. It also has been shown that MSP-1 of *P. falciparum*, *P. vivax* and *P. knowlesi* binds to erythrocytes in an *in vitro* erythrocyte-binding assay (EBA)[36,37]. In recent years, MSP-1 has been evaluated in numerous investigations for its potential as a component of a malaria blood-stage vaccine[38].

Though structural studies at the resolution of electronmicroscopy indicated that the merozoite surface was possibly composed of multiple proteins, other

surface proteins were not discovered until more recently. Three *P. falciparum* proteins, in addition to PfMSP-1, have been identified that are associated with the merozoite surface and have been named accordingly as MSP-2, MSP-3 and MSP-4 (Refs 39,40) (R.L. Coppel, Abstract*); two of these, like MSP-1, are glycosylphosphatidylinositol (GPI) anchored[39] (R.L. Coppel, Abstract*). Similarly, in addition to PvMSP-1, we have identified three *P. vivax* merozoite proteins (PvMSP-2, -3 and -4) that appear to be associated with the surface of *P. vivax* merozoites (M.R. Galinski and J.W. Barnwell, unpublished). It is interesting that, in *P. vivax*, only MSP-1 appears to be GPI anchored. Experiments with metabolic labeling using sugars and fatty acids do not indicate that other major GPI-anchored surface proteins exist in *P. vivax* merozoites. *Plasmodium falciparum* MSP-2 and MSP-4 appear to be distinct from PvMSP-2, -3 and -4. However, features of PfMSP-3 (Ref. 41), also known as SPAM, suggest that there may be a small structural region analogous to a larger structural domain found in PvMSP-3 and PvMSP-4.

Although several discrete proteins are now known to be arranged at the surface of the merozoite, it is not known in which way(s) they interact in order to make up the organized array of complexes noted to be present in ultrastructural studies. The functional role of these proteins is largely unknown. It remains to be seen which, if any, surface proteins other than MSP-1 bind to erythrocytes, as the EBA used to detect the binding of this protein and others may not identify all proteins with initial adhesive functions. The nature and treatment of each culture supernatant, as well as the specific binding conditions utilized, affect the outcome of each EBA. It is also possible that other surface adhesins exist that bind after invasion begins, once appropriate binding sites become exposed, as has been proposed for the epidermal growth factor (EGF)-like domains of the proteolytic processed 19 kDa fragment of MSP-1 (Ref. 42).

The merozoite anterior (apical) pole

Microneme and rhoptry proteins. The first *Plasmodium* molecule found to be located in the microneme organelles of the merozoite was the 135 kDa *P. knowlesi* Duffy-binding protein (PkDBP)[43], which is known to bind to the erythrocyte membrane glycoprotein bearing the Duffy blood group determinants[44,45]. The gene encoding this protein was subsequently used as a probe to clone the corresponding gene from *P. vivax*, PvDBP[46], which encodes a related 140 kDa *P. vivax* merozoite protein that also binds the Duffy glycoprotein of human red blood cells[11]. These two genes were found to be related to the *P. falciparum* gene encoding the merozoite protein, known as erythrocyte-binding antigen-175 (EBA-175)[47,48], which was also found to be localized to the micronemes[49], but bound to sialic acid residues specifically on erythrocyte glycophorin A[50,51] and not the Duffy glycoprotein. The relatedness of these *P. vivax*, *P. knowlesi* and *P. falciparum* proteins, which had been viewed as unique, species-specific molecules with different host-cell binding specificities, underscores the fact that phylogenetically distant malaria species maintain biologically similar proteins, even when divergence may be considerable. Thus, though *P. vivax* and *P. knowlesi* merozoites are dependent upon binding to the Duffy glycoprotein, and *P. falciparum* is dependent upon binding to glycophorin of red blood cells, a distant but nonetheless related molecule performs a similar binding function for each of these malaria species. The red blood cell binding domain of this 'family' of proteins has been located within a conserved cysteine-rich motif common to the proteins of all three species[51,52].

Although many proteins that are localized to the merozoite rhoptries have been characterized and studied from *P. falciparum*[53], only one such protein has been characterized in *P. vivax*. This is the apical membrane antigen-1 (AMA-1)[54], which is homologous to the relatively well-conserved proteins of the same family that have been described in *P. falciparum*[55], *P. fragile*[56], *P. chabaudi*[57] and *P. knowlesi*[58]. The precise role this protein plays in invasion is uncertain, although it has been suggested to be a receptor. Apical membrane antigen-1 is currently being evaluated for its potential as a vaccine candidate. There are other rhoptry-associated proteins in *P. vivax* (J.W. Barnwell, unpublished), but whether some or all will correspond to the rhoptry proteins characterized from *P. falciparum* remains to be determined.

Reticulocyte-binding proteins. The *P. vivax* reticulocyte-binding proteins, PvRBP-1 and PvRBP-2, are high molecular mass molecules that have relative mobilities (*M*) of about 275–280 kDa by SDS-PAGE, are predicted to be largely α-helical in nature, co-localize at the apical pole of merozoites, and bind to reticulocytes in an EBA[8]. Furthermore, there are two RGD amino acid motifs in PvRBP-1, which potentially could serve an adhesive function of ligand binding on the reticulocyte[59,60]. The exact subcellular localization of these proteins remains to be determined and for this reason they have not been categorized above as surface, microneme or rhoptry proteins. However, by immunofluorescence (IFA) they both produce a pattern that is unique when compared to other known proteins viewed in this manner. They do not appear as a 'double dot' pattern typical of rhoptry localized proteins, nor the single 'punctate' apical dot observed for the Duffy-binding protein and sialic acid binding protein, EBA-175, which could be viewed as markers for microneme localization. Both of the PvRBPs give a fluorescent pattern that can be described as an apical crescent or cap that seems to cover the apical pole, extending beyond the conoid process. Each of these large proteins also has a putative transmembrane domain at its C-terminus, which preceeds a short cytoplasmic domain, suggesting that they are membrane-bound and that the major portion of both proteins is externalized.

The binding of these proteins to reticulocytes is dramatic[8]. When reticulocyte-depleted human red blood cell populations are used in an EBA there is no binding of the PvRBPs, whereas when reticulocyte-enriched red blood cells are used, there is abundant binding. Adhesion of these proteins is independent of the presence or absence of the erythrocyte Duffy glycoprotein, indicating that they are binding to a novel receptor(s) on the reticulocyte membrane. Furthermore, binding of these proteins to reticulocytes of

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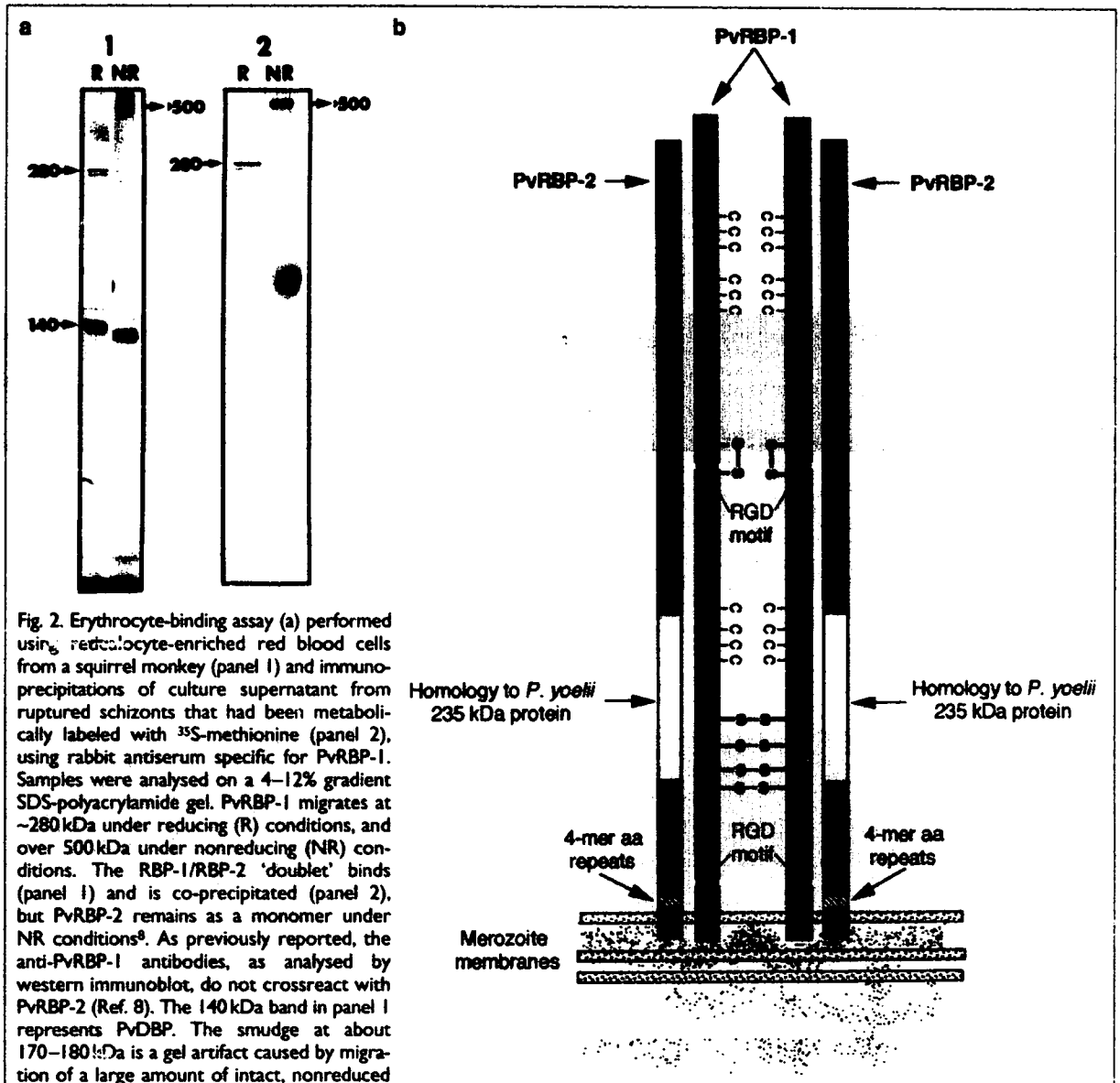

Fig. 2. Erythrocyte-binding assay (a) performed using reticulocyte-enriched red blood cells from a squirrel monkey (panel 1) and immunoprecipitations of culture supernatant from ruptured schizonts that had been metabolically labeled with ^{35}S-methionine (panel 2), using rabbit antiserum specific for PvRBP-1. Samples were analysed on a 4–12% gradient SDS-polyacrylamide gel. PvRBP-1 migrates at ~280 kDa under reducing (R) conditions, and over 500 kDa under nonreducing (NR) conditions. The RBP-1/RBP-2 'doublet' binds (panel 1) and is co-precipitated (panel 2), but PvRBP-2 remains as a monomer under NR conditions[8]. As previously reported, the anti-PvRBP-1 antibodies, as analysed by western immunoblot, do not crossreact with PvRBP-2 (Ref. 8). The 140 kDa band in panel 1 represents PvDBP. The smudge at about 170–180 kDa is a gel artifact caused by migration of a large amount of intact, nonreduced IgG and does not represent a specific immunoprecipitated malaria protein. Pre-stained protein standards (Gibco/BRL) and apolipoprotein B (Sigma) were used as molecular markers. Schematic representing a hypothetical model of the PvRBP complex (b), based on primary amino acid sequence, secondary structure prediction, preliminary localization studies, and biochemical analyses. Both PvRBP-1 and PvRBP-2 polypeptide chains have putative transmembrane domains and short cytoplasmic tails, and as such are depicted embedded in the outermost membrane of the merozoite. The two PvRBP-1 monomers are drawn with intra- and interchain disulphide bonds, consistent with experimental data indicating the presence of these covalent bonds; however, which of the 16 cysteines (Cs) are actually involved in disulphide-bond formation is not yet known. The close proximity and positioning of PvRBP-2 monomers to PvRBP-1 indicate that noncovalent interactions may be responsible for their apparent association. The yellow area of PvRBP-2 represents the region of highest homology with the *P. yoelii* protein, Py235 (Ref. 69) (see text for details). The RGD amino acid motifs of PvRBP-1, and C-terminal 4-mer repeated amino acid motifs of PvRBP-2 are also indicated.

other primate species correlates with their susceptibility to *P. vivax*[8]. For example, the PvRBPs bind to Saimiri monkey reticulocytes, which are invaded by *P. vivax*, but do not bind to rhesus monkey reticulocytes, which are not invaded by *P. vivax*.

One of the most striking features about the PvRBPs is that they seem to form a multimeric complex. The most compelling experiments, supporting a multimeric structure, demonstrate that PvRBP-1 migrates as a homodimer in nonreducing SDS-polyacrylamide gels, while PvRBP-2 migrates as a monomer under these conditions. This is evident in western immunoblot analyses[8], and also when proteins from EBA elutions or immunoprecipitates of ^{35}S-methionine labeled merozoite material are analysed by autoradiography (Fig. 2a). Reticulocyte-binding protein-1 contains 16 cysteine residues, clustered into four regions, some of which could participate in the formation of interchain, as well as intrachain disulphide bonds. Moreover, both of the PvRBP molecules tend to bind red blood cells in EBA analyses and coprecipitate with antisera specific for one or the other proteins. In light of current data, we propose that PvRBP-1 is a homodimeric membrane-bound protein

that is dependent on the formation of interchain disulphide bonds between monomers. Furthermore, since PvRBP-2 often co-precipitates with PvRBP-1, it seems to associate with PvRBP-1 through noncovalent interactions, leading us to tentatively propose a basic model of the PvRBP complex as illustrated in Fig. 2b.

The process of invasion: unfolding a molecular cascade

Plasmodium invasion of red blood cells entails a complex series of interactions between merozoite and host components[22,61]. The adhesive surface molecules of the merozoite may serve the purpose of 'capturing' red blood cells as they make contact in the circulation. Yet, the apical pole is known to be the 'business end' of the merozoite, where invasion is initiated. Somehow, the parasite becomes reorientated such that the apex of its anterior end is juxtaposed directly with the surface of the erythrocyte where formation of a tight junction is initiated. Although microscopic investigations, beginning 25 years ago, elegantly detail the sequence of events that occur as a merozoite attaches to and gains entry into a red blood cell, we are only beginning to understand the encompassing molecular processes. Especially with the discovery and basic characterization of several merozoite proteins that adhere to red blood cells in EBAs, a framework is emerging upon which we can begin to define the series of molecular events that constitute the early phases of invasion: merozoite attachment, apical juxtapositioning with the red blood cell surface, and junction formation between the merozoite and the red blood cell membranes.

It is known from videomicroscopy that, prior to apical contact with erythrocytes, merozoites can attach reversibly at any point along their surface. Videomicroscopy and electronmicroscopy studies also suggest that apical reorientation is facilitated by the adhesive merozoite surface coat[24,25]. These visual images indicate that the merozoite surface assists in 'docking' the merozoite at the surface of a red blood cell, and also aids the merozoite as it comes to be positionally reorientated, as fibrillar extensions between the merozoite surface and red blood cell seem to maintain the necessary associations. In a general scheme for relating adhesive properties of merozoite proteins with function, it is reasonable to suggest that this initial attachment may involve the binding of MSP-1[36,37], perhaps in conjunction with other known or unknown merozoite surface proteins, and may very well facilitate reorientation.

After reorientation, an electron-dense 'tight junction' forms between the apical cone of the merozoite and the erythrocyte membrane[62,63]. This electron-dense junction moves posteriorly and circumferentially over the merozoite as the erythrocyte membrane invaginates and the merozoite enters the host cell. Electronmicrograph analysis of *P. knowlesi* invasion has shown that this junction does not form between *P. knowlesi* merozoites and Duffy-negative red blood cells[64]. The interpretation has followed that the binding interaction of the DBP of *P. knowlesi* or *P. vivax* with the Duffy glycoprotein either initiates the formation of, or forms a part of, the 'moving junction'. By inference, the EBA-175 member of this family of adhesive proteins would serve the same function for *P. falciparum* merozoites.

This generalized 'two step' scheme for describing the initial contact of merozoites with erythrocytes and junction formation, now can be expanded for *P. vivax* to include a third component, namely PvRBP binding, to satisfy the requirement of reticulocyte selection. The predicted structure, position on the merozoite and adhesive feature of the PvRBPs together suggest a multifunctional role for these proteins. We have proposed that the PvRBPs target the reticulocytes for invasion[8]. From this perspective, binding of these proteins would commit a merozoite to an appropriately selected host cell. When a vivax merozoite is released into the blood circulation, it is surrounded by a preponderance of normocytes, cells it does not invade, but presumably comes in direct contact with. If reversible adhesive contacts imparted by the merozoite surface coat occur as normocytes are encountered (PvMSP-1 binds to normocytes, as well as reticulocytes), committed interactions must not occur until a reticulocyte is detected (Fig. 3). The specificity of the PvRBPs for reticulocytes strongly suggests that they bind to a specific reticulocyte ligand(s). This necessary specific adhesion event could possibly serve the added functions of (1) stabilizing the weaker, reversible contact(s) between the merozoite and host cell that could be directly mediated by MSP-1 and/or other unclassified adhesive surface proteins, and (2) enabling the ensuing adhesive reaction(s), leading to junction formation, as well as the invasion process to proceed.

It is likely that some signal is needed to trigger the release of the PvDBP, at the appropriate time, from its sequestered placement in the micronemes[8]. A case could be made to support the possibility that MSP-1 initiates such a signaling event once contact occurs between this GPI surface-anchored protein and a ligand on the erythrocyte, as GPI (free and protein-bound) has been reported to activate intracellular signal transduction pathways[65,66]. This could seem plausible for malaria parasites such as *P. knowlesi* or *P. falciparum* that invade normocytes. In this scenario, merozoites would be 'primed' to initiate junction formation once the appropriate apical orientation occurs. However, this precise series of events would not seem to be appropriate for a parasite, such as *P. vivax*, that is destined to invade reticulocytes. Given the 100-fold greater number of normocytes, premature release of the PvDBP would result in the irreversible abortive binding of the majority of *P. vivax* merozoites to mature erythrocytes (like PvMSP-1, PvDBP binds to both mature erythrocytes and reticulocytes). In this case, and in the absence of other known receptors, we propose that PvRBPs, upon binding to their red blood cell receptor(s), are in a position to trigger the release of the PvDBP. This model is compatible with the postulate that the PvRBPs bind and select reticulocytes prior to the progression of other irreversible processes. Activation of the appropriate signaling pathway(s) by the requisite binding of the PvRBPs would ensure that the PvDBP is only released in a timely manner. This scenario needs to be investigated further[37,67]. What form(s) or mechanism(s) of signal transduction might occur remains largely unknown. What is known, however, is that

staurosporine, an inhibitor of serine/threonine kinases, inhibits entry of *P. knowlesi* merozoites into red blood cells, but not attachment or junction formation[68]. This suggests that a phosphorylation pathway might affect a microtubule/actin-based motor that is involved in merozoite junctional movement and host cell membrane invagination rather than signal transduction events just prior to actual invasion. These mechanisms deserve further investigation, as they hold considerable potential for the development of chemotherapeutic interventions that could target crucial signaling pathways and inhibit parasite multiplication.

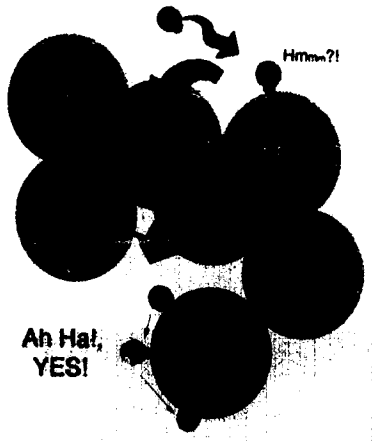

Fig. 3. Cartoon illustrating the plight of the *Plasmodium vivax* merozoite. Ten to 16 *P. vivax* merozoites are produced from each schizont. When released into the circulating blood, they may encounter 50–200 normocytic erythrocytes before 'bumping into' a reticulocyte. Logical reasoning would indicate that homing to a reticulocyte would require a specific mechanism, such as a reticulocyte receptor, 'in place' at the time merozoites are released from an infected cell. The efficiency of *P. vivax* selection and invasion of reticulocytes[16,17] is attested by the fact that, during the acute phase of an infection in either non-immune humans or splenectomized monkeys, *P. vivax* multiplies by eight- to 12-fold between each 48 h cycle of growth.

PvRBP analogs/homologs

If, as postulated, the PvRBP complex serves the multifunctional role of reticulocyte recognition and perhaps signaling for *P. vivax* merozoites, then from an evolutionary perspective it also is reasonable to propose that functionally and structurally analogous merozoite molecules might be present in all species of *Plasmodium*[8]. *Plasmodium cynomolgi*, a reticulocyte-preferring, closely related simian malaria, contains proteins homologous to PvRBP-1 and PvRBP-2. However, concurrent experiments showed that PvRBP gene probes did not cross-hybridize to the DNA of *P. knowlesi*, *P. falciparum* or *P. berghei*, even under conditions of lowered stringency[8]. In addition, antibody crossreactivity was not observed when initial PvRBP antibody preparations, which recognized a limited central portion of each protein, were tested for reactivity with these parasites. However, observations (see below) lend support to the hypothesis that a molecular complex comparable to the PvRBPs is present in other species of *Plasmodium*.

First, a precedent exists in earlier studies of *P. knowlesi* that would suggest that other proteins function apically prior to the interaction that leads to junction formation[62]. In the absence of the erythrocyte Duffy glycoprotein, the merozoite can remain apically attached via long fiber-like macromolecules. What comprises these fibrils? These long-ranging fibrils would be consistent with the large, hydrophilic and α-helical molecular structure, predicted to be characteristic of the PvRBP (or RBP-related) macromolecules. Moreover, it is relevant to note that as the junction between the merozoite and erythrocyte membranes 'moves' around the merozoite as it enters the red blood cell, the apical pole remains 'attached/anchored' via an electron-dense region[25,63] that has the appearance of these fibrillar components. Second, rabbit antisera recently produced against recombinantly expressed partial segments of selected regions of PvRBP-1 recognize *P. falciparum* and *P. knowlesi* merozoites by IFA in a pattern that is identical to that seen on merozoites of *P. vivax* and *P. cynomolgi* (G. Rosas-Acosta, M.R. Galinski and J.W. Barnwell, unpublished). Third, EBA analyses of *P. falciparum* proteins from culture supernatants demonstrate that two high-molecular-mass proteins, reminiscent of the PvRBP high-molecular-weight doublet[8], bind to erythrocytes (J.W. Barnwell, unpublished). We have tentatively called these the *P. falciparum* normocyte-binding proteins (PfNBPs).

The most convincing evidence for the existence of analogous proteins among distantly related species of *Plasmodium* comes from the comparative analysis of the structure of a large-molecular-mass (235 kDa) merozoite protein of *P. yoelii* (Py235), which localizes to the rhoptries and has a role in the invasion of normocytes[69]. Within the deduced peptide sequence encoded by the Py235 gene, there is a region (of 500 amino acids) that exhibits a significant degree of homology to the partial sequence reported for PvRBP-2 (29.6% identity, 51.6% similarity)[69] (Fig. 2b). The significance of this homology is supported further by our more-recent analysis comparing the C-terminal regions of both proteins. Approximately 40% of the PvRBP-2 sequence (3.75 out of 9.5 kb) had been reported previously[8]. This complete gene has since been cloned and analysed (M.R. Galinski, unpublished). Interestingly, there is no additional significant sequence homology between this and the Py235, but structurally they appear to be related. Both proteins contain aproximately 400 amino acids C-terminal to the previously noted homology, and both have a putative transmembrane domain that is immediately preceded by a set of short repeated amino acid motifs and followed by an apparent cytoplasmic domain (Fig. 2b). Thus, Py235 and PvRBP-2 appear to belong to the same protein family. As these two highly diverged species seem to manifest a gene product that is functionally and structurally similar, it is reasonable to assume that a related gene is present in *P. falciparum* and in other species of malaria.

Alternative receptors fulfill multiple requirements

It has become evident over the past decade that *P. falciparum* is capable of utilizing alternative receptors to recognize and gain entry into red blood cells[70–74]. Not all strains of *P. falciparum* require sialic acid or glycophorins for merozoite invasion and it is now believed that at least two merozoite receptors exist that are functional alternatives for EBA-175. Some strains of *P. falciparum* will invade erythrocytes that lack the appropriate glycophorin A ligand, but retain glycophorin B, and others invade erythrocytes lacking

both glycophorin A and B ligands; cells lacking glycophorin A do not bind EBA-175 (Refs 51,74). As these alternative merozoite receptors are not yet identified, it is not known whether or not they belong to the same gene family as EBA-175. The H strain of *P. knowlesi*, on the other hand, is known to contain three genes in its genome that comprise the DBP family (α, β and γ), all of which are expressed[43]. This 'redundancy' provides an explanation as to why either anti-Duffy glycoprotein antibody-mediated blockade or enzymatic removal of the Duffy determinants does not block invasion of *P. knowlesi* into rhesus monkey erythrocytes[75] (J.W. Barnwell, unpublished). Each gene product binds to erythrocytes, but only the α protein binds to the Duffy glycoprotein on erythrocytes of both humans and a simian host of *P. knowlesi*, *Macacca mulatta*[43,52]. The β and γ members of this family bind rhesus monkey erythrocytes, but not human red blood cells, and likely perform the role of the DBP that is necessary for junction formation in this primate host.

In addition, the Py235 gene, which (as noted above) appears to be a homolog of PvRBP-2, is one member of a large gene family[76]. In fact, multiple bands have been observed when immunoprecipitates of Py235 are electrophoresed on 5% polyacrylamide gels[76,77]. *Plasmodium yoelii* is known to comprise subpopulations that can invade normocytes and reticulocytes (lethal parasites) and subpopulations that are restricted to reticulocytes (non-lethal parasites). Proteins expressed by different members of the Py235 gene family may have differing red blood cell specificities, perhaps accounting for the observation that an anti-Py235 monoclonal antibody (mAb) passively protects mice infected with the virulent YM strain of *P. yoelii*, apparently by restricting the parasites to reticulocyte invasion[78]. Interestingly, this would suggest that, in *P. yoelii*, this RBP-2-like protein binds to normocytes. Moreover, as we do not know whether or not a protein similar to PvRBP-1 exists in *P. yoelii*, it remains possible that another distinct protein, such as a PvRBP-1 homolog with reticulocyte specificity, is expressed in the non-lethal parasite population and performs the function of reticulocyte selection/adhesion.

Are alternative adhesion receptors expressed in *P. vivax* merozoites? Prior research has indicated that in humans, susceptibility to *P. vivax* is absolutely dependent upon expression of the Duffy glycoprotein on erythrocytes[79,80]. In keeping with this view, a mAb specific for the Duffy glycoprotein[81] blocks *P. vivax* merozoite invasion of human Duffy-positive reticulocytes *in vitro* by 80–90% of the control cells[10]. However, data now exist that hint at the possibility of alternative receptor molecules with specificities for molecules other than the Duffy glycoprotein. First, PvDBP does not adhere to the Duffy glycoprotein of squirrel monkey erythrocytes, though the squirrel monkey is susceptible to *P. vivax*[11]. In addition, the Duffy glycoprotein mAb only partially blocks invasion of *P. vivax* into owl monkey reticulocytes, despite the fact that the anti-Duffy mAb abolishes binding of the PvDBP to the erythrocytes of this primate[10] (J.W. Barnwell, unpublished). These data strongly suggest that *P. vivax* merozoites can express a protein(s) that does not bind to the Duffy glycoprotein, but may perform the function of the DBP. Redundancy in functionally similar merozoite adhesins could be a 'fact of life' in parasitism by *Plasmodium* species, including *P. vivax*.

We are pursuing investigations of a 145 kDa merozoite protein, named *P. vivax* merozoite apical cone protein, or PvMacP (M.R. Galinski and J.W. Barnwell, unpublished) that, based on binding and location, could be an alternative receptor to the PvDBP. This protein binds specifically to squirrel-monkey and owl monkey reticulocytes. On mature segmented schizonts, PvMacP localizes by IFA to the apical pole of merozoites, sometimes as a small fluorescent double dot, but mostly as a single dot. Immunoelectronmicroscopy shows that, on free merozoites, PvMacP localizes consistently to the apical conoid process.

If PvMacP operates in the invasion of primate host cells as an alternative to the PvDBP, does it also function in merozoite invasion of human red blood cells in vivax malaria-endemic regions? The answer to this question will require further investigation of *P. vivax* isolates acquired directly from humans. However, we have determined that PvMacP was consistently expressed in the majority of merozoites from a sample of 20 *P. vivax* isolates taken directly from human infections. For one thing, this suggests that monkey adaptation did not select a minor population of parasites expressing this protein, or induce expression of a silent gene, as has been suggested to occur in *P. falciparum* during adaptation to an alternative invasion pathway[73], but rather, that PvMacP is another adhesive apical protein that is generally characteristic of *P. vivax* merozoites.

Another question is whether or not there is functional polymorphism in the PvRBPs. Both of the PvRBP genes are present as single-copy genes. Although little is known about the diversity of the PvRBPs, some useful information is available from studies of *P. cynomolgi*. Different strains of *P. cynomolgi* are known to vary in their relative reticulocyte 'preference', which, at best (for *P. vivax* or *P. cynomolgi*), can be defined as restriction of early ring-stage parasites to morphologically identifiable reticulocytes[82]. We also have found that the restriction enzyme pattern differs for the RBP genes of different strains of *P. cynomolgi*. Diversity may be one means to provide alternative binding sites. Also, it is conceivable that the PvRBP molecules could associate (dimerize) in different ways to form the RBP complex, thus creating alternative binding domains (or, perhaps, alternative signaling 'instructions')[83]. Also, high parasitemias (much greater than 1%) occur occasionally in human *P. vivax* infections[84], perhaps indicating that invasion by some strains of *P. vivax* may not be confined strictly to reticulocytes. The notion, that functional polymorphism in the RBPs of *P. vivax* is plausible, is also supported by the precedent of a multigene family for the RBP-2-like analog of *P. yoelii*[76].

Plasmodium vivax merozoite vaccine(s): current considerations

Anti-merozoite vaccines can be envisioned to occur by several different antibody-mediated mechanisms, including merozoite agglutination[28], cytophilic antibody dependent toxicity[85], or interference with biological processes such as merozoite protease activity[86]. The concept of preventing malarial merozoite invasion into erythrocytes by interfering with critical

receptor–ligand interactions through vaccination is also quite attractive.

In developing a vaccine of this nature, a number of factors must be considered, including: (1) the identification of adhesion domains responsible for ligand binding; (2) determination of their structure; and (3) reproduction of this structure through recombinant expression or synthetic technologies. The goal must be to produce immunogens that will induce antibodies able to recognize the corresponding binding domains of the native merozoite adhesins.

Five *P. vivax* proteins (PvMSP-1, PvRBP-1, PvRBP-2, PvDBP and PvAMA-1) could be viewed today as potential candidates for development of an anti-merozoite vaccine that interferes with receptor–ligand interactions. In the case of the PvRBP complex, the reticulocyte-binding domain(s) has not yet been identified, however, and investigations towards this end are actively under way. A few major considerations in this search are that the binding domain(s) (1) may be located on either one or both of the RBP molecules, (2) may be conformational, and (3) possibly only form after dimerization of both proteins. The two RGD domains of PvRBP-1 provide one set of clues that could lead to the identification of actual binding regions, although it remains to be determined if these are functional binding sites. On the other hand, the domain responsible for the binding of PvDBP has been determined[52]. However, an efficient method of production of this domain, which retains the structural basis for both adhesion and immunogenicity, as well as the detailing of the finer structural determinants responsible for the specificity of the adhesion, still needs to be resolved. Further, the structural basis of the adhesion of native (pre-processed) MSP-1 to erythrocytes[36,37] has not yet been thoroughly investigated for *P. vivax* or *P. falciparum*, and the putative receptor function of the AMA-1 protein family[54–58] needs to be clarified and, hence, possible binding domains identified and evaluated.

If our supposition is correct that the PvRBPs target the young red blood cells for invasion, the PvRBPs (and RBP-like analogues) may be one of the critical targets for antibody-mediated neutralization of merozoite invasion. By preventing the 'commitment' step of attachment, the initiation of the irreversible steps of PvDBP binding (or, likewise EBA-175 binding) and junction formation would probably be hindered, and invasion should not occur. Furthermore, if transduced signals are needed for release of the PvDBP from micronemes, and if the binding of the PvRBPs is an initiating component of this signal, then antibody binding to the PvRBPs, theoretically, could induce such signals and cause premature release of the PvDBPs. This would have at least two possible effects. Importantly, the PvDBPs would be much more readily exposed to the action of anti-PvDBP antibodies prior to junction formation. It also is conceivable that this premature signaling action could cause abortive invasion attempts by 'encouraging' the irreversible attachment of merozoites to mature erythrocytes.

However, in the unlikely event that *P. vivax*, under such unusual circumstances can invade (and survive in) normocytes, it must also be considered that the release of DBP might lead to an enhancement of infections due to the promotion of junction formation and invasion. An anti-PvRBP vaccine could also, potentially, select for a minor parasite population that can invade normocytes, perhaps making *P. vivax* a more virulent parasite. We raise these possibilities (not knowing how likely they may be), in part, to highlight the absolute importance of identifying potential receptors that operate as functional alternatives to primary receptors, whether for *P. vivax* or *P. falciparum*. Likewise, it will be necessary to determine the extent and functional significance of polymorphism (structural and functional) in the receptor molecules, and the relationship of this polymorphism to immunogenicity. For example, the adhesion domain of DBP has been found to exhibit amino acid polymorphism in human isolates[87]. Will these amino acid changes affect antibody binding and, thus, neutralization of invasion, similar to that which occurs as a result of variation in the receptors of viruses? In either case, immunization against a major population, potentially, could select minor variants that express alternative receptors or structural polymorphisms, rendering an effective vaccine partially or completely ineffective, while foresight and further basic research may prevent such occurrences.

Regardless of the above considerations, the time between merozoite release and completion of host cell entry is short, a matter of a few minutes at most, and the inclusion of several receptor components together in a formulated cocktail merozoite receptor vaccine cumulatively to hinder successive interactions with host cells will most likely be required to obtain a high level of efficacy. Moreover, the incorporation of other *P. vivax* merozoite molecules, such as epitopes of PvMSP-3 and -4, which may induce merozoite agglutinating and cytophilic antibodies, may help to achieve a greater maximum and broader protection.

Concluding remarks

With five *P. vivax* merozoite vaccine candidate binding proteins now under investigation, there clearly has been significant progress in the field of *P. vivax* vaccine research. In essence, the development of vaccines targeted at *P. vivax* merozoites, given the current state of knowledge, is no longer far behind similar vaccines for *P. falciparum*. In fact, the merozoite receptor antigens that are actively being investigated for *P. falciparum* include at this time only three candidates (MSP-1, EBA-175 and AMA-1). With consideration of the cascade of molecular events that seems to occur prior to *P. vivax* merozoite invasion of reticulocytes (ie. RBP binding preceeds DBP binding), however, we certainly would include RBP-like analogs to the list of *P. falciparum* candidates.

We still have a fair way to go towards understanding *P. vivax* merozoites and the process of erythrocyte invasion. There are also many unknowns with regard to making a highly effective blood-stage vaccine, even if all the 'best' components were in hand. Notwithstanding, there has been considerable movement at many levels on basic research concerning the merozoite and in applied research towards developing effective merozoite vaccines. We have outlined much of the progress of recent years, especially with regard to preliminary research involving the identification and characterization of *P. vivax* merozoite receptors. These molecules are potentially strong vaccine candidates

and further characterization is clearly required. As the more empirical aspects of vaccine development are carried forward, we emphasize the importance of continued fundamental studies to understand malaria blood stages and to elucidate features of their relationship to their host environment. As we uncover more detail about the malaria parasite and its biological processes, we will be in a considerably stronger position to devise more effective immunogenic compounds for vaccination.

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References

1 Institute of Medicine (1991) *Malaria: Obstacles and Opportunities* (Oaks, S.C., Jr *et al.*, eds), National Academy Press
2 Riekmann, K.H. *et al.* (1989) ***Plasmodium vivax* resistance to chloroquine?** *Lancet* ii, 1183–1184
3 Murphy, G.S. *et al.* (1993) **Vivax malaria resistant to treatment and prophylaxis with chloroquine.** *Lancet* 341, 96–100
4 Myat-Phone-Kyaw *et al.* (1993) **Emergence of chloroquine-resistant *Plasmodium vivax* in Myanmar (Burma).** *Trans. R. Soc. Trop. Med. Hyg.* 87, 687
5 Peters, W. (1984) in *Antimalarial Drugs I: History and Current Status of Drug Resistance* (Peters, W. and Richards, W.H.G., eds), pp 437–445, Springer-Verlag
6 Luzzi, G.A. *et al.* (1992) **Treatment of primaquine-resistant *Plasmodium vivax* malaria.** *Lancet* 340, 310
7 Trager, W. and Jensen, J.B. (1976) **Human malaria parasites in continuous culture.** *Science* 193, 673–676
8 Galinski, M.R. *et al.* (1992) **A reticulocyte-binding protein complex of *Plasmodium vivax* merozoites.** *Cell* 69, 1213–1226
9 Waters, A.P., Higgens, D.G. and McCutchan, T.F. (1993) **The phylogeny of malaria: a useful study.** *Parasitology Today* 9, 246–250
10 Barnwell, J.W., Nichols, M.E. and Rubenstein, P. (1989) ***In-vitro* evaluation of the role of the Duffy blood group in *Plasmodium vivax* erythrocyte invasion.** *J. Exp. Med.* 169, 162–167
11 Wertheimer, S.P. and Barnwell, J.W. (1989) ***Plasmodium vivax* interaction with the Duffy blood group glycoprotein: identification of a parasite receptor-like protein.** *Exp. Parasitol.* 69, 340–350
12 Escalante, A.A. and Ayala, F.J. (1994) **Phylogeny of the malarial genus *Plasmodium*, derived from rRNA gene sequence.** *Proc. Natl Acad. Sci. USA* 91, 11373–11377
13 Coatney, G.R. *et al.* (1971) *The Primate Malarias*, US Dept of Health, Education and Welfare, Washington, DC
14 David, P.H., Barnwell, J.W. and Mendis, K.N. (1990) in *New Generation Vaccines* (Woodrow, G.C. and Levine, M.M., eds), pp 531–544, Dekker
15 Krotoski, W.A. (1985) **Discovery of the hypnozoite and a new theory of malarial relapse.** *Trans. R. Soc. Trop. Med. Hyg.* 79, 1–11
16 Kitchen, S.K. (1938) **The infection of reticulocytes by *Plasmodium vivax*.** *Am. J. Trop. Med.* 18, 347–353
17 Mons, B. *et al.* (1988) ***Plasmodium vivax*: *in vitro* growth and reinvasion of red blood cells of *Aotus nancymai*.** *Exp. Parasitol.* 66, 183–188
18 Aikawa, M., Miller, L.H. and Rabbege, J.R. (1975) **Caveola-vesicle complexes on the plasmalemma of erythrocytes infected by *Plasmodium vivax* and *P. cynomolgi*.** *Am. J. Pathol.* 79, 285–300
19 Barnwell, J.W. (1990) **Vesicle-mediated transport of membrane and proteins in malaria-infected erythrocytes.** *Blood Cells* 16, 379–395
20 Luse, S.A. and Miller, L.H. (1971) ***Plasmodium falciparum* malaria: ultrastructure of parasitized erythrocytes in cardiac vessels.** *Am. J. Trop. Med. Hyg.* 20, 665–660
21 Howard, R.J. and Barnwell, J.W. (1984) in *Contemporary Topics in Immunobiology Vol. 12 Immunology of Parasites and Parasite Infections* (Marchalonis, J.J., ed.), pp 127–200, Plenum
22 Ward, G.E., Chitnis, C.E. and Miller, L.H. (1994) in *Strategies for Intracellular Survival of Microbes* (Russell, D., ed.), pp 155–190, Saunders
23 Braun Breton, C. and Pereira Da Silva, L.H. (1993) **Malaria proteases and red blood cell invasion.** *Parasitology Today* 9, 92–96
24 Mitchell, G.H. and Bannister, L.H. (1988) **Malaria parasite invasion: interactions with the red cell membrane.** *CRC Crit. Rev. Onc./Hem.* 8, 255–310
25 Bannister, L.H. and Dluzewski, A.R. (1990) **The ultrastructure of red cell invasion in malaria infections: a review.** *Blood Cells* 16, 257–292
26 Holder, A.A. and Freeman, R.R. (1981) **Immunization against blood-stage rodent malaria using purified parasite antigens.** *Nature* 294, 361–364
27 Holder, A.A. and Freeman, R.R. (1982) **Biosynthesis and processing of a *Plasmodium falciparum* schizont antigen recognized by immune serum and a monoclonal antibody.** *J. Exp. Med.* 156, 1528–1535
28 Epstein, N. *et al.* (1981) **Monoclonal antibodies against a specific surface determinant on malarial (*Plasmodium knowlesi*) merozoites block erythrocyte invasion.** *J. Immunol.* 127, 212–217
29 Holder, A.A. (1988) **The precursor to the major merozoite surface antigens: structure and role in immunity.** *Prog. Allergy* 41, 72–97
30 Miller, L.H. *et al.* (1993) **Analysis of sequence diversity in the *Plasmodium falciparum* merozoite surface protein-1 (MSP-1).** *Mol. Biochem. Parasitol.* 59, 1–14
31 del Portillo, H.A. *et al.* (1991) **Primary structure of the merozoite surface antigen 1 of *Plasmodium vivax* reveals sequences conserved between different *Plasmodium* species.** *Proc. Natl Acad. Sci. USA* 88, 4030–4034
32 Gibson, H.L. *et al.* (1992) **Structure and expression of the gene for Pv200, a major blood-stage surface antigen of *Plasmodium vivax*.** *Mol. Biochem. Parasitol.* 50, 325–334
33 Lewis, A.P. (1989) **Cloning and analysis of the gene encoding the 230-kilodalton merozoite surface antigen of *Plasmodium yoelii*.** *Mol. Biochem. Parasitol.* 36, 271–282
34 Deleersnijder, W. *et al.* (1990) **Molecular cloning and sequence analysis of the gene encoding the major merozoite surface antigen of *Plasmodium chabaudi chabaudi* IP-PC1.** *Mol. Biochem. Parasitol.* 43, 231–244
35 Daly, T.M., Burns, J.M., Jr and Long, C.A. (1992) **Comparison of the carboxy-terminal, cysteine-rich domain of the merozoite surface protein-1 from several strains of *Plasmodium yoelii*.** *Mol. Biochem. Parasitol.* 52, 279–282
36 Perkins, M.E. and Rocco, L.J. (1988) **Sialic acid-dependent binding of *Plasmodium falciparum* merozoite surface antigen, Pf200, to human erythrocytes.** *J. Immunol.* 141, 3190–3196
37 Barnwell, J.W. and Galinski, M.R. (1991) **The adhesion of malaria merozoite proteins to erythrocytes: a reflection of function?** *Res. Immunol.* 142, 666–671
38 Diggs, C.L., Ballou, W.R. and Miller, L.H. (1993) **The major merozoite surface protein as a malaria vaccine target.** *Parasitology Today* 9, 300–302
39 Smythe, J.A. *et al.* (1988) **Identification of two integral membrane proteins of *Plasmodium falciparum*.** *Proc. Natl Acad. Sci. USA* 85, 5195–5199
40 Oeuvray, C. *et al.* (1993) **Characterization of a *Plasmodium falciparum* merozoite surface antigen targeted by defense mechanisms developed in immune individuals.** *C.R. Acad. Sci. Paris (Biol. Sci.)* 316, 395–399
41 McColl, D.J. *et al.* (1994) **Molecular variation in a novel polymorphic antigen associated with *Plasmodium falciparum* merozoites.** *Mol. Biochem. Parasitol.* 68, 53–67
42 Holder, A.A. and Blackman, M.J. (1994) **What is the function of MSP-1 on the malaria merozoite?** *Parasitology Today* 10, 182–184
43 Adams, J.H. *et al.* (1990) **The Duffy receptor family of *Plasmodium knowlesi* is located within the micronemes of invasive merozoites.** *Cell* 63, 141–153
44 Haynes, J.D. *et al.* (1988) **Receptor-like specificity of a *Plasmodium knowlesi* malarial protein that binds to Duffy antigen ligands on erythrocytes.** *J. Exp. Med.* 167, 1873–1881
45 Chaudhuri, A. *et al.* (1993) **Cloning of glycoprotein D cDNA, which encodes the major subunit of the Duffy blood group system and the receptor for the *Plasmodium vivax* malaria parasite.** *Proc. Natl Acad. Sci. USA* 90, 10793–10797
46 Fang, X. *et al.* (1991) **Cloning of the *Plasmodium vivax* Duffy receptor.** *Mol. Biochem. Parasitol.* 44, 125–132
47 Adams, J.H. *et al.* (1992) **A family of erythrocyte binding proteins of malaria parasites.** *Proc. Natl Acad. Sci. USA* 89, 7085–7089

48 Camus, D. and Hadley, T.J. (1985) **A *Plasmodium falciparum* antigen that binds to host erythrocytes and merozoites.** *Science* 230, 553–556

49 Sim, B.K.L. *et al.* (1992) **Localization of the 175-kilodalton erythrocyte binding antigen in micronemes of *Plasmodium falciparum* merozoites.** *Mol. Biochem. Parasitol.* 51, 157–160

50 Orlandi, P.A., Klotz, F.W. and Haynes, J.D. (1992) **A malaria invasion receptor, the 175-kilodalton erythrocyte binding antigen of *Plasmodium falciparum*, recognizes the terminal Neu5Ac(α2-3)Gal sequence of glycophorin A.** *J. Cell Biol.* 116, 901–911

51 Sim, B.K.L. *et al.* (1994) **Receptor and ligand domains for invasion of erythrocytes by *Plasmodium falciparum*.** *Science* 264, 1941–1944

52 Chitnis, C.E. and Miller, L.H. (1994) **Identification of the erythrocyte binding domains of *Plasmodium vivax* and *Plasmodium knowlesi* proteins involved in erythrocyte invasion.** *J. Exp. Med.* 180, 497–506

53 Perkins, M.E. (1992) **Rhoptry organelles of apicomplexan parasites.** *Parasitology Today* 8, 28–32

54 Cheng, Q. and Saul, A. (1994) **Sequence analysis of the apical membrane antigen 1 (AMA-1) of *Plasmodium vivax*.** *Mol. Biochem. Parasitol.* 65, 183–187

55 Peterson, M.G. *et al.* (1989) **Integral membrane protein located in the apical complex of *Plasmodium falciparum*.** *Mol. Cell Biol.* 9, 3151–3155

56 Peterson, M.G. *et al.* (1990) **Apical membrane antigen of *Plasmodium fragile*.** *Mol. Biochem. Parasitol.* 39, 279–284

57 Marshall, V.M. *et al.* (1989) **Structure of the apical membrane antigen I (AMA-1) of *Plasmodium chabaudi*.** *Mol. Biochem. Parasitol.* 37, 281–284

58 Waters, A.P. *et al.* (1990) **A merozoite receptor protein from *Plasmodium knowlesi* is highly conserved and distributed throughout *Plasmodium*.** *J. Biol. Chem.* 265, 17974–17979

59 Kuhn, K. and Eble, J. (1994) **The structural bases of integrin–ligand interactions.** *Trends Cell Biol.* 4, 256–261

60 Joneckis, C.C. *et al.* (1993) **Integrin alpha 4 beta 1 and glycoprotein IV (CD36) are expressed on circulating reticulocytes in sickle cell anemia.** *Blood* 82, 3548–3555

61 Wilson, R.J.M. (1990) **Biochemistry of red cell invasion.** *Blood Cells* 16, 237–252

62 Aikawa, M. *et al.* (1978) **Erythrocyte entry by malaria parasites: a moving junction between erythrocyte and parasite.** *J. Cell Biol.* 77, 72–78

63 Aikawa, M. and Miller, L.H. (1983) in *Malaria and the Red Cell*. CIBA Foundation Symposium 94 (Evered, D. and Whelan, J., eds), pp 45–63, Pitman

64 Miller, L.H. *et al.* (1979) **Interactions between cytochalasin B-treated malaria parasites and erythrocytes: attachment and junction formation.** *J. Exp. Med.* 149, 172–184

65 Brown, D. (1993) **The tyrosine kinase connection: how GPI-anchored proteins activate T cells.** *Curr. Opin. Immunol.* 5, 349–354

66 Schofield, L. and Hackett, F. (1993) **Signal transduction in host cells by a glycosylphosphatidylinositol toxin of malaria parasites.** *J. Exp. Med.* 177, 145–153

67 Coppel, R.L. (1992) **Malaria – revealing the ties that bind.** *Parasitology Today* 8, 393–394

68 Ward, G.E. *et al.* (1994) **Staurosporine inhibits invasion of erythrocytes by malarial merozoites.** *Exp. Parasitol.* 79, 480–487

69 Keen, J.K. *et al.* (1994) **A gene coding for a high molecular mass rhoptry protein of *Plasmodium yoelii*.** *Mol. Biochem. Parasitol.* 65, 171–177

70 Mitchell, G.H. *et al.* (1986) **Invasion of erythrocytes by *Plasmodium falciparum* malaria parasites. Evidence for receptor heterogeneity and two receptors.** *Blood* 67, 1519–1521

71 Hadley, T.J. *et al.* (1987) **Falciparum malaria parasites invade erythrocytes that lack glycophorin A and B (MkMk). Strain differences indicate receptor heterogeneity and two pathways for invasion.** *J. Clin. Invest.* 80, 1190–1193

72 Perkins, M.E. and Holt, E.H. (1988) **Erythrocyte receptor recognition varies in *Plasmodium falciparum* isolates.** *Mol. Biochem. Parasitol.* 27, 23–34

73 Dolan, S.A., Miller, L.H. and Wellems, T.E. (1990) **Evidence for a switching mechanism in the invasion of erythrocytes by *Plasmodium falciparum*.** *J. Clin. Invest.* 86, 618–624

74 Dolan, S.A. *et al.* (1994) **Glycophorin B as an EBA-175 independent *Plasmodium falciparum* receptor of human erythrocytes.** *Mol. Biochem. Parasitol.* 64, 55–63

75 Mason, S.J. *et al.* (1977) **The Duffy blood group determinants: Role in the susceptibility of human and animal erythrocytes to *Plasmodium knowlesi* malaria.** *Br. J. Haematol.* 36, 327–335

76 Keen, J. *et al.* (1990) **Identification of the gene for a *Plasmodium yoelii* rhoptry protein: multiple copies in the parasite genome.** *Mol. Biochem. Parasitol.* 42, 241–246

77 Ogun, S.A. and Holder, A.A. (1994) ***Plasmodium yoelii*: Brefeldin A-sensitive processing of proteins targeted to the rhoptries.** *Exp. Parasitol.* 79, 270–278

78 Freeman, R.R., Trejdosiewicz, A.J. and Cross, G.A.M. (1980) **Protective monoclonal antibodies recognising stage specific merozoite antigens of a rodent malaria parasite.** *Nature* 284, 366–368

79 Miller, L.H. *et al.* (1976) **The resistance factor to *Plasmodium vivax* in blacks: The Duffy blood group genotype FyFy.** *New Engl. J. Med.* 295, 302–304

80 Spencer, H.G. *et al.* (1978) **The Duffy blood group and resistance to *Plasmodium vivax* in Honduras.** *Am. J. Trop. Med. Hyg.* 27, 664–672

81 Nichols, M.E. *et al.* (1987) **A new human Duffy blood group specificity defined by a murine monoclonal antibody.** *J. Exp. Med.* 166, 776–785

82 McWilson, W., Skinner, J.C. and Guinn, E. (1966) **Biology of the simian malarias of Southeast Asia. I. Host cell preferences of young trophozoites of four species of *Plasmodium*.** *J. Parasitol.* 52, 14–16

83 Heldin, C-H. (1995) **Dimerization of cell surface receptors in signal transduction.** *Cell* 80, 213–223

84 Witzig, R.S. and Barker, R.H., Jr (1994) **Atypical *Plasmodium vivax* infection in Peru.** *Trans. R. Soc. Trop. Med. Hyg.* 88, 198

85 Bouharoun-Tayoun, H. *et al.* (1990) **Antibodies that protect humans against *Plasmodium falciparum* blood stages do not on their own inhibit parasite growth and invasion *in vitro*, but act in cooperation with monocytes.** *J. Exp. Med.* 172, 1633–1641

86 Blackman, M.J. *et al.* (1994) **Antibodies inhibit the protease-mediated processing of a malaria merozoite surface protein.** *J. Exp. Med.* 180, 389–393

87 Tsuboi, T. *et al.* (1994) **Natural variation within the principal adhesion domain of the *Plasmodium vivax* Duffy binding protein.** *Infect. Immun.* 62, 5581–5586

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Erratum

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