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2nd, revised and enlarged edition

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Volume Editors

Peter Perlmann *Stockholm*
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Malaria Antigens

Preface

In spite of all the efforts to control this infection, malaria is as frequent and deadly today as it was 12 years ago when this book was first published. However, due to the rapid development of immunology and parasitology, an enormous amount of new results in malaria immunology has come forth during this period and provided a basis for this 2nd edition. The book is now composed of 19 chapters, arranged in four major sections. The first section (3 chapters) deals with the malaria parasite and its interactions both with the vertebrate host and with the mosquitoes which transmit the disease. The second section (6 chapters) presents a detailed account of the many antigens giving rise to important immune responses in the vertebrate host while section three (3 chapters) reports on the mechanisms of immunity and their regulation by environmental and genetic factors. Finally, this volume also contains 7 chapters on malaria vaccine development, dealing both with the application of the most recent vaccine technologies and with the ongoing or planned malaria vaccine trials.

Altogether the 19 chapters, which are authored by well-recognized experts, provide a broad and up-to-date overview of the rapidly expanding field of malaria immunology, an area of critical importance both for an understanding of immunity to infection in general and for coming to grips with one of the biggest and most devastating infectious diseases worldwide.

Peter Perlmann
Marita Troye-Blomberg

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Structure and Life Cycle

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Introduction

Plasmodium species, as members of *Apicomplexa*, share many common morphological features. Each of the developmental stages in the life cycle of malaria parasites exhibits a remarkable conservation and distinct patterns of structural organization [1, 2]. These conservations are supposed to have originated in the special adaptation to the tissues and/or cells in the different hosts of malaria parasites. As the technology of electron microscopy has improved, more detailed electron microscopic observations of the various stages of malaria parasites have been carried out and greatly advanced our knowledge of the life cycle and the fine structure of malaria parasites. Although the significance of the morphological changes is not fully understood, the introduction of the techniques of immunoelectron microscopy to the field of malaria parasites [3] has helped us in the meaningful and dynamic analysis of parasite morphology and cell biology, and our knowledge of the subcellular localization of malaria antigens and their functions in specific parasite organelles has been accumulated. Structural, biochemical and molecular biological aspects are different among the complex cycle comprising the erythrocytic schizogony, mosquito stages, and preerythrocytic (exoerythrocytic) schizogony. In this chapter, we will describe the ultrastructure of each specific stage, and the morphological and functional changes of the host cells induced by malaria parasites.

Life Cycle

The life cycle of the malaria parasite is complex (fig. 1). The sporozoites are transmitted to the vertebrate host by the bite of infected female mosquitoes

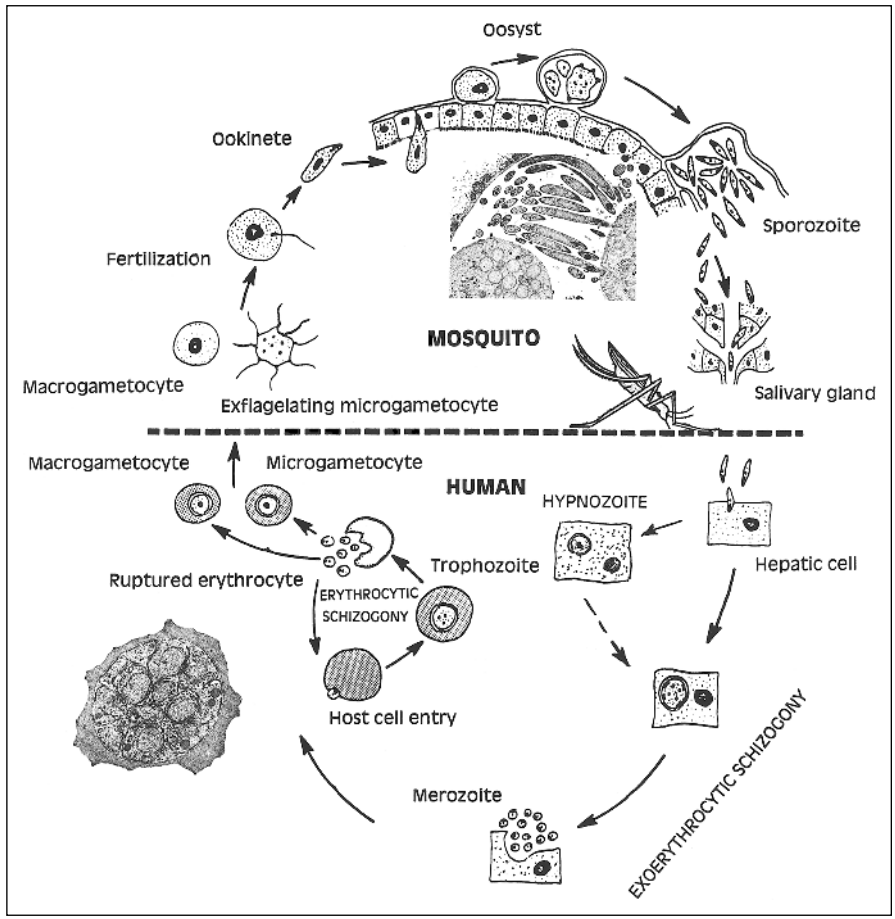
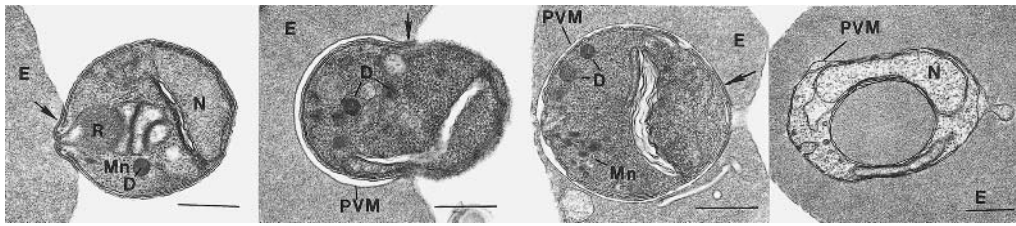


Fig. 1. Schematic drawing of the life cycle of malaria parasites.

of the genus *Anopheles*. The sporozoites enter hepatocytes shortly after inoculation into the blood circulation. This process has demonstrated that sporozoite invasion of hepatocytes involves surface proteins of the sporozoite and host cell surface molecules. Sporozoites infected in the hepatocytes develop into preerythrocytic (exoerythrocytic, EE stage; fig. 9) schizonts during the next 5–15 days depending on the *Plasmodium* species. *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium cynomolgi* have a dormant stage, named hypnozoite [4, 5], that may remain in the liver for weeks to many years before the development of preerythrocytic schizogony. This results in relapses of malaria infection. *Plasmodium falciparum* and *Plasmodium malariae* have no persistent phase. A preerythrocytic schizont contains 10,000 to 30,000 merozoites, which are



a ***b*** ***c*** ***d***

Fig. 2. Malaria merozoite invasion process. ***a*** Apical end of a *P. knowlesi* merozoite attaches to an erythrocyte (E). The erythrocyte membrane becomes thick at the attachment site (arrow). ***b*** Further advanced stage of erythrocyte entry by a *P. knowlesi* merozoite. The junction (arrow), formed between the thickened erythrocyte membrane and the merozoite, is always located at the orifice of the merozoite entry. No surface coat is visible on the portion of the merozoite surface, which has invaginated the erythrocyte membrane, while the surface coat is present behind the junction (arrow) site. ***c*** Erythrocyte entry by a *P. knowlesi* merozoite is almost complete. The junction (arrow) has now moved to the posterior end of the merozoite. An electron-opaque projection connects the merozoite's apical end and erythrocyte membrane. ***d*** A trophozoite (ring form) stage of *P. falciparum* is surrounded by the parasitophorous vacuole membrane (PVM). R = Rhoptry; D = dense granules; Mn = micronemes; E = erythrocyte; N = nucleus. Bars = 0.5 μm. ***a*** and ***c*** are reprinted with permission from Fujioka and Aikawa (1999) [60]; courtesy of Harwood Academic Publisher.

released into the blood circulation and invade the red blood cells. The merozoite develops within the erythrocyte through ring, trophozoite and schizont stages (erythrocytic schizogony; fig. 3a). The parasite modifies its host cell in several ways to enhance its survival. The erythrocyte containing the segmented schizonts eventually ruptures and releases the newly formed merozoites that invade new erythrocytes (fig. 2). Erythrocyte invasion by merozoites is dependent on the interactions of specific receptors on the erythrocyte membrane with ligands on the surface of the merozoite. The entire invasion process takes about 30 s. Concomitantly, a small portion of the parasites differentiate from newly invaded merozoites into sexual forms, which are macrogametocyte (female) and microgametocyte (male). What triggers this alternative developmental pathway leading to gametocyte formation is unknown. Mature macrogametocytes, taken into the midgut of the *Anopheles* mosquito, escape from the erythrocyte to form macrogametes. Microgametocytes exflagellate, each forming eight haploid motile microgametes after a few minutes in the mosquito midgut. The microgamete moves quickly to fertilize a macrogamete and forms a zygote. Within 18–24 h, the non-motile zygotes transform into motile ookinetes. The ookinetes have to cross two barriers: the peritrophic matrix (PM) and midgut epithelium (fig. 7a). After traversing the midgut epithelium, the ookinete reaches the extracellular space between the midgut epithelium and the overlaying basal lamina,

and transforms into an oocyst (fig. 7b). Ten to 24 days after infection, depending on the *Plasmodium* species and ambient temperature, thousands of sporozoites (fig. 8b) are released into the hemocoel and the motile sporozoites invade the salivary gland epithelium. When an infected mosquito bites a susceptible vertebrate host, the *Plasmodium* life cycle begins again.

Erythrocytic Schizogony Merozoites

The erythrocytic merozoite is an ovoid cell and measures approximately 1.5 μm in length and 1 μm in width (fig. 2a). The apical end of the merozoite is a truncated cone-shaped projection demarcated by the polar rings. Three types of membrane-bound organelles, namely, rhoptries (two prominent pear-shaped, 570×330 nm), micronemes (ovoid bodies, 100×40 nm), and dense granules (spheroid vesicles, 140×120 nm), are located at the anterior end of the merozoite [6]. The contents of these organelles play a role in the binding and entry of the merozoite into the host cells. Extracellular merozoites are intrinsically short-lived and must rapidly invade a new host erythrocyte. The merozoite is surrounded by a trilaminar pellicle that is composed of a plasma membrane and two closely aligned inner membranes [1]. The plasma membrane measures about 7.5 nm in thickness. Just beneath this inner membrane complex is a row of subpellicular microtubules which originate from the polar ring of the apical end and radiate posteriorly [2]. It has been suggested that the inner membrane complex and subpellicular microtubules function as a cytoskeleton giving rigidity to the merozoite and may be involved in invasion [7]. The presence of *P. falciparum* myosin A in the apex of the mature merozoites suggests its involvement in merozoite motility during invasion [8]. The outer membrane of the extracellular merozoite is covered with a surface coat of about 20 nm in thickness, and plays an important role in the early stages of merozoite invasion [9]. A mitochondrion is seen in the posterior portion of the merozoites [1]. Mammalian parasites appear to have a few cristate or acristate mitochondrion. An additional structure, referred to as a spherical body, has been identified. A recent study [10] described that the plastid of *P. falciparum* (or 'apicoplast') is the evolutionary homologue of the plant chloroplast. The apicoplast is surrounded by four membranes and is likely to contain many prokaryote-type pathways. Golgi complexes are inconspicuous in the merozoite.

Host Cell Entry

Malaria merozoite invasion process is complex (fig. 2a–c) and involved in the multi-step sequence which can be divided into four phases: (1) initial

recognition and reversible attachment of the merozoite to the erythrocyte membrane; (2) reorientation and junction formation between the apical end of the merozoite (irreversible attachment) and the release of rhoptry-microneme substances with parasitophorous vacuole formation; (3) movement of the junction and invagination of the erythrocyte membrane around the merozoite accompanied by removal of the merozoite's surface coat, and finally (4) resealing of the parasitophorous vacuole membrane (PVM) and erythrocyte membrane after completion of merozoite invasion (fig. 2) [for review see 1, 11, 12]. The initial factor underlying recognition between merozoites and erythrocytes may occur between the merozoite surface coat filament and erythrocyte surface. Multiple different receptor-ligand interactions occur during the merozoite invasion process into an erythrocyte [13, 14]. Merozoite surface protein-1, with a glycosylphosphatidylinositol anchor, (MSP-1; also called MSA1, gp195 or PMMSA) could be involved in the initial recognition of the erythrocyte in a sialic acid-dependent way [15]. Herrera et al. [16] suggested that MSP-1 interacted with spectrin on the cytoplasmic face of the erythrocyte membrane. Three other *P. falciparum*-merozoite surface proteins, named MSP-2, MSP-3 and MSP-4, have been identified [17, 18]. A number of investigators concluded that sialic acid on glycophorins are involved in receptor recognition for merozoite invasion [15, 19–21] after initial attachment. The microneme derived 175-kD erythrocyte-binding antigen (EBA-175) [22] of *P. falciparum* also binds to sialic acids on glycophorin [23]. The gene structure of EBA-175 has striking similarities with the Duffy-binding proteins of *P. vivax* and *P. knowlesi* [23–26]. Phylogenetically distant malaria species, *P. falciparum*, *P. vivax*, *P. knowlesi*, and also rodent malaria parasites [27] maintain species-specific and biologically similar proteins. EBA-175 seems to be the most important ligand for binding of merozoites to glycophorin A on the erythrocytes; however, some *P. falciparum* merozoites can utilize alternative pathways for invasion. Dolan et al. [28] showed that glycophorin B can also act as an erythrocyte receptor. Furthermore malaria merozoites can utilize independent pathways for invasion without sialic acid [29]. Following reorientation of the apical end of the merozoite contacting the erythrocyte membrane, a junction is formed between the apical end of the merozoite and erythrocyte membrane, and moved from the apical end to the posterior end of the merozoite. The junction seems to selectively control internalization of host cell plasma membrane components into the PVM [30, 31]. The merozoite cap protein 1 (MCP-1) [32] with an oxidoreductase domain is localized to the junction. The positive charge cluster in the C-terminal domain of this protein resembles domains in some cytoskeleton-associated proteins, raising speculations that the C-terminal domain of MCP-1 interacts with the cytoskeleton in *Plasmodium* [33]. As the invasion progresses, the depression of the erythrocyte membrane deepens and conforms to the shape of

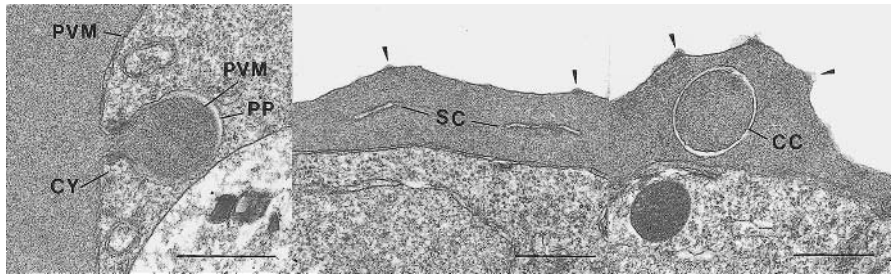
the merozoite. The junction is no longer observed at the initial attachment point but now appears at the orifice of the merozoite-induced invagination of the erythrocyte membrane. Cytochalasin B or D [34, 35] blocks the merozoite invasion step into erythrocyte. Staurosporine also blocks invasion at a step which is morphologically similar to the arrest seen with cytochalasin B or D [13, 14]. From these results, an actin-based motility system, probably within the parasite, might play an important role in the movement of junction during merozoite invasion into erythrocyte [35]. Several proteins have been identified and localized to the apical complex in *Plasmodium* species. The secretion-triggering mechanism seems to be similar to those of many other exocytotic cells. A calcium-dependent second-messenger system may be involved in the secretion of rhoptry-microneme contents [36]. Rhoptries contain high molecular weight proteins, 140-kD Rhop-1, 130-kD Rhop-2 and 110-kD Rhop-3 (Rhop-H) [for review see 37]. The Rhop-H proteins are localized in the electron-dense compartment of rhoptries of *P. falciparum* (fig. 8) [38]. Apical membrane antigen-1 (AMA-1 also called Pf83) is localized in the rhoptry organelles, and is processed to a 66-kD molecule with epitopes expressed on the surface of the merozoite. AMA-1 family proteins are homologous to the relatively well-conserved proteins in *Plasmodium* species [39–41].

During host cell invasion, no surface coat is visible on the portion of the merozoite within the erythrocyte invagination (fig. 2b), whereas the surface coat on the portion of the merozoite still outside the erythrocyte appears similar to that seen on the free merozoites. Biochemical studies demonstrated that the 19-kD fragment is transported into the erythrocyte while other MSP-1 fragments were shed into supernatant during merozoite invasion [42, 43]. When the merozoite has completed entry, the junction fuses at the posterior end of the merozoite, closing the orifice in the fashion of an iris diaphragm. The merozoite still remains in close apposition to the thickened erythrocyte membrane at the point of final closure [1]. After completion of host cell entry, the merozoite is now surrounded by the PVM (fig. 2d). Fluorescent lipid probes have been used to demonstrate that PVM lipids are largely derived from the erythrocyte membrane [44]. This membrane serves as an interface between the parasite and host cell cytoplasm. Molecules such as nutrients must cross the PVM from the host cell to the parasite and other molecules such as metabolites and parasite-synthesized proteins must cross the PVM in the opposite direction. Dense granules of *P. knowlesi* merozoites were shown to move to the merozoite pellicle after merozoite entry into the erythrocyte [6]. These contents were released into the parasitophorous vacuole space and appeared to assist the formation of invaginations of the PVM. The ring-infected erythrocyte antigen (RESA; also called Pf155) [45, 46] is located in dense granules [47]. This antigen appears not to be transferred to the erythrocyte membrane during the initial formation of a

junction between the apical end of a merozoite and the erythrocyte. The transportation process of the RESA/Pf155 protein from the dense granule to the infected erythrocyte membrane is unknown. This antigen is suggested to associate with the erythrocyte cytoskeleton mediated by spectrin [48, 49]. The organelle contents of the merozoite play a role in merozoite entry into the erythrocyte and also appear to have the additional roles of modification of the host cell membrane and PVM. These modifications seem to enable malaria parasites to survive and proliferate within the host erythrocytes. Recently subtilisin-like proteases, PfSUB1 [50] and PfSUB2 [51] from a subset of the dense granules were described. These enzymatically active proteases may function in the initial steps of erythrocyte invasion.

Trophozoites and Schizonts

When the extracellular merozoite invades the erythrocyte, it rounds up due to the rapid degradation of the inner membrane complex and subpellicular microtubules of the pellicular complex, and becomes a trophozoite. Dense granules within the merozoite move to the merozoite pellicle, and the contents of dense granules are released into the parasitophorous vacuole space [6]. The parasite in the erythrocyte is surrounded by the PVM (fig. 2d). This membrane serves as an interface between the parasite and host cell cytoplasm [for review see 52, 53]. Molecules such as nutrients must cross the PVM from the host cell to the parasite and other molecules, such as metabolites and parasite synthesized surface proteins (e.g. knob proteins), must cross the PVM in the opposite direction. The trophozoite survives intracellularly by ingesting host cell cytoplasm through a circular structure named the cytostome [54]. The cytostome (fig. 3a) possesses a double-membrane, consisting of an outer membrane (parasite plasmalemma) and an inner membrane (PVM). Malaria parasites use host hemoglobin as a source of amino acids; however, they cannot degrade the hemoglobin heme byproduct. Free heme is potentially toxic to the parasite. Therefore during hemoglobin degradation, most of the liberated heme is polymerized into hemozoin (malaria pigment), which is stored within the food vacuoles [55, 56]. The trophozoite of *P. falciparum* has several mitochondria with few cristae or acristate mitochondria. Cristate mitochondria, however, have also been observed in the erythrocytic trophozoites of *P. malariae* [57]. Immunoelectron microscopic analysis reveals the distinct mitochondrial localization of *P. falciparum* heat shock protein (PfHsp60) [58]. Ribosomes are abundant in the trophozoites, and most of them are of the free type. Various merozoite organelles, which had disappeared during trophozoite development, reappear at the segmented schizont (fig. 4a).



a *b* *c*

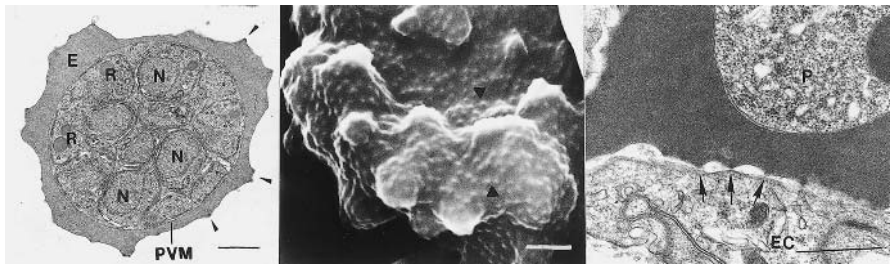
Fig. 3. ***a*** Transmission electron micrograph of a *P. falciparum* trophozoite ingesting the erythrocyte cytoplasm by a cytostome (CY). The cytostome possesses a double membrane consisting of an outer membrane (parasite plasmalemma, PP) and an inner membrane (PVM). ***b, c*** Electron micrographs of *P. falciparum*-infected erythrocytes. Note difference in morphology between slit-like cleft (SC) and circular cleft (CC). The circular cleft appears to develop as a whorl-like extension of the parasitophorous vacuole membrane (PVM). Arrowheads indicate the knobs. Bars = 0.5 μm.

Host Cell Alterations by Malaria Parasites

After invasion into the host erythrocytes, parasites begin to remodel and modify both internal and external membranes of the erythrocyte. These modifications enable the parasites to survive and proliferate in the host. Four basic types of ultrastructural alterations have been described in infected erythrocytes; knobs (fig. 4), tubovesicular complex, cytoplasmic clefts (fig. 3b, c) and caveola-vesicle complexes [59, 60].

Knob Formation and Cytoadherence

Knobs occur on erythrocytes infected by *falciparum*-, *ovale*-, and *malaria*-type parasites, and have been studied intensely in *P. falciparum*-infected erythrocytes because of the important role of the knobs in mediating cytoadherence of infected erythrocytes to the vascular endothelium (fig. 4c). Knobs are cup-shaped, electron-dense protrusions found in the infected erythrocyte membrane, measuring 30–40 nm in height and 100 nm in width by electron microscopic observation (fig. 4a, b). Aikawa et al. [61] investigated the structure of the knobs of unfixed *P. falciparum*-infected erythrocytes by atomic force microscopy. Each knob was found to consist of two distinct subunits. These knobs form focal junctions with the endothelial cell membrane. The phenomenon of cytoadherence is thought to be a mechanism developed by *P. falciparum* to avoid destruction by



a ***b*** ***c***

Fig. 4. Electron micrographs showing the electron-dense protrusions (knobs; arrowheads) on the surface of *P. falciparum*-infected erythrocytes. ***a*** Mature schizont stage (segmenter) of *P. falciparum*-infected erythrocyte has numerous knobs. E = Erythrocyte; N = nuclei of individual merozoite; PVM = parasitophorous vacuole membrane; R = rhoptries. Bar = 1 μm. ***b*** Scanning electron micrograph showing knobs (arrowheads) over the *P. falciparum*-infected erythrocyte surface. Bar = 0.5 μm. Reprinted with permission from Aikawa et al. (1983) [62]; courtesy of the Journal Parasitology. ***c*** Transmission electron micrograph showing adherence (arrows) between a *P. falciparum* (P) infected erythrocyte via knobs and an endothelial cell (EC) of a cerebral microvessel. Bar = 1 μm. Reprinted with permission from Atkinson and Aikawa (1990) [59]; courtesy of Blood Cells.

the spleen. Cytoadherence causes infected erythrocytes to adhere to the vascular endothelium and sequester in postcapillary venules of various organs. The sequestration phenomenon frequently leads to organ-specific damage and lethal syndromes [for review see 63]. At least eight *falciparum* malaria proteins have been identified on the surface or in association with the cytoskeleton of erythrocytes [for review see 60]. These include proteins such as histidine-rich protein-I and II (HRP-I and II; HRP-I is also called knob-associated histidine-rich protein, KAHRP), erythrocyte membrane protein-1, 2 and 3 (PfEMP-1, 2 and 3), ring-infected erythrocyte membrane surface antigen (Pf155/RESA), sequestrin and rosettins.

HRP-1/KAHRP is a 90-kD water-insoluble, histidine-rich protein that has been localized in electron-dense knobs and clefts [64, 65]. This protein is transported to the cytoplasmic face of knobs in association with electron-dense material; therefore, it appears to be involved in the structural formation of the knob [66]. PfEMP-1, encoded by a large family of genes (*var*), is an antigenically diverse 200- to 350-kD surface protein of infected erythrocyte [67–69], and seems to be one of the major cytoadherent proteins, mediating adherence of *P. falciparum*-infected erythrocytes to microvascular endothelial cells in cerebral malaria patients. Immunoelectron microscopic localization has identified the PfEMP-1 molecule at the tip of the knob protrusions of the infected erythrocyte [67]. Knob formation is critically dependent upon the expression of the

HRP-1/KAHRP [70]. This protein is transported through Maurer's cleft to the cytoplasmic face of the erythrocyte membrane where association with spectrin and actin occur beneath the erythrocyte membrane [71]. Waller et al. [72] suggested a direct interaction between HRP-1/KAHRP and the cytoplasmic region of PfEMP-1. HRP-II is a water-soluble, histidine-rich 70-kD protein localized in the erythrocyte cytoplasm, in association with clefts and the erythrocyte cytoplasm [73], and also released into plasma in high amounts. Sequestrin, a CD36 recognition protein, is an approximately 270-kD protein localized on the surface of infected erythrocytes [74]. PfEMP-2 (also called mature erythrocyte surface antigen, MESA) is polymorphic in size, 250–300 kD in different isolates, and it has been localized in the parasitophorous vacuole of the schizont, within membrane-bound vesicles in the erythrocyte cytoplasm, in association with knobs, and the inner face of the erythrocyte membrane covering the knobs [75]. PfEMP-2/MESA is specifically associated with the cytoskeleton of the infected erythrocytes [71, 76], therefore this may serve as an important anchoring element for PfEMP-1. PfEMP-3 is a 315-kD surface antigen which is located on the erythrocyte membrane [77]. PfEMP-3 may be involved in knob formation and it is suspected that it interacts with a protein(s) of the erythrocyte cytoskeleton. Pf155/RESA is a ring-infected erythrocyte surface antigen that has been localized specifically in the dense granules of merozoites [47]. This molecule is translocated to the erythrocyte membrane/cytoskeleton from dense granules of merozoites which have newly invaded the erythrocytes. This molecule is a spectrin-binding protein that forms a complex with actin, spectrin and band 4.1 [48]. Rosettins are 22- to 28-kD rosetting ligands which are located on the erythrocyte membrane [78, 79]. As the receptors of rosettins seem to be both CD36 and ABO blood group antigens [80, 81], these molecules have potential to bind to the endothelial cell receptors.

Cytoplasmic Clefts

Cytoplasmic clefts have been reported from all of the species of primate malaria parasites. These clefts have been demonstrated to be continuous with the PVM, and clearly differ in structure from the erythrocyte membrane skeleton. Freeze-fracture and cytochemical studies of the PVM and clefts have shown that they have a reversed polarity from the erythrocyte membrane skeleton in terms of distribution of intramembranous particles and location of ATPase and NADH oxidase activity. In *P. falciparum*-infected erythrocyte cytoplasm, two morphologically distinct populations of membrane-bound clefts have been identified: (1) flattened and slit-like clefts (fig. 3b), and (2) larger, circular or vesicular clefts (fig. 3c) [59, 82]. The slit-like clefts appear ranging from 100 to 600 nm in length in cross-section and correspond in the structure to Maurer's clefts. The large circular clefts appear to originate from thin pseudopodial

extensions of the parasite cytoplasm and the PVM. Immunocytochemical studies have clearly demonstrated both antigenic and morphological differences among these cytoplasmic clefts [59, 83, 84]. Three different groups of antigen, at least, are transported and/or located on the clefts within the infected erythrocyte cytoplasm: (1) cytoskeletal-associated antigens; (2) water-soluble antigens, and (3) membrane-associated antigens [65, 73, 84].

Tubovesicular Network

In the *P. falciparum*-infected erythrocyte cytoplasm, distinct tubular structures have been demonstrated [85, 86] using laser confocal microscopy. These studies suggested that the tubovesicular network (TVM) was continuous with the PVM, and contained the Golgi-specific protein, sphingomyelin synthase. Lipid biosynthesis is suggested to exist in two distinct sphingomyelin synthase activities in the PVM/TVM network [87]. Halder et al. [52] suggested the relationship of PVM/TVM to Maurer's clefts and loop structure in the infected erythrocyte. However, Maurer's cleft and loop structure have distinct protein components (28- and 45-kD parasite proteins). Therefore, Maurer's cleft and loop structure seem somehow separate in the PVM/TVM network. The TVM is thought to play a part in the metabolism and sorting of membrane lipids.

Caveola-Vesicle Complexes

P. vivax induces morphologic alterations in the infected erythrocytes that are visible by light microscopy in Rhomanovsky-stained blood smears. These morphologic changes are referred to as Schuffner's dots and have been associated with caveola-vesicle complexes by electron microscopy. Caveolae are small flask-like invaginations of the infected erythrocyte membrane skeleton that measure approximately 90 nm in diameter. In *vivax*- and *ovale*-malaria, spherical or tube-like vesicles are associated singly or in small clusters with the base of caveolae to form caveola-vesicle (CV) complexes. It has been suggested that the CV complexes could be involved in the uptake of plasma protein and/or release of specific malaria antigens [59, 66, 88, 89]. Phalloidin-gold complexes were used to localize the distribution of F-actin in erythrocytes infected with *vivax*-type malaria parasites [90]. Studies by Fujioka et al. [90] suggested that an accumulation and a reconstruction of F-actin in the erythrocyte membrane occurred at the site of CV complexes.

Sexual Forms

Gametocytogenesis begins when a merozoite enters into an erythrocyte and, instead of following the stages of asexual replication, develops into a

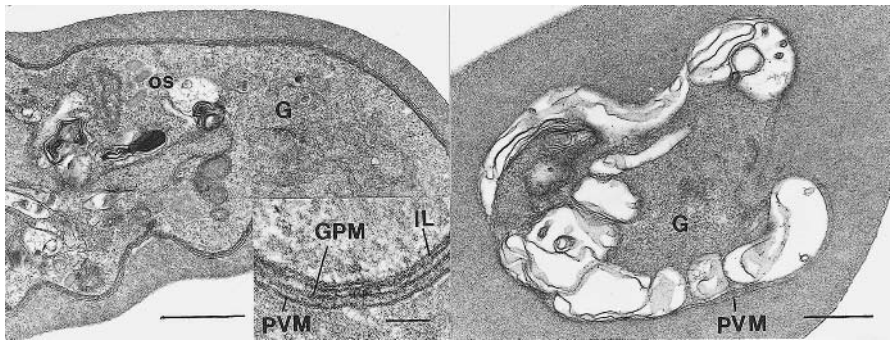


Fig. 5. Transmission electron micrographs of *P. falciparum* wild-type ***a*** and 3'CO clone (lack of Pgf27 locus) gametocytes ***b***. ***a*** Gametocyte (stage III–IV) from wt3D7. OS = Osmiophilic bodies. Bar = 1 μm. Inset: A pellicular complex consists of a parasitophorous vacuole membrane (PVM), a gametocyte plasmalemma (GPM), and an inner layer composed of two closely apposed membranes (IL). Bar = 0.1 μm. ***b*** Vacuolated gametocyte from the 3'CO clone. Vacuolation originates at the plasmalemma of the gametocyte (G). Bar = 0.5 μm. Reprinted with permission from Lobo et al. (1999) [97]; courtesy of Molecular Cell.

micro- (male) or macro- (female) gametocyte in the red blood cells. Infected erythrocytes in the fine capillaries of bone marrow and spleen only develop the gametocytes. The events that trigger sexual development are not well understood. Over 1–2 weeks, the parasite develops through five morphological distinct stages (I–V) in *P. falciparum* gametocytogenesis. The gametocyte is a haploid and uninucleate parasite surrounded by three membranes (fig. 5a). The outermost of the three membranes is the PVM which originates from the erythrocyte membrane. The plasma membrane of the gametocyte forms the central membrane, and the inner membrane is 15–18 nm in thickness and consists of two separate membranes in close apposition. A row of several subpellicular microtubules is observed in the gametocyte cytoplasm. Small, round, electron-dense osmiophilic bodies are seen in the cytoplasm near the pellicle (fig. 5a). They are more frequently present in the macrogametocytes than in the microgametocytes. High female-to-male ratios of parasites on *Plasmodium* infections have been puzzled. Paul et al. [91] suggested that sex determination is adaptive and is regulated by the hematologic state of the host. Macrogametocytes contain abundant ribosomes, whereas microgametocytes contain fewer ribosomes. Early in gametocytogenesis (stage II), four proteins are expressed by the parasite, namely, Pfs230, Pfs2400, Pfs48/45 and Pf155/RESA [92–94]. Pf155/RESA is also transferred to the erythrocyte membrane in the early stage of the asexual parasite. It is of interest that the parasite appears to use the same molecules

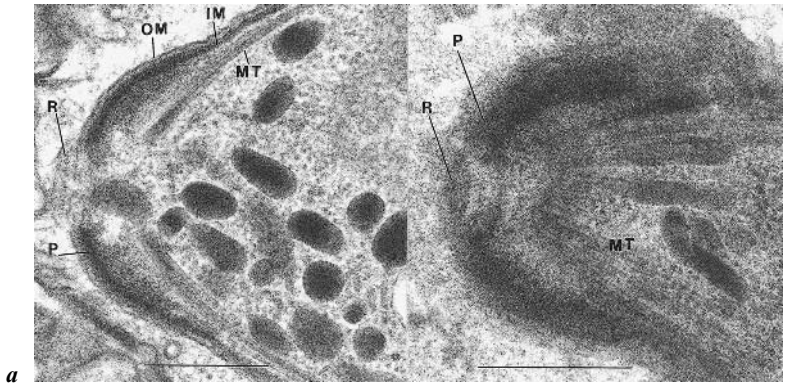
during invasion of erythrocytes and during release of gametes from infected erythrocytes. Pfs230 is identified on the gametocyte until its emergence from the erythrocyte in the mosquito midgut [93], whereas, Pfs2400 is no longer detectable in the fully emerged gametes. Quakyi et al. [94] speculated that Pf155/RESA either directly perturbed the membrane or carried other proteins, such as lipases, to the membrane that lead to erythrocyte lysis during gametogenesis. Sexual differentiation (gametocytogenesis) is multifactorial. It has been indicated that morphological changes associated with the development of the sexual parasite are accompanied by distinct biochemical events like the regulation of stage-specific gene expression at either the transcriptional or translational level. Pfg27 is one of the sexual stage-specific proteins and it is abundantly expressed at the onset of gametogenesis [95, 96]. Recently, Lobo et al. [97] reported the successful disruption of a sexual stage-specific gene locus in *P. falciparum*, Pfg27, and demonstrated that its encoded protein is critical for gametocyte development (Fig. 5b). Therefore, loss of expression of Pfg27 leads to loss of the sexual phenotype in the parasite.

Mosquito Stages

Fertilization and Zygote Formation

When mature gametocytes are ingested by a mosquito, transformation of the gametocytes to gametes is initiated and fertilization of female by male gametes takes place in the lumen of the mosquito midgut. An emerged macrogamete is surrounded by two sets of membranes, a plasmalemma and an interrupted but still extensive double inner membrane similar to that found in the intracellular gametocytes. Gametocyte-activating factor (GAF) has been isolated from the mosquito gut. Gametocyte-activating factor has recently been identified as xanthurenic acid, an intermediate product of tryptophan metabolism [98].

The male gametocyte generates eight haploid motile gametes within 10–20 min after ingestion of the microgametocyte into the mosquito midgut by a process known as exflagellation. Exflagellation can be induced in vitro by reducing the temperature of cultured mature microgametocytes from 37 to 28 °C. Additionally, pH, HCO_3 and xanthurenic acid [98] concentrations together provide permissive conditions for exflagellation. During microgametogenesis, the outermost membrane, which is the membrane derived from the parasitophorous vacuole, disintegrates and the membrane of the gametocyte becomes interrupted. The nucleus becomes irregular in shape, with extended projections. Kinetosomses appear near the centriolar plaques located close to the nuclear membrane. Kinotosome-axoneme complexes develop from the



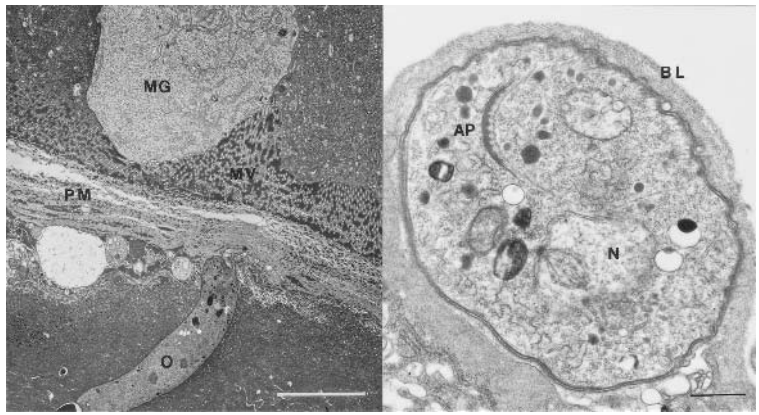
a ***b***

Fig. 6. Transmission electron micrographs of *P. gallinaceum* ookinetes (***a*** and ***b***). The apical region containing apical complex organelles is shown. OM = Outer unit membrane; IM = inner membrane complex; MT = microtubules; P = polar ring; R = conoidal ring. Bars = 0.5 μm. Reprinted with permission from Sam-Yellowe and Fujioka (2000) [37]; courtesy of the Journal of Protozoology Research.

kinetosomes. The axoneme possesses microtubules which are arranged in a 2×9 distribution. After fertilization, resulting diploid zygotes transform into motile ookinetes. The diploid zygote undergoes two meiotic divisions that permit the recombination of male and female genomes. This event is characterized by the striking differences in the morphology. Zygotes are spherical in shape, about 6 μm in diameter, whereas ookinetes have a palmate shape of approximately 15–19 μm in length and 1–2.7 μm in width. By 8–10 h after fertilization, the point where the subpellicular microtubules begin to extend beneath the pellicle and the deposit of electron-dense material, the developing canopy (collar-like structure) extends away from the apex [99]. Elongation of the ookinete continues to completion by 18–24 h after fertilization and is characterized largely by an extension of the pellicular complex and a proliferation of micronemes (fig. 6).

Ookinete and Oocyst

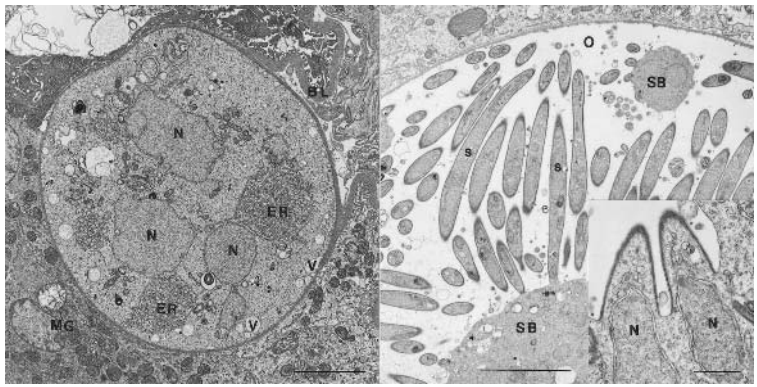
Within ~24 h, the non-motile zygote differentiates into a motile ookinete. Ookinetes have to traverse two barriers: the peritrophic matrix/membrane (PM) and the midgut epithelium (fig. 7a). The PM is made up of a chitin network and ookinetes produce and release pro-chitinase, which is activated by the gut trypsin [100–103]. Chitinases are present within micronemes and appear concentrated within an electron-dense collar of the subpellicular complex during the secretion process [102]. Chitinase may be one of the enzymes involved in the ookinete traversing the PM. After crossing the PM, ookinetes are confronted



a ***b***

Fig. 7. Transmission electron micrographs of a *P. gallinaceum* ookinete ***a***, and early oocyst ***b***. ***a*** An ookinete (O) migrates towards the periphery of the blood bolus and starts to traverse the chitin-containing peritrophic matrix (PM). MG = Midgut epithelial cell; MV = microvilli. ***b*** An ookinete has begun apparently normal transformation to oocyst beneath the basement membrane of the mosquito midgut epithelium. Note apical complex (AP) of the ookinete retracting from outer membrane in resorption. BL = Basal lamina. Bars = 5 μm. Reprinted with permission from Vernick et al. (1995) [105]; courtesy of Experimental Parasitology.

with a second barrier: the midgut epithelium. Ookinete invasion of a midgut cell occurs at its lateral apical surface. Vernick et al. [104] described that an ookinete is apparently traversing laterally from an electron-dense to an electron-lucent epithelial cell. They proposed that the previously undescribed stalk-form ookinete might be an adaptation to facilitate parasite locomotion through the cytoplasm of epithelial cells. After ookinetes reach the extracellular space between the midgut epithelium and the overlaying basal lamina, they become round and begin to transform into oocysts (fig. 7b). Extracellular matrix components of basal lamina may be important for oocyst development in the mosquito hemocoel [106]. A thick electron-dense capsule of 0.1–1 μm thickness surrounds the oocyst. The oocyst enlarges progressively, up to approximately 35 μm in diameter, as the nucleus divides repeatedly. As the young oocyst grows, vacuoles appear beneath the oocyst capsule (fig. 8a). The vacuoles enlarge and form large clefts that subdivide the oocyst cytoplasm into sporoblasts. From these sporoblasts, sporozoites bud into the inter-space of the oocyst (fig. 8b). The circumsporozoite (CS) protein is selectively expressed during the sporoblast formation process. CS-knockout mutants differentiate normally to the early oocyst stage but fail to form viable sporozoites [107], suggesting that the CS protein may be essential for the budding process of sporozoites in the oocyst.



a ***b***

Fig. 8. a Electron micrograph of *P. berghei* oocyst in a mosquito midgut epithelium (MG). The oocyst is surrounded by an oocyst capsule and contains several nuclei (N) and aggregated endoplasmic reticulum (ER). Several vacuoles (V) are observed beneath the oocyst capsule. BL = Basal lamina. Bar = 5 μm. ***b*** Sporozoites (S) are budding from a *P. berghei* sporoblast (SB) in a mature oocyst (O). Bar = 5 μm. Inset: Higher magnification of budding sporozoites from sporoblast. The pellicle of the sporozoites is composed of an outer membrane and double inner membrane, while the sporoblast consists of a single membrane. N = Nuclei. Bar = 1 μm.

The oocyst progresses through a complex developmental process that, after 12–24 days (depending on the *Plasmodium* species), culminates in the release of thousands of sporozoites into the hemocoel.

Sporozoite

The sporozoite has an elongated shape, about 11 μm in length and 1 μm in diameter (fig. 8b). Two types of membrane-bound organelles, rhoptries and micronemes, are located at the anterior half of the sporozoite. The pellicle is composed of an outer membrane, double inner membrane and a row of subpellicular microtubules [1]. One or two mitochondria, which have cristae, are closely associated with the nucleus. A Golgi apparatus, classical coated vesicles, endoplasmic reticulum, and ribosomes have been found. Mature sporozoites exit from the oocyst to the hemocoel and invade only the medial lobe and distal portion of the lateral lobes of the salivary gland, suggesting that there is specificity in the sporozoite-salivary gland interaction. A number of surface proteins are thought to potentially mediate this specificity. Synthesis of the thrombospondin-related anonymous protein (TRAP; also called PfSSP2) [108–110] gradually increases during sporozoite maturation. TRAP-knockout mutants did not infect salivary glands and also could not invade mammalian cells, hepatoma HepG2 cells, in vitro [111, 112]. These sporozoites do not

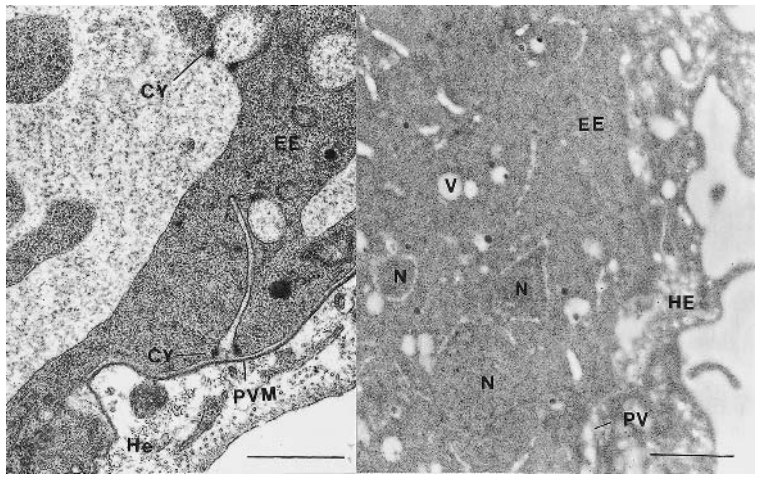
exhibit typical gliding motility. TRAP, a member of a group of *Apicomplexan* transmembrane proteins that have common adhesion domains, is suggested to have functions in gliding motility and infectivity for both the mosquito salivary glands and the liver cells of the vertebrate host. In addition to TRAP, region I of the CS protein seems to be specifically involved in interaction with the medial lobe and distal portion of the lateral lobes of the salivary gland [113]. The studies by Pimmenta et al. [114] provide important information for defining molecular events at the various steps in the journey of the sporozoite through the hemolymph to the salivary gland of the mosquito host.

The surface membrane of sporozoites appears to contain other proteins as well.

To date, a total of five *P. falciparum* sporozoite surface antigens have been described, including CSP, TRAP/SSP2, Pf16 [115, 116], STARP [117] and SALSA [118]. Recently two additional sporozoite surface proteins, such as PfARP and MB2, have been isolated [119, 120]. PfARP is an asparagine-rich protein from *P. falciparum*. Antibodies to PfARP were localized to the surface of sporozoites as well as the intracellular components in all erythrocytic asexual and sexual stages of the parasite. Nguyen et al. [120] reported the identification and characterization of MB2, a novel *P. falciparum* sporozoite surface protein. Interestingly immunoelectron microscopic studies revealed that this protein has differential cellular localization: it is observed on the surface membrane of sporozoites, but is translocated into the nucleus of blood-stage parasites. Evidence reported by Nguyen et al. [120] suggested that protein processing is the mechanism responsible for the differential localization of MB2. However, what role, if any, MB2 plays in sporozoite invasion of mosquito salivary glands and/or hepatocytes is not known.

Preerythrocytic Schizogony

In mammalian malaria parasites, the preerythrocytic (exoerythrocytic) stages occur in the liver of the vertebrate host after the inoculation of sporozoites by an infected female *Anopheles* mosquito. Sporozoites injected by the mosquito remain at the site of the bite for at least 5–15 min after mosquito blood-feeding [113]. A series of complex molecular interactions between sporozoite and hepatocyte molecules has been suggested for sporozoite invasion and subsequent intrahepatic development. The exact route of sporozoites to their target cells is still not fully understood. The sporozoite may enter the space of Disse by gliding [121] through the fenestrated membrane of the endothelial cells lining the liver sinusoids and then bind directly to the receptors on the hepatocytic surface [122]. Over the course of 5–15 days depending on the species, the invaded



a *b*

Fig. 9. Transmission electron micrographs of the preerythrocytic (exoerythrocytic) stage of parasites. ***a*** *P. vivax* preerythrocytic schizont (EE) grown in a HepG2-A16 hepatoma cell (He). An elongated and a round cytostome (CY) are present in the cytoplasm. PVM = Parasitophorous vacuole membrane. Reprinted with permission from Uni et al. (1985) [133]; courtesy of the American Journal of Tropical Medicine and Hygiene. ***b*** LR White section of a *P. falciparum* preerythrocytic schizont (EE). The preerythrocytic (liver stage) parasites were cultured in *Aotus* hepatocytes (HE) in vitro. Internal structures include nuclei (N) and vacuolated area (V). PV = Parasitophorous vacuole. *P. falciparum* preerythrocytic schizont sample was kindly supplied by Dr. Michael R. Hollingdale. Bars = 1 μm.

sporozoites undergo the process of asexual reproduction (preerythrocytic or exoerythrocytic schizogony) and release thousands of merozoites that can invade only red blood cells. The maturing preerythrocytic schizont is clearly surrounded by the PVM (fig. 9a). At an early stage of schizogony, the cytoplasm contains numerous vacuoles (type I and II) [123]. These vesicles originate from the endoplasmic reticulum and Golgi of the parasite and transport flocculent material to the parasitophorous vacuole space [124, 125]. At a late stage of schizogony, the schizont plasma membrane is deeply invaginated into the schizont cytoplasm, creating a highly contoured periphery with numerous interconnected islands (called cytomeres, which resemble the sporoblasts in the oocysts) of parasite cytoplasm. The schizont cytoplasm contains numerous nuclei, scattered electron-dense rhoptry precursors, mitochondria, and electron-dense granules (fig. 9b). At the final stage of differentiation, merozoites bud from the surface of cytomeres. The budding process of the preerythrocytic merozoites resembles that of the sporozoite formation within the oocyst. During the development of uninucleate sporozoites to mature preerythrocytic forms, several antigens have been identified, which include CS antigens of *P. falciparum*, *P. vivax*,

P. cynomolgi and *P. berghei* [125–127], a *P. falciparum* liver-stage antigen (LSA-1) [128], a *P. berghei* protein designated Pb1 [129], and a *P. berghei* antigen called LSA-2 [130]. In addition, a number of erythrocytic stage antigens, MSP-1 [127] and the *P. falciparum*-exported protein-1 (EXP-1) [131, 132] have also been shown to be expressed in the preerythrocytic stages of parasites.

In the vertebrate host, survival of the malaria parasite is absolutely dependent on its ability to invade liver cells and erythrocytes. Upon host cell invasion, the malaria parasite develops a membrane known as the PVM in the respective host cell. Sporozoites penetrate the liver cell and blood stage merozoites actively invade erythrocytes. In both cases the parasite initiates the formation of the PVM during invasion (see 'merozoite invasion'). The biological functions of the PVMs are believed to be substantially different between liver and erythrocytic stages. The reasons are largely based upon the differences of their host cells, for example, (1) the erythrocyte has no nucleus or organelles, (2) the erythrocyte does not endocytose, (3) the erythrocyte lacks the machinery for de novo lipid biosynthesis, (4) the erythrocyte does not present molecules on the surface in the context of MHC class I or II, and (5) the erythrocyte lacks the endolysosomes. The identification of liver-stage specific protein synthesis might be very difficult due to de novo protein synthesis within the nucleated host cell. Therefore, little is known about the formation and maintenance of the PVM, and the trafficking of liver stage-specific proteins during liver stage development. In both stages, the PVM interacts with the parasite plasma membrane and host cell cytosol. The best evidence for the role of the PVM as a barrier to host immune systems comes from the studies of *Toxoplasma* [134]. *Toxoplasma* selectively excludes host cell transmembrane proteins at the junction during host cell invasion, thereby creating a non-fusiogenic PVM [134].

Certain species of malaria parasites (*P. vivax* and *P. ovale*) have the dormant stage, named 'hypnozoite' in the vertebrate liver [4, 5]. The relapse originates from the dormant parasites and causes malaria relapses. Although the presence of *P. vivax* hypnozoite has been indicated in the hepatoma cell culture system [135, 136], details of the ultrastructure of the hypnozoite have not yet been demonstrated and the biological basis of this stage remains unknown.

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Malaria Parasites and the Anopheles Mosquito

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Introduction

Malaria continues to exact an unacceptable toll upon the human population in the tropical and subtropical regions of the world. With the increasing globalisation of recreational activity, it can be anticipated that increasing numbers of cases will be met in non-endemic countries. These sporadic cases may lead to local outbreaks of disease transmitted by local susceptible mosquitoes [1]. Because of the suffering incurred by the human population, huge efforts have been made to alleviate the disease in man; however, it must be recognised that the distribution of the parasite is largely determined by the distribution of the mosquito vectors which, in the case of malarial parasites of humans, are all of the genus *Anopheles*. There is now much theoretical, biological and practical experience that suggests we should focus significant effort to attacking the malarial parasite as it passes through the mosquito vector to achieve the most cost-effective and therefore efficient reduction in malaria distribution.

This chapter will focus on the analysis of the cell and molecular biology of the parasite, and its interactions with the mosquito vector – notably with the innate immune mechanisms. It is now clear that insect immunity includes humoral and cellular systemic reactions that take place in haemocytes and the widely distributed fat body. But immunity also includes local epithelial reactions in organs such as the midgut and the salivary glands [2]. During its elaborate, multistage development in the mosquito, and in particular during its penetration of the midgut and salivary epithelia, and its journey from the

midgut to the salivary glands, the malaria parasite might trigger immune responses. The cell biology of the development of the parasite is complex, thus, we will summarise the morphological stages and molecular components of the parasite that appear to be involved in interactions with the vector. We will then summarise what is now known about mosquito immunity with particular emphasis on reactions that may be pertinent to the parasite's life cycle. Finally, we will use the background of the parasite life cycle and mosquito immunity to discuss possibilities for the future development of effective intervention strategies targeted to the mosquito stages of the parasite's life cycle.

The Biology of Malaria Development in the Mosquito Bloodmeal

Gametocytes

Plasmodium transmission from man to the mosquito can only be initiated by the intra-erythrocytic sexual blood stages of the life cycle – the gametocytes. In *Plasmodium falciparum* commitment of asexual parasites to sexual development takes place in the schizont prior to merozoite release. All merozoites from a committed schizont will give rise to gametocytes [3]. The molecular basis for commitment is unknown. Gametocyte morphology is distinct from that of asexual blood stages and mirrored by an equally distinct transcriptional activity that prepares the long-lived, circulating mature male and female gametocyte sexual precursors for the sudden re-initiation of development in the mosquito midgut. The extent of transcriptional reprogramming is emphasised by recent microarray experiments with *P. falciparum* that demonstrated that of 3,648 DNA clones assessed fully 500 were upregulated in gametocytes [4]. Despite these experiments, gametocytogenesis is generally poorly characterised at the transcriptional level. In part this is a reflection of the lack of genome information, a situation that is obviously changing. Two markers for gametocyte development in *P. falciparum* are Pfg27/25, an intracellular protein whose essential role in gametocyte development was demonstrated by gene disruption studies [5, 6], and Pfs16 encoding a specific surface protein that has no particular function ascribed [7].

The dimorphism of the mature gametocytes presages the very different development the cells will undergo when they are ingested into the bloodmeal of the female mosquito. The female parasite (macrogametocyte) has a greatly expanded endoplasmic reticulum and a dense ribosome population. The ribosomes are assembled on the A form rRNA scaffold. The mitochondrion and plastid are greatly extended and numerous profiles are visible in cell sections. The nucleus, which contains 1.7C DNA [8], is small and in some species contains an electron-dense structure previously considered to be a nucleolus [9], but which is more likely to be a transcription or replication 'factory' [10].

Messenger RNAs of some proteins that are required early (ca. 2 h) in gamete/zygote development (e.g. P25/P28) lie untranslated within the cytoplasm [11]. The molecular mechanism of this repression is not clear; however, both Pbs21 and Pbs25 mRNAs have extensive poly (U) tracts in both the 5′- and 3′-untranslated regions that are known in other systems to be involved in binding proteins that can mediate translational repression [12]. Proteins required even earlier in gamete development (e.g. Pfs230) are often pre-synthesised in the mature macrogametocyte whilst it is circulating in the vertebrate host [13]. All the above reflect the need for rapid biosynthesis and development in the female cell following transfer to the mosquito gut. Other characteristic cytoplasmic organelles include abundant regulated secretory vesicles (termed osmiophilic bodies) that, immediately following activation of the gametocytes in the mosquito gut, discharge molecules such as Pfs230 into the parasitophorous vacuole. These lyse the red cell, leaving the extracellular female gamete available for fertilisation. The female gametocyte may also be prepared metabolically for environmental differences it may encounter in the mosquito midgut [14].

The male gametocyte by contrast has only a sparse ribosome population, almost no endoplasmic reticulum, less numerous mitochondrial and plastid profiles and a large nucleus also containing an enigmatic 1.7C DNA. The physical distinction of the male cell will presumably be reflected in differences in expression of unique proteins, currently only one of which is known, the male gametocyte-enriched form of α-tubulin [15].

These ‘pre-programmed’ mature gametocytes circulate in the peripheral bloodstream arrested in G_1 of the cell cycle until they are taken up by a blood-feeding anopheline, or until they die. Following release of the sequestered immature gametocytes from the deep tissues into the peripheral circulation the natural half-life of the mature gametocyte of *P. falciparum* has been estimated as between 2.5 [16] and 4.7 days [17]. Natural antibody responses are generated to ‘pre-synthesised’ antigens that are normally expressed on the surface of the gametes in the mosquito bloodmeal. Amongst these are Pfs230 and Pfs48/45. Antibodies to Pfs230 result in complement-mediated lysis of macrogametes [18] and those to Pfs48/45 in immunoglobulin-mediated blockade of fertilisation [19]. Additional host-derived factors that modulate the infectiousness of a gametocyte carrier to the mosquito include: blood pH and the related bicarbonate level, which ideally exceeds 20 μ*M,* and IFNγ which regulates the activity of peripheral blood leucocytes which in turn kill parasites by a nitric oxide-mediated mechanism in the bloodmeal [20]. Noting that the enigmatic mosquito-exflagellation factor has now been defined as xanthurenic acid (XA) [21], it will be interesting to determine what role, if any, blood-derived XA may play in the regulation of infectivity.

Gametogenesis

Current evidence suggests that the conditions that prevail in the midgut (i.e. pH 7.4–7.6; $pHCO_3 > 20\,\mu M$) permit induction of gametogenesis to be triggered by a fall in blood temperature of >5°C and the presence of XA. The parasite must, therefore, possess sensing mechanisms and signal transduction systems to relay the change in environment and convert this into appropriate biological development. It remains a challenge to find rational inhibitors for what must be some unique regulatory events.

Both male and female gametocytes are induced to escape the host erythrocyte. Whilst little is known of the molecular mechanisms involved, regulated secretion of the osmiophilic bodies into the parasitophorous vacuole is at least partly responsible [22], as may be the increase in volume of the parasite [23]. The emergent female cell is now the macrogamete. The male additionally undergoes exflagellation (microgametogenesis) leading to the simultaneous assembly of 8 flagella. Exflagellation is achieved routinely within 15 min and involves activation of multiple replication forks on each of the 14 chromosomes to achieve the high rates of DNA replication required [24], rapid assembly of the flagella and the formation of 8 haploid genomes. Following the third mitosis the 8 microgametes rapidly emerge from the cell surface. Each contains just a nucleus, an axoneme and an apical organelle of unproven function [25]. The absence of a mitochondrion and plastid [25] clearly indicates these organelles are inherited maternally, an observation confirmed by genetic studies [26]. Microgametes swim away into the viscous concentrated bloodmeal where they may interact with red blood cells [27] and contact and fertilise the female cell (usually within 1 h of blood feeding). Fertilisation is largely dependent upon the expression of P48 on the surface of microgametes – despite the protein being distributed in both male and female gametes [28]. Recombinant *Plasmodium* deficient in P48/45 produces normal gametes but has a reduced capacity to infect mosquitoes as judged by oocyst production.

Zygote-Ookinete Differentiation

Contact between the gametes induces a characteristic change in motile behaviour of the male gamete that precedes fertilisation [23]. Following fusion of the plasma membranes of the two cells, the axoneme and condensed male nucleus enter the cytoplasm of the female gamete. The axoneme rapidly depolymerises and the male nucleus enlarges and fuses with its female counterpart [25]. Zygote development follows over the subsequent 9–12 h. In this period the surface proteins are radically changed, reportedly all proteins of >55 kD are lost [29], at the same time the parasite becomes sensitive to the declining complement levels in the bloodmeal [30]. The zygote surface is now dominated by two highly immunogenic proteins, P25 and P28, encoded by related and

closely linked genes [31]. These multifunctional molecules confer protection against proteolytic degradation of the parasite surface that makes the parasites susceptible to attack by peripheral blood lymphocytes [32]. Proteolytic activity in the mosquito midgut is intense; seven trypsins are sequentially expressed after blood feeding together with chymotrypsin and aminopeptidase [33].

The diploid zygote begins the assembly of the complex organelles required to form the motile and invasive ookinete [34]. The parasite simultaneously undergoes an immediate two-step meiotic division of the genome within a single nucleus; meiosis is completed before the mature ookinete is formed [35]. The resulting nucleus contains 4 separate haploid genomes that may be of both parental and recombinant composition. Formation of the ookinete follows a conserved cell strategy found also during blood stage merozoite and sporozoite formation, the major difference being that only a single motile ookinete is formed (as opposed to tens of merozoites or thousands of sporozoites). Ookinete formation begins with the movement of the microtubule organising centre toward the plasma membrane of the presumptive anterior tip of the cell. From this, the numerous (ca. 90) subpellicular microtubules elongate posteriorly. Between the microtubules and the plasma membrane a single flattened vacuole is formed from a diverticulum of the endoplasmic reticulum, this elongates in exact register with the microtubules and together these structures determine the shape of the retort-like extension. The inner membrane complex is contiguous at its anterior edge with a rigid collar (where chitinase is later found in abundance) [36].

Within the cytoplasm two major organelle systems develop from the Golgi apparatus, the micronemes and two crystalloids. Micronemes are found in the anterior third of the cell, they are membrane-bound electron-dense secretory vesicles that connect via ducts to the cell surface through the apical collar. Micronemes contain both CTRP, a transmembrane molecule required for ookinete motility and invasion of the midgut wall [37], and chitinase [38], an enzyme secreted as an inactive pro-enzyme which is reportedly activated by proteases in the midgut. The activated enzyme then digests the chitin polymer of the peritrophic matrix (PTM) [38]. In some species of mosquito (e.g. *Aedes*) the PTM is a severe impediment to the migration of the ookinete out of the bloodmeal, and inactivation of chitinase by the competitive inhibitor, allosamidin [39], or inactivation of trypsin by antibodies [40] reduces infections significantly.

Protein synthesis is significantly upregulated about 9 h after blood feeding and includes the de novo transcription of a wide range of genes including CTRP and Pbs70. An unusual aspect of malarial biology found at about this time is that the parasite switches its ribosomal RNA (rRNA) from the A form of the sSUrRNA (found in the blood stages in the vertebrate host) to the S form [41]. In some

species a third transient ribosomal O form is expressed in the ookinete and oocyst [42]. It is assumed that these changes are of biological advantage to the parasite; however, some mRNA species expressed in the ookinete (e.g. P28) can be translated in the blood stages in appropriately transformed parasites, which suggests that mRNA species are not translated exclusively on one ribosome type [43].

Biology of Malaria Development within and beyond the Midgut Epithelium

The ookinete, having penetrated the PTM (which in some species is chitin-rich), crosses a protease-rich space and contacts the microvillar surface of the midgut wall. Initial contact has been described as being with a fibrous network which overlays the microvillar surface of the midgut epithelium [44]. Recognition of the midgut surface may involve mucin [45]. As yet no parasite receptors have been characterised for any mosquito ligand on the midgut surface. It has been suggested that the ookinete selectively invades a small subclass (15%) of the midgut cells, termed Ross cells, that exclusively express vacuolar ATPase [46]. These studies, however, failed to take into account the significant disruption caused to the invaded host cell [47–50] that may lead to it being pinched out of the epithelium [50]. Invasion of the midgut cell appears to result in the transient formation of a parasitophorous vacuole, which rapidly degrades such that the ookinete lies directly in the cell cytoplasm. Here the parasite sheds large quantities of P28, and secretes subtilisin into the host cell cytoplasm [50].

Biology of Oocyst Differentiation

The ookinetes that survive immune attack in the midgut epithelium and emerge through the basal plasma membrane then come into contact with the collagen/laminin-rich basal lamina. Here the ookinetes eventually cease moving and round up to form oocysts, though a fraction may migrate many microns over the surface of the epithelial cells. Evidence is accumulating that the P25/28 family of proteins may play a role in the initiation of oocyst development. P28 from *P. gallinaceum* has been implicated in binding to extracellular matrix proteins, laminin and collagen IV [51]. Furthermore, mutant *P. berghei* that are deficient in expression of both members of the P28/25 family show a remarkably reduced ability to form mature oocysts. These mutants are apparently able to invade midgut cells and engage the basal lamina complex and can become targets for melanisation; however, only very rarely do they produce oocysts. Thus although P25/28 proteins appear to play a role in maintaining ookinete survival in the gut lumen they may also have an important but non-essential role in ookinete traversal of the epithelium. Deletion of a single gene encoding either

P25 or P28 proteins generates a parasite that is still capable of a significant level of oocyst formation implying that there is functional redundancy between these closely related proteins.The presence of two distinct proteins with functional overlap may also serve to widen the range of vectors that the parasite is able to colonise.

Differentiation of the ookinete into an oocyst beneath the basal lamina begins with the resorption of the apical complex and the crystalloid. Subsequent vegetative growth and replication in the oocyst is remarkably similar to schizogony of both the liver and blood stream forms. The genome in the persistent nucleus syncytial, divides rapidly and the nucleus becomes highly lobed. Some 11 synchronous mitotic divisions result in the formation of about 8,000 sporozoites in each oocyst. In the cytoplasm distinct islands of endoplasmic reticulum develop, each associated with a Golgi-like region of vesicles and smooth reticulum. The mitochondria and plastids proliferate in the expanding cell, which is some 2–4 μm in diameter on day 2 and about 50 μm in diameter by day 8 after fertilisation. Usually by day 6 the cytoplasm of the oocyst is subdivided by deep clefts, which originate by the expansion of the cisternae of the endoplasmic reticulum [52]. The circumsporozoite protein (CSP) is expressed on the surface membrane at this time. Failure to express the protein significantly depresses sporozoite production [53]. Sporozoite differentiation is comparable to ookinete formation with the exception that sporozoites form at every point where spindle poles (MTOC) of the dividing nuclei come into close apposition to the sporoblast surface, thus each nucleus initiates the formation of 2 haploid sporozoites. Within a single mosquito each oocyst will produce 2 unique recombinants, plus two parental genotypes and one oocyst could produce equal sporozoite numbers of each possible genotype – if they are of equal fitness during sporogonic development. The emergent sporozoites are rigid and straight until fully formed, whereupon they become more flexible and motile. Like the ookinete, the sporozoite first forms an apical ring (MTOC) and then subpellicular microtubules and pellicle vacuole. The pellicle vacuole membranes have linear rows of intramembranous particles (IMPs) on the membrane face attached to the microtubules, and probably represent the points of attachment of the microtubules [54]. These IMPs in turn are believed to organise the molecular motor (actin and myosin) that drives the sporozoite forward during gliding locomotion and invasion [55]. The secretory organelles (micronemes and rhoptries) are initially formed as two ‘empty’ vacuoles, which become progressively more electron-dense and numerous forming the micronemes. CSP and thrombospondin-related anonymous protein (TRAP) are stored in the micronemes until secretion onto the parasite surface where they are essential for locomotion and invasion. A single mitochondrion and plastid are drawn into the sporozoite body alongside the nucleus. The postnuclear region is occupied almost exclusively by endoplasmic reticulum.

The more mature oocyst is surrounded by an amorphous matrix, which appears to be secreted from vesicles in the young cell. Outside this lies the basal lamina of the midgut. Should the oocyst die, a not infrequent occurrence, the oocyst will become either highly vacuolated with 'apoptotic' nuclei and/or melanised. The amorphous matrix and the surface membranes are the primary targets of this immune response [56]. Melanised oocysts are also known as 'Ross's black spores' [57].

Biology of Sporozoite Development

Motile sporozoites burst out of the constraining oocyst, and escape through large holes torn in the basal lamina by the massively enlarged parasite. During gliding locomotion the sporozoite leaves a trail containing surface proteins (including CSP and TRAP) and lipids suggesting significant membrane attachment to the substratum [58, 59]. TRAP is a transmembrane surface protein that contains two distinct extracellular domains that are implicated in protein–protein reactions associated with cell adhesion, notably the thrombospondin motif and a MIDAS domain that co-ordinates magnesium ions. TRAP also has a conserved positively charged cytoplasmic domain [60]. TRAP therefore bears significant structural similarities with the secreted ookinete protein CTRP, and related proteins can be found in the motile stages of numerous other Apicomplexa (e.g. *Toxoplasma*, *Cryptosporidium* and *Eimeria*). TRAP-disrupted *P. berghei* sporozoites are non-motile and fail to invade the salivary glands [61]. Gene conservation is emphasised by the fact that the similarly charged but otherwise divergent cytoplasmic domain of the *Toxoplasma gondii* TRAP homologue can functionally replace that of *P. berghei* TRAP [62]. Thus, one role of TRAP is thought to be as a force transduction protein coupling movements of the cytoskeleton and an external anchoring role. A consequence is an anterior to posterior relocation of TRAP and the observed posterior shedding of the protein/lipidaceous trails mentioned above. Following oocyst rupture large quantities of the dominant sporozoite surface protein CSP can be found adhering to the midgut surface. A minority of sporozoites engage in retrograde invasion and can be found in the epithelial cells, and in the midgut lumen. The majority of sporozoites enter the haemocoele; of these only 10–25% are subsequently found in the salivary glands. There is clear evidence that the sporozoites specifically recognise the basal lamina overlying the salivary glands suggesting that invasion of the cells is a receptor-mediated event [63]. Sporozoites from the haemocoele are poorly infectious to the vertebrate host but invade the salivary gland, those from the salivary gland will not re-invade the glands but are infectious to liver cells [64]. Whether this is a time-dependent or a tissue-specific correlation is unclear; however, in those insects where the sporozoites do not enter the glands (including *Drosophila*) infectious sporozoites can be

found in the haemocoele late in the infection suggesting differentiation might be temporally regulated [65].

During invasion of the salivary gland the sporozoites initially form a parasitophorous vacuole, around which high concentrations of mitochondria can be found [66]. The sporozoites (like the ookinetes) then emerge from the vacuole, and enter the acinus where, in highly infected cells, they can be found intimately packed in parallel bundles [66]. Sporozoites are found only in the median, and the distal lateral lobes, the part where the salivary duct is least chitinised (if at all). Sporozoites and saliva then move into the salivary ducts where they remain until blood feeding. At each blood feed laboratory-infected mosquitoes (which may have tens of thousands of sporozoites in the glands) deliver about 20 sporozoites into the vertebrate [67]. Infected mosquitoes are described as being less capable of taking a full bloodmeal, with the result that they probe more frequently than uninfected mosquitoes [68]. This change of behaviour would be of clear evolutionary advantage to the parasite, but to the disadvantage of the hapless vector.

Insect Innate Immunity

Insects posses several defence mechanisms. The first line of defence consists of the structural barriers of the surface exoskeleton (cuticle) and the peritrophic matrix of the midgut, which is an important entry-point of micro-organisms. Subsequent defence mechanisms are collectively known as the innate immune system and operate at the humoral and cellular levels. Well-recognised mechanisms of insect immunity include cellular phagocytosis and melanisation, serine protease cascades leading to coagulation and humoral melanisation, and transient production of antimicrobial peptides by the fat body, haemocytes and epithelial tissues. Implementation of these defence mechanisms requires specific recognition of the micro-organisms by insect receptors that can bind to the microbial surfaces. These receptors may be part of the insect cell membrane, or may bind to that membrane upon recognition by membrane-associated receptors. In either case, microbial attachment to the insect membrane is thought to trigger intracellular signal transduction and transcriptional activation. Therefore, the important components of innate immunity would also include receptors for microbial pattern or indirect receptors for opsonins, and the extracellular and intracellular machinery whereby the cells respond to this recognition. A number of potential pattern recognition receptors have been characterised in insects and include proteins that can bind LPS, peptidoglycan, β-1,3-glucan and chitin. The signal transduction and transcriptional control pathways of innate immunity effector genes have been thoroughly studied in *D. melanogaster*. Microbial challenge leads to nuclear translocation of Rel family transcription factors such as Dorsal, Dif and Relish through two or more

signalling cascades that are specifically activated by various microbial classes. Fungal infection activates the *Toll* cascade leading to activation of anti-fungal (and to some extent antibacterial) genes. Bacterial infection partially activates the as yet undefined *imd* pathway leading to antibacterial gene expression. The presence of other Toll-like and STAT-like pathways has also been demonstrated, and they appear to control expression of both unique and shared sets of genes involved in multiple defence reactions [69, 70].

Mosquito Antimalarial Defence

In nature most parasite–mosquito combinations are incompatible for diverse reasons. Feeding preferences may not expose the mosquito to particular *Plasmodium* species, and mosquito longevity may be inadequate for completion of the parasite lifecycle. Molecular incompatibilities may prevent essential interactions between these two organisms, as in the case of the *Anopheles freeborni* and *Plasmodium knowlesi* combination where the parasite fails to invade the salivary glands. Importantly, incompatibilities caused by the mosquito's innate defence reactions against the parasite have also been documented [71, 72].

The best studied mosquito defence mechanism against macroparasites is melanotic encapsulation, which leads to the deposition of electron-dense melanin-containing material that immobilises and kills the parasite on the basal side of the midgut epithelium [73]. Another less studied refractory mechanism is the lysis of ookinetes in the midgut epithelium of a selected *A. gambiae* strain [74]. However, as indicated above large parasite losses are not limited to refractory strains. Studies on parasite development in fully susceptible mosquitoes have clearly indicated significant parasite losses occurring during critical stages in the midgut and haemolymph. In the midgut lumen, only a small number of ingested gametocytes develop into mature ookinetes, and only a minority of ookinetes ever develop into oocysts after crossing of the midgut epithelium and lodging on its basal surface separated from the haemolymph by the mosquito basal lamina. Later on when the oocyst ruptures only 10–25% of the released sporozoites end up in the salivary glands [71, 72]. The magnitudes of these losses can vary between different mosquito–parasite combinations and their molecular basis is presently unknown, but are thought to result at least partly from killing mechanisms of the mosquito.

Melanisation and Encapsulation of Malaria Parasites

Melanisation reactions in insects are mainly involved in protection through the formation of a sclerotised cuticle, wound healing and encapsulation of infecting micro-organisms. These different melanisation reactions are carried out by similar mechanisms, but the implicated enzymes, such as prophenoloxidases

and serine proteases, are likely to differ. At least six different PPO genes are present in the *A. gambiae* genome and they are differentially regulated during development and in the different mosquito tissues [75].

In a selected *A. gambiae* laboratory strain, L3-5, all late ookinetes and early oocysts are encapsulated as they reach and exit the basal side of the midgut epithelium [73]. A colourless proteinacious layer is first deposited on the parasite surface, which later on becomes pigmented and gives rise to the melanised capsule. The absence of haemocytes around the melanisation site and its polarisation towards the haematocoele suggest that the melanotic encapsulation of *Plasmodium* in refractory mosquitoes is of the humoral type [76].

Activation of the melanisation reaction upon recognition of a micro-organism by a receptor molecule is mediated by a serine protease cascade leading to the cleavage of PPO to PO [77].

After proteolytic activation, PO catalyses oxidation of tyrosine to the melanin precursor indolequinone through several steps. Dopachrome conversion enzyme and DOPA decarboxylase are also implicated in melanin synthesis [78]. The parasite may be killed by toxic by-products of the reaction such as free radicals, or by starvation within the capsule.

The significantly increased thickness and darkness of the melanotic capsule at the ookinete's apical end facing the haemolymph suggests that key components of the melanisation reaction originate from the haemolymph and may be diffusing into the midgut epithelium through the basal labyrinth. No differences in transcriptional expression of PPO genes was detected between the susceptible and refractory mosquitoes suggesting that the refractoriness trait may be determined by other upstream factors, such as receptor molecules or regulatory serine proteases and their inhibitors.

Several pattern recognition receptors have been linked to PPO activation in moths and the crayfish, including a lectin, a β-1,3-glucan recognition protein, and PGRP. Apart from triggering the PPO cascade, these proteins may also serve as components of the proteinacious layer that forms the capsule after cross-linking and melanisation [79–81].

Three serine proteases, from moths and the earthworm, have been implicated in the PPO activation system [82–84]. All of them contain one or more cysteine knot domains in their amino-terminal portion and share other unique features not found in other serine protease families, such as two glycines involved in determining trypsin specificity and an aspartic acid residue in the non-catalytic part. Serine protease inhibitors (serpins) from moths and fall webworm exhibit inhibitory effects on the melanisation reactions and are likely to be involved in regulating and restricting the melanisation reaction close to the encapsulation site [85, 86].

Although melanotic encapsulation appears to be a general response against malaria in the selected *A. gambiae* strain 3–5, a certain degree of specificity

seems to exist. The sympatric (African) strains of *P. falciparum* are not melanised as frequently as the allopatric South American strains [73]. Furthermore, it has been shown that negatively charged carboxymethyl-Sephadex beads are exclusively encapsulated in the refractory strain while not in the susceptible strain. When these beads were pre-incubated in susceptible mosquitoes they were not encapsulated in the refractory strain. A 'protective' substance of the susceptible mosquito is likely to have made them invisible to the PPO activation system in the refractory strain [87]. Infections with some *Plasmodium* clones leads to a partial killing by encapsulation in the refractory strain yet most of the identical ookinetes will nonetheless develop into mature oocysts [F.H. Collins, personal commun.]. This selective killing is likely to result from differences in the micro-environmental concentrations of active components in the ookinete surrounding which in turn may depend on differences in the invaded cell types of the midgut epithelium. Attempts towards the characterisation of genes that control melanotic encapsulation of *Plasmodium* and 'inert' Sephadex beads has been implemented through genetic mapping experiments [88, 89]. Three quantitative trait loci (QTL) controlling this trait have been located, and the major locus, *Pen1*, which maps in region 8C of the 2R chromosomal arm, controls more than 65% of the phenotype. Identification of the genetic element within the *Pen1* locus controlling this trait is currently underway in the laboratories of F.H. Collins and F.C. Kafatos, through positional cloning and determination of sequence differences between the refractory and susceptible strains.

Spatio-Temporal Immune Responses to Malaria Infection

Expression profile analysis has identified numerous innate immunity genes in *A. gambiae* (see below) that are known from other organisms to be involved in recognition, signal transduction and amplification and microbial killing. These immune genes have been extensively used to characterise the innate immune responses at the transcriptional level in the course of malaria infection. Malaria infection is accompanied by expression of immune markers in a temporal and spatial pattern that correlates with the parasite's life cycle in the mosquito. At the early stages during midgut invasion, immunity genes are upregulated in the midgut; at the later stages when sporozoites are spread in the haemolymph and invade the salivary glands, markers are activated in the salvary glands. Systemic induction at both times occurs in the abdomen part where the fat body and haemocytes are located [2, 90, 91]. In *Anopheles stephensi* the upregulation of NOS was directly linked to elevated NO concentrations both in the midgut and carcass and to a significant reduction in parasite prevalence [92]. Other studies have shown that pre-immune activation of mosquitoes by bacterial challenge, prior to feeding on malaria-infected hosts, restricted parasite development by 40% during their passage from the oocyst to the salivary glands [93].

Immunity Genes of A. gambiae

A significant number of genes belonging to all known innate immunity functional classes have been isolated, including antimicrobial effectors, pattern recognition receptor candidates, serine proteases and their inhibitors, prophenoloxidases, signalling pathway components, iron metabolism components and NOS. The initial case-by-case identification was recently complemented with a systematic genomic approach. In a pilot expressed sequence tag (EST) project, the sequencing of ESTs from a normalised subtracted library led to the discovery of approximately 2,300 new mosquito genes, of which 38 showed significant similarity to known innate immunity genes of other organisms [94]. The majority of the presently available *Anopheles gambiae* putative innate immunity genes and their characteristics are presented below.

Pattern Recognition Receptors

Several genes encoding putative pattern recognition receptors involved in triggering immune responses upon microbial and parasitic recognition have been identified and studied. Some of these genes encode components shown to be involved in PPO cascade activation such as a *Manduca sexta* lectin [79], a peptidoglycan recognition protein (PGRP) [80] and a β-1,3-glucan recognition protein [81]. Other genes such as the gram-negative bacteria-binding protein (GNBP), gal-lectin IGALE20, the chitin-binding domain encoding mucin, ICHIT and the fibrinogen-like domain encoding immunolectins AgFBN-1 to 7 encode components known to be involved in opsonisation and agglutination of micro-organisms [91, 95]. A *D. melanogaster* GNBP homologue was recently shown to be involved in mediating κB-dependent transcriptional activation of antimicrobial peptide genes upon binding to LPS and β-1,3-glucan [96].

The vertebrate ficolins, also encoding fibrinogen-like domains at their carboxy terminus, can activate MASP1 and MASP2 in the complement pathway as does the mannose-binding lectin (MBL) [97]. Horseshoe crab fibrinogen-like domain encoding immunolectin, apart from agglutinating bacteria, can also activate and enhance the antimicrobial activity of 'big' defensin [98].

Other putative pattern recognition receptor genes identified as ESTs [95] include a putative homologue of the *D. melanogaster* dSR-C1 protein, and a haemomucin which in *D. melanogaster* is a midgut and haemocyte protein believed to be involved in opsonisation [99, 100].

Serine Proteases and Serpins

Serine proteases are involved in proteolysis through a common enzymatic mechanism and are produced as inactive zymogens that are specifically activated by proteolytic cleavage. Serine proteases constitute key components of activation

and amplification cascades in innate immune responses where they can activate defence mechanisms such as melanisation, coagulation and trigger production of effector molecules through activation of intracellular signalling pathways [77, 101].

An increasing number of immune-related serine proteases are now being characterised in *A. gambiae* [93, 101–104]. Immune-related serine proteases can often be distinguished from the numerous digestive proteases by their modular amino-terminal part, which contains additional functional domains possibly involved in regulation. The clip domain (disulphide knot), consisting of 7 cysteines with a conserved spacing, is the most common of these amino-terminal domains. Their function is not fully understood: it may mediate protein–protein interactions, or possess antimicrobial activity, based on its similarity to big defensin [105]. The mosaic amino-terminal portion of the immune responsive serine protease SP22D contains adhesive domains known to be involved in interactions and may thus be of regulatory significance: Binding to bacterial surfaces may lead to activation of serine protease [106, 107]. Two immune responsive serine protease-like genes, ISPL5 and AgISPR5, possess non-functional catalytic triads rendering them incapable of performing proteolytic cleavage [91, 104]. Similar immune-inducible serine protease-like molecules from crayfish and a coleopteran insect are involved in cell adhesion and PPO-cascade activation, respectively [107, 108]. The non-functional catalytic domain may mediate protein–protein interactions, and act as antagonists to other serine proteases for substrate binding.

Most *A. gambiae* immune-related serine proteases are found in the haemolymph and/or haemocytes. This may indicate a potential role in cascades leading to the activation of defence reactions in the haemolymph. AgSP14D2, AgSP11A and two ESTs encoding immune-inducible serine proteases are likely to be involved in PPO cascades having the specific sequence features of PPO-activating enzymes: clip domains; two glycines determining trypsin specificity in the catalytic part, and two cysteines in the non-catalytic part [95, 102, 103]. Four *A. gambiae* serpins have been cloned, two of which are immune responsive [95, 104].

Transcriptional Control of Immune Responses

A. gambiae putative immune-signalling cascade components include homologues involved in the *D. melanogaster* developmental and immune signalling Toll pathway. Two Toll-like genes have been cloned from *A. gambiae* [110]. Ag-Toll and Ag-Trex, belonging to the *D. melanogaster* Toll group and Tollo/Trex group, respectively, are probably members of a larger family of which at least some members will be implicated in immune-response activation. One Rel family transcription factor, Gambif1, and a STAT family transcription factor, Ag-STAT, have been isolated and studied *A. gambiae* [111, 112]. Gambif1 is closely related to *D. melanogaster* Dorsal, and Ag-STAT is most similar to

D. melanogaster D-STAT and the vertebrate STAT 5 and STAT 6. Bacterial infection leads to nuclear translocation of both transcription factors in fat body cells. Malaria infection does, however, not affect Gambif1 or Ag-STAT, clearly pointing to the existence of distinct antibacterial and antimalarial immune responses in mosquitoes. The evolutionary conservation of the mechanisms involved in regulatory control of innate immunity genes is highlighted by the fact that Gambif1 can bind to κβ-like DNA motifs of both *A. gambiae* and *D. melanogaster* antimicrobial gene regulatory regions.

Other genes potentially involved in immune signalling pathways are represented by ESTs showing high similarity to a Pelle-associated protein, Cactus and a κβ-motif binding protein [95]. The dissection of immune signalling pathways responding to different elicitors in the mosquito may facilitate the definition and discrimination between antimalarial from antimicrobial defence mechanisms.

Antimicrobial Peptides

Four antimicrobial peptide genes have been isolated from *A. gambiae*: one defensin, two cecropins and a novel peptide, gambicin [113, 114; Vizioli, personal commun.]. Defensin, cecropin-1 and gambicin show strikingly similar expression patterns being highly transcribed in the fat body-containing abdomen and thorax as well as in the cardia of the anterior midgut; defensin is also present in specific cells of the posterior midgut [Richman and Vizioli, personal commun.]. Two insect defensins from *Aeschna cyanea* and *Phormia terranovae* can kill the oocyst and sporozoite stage *Plasmodium* in vitro [115].

Prophenoloxidases

Six prophenoloxidase genes have been characterised from *A. gambiae* [75, 116, 117]. They show marked variations in their developmental profiles and patterns of response to the hormone ecdysone, supporting differences in function. PPOs are quick-acting defence enzymes, which are largely regulated at the post-translational level. Even though transcription is not induced by infection, some of the adult-expressed PPOs are induced by blood feeding and are good candidates for mediating encapsulation of parasites.

Parasite Development in the Mosquito as a Target for Malaria Control

The enormous clinical impact of malaria on the human host provides clear justification for the sustained efforts to alleviate human infection by the development of drugs and vaccines targeted to the pathogenic asexual blood-stage parasites, and/or to the disease-causing mechanisms in man. However, to use

these approaches to attempt control in endemic areas may be less effective than targeting the parasite in the mosquito vector. Theoretical reasons for targeting the parasite in the vectors are based on the following secure biological premises.

(1) The number of parasite oocysts surviving in a single mosquito is usually very small; only 1–4 oocysts are formed from about 60 ookinetes that develop in the gut. Therefore the relative impact of any measure will be greater than attacking the 10^9 parasites found in the blood of an infected patient.

(2) The parasites in the mosquito are relatively long-lived and are therefore potentially exposed to attack for long periods.

(3) During much of this time the parasite is extracellular and therefore highly vulnerable to immune attack.

(4) An additional advantage discovered during the development of transmission-blocking vaccines is that important parasite surface antigens are expressed de novo in the vector (e.g. CTRP, P25/P28) and therefore are not normally challenged by specific vertebrate antibodies. Perhaps as a consequence of this lack of immune pressure these antigens show less polymorphism than many blood-stage antigens. These mosquito-stage antigens are therefore relatively stable and vulnerable targets. It is clear that new antigens in this category should be sought as a priority in the development of new vaccines.

(5) If integrated malaria control programmes include transmission-blocking vaccines directed against mosquito-stage antigens, it should be possible to prevent transmission of escape mutants that arise from vaccination directed against pre-erythrocytic and erythrocytic stages.

Antibodies and Drugs Delivered from the Host

The gametes and ookinetes are bathed in 'undiluted' blood for 24 h. Antibodies have been shown to remain effective throughout this period, nevertheless complement is destroyed within 8 h [118] and PBLs within 12 h [119]. Thus to target these long-lived extracellular parasites in the bloodmeal should be a priority. It is encouraging that experimental transmission-blocking vaccines to midgut-stage parasites have been found to be very effective [120, 121].

Parallel reasoning should have driven the development and evaluation of transmission-blocking drugs, yet inexplicably these have been a low priority in previous work. Current drugs with proven or suspected transmission-blocking potential include the artemisinin-based compounds, Malarone™ and sulphadoxine-pyrimethamine. Future drug combinations must recognise that transmission is a potential target.

Engineering Parasite Refractory Mosquitoes

With the advent of genetic manipulation of the mosquito vector [122], it is clear that a variety of new strategies to control malaria transmission may emerge

and should be studied. First is the construction of mosquitoes with enhanced innate immunity directed against the ookinete in the midgut, or the oocyst and sporozoite in the haemocoele. Second, detailed molecular knowledge of the preparation for and development of *Plasmodium* within the vector will allow further intervention measures to be developed, for example mosquitoes could be engineered that lack essential molecules that are required to regulate parasite development, e.g. xanthurenic acid [21], or midgut or salivary gland receptors (when found). Finally mosquitoes could be engineered that secrete heterologous toxic molecules at appropriate times in parasite development. Such concepts have been modelled in studies on the secretion of scFv antibody directed against P25/28 [123], or hybrid toxins based upon the use of ScFv to target the toxins [124]. Alternatively one might consider engineering mosquitoes that deliver powerful immunogens in their saliva and thus contribute to the general elevation of the 'natural' immune response found in individuals in endemic areas [125]. The ultimate application of any of these concepts will be critically dependent upon perceived environmental and safety issues, and the development of appropriate mechanisms to drive the advantageous genes through the heterogeneous vector population [126].

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Malaria: Pathogenicity and Disease

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Introduction

Falciparum malaria is the most important cause of morbidity and mortality resulting from the four human plasmodium species. This chapter deals almost exclusively with this pathogen, although work on other species has contributed to our understanding of the pathogenicity of malaria. Currently approximately 400 million people world-wide are infected with malaria and there are thought to be 130 million new cases of malaria each year. Estimates of attributable mortality suggest that over 1 million people die each year from malaria [http://www.nature.com/nm/specialfocus/malaria, Wahlgren M, 2000]. The great burden of disease is in sub-Saharan Africa where 90% of all deaths occur, the majority of these in children. For this reason the principal focus of this chapter will be on our understanding of the pathogenicity resulting in disease in African children.

Epidemiology of Disease

Epidemiological observations continue to challenge the scientific community to provide explanations for the disease pattern seen in malaria endemic areas. Although most individuals living in endemic areas will have had multiple episodes of infection during childhood, not all of these will result in clinical disease and only a minority will develop manifestations of severe disease. More recently it has been demonstrated that the intensity of transmission has a major impact on disease spectrum and, in an apparent paradox, it has been shown that under the highest levels of transmission, disease severity (at least as judged by mortality rates) may be lower than under moderate levels of transmission [1].

A complex interplay between the parasites and possibly their variants, the microenvironment affecting risk of exposure and the host, particularly an individual's ability to develop a protective immune response is likely to account for this variability in disease pattern. However, while explanations are awaited for these observations, one should be wary of generalising uncritically the results of investigations into pathophysiological mechanisms from one setting to another.

Clinical disease resulting from malaria can be pragmatically divided into three categories: disease amongst children in endemic areas; disease in pregnant women, and disease in non-immune individuals. For reasons discussed above the focus of our attention will be on disease in children from endemic areas. In these same areas, disease in pregnancy, especially amongst primigravidae, is an important cause of adult mortality and morbidity [2]. This is supported by the disease pattern in low transmission areas where pregnancy considerably increases the risk of a poor outcome in non-immune adults with malaria [3]. In the following sections we shall describe disease attributed to malaria and summarise some of the proposed pathophysiological mechanisms at work. We make no attempt to cover the large area of the literature concerned with treatment.

Virulence of Falciparum Malaria

Clinical symptoms and signs of malaria occur when *Plasmodium falciparum* multiplies within erythrocytes. The hepatic stages and gametocytes are asymptomatic. The cause of *P. falciparum*'s virulence in comparison with other human parasites is unknown, but its multiplicative capacity and ability to sequester in the deep vascular beds are thought to contribute. Indeed the rapid multiplication rate of falciparum may mean that a lethal parasite burden is reached in 3–4 parasite life cycles in a child.

Mediators of Severe Disease

A number of substances have been proposed to be responsible for severe disease. Most recently reactive oxygen species (ROS), cytokines and nitric oxide (NO) have been implicated, with the possibility of a dual role for some, in which these substances are involved in both the pathogenesis of severe disease and killing the parasite.

Reactive Oxygen Species

Clark et al. [4] proposed that ROS may play a role in the pathogenesis of severe malaria. They subsequently suggested that extravasated erythrocytes may

lead to ROS generation, causing coma and damage to local tissues, including the brain. ROS produced by leucocytes may also kill the parasites.

In humans there is some evidence that ROS play a role in pathogenesis. Monocyte generation of ROS was increased in adults with falciparum malaria, especially those with severe disease [5, 6]. In children, ROS were associated with anaemia [7, 8] and products of lipid peroxidation were found in the cerebrospinal fluid (CSF) in cerebral malaria (CM) [9]. Thus ROS appear to be generated in acute malarial infections, but their role in the pathogenesis of complications is unclear.

Cytokines

In 1987, Clark et al. [10] suggested that tumour necrosis factor (TNF)-α was a possible mediator of severe malaria. Since then a number of cytokines have been implicated, with the pro-inflammatory cytokines, e.g. TNF-α, interferon (IFN)-γ, interleukins (IL)-1B and IL-10, thought to be responsible for cerebral symptoms, whilst anaemia is associated with low levels of IL-12 and transforming growth factor-β [11].

P. falciparum-infected erythrocytes produce pyrogenic material which triggers the release of TNF (and other cytokines) from host mononuclear cells [12]. Elevations of TNF result in pyrexia in both falciparum [12] and vivax malaria [13]. A monoclonal antibody against TNF attenuates fever in children with CM [14]. In the relatively benign vivax malaria (without cerebral symptoms), very high (up to 3,000 pg/ml) TNF levels have been measured transiently in serum [13], suggesting that such high systemic levels do not themselves cause serious disease. Furthermore, elevations in TNF can exert antiparasitic effects by inhibiting parasite multiplication and synergising with other factors to produce gametocidal effects [15].

Parasite-derived material is responsible for TNF release from host cells. Parasites vary in their ability to induce TNF release and those obtained from patients with cerebral disease induced more TNF release ex vivo than those from patients with uncomplicated disease [16]. However, there was considerable overlap in TNF-releasing activity between disease groups and only ~60% of parasite isolates from patients grew sufficiently to allow assay [16].

Host factors are also important in determining the TNF released in response to infection. A TNF-α promoter polymorphism has been shown to be associated with disease severity in Gambian children [17] with a homozygous polymorphism at −308 bp relative to the start of TNF transcription, associated with a 7.7-fold increase in the relative risk of death or neurological sequelae in CM. Other TNF-promoter polymorphisms are now the subject of detailed assessments in case-control studies in malaria as well as other diseases. While TNF is the best-researched inflammatory mediator in malaria, there is also evidence

of increased IL-10 production in severe malaria, associated with elevations in TNF levels [18] that may contribute to severe disease.

The timing and amounts of different cytokines that are released, particularly TNF, may be important determinants of subsequent pathophysiological events and perhaps mortality. Thus increased production of TNF early in malarial infection may be protective, whereas prolonged, high TNF levels may be detrimental. This temporal relationship has been difficult to identify in humans. One of the difficulties in interpreting immunologically measured elevations in cytokines, particularly when receptor binding can influence the unbound concentrations, is that the biologically active moieties (either circulating or local) may not be represented in these measurements. Thus bioassays of circulating cytokines may be more relevant, but are more laborious to perform.

In African children, circulating cytokines (particularly TNF) are much higher in severe malaria than controls [19–24]. Most studies detected higher levels in children with CM rather than non-CM. These elevations in TNF may exacerbate the tendency to sequestration by the upregulation of host ligand molecules responsible for cyto-adherence of parasites (especially ICAM-1, vascular cell adhesion molecule-1 (VCAM-1) and endothelial-selectin), increasing mechanical obstruction of cerebral or other blood vessels. Localised TNF production may not be detected by plasma assays but may nevertheless have important pathophysiological consequences. Increased TNF may also have consequences on the metabolic status of patients or in the pathophysiology of anaemia.

An attempt to reduce TNF activity in vivo using a mouse monoclonal anti-TNF antibody (B-C7) did not significantly reduce the mortality in African children with severe malaria, despite decreasing the temperature [14]. Thus the increased circulating TNF associated with CM may be an epiphenomenon rather than indicating a causal role of TNF in mortality. Thus, although TNF is likely to be important, it may be just one part of a wider process. It is known that there are associated increases in IL-1 and IFN-γ concentrations as well as increases in circulating TNF receptors in acute infection. Circulating IL-6 and IL-8 levels [25] are also increased in falciparum infection. In relatively uncomplicated infections, IL-8 levels remained elevated for up to 4 weeks after the acute infection has been cured [25].

Both falciparum and vivax infections are associated with increases in circulating markers of endothelial cell activation (sICAM-1, sVCAM-1 and sELAM-1) and these markers were significantly higher in Gambian children with severe falciparum malaria compared with uncomplicated infections [26]. These markers were also elevated in non-immune subjects, suggesting that elevations are a consequence of cytokine activation by TNF. However, in a recent study in Gambian children, malaria was associated with elevations in circulating ICAM-1 levels (which correlated with TNF and IL-1a levels), but elevations

were not related to disease severity [27]. Thrombomodulin, another marker of endothelial cell damage, was also significantly higher in non-immune adults with severe malaria compared with those with uncomplicated infections [28].

Nitric Oxide

Since the cytokine hypothesis was first elaborated, NO has been identified as a potential mediator for TNF. NO is involved in host defence by killing intracellular micro-organisms [29, 30], in maintaining circulatory status by its action on endothelial cells, and in neurotransmission [31]. It is produced both constitutively in certain tissues, and in response to inflammatory stimuli through the action of cytokines which upregulate the synthesis of inducible NO synthase (iNOS or NOS2).

Thus NO is thought to be produced in cerebral endothelial cells, and to diffuse into brain tissue interfering with neurotransmission [31]. It may also cause neurological damage by forming peroxynitrite, although this was not part of the original hypothesis. An alternative suggestion for the role of NO in malaria is that it may be important in host defence, particularly in intracellular killing of parasites. However, testing the hypothesised actions of NO in malaria is challenging because of its evanescent nature, and the inapplicability of results from many animal studies to human infection. Instead, indirect measures have been used. NO is metabolised to nitrate and nitrite, and since these products are stable in plasma and urine they are used as surrogate endpoints that reflect increased iNOS activity.

In Tanzanian children with CM, NO synthesis was decreased, compared with patients with uncomplicated or subclinical infections [18]. Changes in plasma TNF profiles were consistent with previous reports that progressively higher levels were associated with increasing disease severity with highest levels in fatal cases. Levels of the anti-inflammatory cytokine, IL-10, were also increased in more severe disease, suggesting a mechanism by which NO synthesis could be suppressed in the patients with CM. In this study, increased NO synthesis was interpreted as being protective of the development of cerebral and fatal disease, and therefore important in defence rather than as a contributor to cerebral pathology. Some support for this suggestion comes from work with desferrioxamine in children with CM, where NO synthesis was increased in desferrioxamine recipients compared with placebo recipients [32].

In contrast, a study from Papua New Guinea noted elevations in reactive nitrogen intermediates in children with CM compared with conscious patients, and higher levels in fatal cases compared with survivors [33]. However, African studies have failed to detect such an association [34, 35]. In order to assess the intracerebral synthesis of nitrogen oxides, which may arise through local diffusion of NO if it is produced by cerebral endothelial cells, CSF measurements

were performed in a subset of patients with CM. Again, no elevations in nitrogen oxides was observed, suggesting that coma in malaria may not be due to derangements in NO metabolism [35].

Red Cell Deformability

Early studies of *P. knowlesi* parasitised red blood cells (PRBCs) showed infection increased resistance to flow through 5-μm polycarbonate sieves, and obstructed pores at high parasitaemia. Infection was also associated with a decrease in red cell deformability (RCD), and these changes were suggested to contribute to the development of microcirculatory obstruction [36]. Subsequently, more sophisticated studies have confirmed that there is a stage-dependent decrease in the deformability of red cells as *P. falciparum* matures [37], and that mature parasites require correspondingly larger pressures (4- to 6-fold, compared with controls) to allow entry of PRBCs into small (3 μm) capillaries. These changes, could reduce the circulatory flow in downstream (post-capillary) venules and contribute to other pathophysiologically important processes such as cyto-adherence. The molecular basis for adhesion at physiological flow rates has also been studied in cultured cell receptors [38, 39], but relating changes ex vivo to patients' clinical status is not straightforward [40, 41].

The reduction in RCD not only occurs in PRBCs, but also the non-PRBC as well. Microvascular perfusion in severe falciparum malaria is therefore limited by mechanical obstruction, adherence of other red blood cells and the stiffness of the non-adherent red blood cells. RCD measured at low shear rates encountered in capillaries and venules, is the most powerful prognostic indicator of death in adults [42], although not associated with the syndrome of CM. Similar studies in Kenyan children also showed a strong association with severe disease, but the increase in the RCD with blood transfusions raises therapeutic possibilities [Dondorp, unpublished observations].

Disease Caused by Malaria

Mild Malaria

The most common clinical manifestation of malarial infection is a non-specific febrile illness. The fever rarely follows classical descriptions of cyclical fevers with rigors and chills. Although this pattern is more likely to be found in adults than children, even in adults the clinical presentation is rarely specific. Furthermore in most situations in Africa, malaria is so ubiquitous, both as infection and as a disease, that for practical purposes it cannot be diagnosed using clinical features with any degree of certainty. In many areas the background

prevalence of parasitisation is also high so that even the addition of microscopic diagnosis may often not help.

Syndromes of Severe Malaria

In the last decade there has been considerable debate about the classification of severe malaria. A proposed World Health Organisation (WHO) scheme for classifying disease, which was largely based on accumulated experience of severe malaria amongst South East Asian adults [43], has now been shown in a number of settings to have limited application to severe disease amongst children [44–46]. Nevertheless the systematic WHO classification retains some value in emphasising the multi-system nature of severe malaria. We will describe both the pragmatic, syndromic classification of severe malaria largely based on work in children from endemic areas and use some of the WHO categories to emphasise particular areas of important difference in the disease pattern seen in non-immune adults or areas of particular pathophysiological interest.

The advantage of the syndromic approach to classification is that life-threatening disease may be identified on the basis of simple, bedside clinical features (fig. 1). While the absolute proportions of particular syndromes appear to vary from site to site, it has now been demonstrated in a number of settings that the sensitivity of the *combination* of clinical features for detecting severe disease remains high [44–46]. Another major advance is the explicit acknowledgement that what were once considered specific disease presentations (i.e. CM) may be observed in children with a range of additional problems. In fact in some cases it appears likely that quite separate pathophysiological processes may result in a similar syndrome [47, 48]. Thus the clinical criteria for 'CM' may be satisfied by a child with a malaria-associated severe convulsive disorder, a child with an apparently primary encephalopathy without seizures, or a child who's primary problems are systemic metabolic abnormalities. This recent realisation should encourage caution when results from different research sites are interpreted, and may significantly alter the generalisability of research findings.

Severe Malarial Anaemia

All children with significant clinical malaria have some degree of anaemia. The pathogenesis of malarial anaemia is complex. PRBCs are destroyed within 72 h, either because they burst during schizogony or are removed by the spleen. However, there is increasing evidence to suggest that non-PRBC have a shortened life span. Possible mechanisms of destruction of erythrocytes include immune-mediated mechanisms, reduced RCD and ROS damage to the erythrocyte membrane [47]. Data on the relative importance of immune sensitisation of uninfected cells is contradictory in studies carried out in different areas [47, 49]. In addition to the loss of cells, malaria infection is also associated with a degree

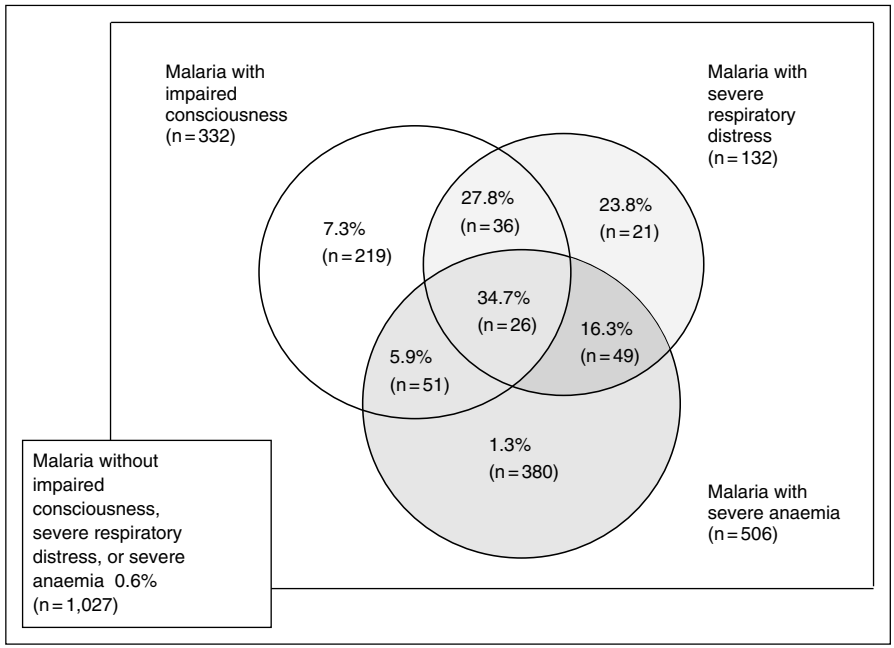

Fig. 1. Clinical features on presentation and outcome of children admitted with malaria to Kilifi District Hospital, Kilifi, Kenya.

of marrow suppression [50] though this may be less specific for malaria in African children than has previously been thought to be the case [51]. Marrow suppression is not related to erythropoietin deficiency but may result from an altered balance of cytokines, particularly IL-10 and TNF.

In addition to the complexity of processes involved in malarial anaemia, it has to be recognised that in most malaria endemic areas there are several other causes of anaemia [51], most importantly iron deficiency, and the contribution of each is hard to determine in an individual. This has led to a certain amount of confusion in the definition of severe malarial anaemia. From a pragmatic point of view, however, it is the clinical state of the child that defines the condition. Lackritz et al. [52] reported that respiratory distress is the single most important prognostic factor, and this probably represents the main indication for blood transfusion in children with malaria and severe anaemia [53]. This is supported by our experience: in figure 1 it can be seen that the majority of severely anaemic children have a relatively low mortality (it was the policy to treat these children conservatively, without transfusion) unless the problem is complicated by respiratory distress.

Respiratory Distress

This syndrome has not figured in standard descriptions of clinical malaria in children. In the past respiratory distress has been attributed to congestive cardiac failure in children with severe malaria. However, this may not be the case. In the vast majority of cases respiratory distress in severe malaria seems to be a reflection of underlying metabolic acidosis [44, 54]. Subsequent studies have confirmed that increased depth of breathing (analogous to Kussmaul's respiration) is a key sign and one that with appropriate training has excellent sensitivity and specificity for severe metabolic acidosis [55].

In general metabolic acidosis is associated with high lactate levels [24, 56], although acidosis in severe malaria is not necessarily synonymous with lactic acidosis as in a proportion of children the lactate is not particularly high, and even in those where it is, it can rarely account entirely for the observed high anion gap [56]. Acidosis may be caused by hypovolaemia secondary to dehydration and possibly systemic vasodilatation, renal impairment (see below), salicylate intoxication [57], ketosis [Waruiru C, submitted] and the specific effects of certain cytokines, notably TNF.

Although the underlying pathophysiology of metabolic acidosis is likely to be complex, from a clinical point of view two factors seem to be of major importance: reduced circulating volume, and reduced oxygen-carrying capacity. The fact that profound anaemia can be tolerated at all in malaria with a minimum of obvious symptoms indicates: (i) that a large resting reserve exists within the oxygen supply/demand relationship, and (ii) that a sophisticated array of compensatory mechanisms exist for this purpose. Such compensatory mechanisms include the abilities to: increase total blood flow (cardiac index); extract more oxygen from blood flowing through tissues (increase the oxygen extraction ratio), and re-distribute flow to areas of greatest need. In the relatively small proportion of children who become severely symptomatic with respiratory distress it is likely either that compensatory mechanisms are impaired or that oxygen demand has outstripped supply or that a combination of the two problems is present. In either case one consequence of a net failure of oxygen delivery is a shift from aerobic to anaerobic metabolism in tissues with the production of lactate and an associated acidosis. The idea that a tissue oxygen debt plays an important role in the generation of metabolic acidosis is supported by the demonstration that total oxygen consumption of children with severe malarial anaemia rises markedly during the course of blood transfusion and in proportion to the lactate level on admission [58].

Although much undoubtedly remains to be found out about the pathogenesis of metabolic acidosis in severe malaria, the clinical implications of what is already known may be important. Acidotic children require immediate and rapid attention to circulating volume and oxygen delivery. The ideal resuscitation fluid

is fresh blood and management would be simplified if there were a limitless safe supply. However, significant and large areas of ignorance remain in our understanding of anaemia/acidosis. Almost nothing is known about cardiac or pulmonary function in severe malaria (see below) despite the fact that sequestration of PRBCs is common in these organs [58].

While respiratory distress in association with severe malaria is most likely to be an indication of metabolic acidosis, genuine congestive cardiac failure may occur possibly in children with more chronic anaemia. A second important cause of 'malarial respiratory distress' may be co-existent lower respiratory tract infection [55]. In some cases the primary problem may, in fact, be a lower respiratory tract infection but confusion arises because the child, like the majority of children in an endemic area, happens to be parasitaemic. Alternatively, there is increasing evidence that children with severe malaria may at the same time have true dual pathology [59].

Neurological Manifestations of Malaria

Falciparum malaria can cause a variety of neurological manifestations [60], which include impairment of consciousness (including CM), seizures, cranial nerve neuropathies and a number of syndromes that develop after a malaria infection, e.g. post-malarial syndrome, cerebellar ataxia. Here we shall concentrate on CM and seizures, since these are the most common manifestations and contribute to the mortality.

Disturbances of Consciousness and CM

The term 'CM' has often been used loosely in the medical literature to describe any disturbance of the CNS in a malaria infection. Since disturbances of consciousness can be caused by systemic complications, e.g. fever, hypoglycaemia, hyponatraemia and uraemia, a strict definition of CM was suggested [43, 61], which has recently been modified as a deep level of unconsciousness (inability to localise a painful stimulus and an incomprehensible vocal response) [62] in the presence of a *P. falciparum* asexual parasitaemia, after the correction of hypoglycaemia and exclusion of other encephalopathies, especially bacterial meningitis and locally prevalent viral encephalitides. To exclude a transient postictal state in adults, coma was required for more than 6 h after a generalised convulsion, although in children this was reduced to 1 h. In clinical practice, any impairment of consciousness or other sign of cerebral dysfunction is an indication for parenteral treatment and intensive care management.

CM has features more similar to a diffuse metabolic encephalopathy, rather than other infective encephalopathies. Focal neurological signs do not usually accompany the depression in consciousness, except in those children who develop hemiparesis or the appearance of brainstem signs during the agonal period.

However, focal motor and generalised tonic-clonic convulsions are the most common clinically detected seizures [63], but subtle or subclinical seizures detected with electroencephalography (EEG) are also common [48]. In some children, the level of consciousness improves with the administration of anti-convulsants, suggesting that seizures contribute to the coma. Between seizures the EEG shows bilateral diffuse slowing of the brain waves, often asymmetrical (not inevitably associated with clinical signs) [63–65].

CM in African Children

African children with CM are older (40–45 months of age) than children with other complications of malaria [47, 66], but CM is rarely encountered after the age of 10 years in people exposed to *P. falciparum* since birth. CM presents usually with a 1- to 4-day history of fever and convulsions, the latter often precipitates coma [67]. Most African children with CM survive, regaining consciousness within 48–72 h of starting appropriate treatment [45, 47, 67, 68]. The median time for recovery of consciousness is 32.3 h (95% CI 23.4–41.1). In children, a median of 10.9% (95% CI 8.3–13.5) have neurological sequelae, a median of 18.7% (95% CI 16.3–21.0) die [47]. Most deaths occur within 24 h of starting treatment [44, 45, 68, 69], usually with brainstem signs, respiratory arrest and/or overwhelming acidosis [47].

Pathogenesis of CM

The pathogenesis of the clinical syndrome of CM is complex, and the syndrome may result from a number of interacting mechanisms [47].

Brain Swelling

Opening CSF pressures are raised in most African children with CM [47, 70] and there is evidence of brain swelling on computerised tomography scans [71] and at post-mortem [68]. Kenyan children dying with CM had clinical signs compatible with transtentorial herniation [69] and, half of the children had sonographic features of progressive intracranial hypertension during the agonal phases [69]. In a post mortem study of 7 Nigerian children dying of CM, transtentorial herniation was seen in 1, whilst 3 others had evidence of brain oedema [68]. Monitoring intracranial pressure (ICP) confirmed that children deeply unconscious from CM had raised ICP [72] and those children who developed severe intracranial hypertension, either died or survived with severe neurological sequelae.

The most likely cause of raised ICP in CM is an increase in cerebral blood volume [69] due to sequestration of PRBCs in the vascular compartment, or obstruction of venous outflow. An increase cerebral blood flow, could be caused by other features of CM, such as seizures, hyperthermia and anaemia. Kenyan

children with severe neurological sequelae have tomographic evidence of cytotoxic oedema during recovery that may contribute to the severe intracranial hypertension. Whether intracranial hypertension is a primary pathophysiological process and the possible role of mannitol remain to be established [72].

Neurological Sequelae

Neurological sequelae are associated with protracted seizures [73], prolonged and deep coma [73], and hypoglycaemia [73, 74]. Some neurological deficits are transient (e.g. ataxia), whilst others, e.g. hemiparesis and cortical blindness, often improve over months, although may not completely resolve. More subtle deficits, e.g. cognitive difficulties, language and behaviour problems, are increasingly being recognised [75]. The incidence of epilepsy after CM is not determined, although often reported. Furthermore, since the seizures that occur during the acute illness are often focal, repetitive or prolonged, damage to the hippocampus may occur, producing memory impairment and complex partial seizures [76].

The causes of the sequelae are largely unknown and are likely to be multifactorial [47] and include: severe intracranial hypertension; stenosis or occlusion of the basal cerebral arteries demonstrated by angiography [77] or transcranial Doppler (possibly related to vasospasm or underlying conditions such as haemoglobinopathies), and hemiconvulsion-hemiparesis syndrome, which is associated with prolonged seizures. Additional mechanisms include mismatch between the delivery of oxygen and glucose in the presence of increase demand (seizures, fever), excitotoxins, ROS or toxins produced by the parasite [47].

Seizures and Malaria

P. falciparum appears to be particularly epileptogenic, for it was the most common cause of seizures in children admitted to a Kenyan hospital [78] and more frequently associated with seizures compared to *P. vivax* infections in Thai children [79]. Although fever may precipitate some seizures, most seizures occur when the rectal temperatures are less than 38.0 °C. In comparison to simple febrile seizures, the seizures in malaria are often recurrent, and 84% of the seizures are complex, most often with a focal nature [78]. The seizures appear not to be associated with hypoglycaemia and hyponatraemia.

Other Manifestations of Severe Disease

Renal Disease

Acute tubular necrosis leading to established renal failure is rare in African children with severe malaria, in contrast to non-immune patients. However, lesser

degrees of renal dysfunction are potentially important. A moderately raised serum creatinine or urea and non-specific abnormalities on urinalysis are not uncommon in children with severe malaria [45, 66]. Such changes probably reflect reductions in circulating fluid volume leading to pre-renal impairment. However, a salt-losing nephropathy [80, 81] and reduced clearance of inorganic acids may occur [56].

While an active glomerulonephritis is frequently reported in adult histological studies [82], the clinical features are not consistent with this [83]. The clinical picture is in fact much more in keeping with the development of acute tubular necrosis [84] with or without oliguria. The cause of acute tubular necrosis in adults remains largely unknown despite the fact that it is the most important cause of death from malaria in many South East Asian countries [85]. Reduced renal microvascular blood flow is presumed to be responsible [86] possibly secondary to sequestration of PRBCs and exacerbated by hypovolaemia and haemoglobinuria [83].

Shock, Cardiovascular and Pulmonary Disease

Circulatory collapse is infrequent as a presenting feature of severe malaria in children [44] but has a very poor prognosis. In adults shock may occasionally be a presenting feature of severe malaria, but more commonly shock is associated with other evidence of multi-organ impairment [87]. Management usually requires aggressive but careful fluid resuscitation and correction of specific abnormalities such as hypoglycaemia. There is emerging evidence that concomitant bacterial infection, both gram-negative and gram-positive, which has been well documented in adults, is more prevalent in severe malaria in children than previously realised. Indeed it may well be that this plays an important role in the pathogenesis of a spectrum of diseases including metabolic acidosis and multi-organ impairment that culminates in circulatory collapse.

No studies of cardiac 'pump' function have been performed in either 'CM' or severe malaria anaemia in African children, although for many years congestive cardiac failure has been considered a potentially life-threatening complication of the latter [43, 88]. A small number of children have had electrophysiological function investigated using 24-hour taped electrocardiograms but no abnormalities could be attributed to malaria [89]. In adults, despite intense sequestration of PRBC in the myocardial vasculature [90], cardiac function is well preserved [91] and most patients have an elevated cardiac index and low or normal right- and left-sided filling pressures [85].

Although respiratory distress is common in severe childhood malaria, pulmonary oedema seems rare, other than as a terminal event even in children receiving relatively aggressive fluid therapy [92]. Evidence for other forms of lung involvement in malaria also seems sparse. Transcutaneous oxygen saturation

are normal even in severe malaria. Chest X-rays in children with falciparum malaria do not demonstrate any specific abnormality [55].

In adults sequestration in the lung microvasculature has been observed post-mortem [90], but the predominant form of lung injury in this group is pulmonary oedema which appears to occur primarily in the context of the adult respiratory distress syndrome [93] to which fluid overload may contribute [94]. Given the frequency of acute renal failure in adults and the propensity to adult respiratory distress syndrome, great care needs to be taken with the fluid management of adult patients with severe malaria.

Hypoglycaemia

Hypoglycaemia has been reported to complicate severe malaria in children in 13–32% of cases [44, 45, 66] and is associated with a higher risk of poor outcome, either the development of neurological sequelae or death [66, 73]. Hypoglycaemia is not a specific complication of severe malaria but rather is a complication of many severe childhood illnesses in the tropics [95], possibly being related to prolonged fasting [95]. Studies in which some of the gluconeogenic precursors have been directly measured indicate that levels of alanine and lactate are normal or high [96, 97] while levels of the counter-regulatory hormones (glucagon, cortisol and growth hormone) are also elevated. In these studies levels of insulin were appropriately suppressed [24, 96, 97]. Although studies in mild malaria suggested that a relative deficiency of gluconeogenic substrate supply might contribute to the development of hypoglycaemia in children [98], these findings were not supported by studies in children with severe disease [99]. Overall impairment in hepatic gluconeogenesis remains the most likely mechanism for hypoglycaemia in severe childhood malaria, although its pathogenesis is unknown.

Non-immune adults seem to be much more susceptible to the insulin-inducing effects of quinine with concomitant hypoglycaemia [100, 101] and this is particularly true of pregnant women. However, hypoglycaemia also occurs in the presence of appropriate insulin levels as a direct complication of the disease process [102].

Disease in Pregnancy

In non-immune populations pregnancy predisposes to particularly severe manifestations of malarial disease. Pregnant women are particularly prone to hypoglycaemia and pulmonary oedema [103]. The risk of hypoglycaemia involves both an increased risk of disease-associated hypoglycaemia and increased susceptibility to quinine-induced hyperinsulinaemia. Although often part of a

multi-system severe disease, hypoglycaemia can be an isolated finding in pregnant women with otherwise mild disease, either as an asymptomatic finding or as a cause of sudden deterioration. In addition to the increased risk to the mother's life, malaria in pregnancy in non-immune women has a range of effects on the foetus. Acute disease is associated with abortion, still birth and premature delivery. Even when pregnancy is closely monitored and treatment of disease prompt, there is a significant reduction in birth weight [104].

In areas of higher transmission pregnant women are also at particular risk of malaria. There is an apparent loss of immunity, particularly evident in the first pregnancy [105]. This does not however manifest as a complete loss of immunity and such women rarely present with acute severe disease. Rather there is increased frequency and density of parasitaemia, which in turn reflects often heavy infection of the placenta, which seems to act as an immunologically naive site. From the mothers point of view the major risk is of maternal anaemia [106]. Placental infection is associated with low birth weight, which is presumed to be due to intrauterine growth retardation [107].

Future Approaches to the Treatment of Clinical Malaria

Over the last decade it has become increasingly apparent that significant gains in terms of mortality from improved treatment of malaria are unlikely to be made as a result of the development of novel, specific anti-malarial drugs. Thus in many sites it has been demonstrated that a majority of deaths occur within 24 h of admission to hospital [44]. Furthermore in large comparative trials of rapidly acting qinghaosu-derived anti-malarials with the slower acting quinine, no survival advantage was observed [108]. This has focused attention on adjuvant therapy when treating clinical disease and obviously further supports the goal of developing a workable preventative strategy.

There are three broad areas that might successfully be exploited to reduce case fatality rates. Firstly, an improved recognition of simple clinical features associated with an increased risk of mortality to ensure that optimum treatment is targeted appropriately. Secondly the possibility that current resources might be better used, and lastly the development of novel adjuvant therapies.

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Sporozoite Antigens: Biology and Immunology of the Circumsporozoite Protein and Thrombospondin-Related Anonymous Protein

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Introduction

The sporozoite's life is a life of cell invasion: in the mosquito, sporozoites from mature oocysts invade salivary glands. During blood feeding, these salivary gland sporozoites are injected into the skin of the mammalian host and rapidly invade hepatocytes, thus initiating malaria infection.

Early studies demonstrating an association between invasive capability and parasite motility led to the hypothesis that *Plasmodium* and other Apicomplexan protists actively invade cells [1, 2]. Recent findings demonstrating that motility is required for cell invasion by Apicomplexan parasites have confirmed this hypothesis [3, 4]. When cell invasion is visualized by electron microscopy, investigators observe that initial attachment between the parasite and the host cell is followed by the formation of a close association/tight junction between the host cell plasma membrane and the anterior pole of parasite. The parasite then induces a parasitophorous vacuole and moves forward into the vacuole [for review see, 5].

Organisms of the phylum Apicomplexa, of which *Plasmodium* is a member, move by gliding motility, a substrate-dependent form of locomotion that does not involve a change in cell shape. It is thought that parasite motility results from posterior translocation, via linkage to an intracellular motor, of surface molecules bound to substrate or cell surface [6]. The invasive stages of these organisms all have structural similarities that are the basis for inclusion in the

phylum Apicomplexa. These structures, collectively called the apical complex, include an array of subpellicular microtubules and specialized secretory organelles (e.g. micronemes, rhoptries and dense granules) which secrete their contents during invasion [for review see, 5]. Comparison of the proteins secreted from apical organelles of different Apicomplexan parasites often reveals the presence of conserved domains, suggesting similar function. However, these proteins are not identical and vary among different stages of the same organism as well as among different Apicomplexan species. These differences likely reflect the different biology and different life histories of each organism.

Investigations into the mechanism of cell invasion and gliding motility have provided new insights into the structure/function of sporozoite antigens that have direct application for the development of malaria vaccines. This chapter focuses on two protective antigens that have been characterized over the last several years, the circumsporozoite (CS) protein and thrombospondin-related anonymous protein (TRAP). Recent cell biological and molecular studies have begun to define the role of these proteins in motility and host/parasite interactions in the mosquito vector and the mammalian host. An understanding of the unique cellular localization and function of sporozoite proteins provides a rationale for the design of subunit vaccines to effectively target the parasite in the vertebrate, as well as in the invertebrate host.

Identification of Sporozoite Antigens

Although only a limited number of sporozoite antigens have been identified to date, this number will undoubtedly increase as a result of the *Plasmodium* genome-sequencing projects. In this chapter, we have focused on the first two protective sporozoite antigens identified, the CS protein and TRAP. The structure and immunogenicity of other sporozoite antigens, as well as additional antigens unique to liver stages, are discussed in the chapter by M. Hollingdale [pp 97–124].

CS Protein

The first sporozoite antigen identified, and in fact the first protozoan parasite antigen cloned, was the CS protein [for review see, 7, 8]. It is a major component of the sporozoite surface, and is also found intracellularly within vesicular structures morphologically indistinguishable from micronemes. Protein expression can first be detected during the oocyst stage and persists in the intracellular exoerythrocytic forms (EEFs), although levels decrease as EEFs develop into mature hepatic schizonts.

The single copy CS gene encodes a protein of 300–400 amino acids with similar molecular structure in rodent, simian and human species of *Plasmodium*

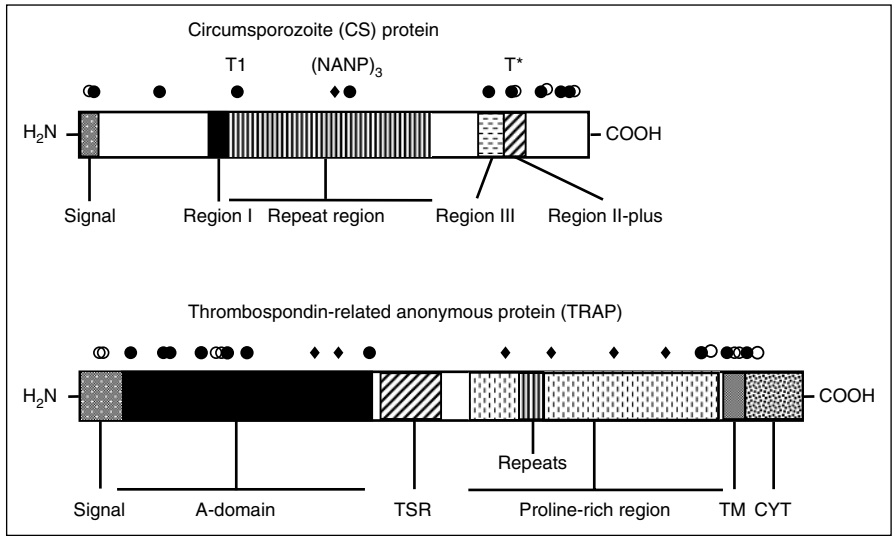

Fig. 1. Schematic diagram of *P. falciparum* CS protein and TRAP. Structural and functional domains of each protein are indicated below the figure. TM = Transmembrane domain; CYT = cytoplasmic domain; TSR = type-I thrombospondin repeat. Symbols above each figure represent CD4+ T cell epitopes (●), CD8+ T cell epitopes (○), and B cell epitopes defined by mAb (♦). The CS protein Th epitopes T1 and T* in combination with the B cell epitope $(NANP)_3$ have been tested as synthetic peptide vaccines in phase I trials.

(fig. 1). All CS proteins contain a putative hydrophobic signal sequence at the amino end and a carboxy terminal sequence consistent with the addition of a glycosylphosphatidylinositol (GPI) anchor, although a characteristic GPI moiety has yet to be demonstrated in the native protein. CS proteins of mammalian malaria species share two highly conserved domains, region I and region II-plus. The region I sequence, KLKQP, is not found in the avian malaria species, *Plasmodium gallinaceum* [9]. Region II-plus contains a motif that is part of the type-I thrombospondin repeat (TSR) found in host proteins involved in cell–cell and cell–matrix interactions. Sequences with homology to region II-plus are also found in a surface protein of the motile ookinete, CTRP the CS- and TRAP-related protein [10, 11], as well as in proteins of other Apicomplexan parasites [12–14]. Region III, found 5′ of region II-plus, has little sequence homology among CS proteins, but has a conserved pattern of aliphatic amino acids predicted to form an amphipathic α-helix.

The conserved regions of the CS protein flank a central repeat region which is antigenically distinct for each species of *Plasmodium*. The simplest repeat motif, found in *Plasmodium falciparum*, consists of a NANP tetramer that is

repeated 30–40 times in different isolates. CS proteins also contain minor variants in the 5′ repeat region which differ at 1–2 amino acids; in *P. falciparum*, this consists of three to four copies of an NVDPNANP sequence. While the CS protein repeats are unique for each *Plasmodium* species, the repetitive sequences are biased toward the inclusion of hydrophilic amino acid residues, in particular N, A, D, P, E, G and Q. Variation in the number of major and minor repeats contained within each CS protein gives rise to the size polymorphisms noted in different parasite strains. In the case of *Plasmodium vivax*, the analysis of the CS protein repeats has led to the identification of distinct isolates and *P. vivax*-related strains.

Thrombospondin-Related Anonymous Protein

In contrast to the immunological methods used to identify the CS protein, TRAP was first identified by genomic Southern blot of *P. falciparum* DNA using an oligonucleotide probe specific for TSR repeats [15]. Subsequent studies, using a mAb derived from a *Plasmodium yoelii* sporozoite-immunized mouse, identified the murine malaria homolog, sporozoite surface protein-2 (SSP2) [16, 17].

TRAP proteins of human and rodent malaria species have similar structure (fig. 1) but differ in molecular weight; *P. falciparum* TRAP contains 559 amino acids while *P. yoelii* TRAP contains 826 amino acids. In addition to the type-I TSRs, TRAP also contains a region with structural homology to the A-domain of integrins and other proteins involved in cell–cell and cell–matrix interactions. In mammalian proteins, the A-domain contains a ligand-binding metal ion-dependent adhesion site (MIDAS), characterized by a DXSXS sequence and conserved downstream threonine and aspartic acid residues [18]. TRAP proteins of all *Plasmodium* species contain this motif [19], suggesting that it is critical for protein function. A-domains are also found in other *Plasmodium* proteins, such as CTRP, as well as in proteins of other Apicomplexan parasites [10, 12, 13].

In contrast to CS protein, TRAP is a typical type-1 molecule, containing a carboxy terminal transmembrane domain and a well-defined cytoplasmic domain that has conserved tryptophan and acidic amino acid residues. In contrast also to CS protein, the central region of *P. falciparum* TRAP contains only a small number of tandem repeats, while numerous repeats are found in the rodent malaria TRAPs [19].

TRAP is found primarily within the micronemes, with a small amount of TRAP on the sporozoite surface [16, 20]. When stained by fluorescence, TRAP gives a patchy polar distribution that is distinct from the linear pattern observed with the CS protein. Similar to CS protein, TRAP is first detected in the oocyst stage and persists in the EEF [4, 17]. Early studies originally detected TRAP in blood stages of *P. falciparum* [15, 20], while in later studies

it was predominately found in the pre-erythrocytic stages of human and rodent malaria species [17, 21, 22].

Biological Function of Sporozoite Antigens

Sporozoites differentiate in the mosquito and both CS protein and TRAP function in the development of infectious sporozoites. Molecular and biological studies have defined the role of these antigens in motility as well as in interactions with host cells, in both the invertebrate and vertebrate hosts.

Sporozoite Development in the Mosquito

When a mosquito takes a bloodmeal from a malaria-infected individual, gametocytes in the bloodmeal differentiate in the mosquito midgut and fuse to form a zygote, which develops into a motile ookinete. The ookinete penetrates the midgut wall and comes to rest under the basal lamina of the midgut where it transforms into an oocyst. Sporozoite development in oocysts takes between 10 and 25 days depending on the species of *Plasmodium* and environmental factors such as temperature. The avian malaria parasite *P. gallinaceum*, can complete this process in vitro when ookinetes are seeded onto matrigel (a gel containing components of extracellular matrix) and allowed to develop in the presence of the insect cell line, Schneiders L-2 [23]. More recently, it has been shown that injection of *P. gallinaceum* ookinetes into the hemocele of *Drosophila melanogaster* results in the production of infectious sporozoites [24]. These studies suggest that oocyst development requires anchorage of the ookinete and young oocyst onto a substrate as well as adequate nutrition.

Sporogonic development in the oocyst involves an asexual process called schizogony in which nuclear division precedes the formation of daughter cells [for review see, 25]. The early oocyst contains one or more cytoplasmic islands called sporoblasts. Sporozoites bud from the sporoblast at sites where an inner membrane complex (IMC) has been deposited along the inner face of the sporoblast plasmalemma. As the sporozoite buds, the IMC grows with the parasite and various cytoplasmic components such as a nucleus, mitochondria and other organelles are pulled into the forming sporozoite. Ultimately, the oocyst is full of sporozoites and the sporoblast resembles a residual body.

Very little is known of the molecular events that control sporogony. Recently it was found that when the gene encoding the CS protein was replaced with the selectable marker dihydrofolate reductase, sporozoites failed to develop [26]. By electron microscopy, these CS protein-negative oocysts were aberrant early in sporogonic development [27]. The formation of the IMC, which normally occurs underneath selected areas of the sporoblast plasmalemma

(the bud sites), was dysregulated and was found lining extensive areas of the plasma membrane. Occasionally buds formed under the IMC of the CS protein-negative oocysts but they had an abnormal shape and were not functional sporozoites. Although the precise role of CS protein in sporozoite development cannot be concluded from these studies, it has been suggested that its expression in early oocysts regulates the docking of cytoplasmic vesicles responsible for the formation of the IMC [27].

Sporozoite Invasion of Salivary Glands

Sporozoites are released from mature oocysts over a 3- to 4-day period, usually occurring between 10 and 14 days after mosquitoes have received an infective bloodmeal. During this time, the parasites are found dispersed throughout the mosquito hemocele, suggesting that they are passively transported by the action of the mosquito's open circulatory system [28]. Experiments with human and rodent *Plasmodia* have found that approximately 20% of oocyst sporozoites reach the salivary glands [29]; a high percentage considering the size of salivary glands compared to the other organs encountered by sporozoites in the hemocele. These data suggest that sporozoites specifically recognize salivary glands. Although the identity of the molecules involved in salivary gland recognition by sporozoites is not yet known, antibodies raised against *Aedes aegypti* glands can block invasion by *P. gallinaceum* sporozoites [30]. In addition, it has recently been demonstrated that in *Anopheles gambiae*, a vector that transmits human malaria, a monoclonal antibody (mAb) to a 100-kD salivary gland protein can block *P. yoelii* sporozoite invasion of the glands [31].

The abundance of CS protein on the oocyst sporozoite surface makes it a good candidate ligand for salivary gland binding. Recent in vitro studies found that CS protein binds to mosquito salivary glands and not to other organs exposed to the hemolymph such as midgut, ovaries and Malpighian tubules [32]. In addition, a peptide encompassing region I (fig. 1) inhibits CS protein binding to salivary glands. These findings suggest that CS protein may mediate sporozoite attachment to salivary glands, possibly through the interaction of region I with a molecule on the basal lamina of the glands.

After sporozoites bind to the glands, they must enter. Apicomplexan protists actively enter cells and it has been hypothesized that TRAP family proteins have a central role in this process. TRAP contains two cell adhesive domains in its extracellular portion plus a transmembrane domain and a cytoplasmic tail. The structural properties of TRAP suggest that it may bind to extracellular matrix/cell surface receptors and link these molecules to the internal motility machinery of the parasite.

Recent genetic transformation experiments have generated sporozoites without TRAP, and sporozoites with subtle mutations in TRAP, in order to test

this model. It was found that TRAP null sporozoites were not motile and invaded salivary glands poorly, if at all [4]. Following this, structure/function analyses of TRAP were performed by creating sporozoites with mutations in different regions of the protein. Sporozoites with truncations in the cytoplasmic tail of TRAP, while appearing to express TRAP normally, were not motile and did not invade salivary glands [33]. These investigators also replaced the TRAP cytoplasmic tail with the sequence from MIC2, a TRAP homolog in *Toxoplasma gondii*, another Apicomplexan parasite. Sporozoites with these hybrid TRAP molecules had normal gliding motility and a wild-type phenotype. These results suggest that the cytoplasmic tail of TRAP interacts, either directly or indirectly with the parasite's actin-myosin motor and that this motility machinery is conserved among Apicomplexan protists.

Sporozoites bearing mutations in the cell-adhesive sequences of TRAP, namely the A-domain and the TSR, were also generated and analyzed. Experiments by two different groups showed that sporozoites with mutations in the MIDAS motif of the A-domain were severely impaired in their ability to invade salivary glands [34, 35]. Interestingly, in both of these studies, oocyst sporozoites had the same gliding phenotype as wild-type oocyst sporozoites. Sporozoites with mutations in the TSR of TRAP were generated and their phenotype depended on how extensive the mutation was. If only the downstream basic residues of this motif were changed, sporozoite invasion of glands was inhibited by 20% and gliding motility was not affected [35]. However, if the basic residues and the CSVTCG portion of the motif were eliminated, salivary gland invasion and gliding motility were abolished [34].

Taken together, these studies demonstrate that TRAP is required for gliding motility and likely provides a critical ligand during salivary gland invasion. Although these data suggest that salivary gland invasion cannot occur without gliding motility, the A-domain mutants demonstrate that gliding motility is necessary but not sufficient for cell invasion. Presumably during invasion, the A-domain of TRAP associates with a cell surface receptor and this association is required for cell entry.

Development of Infectivity by Sporozoites

Sporozoites must invade cells twice in their lifetime: once in the mosquito and once in the vertebrate host. Although the sporozoites that are released from oocysts and successfully invade salivary glands are the same parasites that will later invade hepatocytes, the two populations of sporozoites are different. Early studies with the rodent malaria parasite *P. berghei* showed that sporozoites from mosquito salivary glands were 10,000 times as infective for the vertebrate host as oocyst sporozoites from the same mosquitoes [36]. More recently, using the avian malaria parasite *P. gallinaceum*, investigators demonstrated that salivary

gland sporozoites cannot reinvade the glands [37]. In addition to these differences in infectivity between the two populations of sporozoites, there are also more subtle differences within each group of sporozoites. It has been observed that only 1–5% of oocyst sporozoites are capable of gliding motility. Since gliding motility is likely required for salivary gland invasion [4], the inability of most oocyst sporozoites to glide is puzzling. One explanation is that sporozoites mature as they reside in oocysts and continue this maturation process after their release into the hemolymph. This is supported by the finding that sporozoites isolated from hemolymph exhibit a higher rate of gliding motility compared to oocyst sporozoites [36; Sinnis P, Kappe S, Matuschewski K, unpublished findings]. It is also possible that final maturation of midgut (i.e. oocyst and hemolymph) sporozoites into parasites capable of salivary gland invasion does not occur until the sporozoite contacts the salivary gland. Once in the salivary gland, sporozoites continue to mature and gain infectivity for the vertebrate host over the course of their first week in the glands [36].

Sporozoite Inoculation into the Skin of the Vertebrate Host

After sporozoites enter salivary glands, they migrate through the cells and enter the lumen of the gland where they reside until their injection into a vertebrate host during a bloodmeal [for review see, 25]. Blood feeding by the mosquito is a complex process that involves probing for a blood source followed by ingestion of blood. Release of saliva by mosquitoes is known to occur during probing and the majority of sporozoites enter the host during this time. Sporozoites are injected predominantly into the avascular components of the skin and, within 15 min of blood feeding, sporozoites begin to migrate out of the skin and into the blood stream [38]. How many sporozoites are injected during a bloodmeal? Although it is difficult to precisely mimic the feeding behavior of a mosquito in the laboratory, several different approaches to this question have all shown that a small proportion of the total sporozoite load is injected during a single bloodmeal, likely between 25 and 50 sporozoites [for review see, 25].

Sporozoite Invasion of Hepatocytes

The low number of sporozoites injected by an infected mosquito suggests that sporozoite infection of the vertebrate host is an efficient process. In order to establish malaria infection, these sporozoites must make their way to the liver and invade hepatocytes. Once sporozoites are in the circulation, this process occurs within minutes [for review see, 5]. The rapidity and efficiency of hepatocyte invasion by sporozoites suggests that sporozoites are arrested in the liver by a specific recognition event.

Previous studies have shown that CS protein binds to heparan sulfate proteoglycans (HSPGs) on the hepatocyte surface and in the space of Disse

[for review see, 5]. Could the binding between CS protein on the sporozoite surface and hepatic HSPGs be responsible for the rapid and specific arrest of sporozoites in the liver? When radiolabelled CS protein is injected intravenously into mice, 70–80% of the protein is found in the liver 2 min after injection [39]. The CS protein associated with the liver is in direct contact with hepatocyte microvilli and the space of Disse. In addition, the staining pattern observed is identical to that seen when CS protein is incubated with liver sections in vitro, suggesting that CS protein is cleared from the circulation by hepatic HSPGs. This was confirmed with the finding that physiologic ligands for hepatic HSPGs, namely lactoferrin and lipoprotein remnants, could delay CS protein clearance from the circulation [40].

The question remains whether sporozoites themselves are arrested in the liver by the same mechanism as recombinant CS protein. It is likely that the CS protein–HSPG interaction is important in the life of the sporozoite because inhibitors of this interaction (such as remnant lipoproteins and sulfated glycoconjugates) decrease sporozoite infectivity in vivo [40–42]. However, an inhibitory effect on sporozoite infectivity does not indicate whether the binding of CS protein to HSPGs is required for sporozoite attachment, invasion, or subsequent development in hepatocytes. The precise stage at which the CS protein–HSPG binding event functions has been investigated using in vitro assays. Initial studies found that the removal of the majority of cell surface HSPGs had a minimal inhibitory effect on sporozoite invasion of cells, suggesting that the CS protein–HSPG interaction does not function during sporozoite invasion [43]. A more recent study, however, found that heparin was a potent inhibitor of sporozoite attachment to cells when the assay was performed under conditions of shear flow [44]. In agreement with previous studies, these investigators found that heparin had little inhibitory activity on sporozoite attachment to cells under static conditions. These data suggest that the binding between hepatic HSPGs and the dense CS protein coat of the parasite may function in the dynamic flow conditions of the blood circulation and may be responsible for the arrest of sporozoites in the liver.

HSPGs are ubiquitous molecules found on the surface of most mammalian cells. How then do we account for the specificity of sporozoites for hepatic HSPGs? Previous studies showing that intravenously injected CS protein is cleared by hepatic HSPGs [39, 40], suggest that CS protein binds to either a unique glycosaminoglycan (GAG) chain structure or to a subset of GAGs found only in the liver. The GAGs of HSPGs are based on repeating disaccharide units of N-acetyl-glucosamine and glucuronic acid which can undergo an extensive series of modifications, giving rise to a large amount of structural heterogeneity. It is known that CS protein binds preferentially to more highly sulfated regions of HSPG GAGs [45] and that sporozoite attachment to cells decreases

as the degree of GAG chain sulfation decreases [44]. Previous work has shown that compared to heparan sulfate of other organs, liver heparan sulfate is more extensively modified and highly sulfated [46]. These findings suggest that the degree of GAG chain sulfation could account for the selective targeting of sporozoites to the liver.

One confounding issue is that the CS protein-binding HSPGs are found in the space of Disse and on hepatocyte microvilli that are behind the endothelial cell barrier. The endothelial cells of the liver sinusoids, however, have permanently open fenestrations that allow for direct contact between the blood circulation and hepatocytes. Although these fenestrations are too narrow to allow the free passage of sporozoites, HSPGs of the underlying space of Disse and on hepatocyte microvilli may protrude through these holes and thereby have access to circulating sporozoites.

After their arrest in the liver sinusoids, sporozoites must cross the continuous layer of sinusoidal cells to reach their destination, the hepatocyte. The parasite therefore goes through either Kupffer cells, the professional phagocytes found in the liver sinusoids, or endothelial cells. Although there are no data demonstrating any interaction between sporozoites and endothelial cells, morphological studies of livers from sporozoite-injected animals have documented sporozoites inside Kupffer cells at early time points after sporozoite injection [47]. More recent observations on the interaction between sporozoites and sinusoidal cells found that sporozoites attach to and enter Kupffer cells, but not sinusoidal endothelia [48]. The sporozoites are not phagocytosed but actively enter the cells and remain structurally intact, suggesting that they do not elicit a respiratory burst. These findings support the notion that sporozoites may use Kupffer cells as a gate for hepatocyte infection.

After the sporozoite reaches the hepatocyte, it must invade. It is likely that TRAP plays a critical role in this process, similar to its role in salivary gland invasion. Because TRAP mutant sporozoites do not enter salivary glands in significant numbers and only salivary gland sporozoites are infectious for vertebrates in any biologically meaningful way, experiments in which these midgut sporozoites are used for infectivity studies in vertebrate hosts are difficult to interpret. Nevertheless, sporozoites without TRAP or with mutations in TRAP that decrease their infectivity for salivary glands, have little infectivity in the vertebrate host [4, 33–35]. In addition, a recent study by Gantt et al. [49] shows that cell contact stimulates salivary gland sporozoites to secrete TRAP onto their surface, suggesting that TRAP plays an important role in hepatocyte invasion. The cell surface receptor(s) for TRAP has not been determined although it has been shown that the TSR of TRAP binds to hepatic HSPGs associated with the space of Disse [50, 51] and the A-domain of TRAP binds to heparin [52]. More work is required to demonstrate the role of these binding events during sporozoite invasion of cells.

The sporozoites' journey to the hepatocyte is not simple. They are injected into the skin, far from their target organ, and must go through several cell barriers before they reach their destination, a hepatocyte [38, 48, 53]. First they traverse an endothelial cell barrier, in order to enter the blood circulation, and then travel to the liver sinusoids where they cross the continuous layer of sinusoidal cells and enter hepatocytes. Video microscopy of interactions between sporozoites and cultured cells such as macrophages and HepG2 cells demonstrate that sporozoites can actively enter and exit cells [54]. Recent data show that sporozoites go through many cells before developing into an EEF [53]. They poke holes in the cells as they go through, resulting in the formation of a wound that the cell repairs. It appears that this ability to traverse cells is uniquely suited to the journey that the parasite must take after its injection into the vertebrate host. How a sporozoite 'knows' that it is in the correct place and 'decides' to stay and develop in a particular cell is not known and awaits further investigation.

Immunologic Responses to Sporozoites

Experimental and Naturally Acquired Immunity to Sporozoites

Immunization with an attenuated parasite, the irradiated sporozoite, has been shown to elicit protective immunity not only in animal models, but also in human volunteers [for review see, 7, 55, 56]. It was necessary to deliver this 'prototype' malaria vaccine either by exposure to the bites of irradiated malaria-infected mosquitoes or by intravenous inoculation, as irradiated sporozoites administered by other routes were not protective. Consistent with the low numbers of sporozoites injected by the mosquito, multiple exposures to the bites of large numbers (>1,000) of irradiated malaria-infected mosquitoes were required to elicit solid protection in most volunteers.

In addition to relatively high doses of sporozoites, the induction of long-term protective immunity also depended on administration of attenuated, but not killed, parasites. Sporozoites that were nonviable, due to heat or formalin treatment or exposure to high doses of irradiation, failed to invade hepatocytes and did not elicit a protective immune response. In the *P. berghei*/rat model, the persistence of the irradiated parasite within the hepatocyte for at least 1 week was required to establish long-term immunity [57]. Elimination of residual EEF parasites by drug treatment at day 7, but not day 30, resulted in only short-lived protection. However, prolonged persistence of the parasite is not required in all rodent malaria models as viable *P. berghei* sporozoites, which complete EEF development in 48 h, can also elicit protective immunity [58, 59].

Sporozoite-immunized volunteers and experimental rodent hosts developed sterile immunity, that is blood stages responsible for clinical disease were not

detectable after challenge with viable sporozoites. In the rodent malaria model, immune effector mechanisms could be shown to specifically target the pre-erythrocytic stages of the parasite, as measured by the absence or significant reduction of EEFs detected following sporozoite challenge. As a consequence of this stage specificity, sporozoite-immunized hosts remain fully susceptible to challenge with blood-stage parasites. Immunity is also species-specific, as volunteers immunized with irradiated *P. falciparum* sporozoites are protected against challenge with *P. falciparum*, but not *P. vivax*, infected mosquitoes. Of particular relevance to vaccine development, sporozoite immunization protected against all isolates and strains of *P. falciparum* tested, despite the polymorphisms that are known to exist in many sporozoite antigens, including CS protein and TRAP [60; for review see, 56].

Consistent with the studies in rodent hosts and human volunteers, individuals living in endemic areas, who are naturally exposed to the bites of *Plasmodium*-infected mosquitoes, also develop immune responses to sporozoites [8]. In Africa, the anti-sporozoite antibody titers gradually increase throughout childhood until by early adulthood the majority of individuals are seropositive. The delayed and relatively low magnitude of this naturally acquired immunity is in contrast to the anti-sporozoite titers detected in sporozoite immunized volunteers. This reflects, in part, the low antigen dose, as individuals in most endemic areas receive <1 infected bite/night. In contrast, sporozoite-immunized volunteers are exposed to the bites of hundreds of irradiated, infected mosquitoes over a relatively short period of time.

The suboptimal anti-sporozoite responses observed following natural infection may also reflect the immunosuppressive effects of high parasitemia experienced during childhood. Acute malaria infection can reduce anti-sporozoite antibody responses in the rodent malaria model [61] as well as inhibit the response to vaccines in children living in endemic areas [62]. One mechanism of immunosuppression is suggested by the recent finding that a blood-stage antigen, PfEMP-1 [see previous chapter], can bind to CD54 (I-CAM-1)/CD36 on immature dendritic cells and prevent their maturation to functional antigen-presenting cells [63]. The gradual acquisition of anti-blood-stage immunity and the concomitant reduction in parasitemia during childhood, may reduce this immunosuppression, allowing immune responses to other stages of the parasite to gradually increase. The role of anti-sporozoite responses in naturally acquired immunity, however, is difficult to determine in light of the parallel increases in blood-stage immunity and the inability to measure reductions in liver-stage parasites in the human host.

Immune Effector Mechanisms

In the sporozoite-immunized host, however, anti-sporozoite antibodies, as well as cellular immune mechanisms, clearly have been shown to function in

protection. By necessity, these studies have been carried out in the rodent malaria model, since the hepatic sites of sporozoite invasion and EEF development are not accessible in humans. Murine studies have been interpreted with the understanding that the rodent malaria models vary, depending on the parasite species and murine strain [64–67].

Despite these limitations, the rodent studies have significantly advanced our understanding of the multiple immune effector mechanisms that can function in resistance [for review see, 7, 56, 68]. Early studies, using mAb specific for CS repeats, provided the first demonstration that antibody alone could protect against sporozoite challenge. More recently, CD8+ T cells have been shown to play a critical role, as elimination of these cells abrogates sporozoite-induced immunity in many rodent strains. In addition, the passive transfer of CD4+ as well as CD8+ T-cell clones derived from sporozoite immunized mice can also confer immune resistance.

A common effector mechanism, mediated by both CD4+ and CD8+ T cells, is the ability to produce interferon-γ (IFNγ). This cytokine induces nitric oxide (NO) synthase (iNOS) to generate intracellular NO, a potent inhibitor of EEF development [for review see, 56]. Cells treated with iNOS inhibitors and sporozoite-immunized iNOS knockout mice were unable to eliminate the hepatic EEF. iNOS inhibitors also reduced hepatic CD8+ T-cell infiltration, suggesting a role for NO in trafficking of effector cells, as well as a direct antiparasitic effect on the EEF [69]. However, alternative immune effector mechanisms also exist, since IFNγ and iNOS are not required in all strains of mice, nor at all time points during the course of sporozoite immunization [68, 70]. The role of direct cytotoxicity in the destruction of parasitized hepatocytes remains to be shown, as *P. berghei* EEF can be effectively eliminated in sporozoite immunized Fas (CD95/Apo1) or perforin knock-out mice [71].

In addition to CD4+ and CD8+ T cells, various subpopulations of lymphoid cells found in the liver can also play a role in resistance to sporozoite challenge. Natural killer T (NKT) cells, which co-express the NK1.1 cell marker and a semi-invariant TCR, produce IFNγ that can inhibit EEF in vivo and in vitro [72, 73]. These NKT cells can be stimulated either by an acute blood-stage malaria infection or by ligation of their CD1 receptor with a specific ligand, such as the glycolipid, α-galactosylceramide. NK cells, a subpopulation of NK cells lacking TCR, also produce inhibitory IFNγ, as well as IL12 which is critical for development of CD8+ effector T cells in sporozoite-immunized mice [68, 74]. In sporozoite-immunized αβ TCR knockout mice, γδ T cells can produce IFNγ that also inhibits EEF [75, 76]. These cells, however, are not required for the development of adaptive immunity, as comparable levels of protection were induced in γδ knockout and wild-type mice following sporozoite immunization [77].

The ability to elicit protection in knockout mice that lack specific cellular subsets or various immune mediators suggests that sporozoite-induced immunity is not dependent on a single effector mechanism. The complexities of the immune response found in different murine strains is somewhat daunting and limits our ability to extrapolate these findings to identify protective effector mechanisms in sporozoite-immunized humans. Nevertheless, the clear demonstration that sporozoite-induced humoral and cellular immunity can protect in vivo provides the rationale for multiple approaches to vaccine development.

Subunit Vaccines Based on Sporozoite Antigens

It remains to be shown whether antibody or cells alone are sufficient to protect human hosts against sporozoite challenge. However, a combination of immune effector mechanisms, i.e. antibody to target the extracellular parasite and cellular immunity to target the intracellular EEF, would be expected to increase the efficacy of malaria subunit vaccines. Recent phase I/II trials have provided valuable information that can be used not only to optimize CS subunit vaccines but also to facilitate development of malaria vaccines containing other sporozoite, as well as blood stage, antigens.

Targeting the Extracellular Sporozoite

T and B Cell Epitopes of CS Proteins

The CS protein is an ideal target for antibody-mediated immunity as it is a major surface protein that has been shown to function in parasite host cell interactions. However, efforts to design vaccines that can elicit inhibitory antibodies against functional adhesions domains, such as region II-plus, have had limited success [78, 79]. The detection and/or induction of antibodies specific for these domains may be limited by the presence of conformational epitopes or by homology with TSR in host cell proteins [80, 81].

The CS repeats remain the major target for vaccines designed to elicit protective humoral immunity, as they contain the only known B cell epitope that is recognized by highly inhibitory monoclonal and polyclonal antibodies [for review see, 7, 8]. While the structural function of CS repeats is unknown, recent molecular studies have shown that *P. berghei* repeats can be exchanged with *P. falciparum* repeats without loss of *P. berghei* function in vivo or in vitro [82]. These unique chimeric sporozoites will make it possible to use the rodent host to evaluate the neutralizing capacity of vaccine-induced immunity to *P. falciparum* CS repeats.

The mechanisms by which anti-repeat antibodies protect the host remain to be precisely defined. MAb of all IgG subtypes can passively protect in vivo and

can inhibit sporozoite invasion of hepatoma cells in vitro. These studies indicate that Fc-mediated effector mechanisms and accessory cells are not required to neutralize sporozoite infectivity. Antibodies bound to CS repeats may function by sterically hindering receptor/ligand interactions mediated by other regions of the protein, such as region II-plus. Alternatively, anti-repeat antibodies may inhibit sporozoite motility required for host cell invasion [4, 83]. Apicomplexan parasites shed surface proteins as they glide along the substrate [84–86]. The binding of high concentrations of anti-repeat antibodies to CS on the sporozoite surface may block the translocation of molecules required for gliding, thus immobilizing the parasite and preventing host cell invasion [83].

Despite their repetitive structure which suggests a T-independent antigen [87], the CS repeats have been shown to be T-dependent antigens. Knockout mice that are T cell deficient, or that lack αβ TCR or class-II molecules, fail to develop anti-repeat antibodies following sporozoite immunization [75, 88]. In recent studies, cytokines from NKT cells, stimulated by interaction of surface CD1 molecules with GPI anchors of malaria proteins, were also hypothesized to play a critical role in the anti-repeat antibody response [89]. However, knockout mice lacking CD1 molecules develop anti-CS antibodies comparable to wild type, indicating that B-cell responses are dependent on MHC class II/CD4+ T cell interactions [88].

The inclusion of class-II-restricted Th cell epitopes that can function in eliciting high levels of anti-repeat antibodies is a critical component of vaccine design. While the immunodominant B-cell epitope is localized in the repeat region, multiple CD4+ T-cell epitopes have been identified throughout the CS protein, with the exception of the repeat region [for review see, 56]. T cells of individuals living in endemic areas preferentially recognize polymorphic sequences of the CS protein, suggesting immune pressure by the host. However, only a restricted subset of amino acid residues are found in polymorphic sequences indicating that structural or functional constraints limit the ability of the parasite to alter these regions. The effect of these polymorphisms on immunity to sporozoites remains to be clearly defined. CS polymorphisms did not abrogate the ability to bind to HLA class-II molecules and cells of immune individuals proliferated when challenged with multiple variants in vitro [90, 91]. In contrast, polymorphic cytotoxic T lymphocyte (CTL) epitopes functioned as altered peptide ligands and failed to stimulate CD8+ T cells of naturally infected individuals [92, 93].

The majority of HLA class-II-restricted epitopes in CS protein have been identified empirically using peripheral blood lymphocyte and/or CD4+ T cell clones derived from naturally infected or sporozoite-immunized volunteers. More recently, the extensive analysis of peptide/HLA interactions have led to the development of algorithms to facilitate identification of potential T epitopes

in proteins of *Plasmodium,* and other pathogens [94, 95]. However, binding to HLA in vitro does not necessarily predict T-cell function in vivo. Empirical studies are still required to determine whether these predicted epitopes can elicit Th cells that enhance production of high titer anti-repeat antibodies by B cells [for review see, 96].

Synthetic Peptide Vaccines Based on the CS Protein

Phase-I studies of CS synthetic peptide vaccines are summarized to illustrate the iterative process involved in the development of malaria subunit vaccines [for review see, 96]. Alternative vaccine platforms and approaches, including recombinant viral vectors, particulate proteins and DNA, as well as multicomponent vaccines containing combinations of pre-erythrocytic and erythrocytic stage antigens, are discussed in the chapters by Lal, Ballou and Hoffman [pp 253–261].

The first phase-I/II trials of a *P. falciparum* synthetic peptide malaria vaccine, carried out over a dozen years ago, assessed the immunogenicity of a peptide-protein conjugate containing the $(NANP)_3$ B-cell epitope conjugated to tetanus toxoid (TT) as carrier (fig. 2a). While the magnitude of the antibody response elicited by immunization with $(NANP)_3$-TT was not optimal, partial or total protection was obtained in a small number of volunteers challenged with viable *P. falciparum* sporozoites. This was the first demonstration in humans that antibodies elicited by a small tetramer repeat peptide could totally eliminate, or significantly reduce, an infectious sporozoite inoculum, consistent with previous studies using rodent malaria [97, 98].

Immunogenicity of this first-generation vaccine was limited by potential problems of toxicity and epitopic suppression due to the foreign protein TT carrier. Moreover, epitope density is a critical factor in eliciting protective antibody titers but $<20\%$ of the total mass of the vaccine was malaria epitopes [98]. Most important, since T-cell help was provided by the TT carrier, the peptide-protein conjugate did not induce a strong parasite-specific T-cell response.

Malaria-specific T cells are necessary, not only to elicit anamnestic responses in naturally infected individuals and maintain vaccine-induced immunity, but also to provide a source of inhibitory IFNγ, following exposure to sporozoites under natural conditions. In order to replace the foreign protein TT with parasite-derived T helper (Th) epitopes, carrier-free multiple antigen peptides (MAPs) were developed as a second-generation vaccine. Immunization with MAPs containing CS repeats elicited high levels of protective immunity in mice and non-human primates that directly correlated with high titers of anti-repeat antibodies [99–101].

A *P. falciparum* MAP was constructed by first testing numerous CS-derived Th epitopes for their ability to provide help for repeat specific

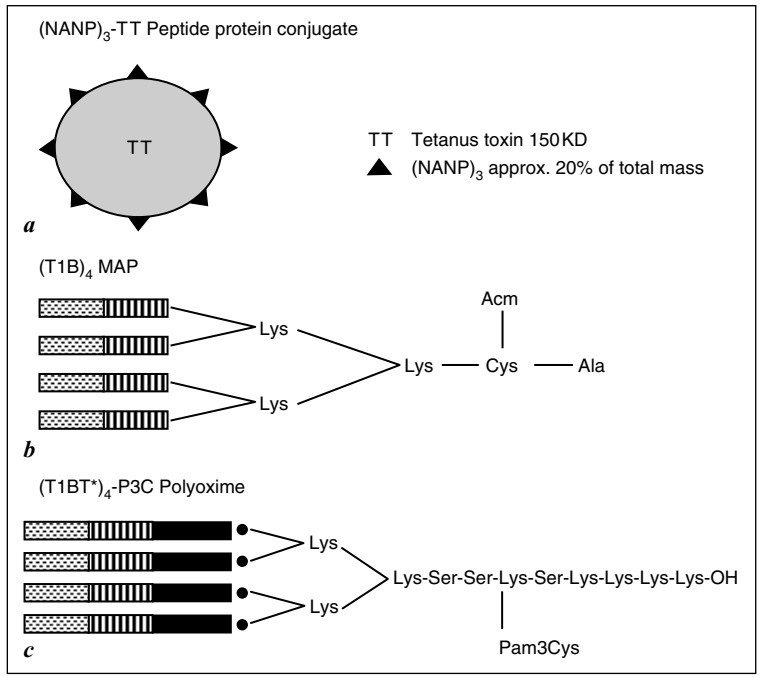

Fig. 2. Illustration of *P. falciparum* CS protein synthetic peptide vaccines that have undergone phase I trials. ***a*** $(NANP)_3$-TT peptide protein conjugate. ***b*** $(T1B)_4$ MAP. ***c*** $(T1BT^*)_4$-P3C polyoxime. ***b***, ***c*** The T1 Th cell epitope (DPNANPNVDPNANPNV) is shown as a stippled bar, the B-cell epitope $(NANP)_3$ as a striped bar, and the T* universal Th epitope (EYLNKIQNSLSTEWSPCSVT) as a solid bar.

B-cells (fig. 2b) [for review see, 96]. The optimal MAP, designated $(T1B)_4$, contained the B-cell epitope $(NANP)_3$ and a Th cell epitope, T1, from the CS repeat region (fig. 1). The T1 epitope was the first T-cell epitope defined by CD4+ T-cell clones derived from a *P. falciparum* sporozoite-immunized volunteer. Administration of $(T1B)_4$ MAP to *P. falciparum* sporozoite-primed mice and monkeys elicited strong anamnestic antibody responses, suggesting that the vaccine would enhance the suboptimal immune responses of individuals living in endemic areas. The T1 epitope is conserved in all *P. falciparum* isolates, and thus could elicit immune responses that would be functional in diverse geographical locations.

A phase-I trial of $(T1B)_4$ MAP was carried out in volunteers of known class-II genotypes [102]. High anti-repeat and anti-sporozoite antibody titers were obtained in the vaccinees who expressed class-II DR or DQ molecules known to bind the T1 epitope (fig. 3). Of particular importance, positive CS

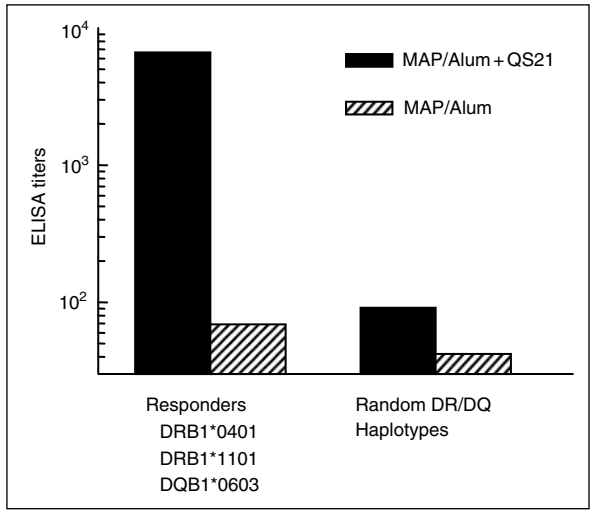

Fig. 3. Anti-repeat antibody titers elicited in volunteers of defined or random class-II genotypes following immunization with $(T1B)_4$ MAP/alum with, or without, the purified saponin QS-21 as co-adjuvant. Adapted from Nardin et al. [102].

precipitin (CSP) reactions were observed when viable *P. falciparum* sporozoites were incubated in sera of the MAP vaccinees, demonstrating the presence of high-affinity anti-peptide antibodies of biological relevance.

It was particularly remarkable that the MAP vaccine, composed of only five amino acids (N, V, A, D, P), could elicit titers comparable to those induced by exposure to hundreds of infected irradiated mosquitoes. Of relevance to vaccine development, these high antibody responses were dependent on adjuvant formulation, requiring the inclusion of a purified saponin, QS-21, as co-adjuvant. When individuals of high-responder genotypes were immunized with MAP/alum without QS-21, they failed to develop significant antibody titers (fig. 3, hatched bars). Therefore, in the absence of correct adjuvant formulation, even a potent antigen might be mistakenly judged to be non-immunogenic.

The $(T1B)_4$ MAP vaccine, however, elicited high titers of antibodies in volunteers of a limited number of class-II genotypes (DRB1*0401, DRB1*1101 and DQB1*0603), which are found in approximately 30% of the population. To overcome genetic restriction, peptide vaccines should contain 'universal' T-cell epitopes which can bind to most, if not all, class-II molecules, in order to ensure immune responses in individuals of diverse genetic backgrounds.

MAPs containing universal T-cell epitopes derived from TT, when combined with CS repeats, elicited protective anti-repeat antibody responses in the *P. yoelii* rodent model, as well as a *P. vivax*/Saimiri monkey model [100, 101].

In addition, non-natural universal T-cell epitopes, developed by altering amino acid residues to increase class-II binding affinity, elicited T help for anti-repeat antibody responses to human and rodent CS proteins [103, 104]. These peptide-induced antibodies were protective against *P. yoelii* sporozoite challenge.

Protection obtained using vaccines containing the non-malarial universal T-cell epitopes demonstrate that anti-repeat antibodies alone are sufficient to protect murine and primate hosts against sporozoite challenge. However, a universal malaria T-cell epitope is required to elicit parasite-specific T cells that could be stimulated by exposure to sporozoites to boost vaccine-induced immunity and provide inhibitory cytokines. CD4+ T-cell clones from sporozoite-immunized and protected volunteers were used to identify a universal T-cell epitope in the C terminus of the *P. falciparum* CS protein (fig. 1). This epitope, termed T*, was recognized in the context of DR 1, 4, 7 or 9 class-II molecules and bound to multiple DR and DQ molecules in vitro [90, 91]. Moreover, peptides containing the T* epitope elicited immune responses in all inbred murine strains tested.

A third-generation malaria peptide vaccine was designed to combine the T* universal epitope with the T1 and $(NANP)_3$ CS repeats (fig. 2c). This tri-epitope vaccine was constructed by ligating a purified 48mer epitopic module to a branched lysine core via oxime bonds [105]. The branched core was modified by the addition of a lipopeptide adjuvant, tripalmitoyl-S-glyceryl cysteine (Pam3Cys), to provide a totally synthetic vaccine which could be characterized by mass spectrometry, gel electrophoresis and Western blots. The $(T1BT^*)_4$-P3C polyoxime, without addition of exogenous adjuvant, was immunogenic in multiple strains of mice [105].

In a small phase-I trial, the polyoxime was found to elicit antibody responses in individuals of diverse class-II haplotypes [106]. The peptide-induced antibodies efficiently reacted with native CS protein on *P. falciparum* sporozoites, as shown by high IFA titers and positive CS precipitin reactions. CD4+ T cells specific for the universal T* epitope, but not the T1 or B repeat epitopes, were detected in volunteers with strong anti-sporozoite antibody responses. The correlation of T*-specific cellular responses and high antibody titers suggests that T* functioned as a universal Th epitope in humans, as predicted by peptide/HLA-binding assays and immunogenicity in mice of diverse H-2 haplotypes [90]. This correlation of class-II binding in vitro with immunogenicity in vivo supports the use of algorithms based on peptide/HLA binding to identify functional Th epitopes [94].

The induction of inhibitory antibodies specific for CS is not limited to the mammalian host, as new molecular approaches now provide the potential to use antibodies to target the parasite in the invertebrate host. Passive transfer studies have shown that salivary gland infections could be totally prevented in mosquitoes given two injections of a mAb specific for the CS protein of an avian

malaria parasite, *P. gallinaceum* [107]. Transgenic mosquitoes expressing fragments of anti-repeat mAb are being developed to specifically target the parasite CS protein ligand during salivary gland invasion in order to prevent transmission of infection [108, 109].

Targeting the Intracellular Parasite

In contrast to the CS protein, the development of immunogens to elicit high levels of neutralizing antibodies specific for TRAP on the sporozoite surface has been more difficult, as a protective immunodominant B-cell epitope has not been identified. mAb specific for TRAP repeats did not efficiently neutralize sporozoite infectivity in vivo or in vitro [17, 21, 49, 51]. Moreover, high concentrations of mAbs specific for predicted functional sequences of the TRAP A-domain (fig. 1), did not inhibit sporozoite infectivity or motility [49]. Mice immunized with TRAP A-domain peptides were not protected against *P. yoelii* sporozoite challenge.

The activity of anti-TRAP antibodies may be limited by the low levels and/or patchy distribution of TRAP on the sporozoite surface or by the inaccessibility of TRAP localized at the parasite/host cell interface [49]. TRAP expressed in the intracellular hepatic stage provides an alternative target for vaccine development. Peptides containing *P. yoelii* TRAP repeats can, in fact, elicit IFNγ-dependent CD4+ -mediated protective immunity in A/J mice [110]. Strains of mice that were not protected following peptide immunization developed predominantly a Th2-type cytokine response.

The ability of peptides to elicit protective CD4+ T cells that can effectively target EEF is not limited to TRAP [for review see, 8]. Peptides containing Th epitopes from *P. berghei* and *P. yoelii* CS proteins elicited Th1 and Th2-type CD4+ T cells that inhibited EEF development both in vitro and in vivo. Moreover, peptides containing an epitope of *P. yoelii* Hep17, a liver stage protein, have also been shown to elicit IFNγ-dependent protective CD4+ T-cell responses [111].

The potential efficacy of class-II-restricted immunity, that is a combination of parasite-specific antibody and CD4+ T cells, has also been shown in recent phase-I/II trials. Immunization with a hybrid CS/Hepatitis B surface protein, termed RTS,S, protected 6/7 volunteers against *P. falciparum* sporozoite challenge [112]. Clinical trials of the RTS,S vaccine are discussed in detail in the chapter by Ballou [pp 253–261]. High levels of antibodies and malaria-specific Th1-type CD4+ T cells, but not CD8+ CTL, were detected in volunteers immunized with RTS,S [113]. These findings suggest that class-II-restricted immunity may be protective in humans, as has been found in some rodent malaria models.

In addition to class-II-mediated immunity, murine class-I-restricted CD8+ T cells can also efficiently target the intracellular parasite and play a critical role

in sporozoite-induced protective immunity [for review see, 8]. Recombinant viruses expressing CS epitopes, delivered in a prime-boost protocol, provided the first subunit vaccine that could elicit high levels of protective CD8+ T cells in rodent malaria models. A recombinant vaccinia virus expressing *P. falciparum* CS protein elicited a 10- to 20-fold increase in the CD8+ T cells of mice primed with *P. falciparum* sporozoites, suggesting that this approach would be useful to booster the low level of CD8+ T cells in individuals in endemic areas [114]. Transgenic mice expressing TCR derived from a protective *P. yoelii* CTL clone have recently been used to define the immunological requirements for the induction of protective CD8+ T-cell-mediated immunity [115].

In addition to recombinant viral vaccines, malaria peptides can also be used to elicit class-I-restricted CD8+T-cell responses. MAPs containing the *P. yoelii* CS immunodominant CTL epitope elicited protective CD8+T cells that targeted EEF both in vivo and in vitro [116, 117]. Moreover, mice and monkeys immunized with a large CS peptide, containing 102 amino acids of the C terminus of *P. falciparum* CS protein, developed CD8+ T cells, as well as CD4+ T cells and antibodies [118]. In a recent phase-I trial, the 102mer peptide, adjuvanted with either alum or Montanide ISA-720, elicited CD8+ T cells in HLA-A*0201-positive vaccinees [119]. Vaccinees receiving the peptide-/Montanide ISA 720 formulation, but not alum-adjuvanted vaccine, also developed positive anti-sporozoite antibody titers.

Conclusions

Molecular and cell biologic studies of sporozoites and their interactions with both mosquito and vertebrate hosts have underscored the similarities between *Plasmodium* parasites and other Apicomplexan protists. These organisms actively invade cells and have unique apical organelles that contain proteins required for invasion. However, each species, and each stage of each species, live in different hosts and have different target cell specificities. These differences are reflected in the biology of each parasite.

It is our challenge to better understand the biology of the *Plasmodium* parasite and to apply this knowledge to the design of vaccines for combating malaria. While multifactorial immune responses are induced in the sporozoite-immunized host, the primary effector mechanisms defined to date are antibodies and IFNγ. This knowledge provides the scientific basis for the development of vaccines to target the extracellular and intracellular stages of the parasite in the mammalian host. Algorithms are now available to predict potential class-I- and II-restricted T-cell epitopes in the vast array of new sporozoite and EEF proteins that will be identified through sequencing of *Plasmodium* genomes. A basic

understanding of the biology and function of these new proteins, as well as previously defined proteins such as CS and TRAP, and of the role each plays in sporozoite-induced immunity, will facilitate the design of efficacious malaria vaccines.

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Immune Responses to Liver-Stage Parasites: Implications for Vaccine Development

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Introduction

Induction of sterile immunity in humans by immunization with irradiated sporozoites has focused attention on the sporozoite and liver stage of the malaria parasite. These experiments were first performed in the 1970s, and were repeated in the 1990s to better understand the immune mechanisms involved. Most focus was originally on antibody-mediated mechanisms against the major protein on the surface of sporozoites, the circumsporozoite protein (CSP). This was despite observations in the late 1970s that cellular mechanisms were critical mediators of protective immunity, thereby implying an intracellular target. However, the low efficacy of CSP vaccines, and the identification of protective cellular mechanisms against CSP, redirected attention to the intracellular liver-stage parasite. This review will highlight the immune mechanisms that target malaria-infected hepatocytes, the antigens involved, and the development of vaccine strategies to induce these protective mechanisms. Although the first in vitro culture of a mammalian liver-stage parasite was achieved 20 years ago [1], little is known regarding its structure and function, as well as expression of specific liver-stage antigens. It is probably true that the liver-stage parasite is better described immunologically than biologically. The imminent completion of the malaria genome sequence will be invaluable in identifying the full range of antigens expressed during liver-stage malaria infection.

Biology of Liver-Stage Malaria Parasites

Sporozoites are injected by the bite of a female *Anopheles* mosquito and localize in liver sinusoids. This is efficient, as only small numbers of sporozoites, perhaps 10–100 per bite are inoculated [2, 3]. This implies that localization is mediated by specific sporozoite–host molecular interactions [for review see, chapter by P. Sinnis and E. Nardin, pp 70–96], which include recognition of hepatocyte-specific sulfated glycosaminoglycans by the relatively conserved region II of sporozoite antigens such as CSP [4] and the thrombospondin-related adhesion protein (TRAP) [5]. However, the mechanisms that lead to sporozoite invasion of hepatocytes, and the molecular pathways that trigger transformation into liver stages are not understood. It is likely that a cascade of interactions between sporozoites and host molecules probably mediates infectivity [6].

Ultrastructurally, the liver stages of *Plasmodium falciparum* resemble more closely those of avian malaria than other mammalian or primate malarias [7]. The parasite develops within a parasitophorous vacuole (PV) bounded by a PV membrane (PVM) that is at least partially derived from the hepatocyte plasma membrane that invaginates during sporozoite invasion [8]. Unlike other intracellular pathogens such as *Leishmania* or *Mycobacterium*, the function or even the pH of the *Plasmodium* PV is unknown. As the parasite increases in size, the PVM extends finger-like projections throughout the hepatocyte cytoplasm, especially adjacent to the hepatocyte nucleus, and extending to, but not fusing with, the hepatocyte plasma membrane [8]. This is presumably to increase uptake of nutrients from the host hepatocyte. Parasite antigens are inserted into the parasite membrane, the PVM, and in the hepatocyte cytoplasm [9] but, apart from one report [10], are not found on the hepatocyte surface. Nuclear division is rapid, giving rise to up to 20,000 merozoites which bud off from the centromere during segmentation. After about 5–6 days in humans, *P. falciparum* parasites rupture releasing the merozoites into the liver sinusoid where they invade red blood cells. During *Plasmodium vivax* development, some liver-stage parasites appear arrested as small (5 μm) uninucleated hypnozoites from which relapses of vivax malaria may be triggered by as yet unknown mechanisms [11].

Following exposure to gamma irradiation, attenuated sporozoites (γ-spz) of both murine and human malarias including *P. falciparum* continue to invade hepatocytes, both in vitro and in vivo [12]. Like non-irradiated infectious sporozoites, γ-spz also transform and initiate development into liver-stage parasites and express liver-stage antigens [13], but nuclear division does not occur and most parasites eventually disintegrate [12]. However, at least in murine malaria, some irradiated parasites may persist for weeks or longer [14], as antigen depot for continuous antigen stimulation of reactive lymphocytes (see section, 'Mechanisms of Protective Immunity to Liver-Stage Parasites').

Table 1. Candidate vaccine antigens of the liver stage of malaria

Antigen	Molecular weight kD	Localization		
		sporozoite	liver stage	blood
CSP	42–67	Surface, microneme	PM, PVM	No
TRAP/SSP2	90–140	Microneme	PM	No?
LSA-1*	200	No	PV	No
LSA-2*	230	No	PVM	No
LSA-3*	200–205	Surface, interior	Periphery	No
SALSA*	70	Surface	PVM	?
STARP*	78	Surface	Periphery	Yes
PfEXP-1/PyHEP17*	17–23	No	PVM, hepatocyte cytoplasm	Yes
GLURP*	200	No	PV	Yes
CSP-2	42–54	Surface, microneme	PVM, ER	No
Pbl.1*	35	No	PVM	No
MSP-1	200	No	Merozoite surface	Yes
GRP78*	78	No	Nucleus, cytoplasm	Yes
Hsp70*	70	No	Nuclear membrane, ER	Yes

* Discussed in this chapter.

PM = parasite membrane; PVM = parasitophorous vacuole membrane; PV = parasitophorous vacuole; ER = endoplasmic reticulum.

Antigens Detected in Liver Stages

Several antigens expressed by either sporozoites or blood-stage parasites may also be expressed in liver stages, but all available evidence suggests that liver-stage antigen-1 (LSA-1) is the only malaria antigen that is specifically expressed in liver stages. Sporozoite antigens may be carried into the hepatocyte during invasion and some antigens may be expressed by sporozoites and liver-stage parasites. Blood-stage antigens, especially those required for red blood cell invasion, are expressed late in liver-stage development. The limited efficacy of CSP vaccines has therefore focused attention on the identification of antigens expressed in liver stages that might be combined with CSP in second-generation vaccines.

Sporozoite antigens are reviewed by P. Sinnis and E. Nardin [pp 70–96] and therefore only those antigens that are targets predominantly of cellular mechanisms targeting the infected hepatocyte are discussed here (table 1).

Liver-Stage Antigen-1

LSA-1 was first identified by screening a *P. falciparum* genomic library with serum from an individual on prolonged malaria chemoprophylaxis [15].

The full-length gene was cloned and sequenced in 1991, and encodes a protein predicted as 230 kD [16] and found by polyacrylamide gel electrophoresis to be 200 kD [17]. The protein contains a large central region of amino acid sequence repeats that is flanked by highly conserved non-repeat regions [16]. The central repeat region contains variable numbers (86.5 in the NF54 standard laboratory strain of *P. falciparum*) of a 17-amino acid (aa) repeat based on EQQSDLEQERLAKEKLQ. It does not contain a hydrophobic transmembrane sequence and appears to be secreted by the parasite into the PV. Expression of LSA-1 occurs soon after sporozoite invasion and increases as the liver stage develops, indicating active synthesis. LSA-1 is not expressed in sporozoites or blood stages. LSA-1 is localized in flocculent material within the PV and may also adhere to the merozoite surface suggesting a crucial role during liver schizogony, perhaps protecting the merozoite surface [18]. When the hepatocyte ruptures, merozoites are released in this flocculent mass containing LSA-1 into the liver sinusoid where they invade red blood cells [19].

Liver-Stage Antigen-2

Antibodies to the repeat region of LSA-1 reacted with *P. berghei* liver stages in vitro, and detected a 230-kD protein (LSA-2) by Western blot [20]. Unlike LSA-1, LSA-2 is localized to the PVM, and it remains unclear whether LSA-2 is the murine malaria analog of LSA-1. However, mice immunized with LSA-1 peptides were protected against challenge with *P. berghei* sporozoites [20; Hollingdale MR, unpublished data]. The relationship between LSA-1 and LSA-2 was supported by the finding that spleen cells from mice immunized with *Plasmodium berghei* sporozoites proliferated with an LSA-1 peptide (epitope T2, aa 1742–1760; see section, 'LSA-1'), and mouse antisera raised to *P. berghei* liver stages recognized another LSA-1 peptide (epitope T1; see section, 'LSA-1'), suggesting that LSA-2 is antigenically related to LSA-1 [21].

Liver-Stage Antigen-3

LSA-3 is a 200-kD protein that is expressed in sporozoites and liver stages, but is not found in blood stages [22, 23]. It contains a 5′region containing repeats of 8 aa arranged in 4 different motifs, and a 3′region containing a block of 4-aa repeats. LSA-3 is localized to internal organelles and the surface of *P. falciparum* sporozoites and is associated with LSA-1 in the liver-stage PV. Antibodies to LSA-3 recombinant proteins and peptides recognized *Plasmodium yoelii* but not *P. berghei* sporozoites and liver stages and detected a 205-kDa *P. yoelii* sporozoite protein [22].

P. falciparum *Sporozoite and Liver Stage Antigen*

Using the same strategy by which LSA-1 was cloned, screening a genomic library with human immune sera identified a novel 70-kD protein found on sporozoites and liver stages [24]. It is actively synthesized during liver-stage development and is associated with the flocculent material in the PV [18, 22]. Like LSA-1 [25], sporozoite and liver-stage antigen (SALSA) is ingested by infiltrating macrophages and neutrophils [25].

Sporozoite Threonine- and Asparagine-Rich Protein

Sporozoite threonine- and asparagine-rich protein (STARP) is a 78-kD protein found in discontinuous patches on the surface of *P. falciparum* sporozoites, the expression of which was confirmed by Northern blotting [22]. It is detected in liver stages by immunofluorescence assays but it is not known if it is expressed or is of sporozoite origin. It contains a repeat region of a variable number of tandem 45- and 10-aa repeats flanked by conserved 5′and 3′non-repeat regions. STARP was also detected in the primate malaria species *Plasmodium reichenowi*, with overall homology with *P. falciparum* at the DNA and protein levels of 94 and 88%, respectively. DNA hybridization indicated that a similar STARP gene may also occur in murine malarias.

PfEXP-1/PyHEP17

A monoclonal antibody (mAb) to *P. yoelii* liver stages recognized developing *P. yoelii* liver-stage parasites as well as rings and trophozoites of infected red blood cells. Immunoelectron microscopy detected this antigen (PyHEP17) on the surfaces of the PV and in the cytoplasm of the host cell of both stages [26]. By Western blot, the mAb detected a 17-kD antigen in blood-stage extracts [24]. Expression of PyHEP17 was similar to that of *P. falciparum* exported protein-1 (PfEXP-1). The sequence homology (37% at the aa level) with PfEXP-1 suggests that PyHEP17 is the murine analog [22, 27].

Glutamate-Rich Protein

The gene is located on chromosome 10 and encodes a 1271-aa protein localized to the PV of both liver-stage and red blood-stage parasites [28]. Antibodies to a recombinant glutamate-rich protein (GLURP) protein immunoblotted a 220-kD protein from blood stages. It contains two tandem repeats, R1 and R2, that are highly conserved in isolates from different geographical areas.

Other Antigens

CSP-2. Mice immunized with *P. falciparum* sporozoites were protected against challenge with *P. berghei* sporozoites [29]. A mAb from *P. falciparum*-immunized mice recognized a 42/54-kD antigen (CSP-2) found on both

P. falciparum and *P. berghei* sporozoites and liver stages, and passively protected mice against *P. berghei* challenge [30]. The gene has not been cloned.

Pbl.1. A mAb from mice immunized with *P. berghei* liver-stage parasites identified a 35-kD antigen localized to the PV and PVM [31].

Heat Shock Proteins. The 70-kD Pfhsp and the 78-kD glucose-regulated protein Pfgrp have been cloned from *P. falciparum*, and localized to the nucleus and cytoplasm and to the nuclear membrane and endoplasmic reticulum of liver stages, respectively [32].

Blood-Stage Antigens. Several antigens that are predominantly expressed by blood-stage parasites have also been detected in liver stages. These include merozoite surface protein (PfMSP-1), serine-rich protein, erythrocyte membrane protein 2 (PfEMP2), the acidic-base repeat antigen, and rhoptry antigen-1 (ROP-1) [33].

Mechanisms of Protective Immunity to Liver-Stage Parasites

Mouse studies have provided important insights into the nature of protective immunity directed against liver-stage malaria. Irradiation-attenuated sporozoites (γ-spz) induce stage-specific protective immunity in mice, monkeys and humans that is directed against sporozoite challenge but not against blood stages. Therefore, protective immunity must be directed against pre-erythrocytic parasites, sporozoites and/or liver stages. Sera from mice immunized with γ-spz do not passively protect naïve mice from sporozoite challenge, whereas adoptive transfer of immune spleen cells does confer protection. These studies strongly suggest that γ-spz-induced protective immunity is mediated by cellular mechanisms [see, chapter by P. Sinnis and E. Nardin, pp 70–96; and recent reviews e.g., 22, 34, 35]. However, as also discussed therein, mAbs to CSP repeat region neutralized sporozoite infectivity in vitro [36] and passively protected mice [37] and monkeys [22] against sporozoite challenge, indicating that anti-sporozoite antibodies could also contribute to protection.

In most murine malaria systems, the critical role of CD8+ T cells was demonstrated in studies in which depletion of CD8+ T cells abolished protective immunity induced by immunization with γ-spz, whereas depletion of CD4+ T cells had no effect [22, 34, 35]. Spleen cells from γ-spz-immunized mice killed liver-stage parasites in cultured hepatocytes in a manner that was major histocompatability class (MHC)-specific and species-specific, suggesting that protection is mediated by CD8+ cytotoxic T lymphocytes (CTL) [38]. However, γ-spz immunity in A/J [39] and BALB/c mice [40] is abolished after their treatment with antibodies to the pro-inflammatory cytokine interferon-γ (IFN-γ), and protection after adoptive transfer of a CD8+ T-cell clone against

P. yoelii CSP was abolished after treatment with anti-IFN-γ [41]. The role of IFN-γ was further supported in experiments in which its systemic administration protected mice [42] and monkeys [43] against murine and monkey malarias, or by administration of N^G-monomethyl-*L*-arginine to inhibit nitric oxide synthesis in vitro [44] or in vivo [45]. In addition, administration of interleukin (IL)-12 to mice [46] or monkeys [47] protected completely against sporozoite challenge. Support for the crucial role of IFN-γ-induced nitric oxide was provided by the finding of a mutation in the inducible nitric oxide synthase gene that was more frequent in children in Gabon with mild malaria than in those with severe malaria [48]. Finally, the roles of perforin and the receptor/ligand system CD95/CD95L, known to mediate elimination by CTL of target cells [49], were investigated in wild-type, perforin-deficient, CD95-mutant, CD95L-mutant and perforin-deficient/CD95L-mutant mice [50]. All mice immunized with *P. berghei* γ-spz were protected against a sporozoite challenge, including perforin-deficient/CD95L animals. These findings suggest that T cells do not kill malaria-infected hepatocytes via the perforin/Fas pathways. The concept that CD8+ T cells act independent of perforin/Fas is well established in other systems, such as in transplantation [51].

Taken together, these results suggest that a major protective mechanism induced by γ-spz is that IL-12 derived from macrophages, dendritic or Kupffer cells induces CD8+ T cells and natural killer (NK) cells to produce IFN-γ. IFN-γ then induces infected hepatocytes to produce nitric oxide that kills the intrahepatic parasite. We, like many others, are pursuing liver-stage vaccine development based on this hypothesis, and specifically that IFN-γ plays a central role in liver-stage protective immunity. IFN-γ has a wider protective role in malaria and may be an important mediator of protection against blood-stage parasites [52].

However, at least in murine models, immunity to γ-spz is complex and certainly multifactorial [35]. For example, IFN-γ receptor-deficient mice are also protected against sporozoite challenge [53]. Further, despite the importance of CD8+ T cells, CD4+ T cells are also involved in γ-spz-induced immunity, and the balance of importance between CD8+ and CD4+ T cells is often dependent on the MHC background of the mice and the species of *Plasmodium*. Thus, a CD4+ cytolytic T-cell clone adoptively conferred protection in mice [54], and CD4+ T-cell-restricted epitopes that correlate with protective immunity induced by IFN-γ have been identified in CSP [55]. In addition, γδ T cells also contribute to protective immunity, as a γδ-T-cell clone adoptively protected mice [56]. T-cell receptor (TCR) γδ-deficient mice challenged with sporozoites showed a significant increase in liver parasite burden compared to similarly challenged immunocompetent mice, and were also found to be more susceptible to sporozoite challenge [57]. These reports collectively support the hypothesis

that γδ T cells are a component of early immunity directed against malaria pre-erythrocytic parasites [57].

Much of the earlier work to dissect γ-spz-induced protective immunity focused on CSP, which is expressed only on sporozoites, although it is carried into the hepatocyte by sporozoites and may be detected during the early part, at least, of liver-stage development [9]. It is thus reasonable to suggest that sporozoite antigens such as CSP or TRAP may be presented to the immune system differently from de novo synthesized liver-stage antigens. Indeed, mice and humans immunized with γ-spz do not develop recall, proliferative T-cell responses following stimulation with CSP peptides, but do with LSA-1 [58]. This is quite different to naturally acquired immunity, when humans develop recall responses to both CSP [59] and LSA-1 [60]. However, natural transmission elicits a protective immunity in humans that is much shorter lived and which less frequently induces sterile immunity than do γ-spz. Nonetheless, studies in endemic areas have provided significant evidence for the role of liver stage antigens in protective immunity (see section, 'Evidence that Liver-Stage Immunity Is Protective in Humans').

The critical role for liver stage antigens in γ-spz-induced immunity was shown in experiments using β_2-microglobulin (β_2m) knockout (−/−) mice which are MHC class-I-deficient [61]. γ-spz failed to elicit protection in β_2m−/−mice, although immunization with γ-spz induced IL-2 and IFN-γ, T-cell proliferation to sporozoite lysates, and anti-CSP antibodies. Splenic cells from β_2m+/+-competent mice failed to confer protection to β_2m−/− recipients, although 80% protection was achieved in β_2m+/− mice. These results confirmed earlier work that suggested that CD8+ T cells induced by γ-spz immunization must recognize liver-expressed antigens presented by MHC class-I molecules to confer protection [62].

There is no pathology associated with malaria liver-stage development, which according to one hypothesis prevents generation of 'danger signals' that might initiate an immune response [63]. The environment of the liver is normally anti-inflammatory [64], and this persistent tolerant state might promote the development of liver-stage parasites by controlling an inflammatory or immune response [65]. Constitutive production of IL-10 within the liver may downregulate intrahepatic lymphocytes and macrophages. However, vaccination by γ-spz induces an infiltration of inflammatory cells into the mouse liver [66] and confers protection against subsequent sporozoite challenge. The significance of the reversal of tolerance in the liver has been the subject of a recent review by one of us [67]. It was proposed that the constitutive production of IL-10 by hepatocytes may be reversed by liver-stage parasites, so creating a local inflammatory state. IL-12, which itself induces liver-stage clearance, could facilitate CD8+ T-cell responses and expansion of CD4+ cells of the T-helper (Th) 1 phenotype. In

turn, the activated CD4+ T cells produce IL-2 and IFN-γ. Since IL-12 was only effective if administered before challenge [46, 47], this is consistent with the argument that an inflammatory state in the liver must be established prior to infection. Thus, the fate of malaria in the liver depends on the cytokine milieu, specifically the balance between IL-10, IL-12, IFN-γ and IL-2, which induce a local inflammatory condition [67].

It is only recently that studies of liver-resident lymphocytes during liver-stage development have been undertaken [67–71]. Immunization with γ-spz induced hepatic lymphocytes to undergo blast transformation, and the numbers of CD3+ CD8+ T cells increased, and the numbers of these cells decreased in livers of mice challenged with *P. berghei* sporozoites [70]. Characterization of intrahepatic CD4+ T cells revealed an immediate response to γ-spz, while the response of CD8+ T cells was delayed until acquisition of protection [67, 70]. Liver-resident T cells from *P. berghei* γ-spz-immunized mice adoptively transferred protection to naïve recipients [67]. These studies are in agreement with the widely held view that CD8+ T cells are important mediators of protection.

According to the responder trap hypothesis [67, 71], activated CD8+ T cells that migrate to the liver will undergo apoptosis [72, 73]. However, γ-spz-induced CD8+ CD45RB^{lo} CD44^{hi} T cells persist in the liver, but not in the spleen, during prolonged protection [71]. The association between CD8+ CD45RB^{lo} CD44^{hi} T cells and protection has been verified using MHC class-I and CD1 knockout mice [61]. γ-spz invade hepatocytes [12] and express LSAs [13], but do not progress to infect red blood cells and become a source of persistent intrahepatic antigen [74]. Elimination of irradiated parasites from the liver by treatment with the antimalarial drug primaquine abolished protection in mice [74]. Primaquine treatment also caused the loss of memory phenotype CD8+ T cells [70] that home to the liver where they persist as CD44^{hi} T cells during prolonged protection [71]. Clearly, CD8+ T cells are likely only to mediate protection if they localize within the liver [75]. Adoptive transfer experiments in mice demonstrated that protective T-cell clones appreciably express CD44, a homing marker, whereas clones showing little or no expression of CD44 were not protective [76]. Taken together, these studies support the hypothesis that continuous stimulation of CD8+ CD45RB^{lo} CD44^{hi} T cells by persistent liver-stage antigens reverses apoptosis in the liver, and thus these cells are able to confer protection against sporozoite challenge.

In addition to intrathymic T cells within the liver, there are also extrathymic T cells that are CD4− CD8−, express TCRαβ at the intermediate level (TCRαβ^{int}) and express the NK1.1+ surface marker; these are known as NK-T cells. NK-T cells represent about 40% of the total mature T-cell population in the liver [66] and are rarely found elsewhere except in bone marrow. The NK-T

cell receptor repertoire is biased towards Vα14 with Vβ8, Vβ7 and Vβ2 [77, 78]. These are cytotoxic cells within the liver and are induced by IL-12 [79], which has a protective regulatory role in protection induced by γ-spz [46]. The complexity of protective immune mechanisms induced by γ-spz was shown in mice in which the requirement for CD8+ T cells was absolute, and which trigger a novel mechanism of adaptive immunity that is dependent on IFN-γ and IL-12, and requires NK-T cells but not CD4+ T cells [80]. NK-T cells also produce IL-4 and could induce Th2 responses [67]. They are known to regulate immune responses to intracellular pathogens and tumors [81, 82] and probably tolerance [78]. Levels of NK-T cells declined during induction of immunity by γ-spz and reappeared after sporozoite challenge [67]. It has been suggested that disappearance of these cells might promote development of Th1 immune responses [66]. Thus, downregulation of NK-T cells may be required to induce a Th1 response in the liver that is essential for protective immunity [67]. This is similar to *Listeria* infection where CD8+ T cells are also required for protection and IL-4-producing CD4+ NK-TCR$\alpha\beta^{int}$ cells are also downregulated early in infection [83]. In malaria, γ-spz-induced protection is accompanied by production of IL-4 by memory CD45RO + CD4 + T cells [84]. In contrast, according to a recently published study, experimental infection with *P. yoelii* sporozoites caused an increase of liver CD4− CD8− NK T + TCR^{int} cells that preferentially expressed Vβ2 and Vβ7 TCR [85]. In vitro analysis revealed that these cells are able to inhibit parasite growth within hepatocytes [85]. However, their role in vivo has not been firmly established, and it is possible that their late appearance and persistence for 30 days may be related to other functions as well, including maintenance of effector CD8+ T cells [70].

Recent investigations of the allele frequencies for four variable region genes of the TCR β chain showed that there was a loss of haplotype diversity in Africans (Zambia) compared to Caucasians [86], which was largely due to the unexpected high frequency of haplotype d (8S3*1–2S1*2–15S1*1–3S1*1). Thus, 29% of this population were homozygous for this common haplotype. This was unexpected, as Africans show an increased diversity of human leukocyte antigen (HLA) haplotypes compared to Caucasians (see section, 'LSA-1'). As a consequence, increased homozygosity reduces the numbers of TCR variants able to recognize HLA-presented malaria peptides, and may be a result of selection by malaria infection [86]. Whether this is related to a role for NK-T cells remains to be determined.

From these studies, we believe that CD8+ T cells releasing IFN-γ are critical components of immunity induced by γ-spz in murine models of malaria, and that successful vaccination of humans with vaccines designed to elicit protective liver-stage immunity will require induction of specific CD8+ T cells that home to the liver.

Evidence that Liver Stage Immunity Is Protective in Humans

LSA-1

Studies in malaria-endemic areas have identified a series of B- and T-cell epitopes in LSA-1 that are associated with protective immune responses. These are found in the N-terminal and C-terminal non-repeat regions, and in the repeat region. This distribution of immunologically significant epitopes provides the rationale for development of LSA-1 vaccines using recombinant proteins, peptides and DNA.

In human volunteers immunized with γ-spz, proliferative T-cell responses to three LSA-1 epitopes (T1 aa 84–107, T3 aa 1813–1835, and T5 aa 1888–1909) were between 3- and 5-fold higher in protected volunteers compared to a non-protected volunteer [58]. These studies provided the first important link between anti-LSA-1 responses and sterile immunity in humans. Naturally acquired responses to LSA-1 were first implicated in human protection in a population in Gambia [87]. Children with mild malaria expressed the HLA B53 allele more frequently than did those with severe malaria, and adult Gambians could mount B53-restricted CTL responses to a peptide from LSA-1 (ls6 aa 11786–1794) but not to peptides from three sporozoite antigens [88]. They suggested that HLA B53-restricted CTL to LSA-1 conferred protection to severe malaria in this population. Further studies have identified a series of HLA class-I-restricted epitopes in LSA-1 and other sporozoite and liver stage antigens (see section, 'HLA and Genetic Resistance to Liver-Stage Antigens').

Protection in naturally exposed populations has been directly linked with anti-LSA-1 immune responses in eight independent studies [89–96]. Five of these studies have linked naturally occurring protection with IFN-γ responses to LSA-1 [89–93]. In Papua New Guinea, IFN-γ production (principally by CD8+ T cells) in response to the T1 epitope of LSA-1 was correlated with the absence of parasitemia over 6 months in adults [89]. In this study, anti-LSA-1 T-cell proliferative responses and immunoglobulin (Ig) G antibody levels were not associated with resistance. In addition, no correlation was found in any assay with T3 or T5 peptides [89]. This was the first direct association between anti-liver-stage responses and resistance to *P. falciparum* in naturally exposed humans and identified LSA-1 as a lead malaria vaccine candidate. The T1 peptide variant most commonly found in the study area binds to HLA*A1101, which has a frequency of 67.5% in this population [90], suggesting HLA class-I allele selection by malaria [87]. Two additional LSA-1 T epitopes have been recently identified in the C-terminal region, T2 (aa 1742–1760) and T4 (aa 1836–1849), and cytokine responses to T1, T2, T3, T4 and T5 epitopes were higher during transmission than in the dry season in highland Kenya [91].

In a separate study group in Gabon, an association was identified between protection and production of IFN-γ in children in an area of perennial transmission [92]. Children who were admitted with mild malaria and who mounted IFN-γ responses to either the LSA-J epitope (aa 1613–1636) or the B53-restricted ls6 epitope of LSA-1 subsequently had a prolonged time to reinfection, a reduced risk of developing malaria-related anemia and a lower annual frequency of parasitemia than did children without these responses [92, 93]. Longitudinal studies in geographically distinct regions of sub-Saharan Africa [91–93] have, therefore, identified epitopes in the non-repeat N-terminal region (T1) and C-terminal region (J and ls6) that are associated with protective T-cell responses mediated via IFN-γ expression.

Three studies in endemic areas have suggested that anti-LSA-1 immune responses other than IFN-γ production may also contribute to naturally occurring protection [94–96]. In a treatment-reinfection study of young males in a malaria-holoendemic area of Kenya over two transmission seasons, IL-10 responses to LSA-1 recombinant N-terminal or C-terminal regions (containing the T1 and J/ls6 epitopes, respectively) expressed in *Escherichia coli* predicted delayed time to reinfection, reduced frequency of parasitemia, and reduced parasite densities [94]. Resistance to malaria was not predicted by IFN-γ or TNF-α responses in either season. An earlier study in Gabonese children reported that IL-10 responses to LSA-J and ls6, and antibodies against a 41-mer LSA-1 repeat peptide, correlated with decreased time to parasite clearance after treatment [95]. A study in another holoendemic area of Gabon reported that children with detectable anti-LSA-J antibodies had significantly longer times to reappearance of parasitemia compared to those without such antibodies [96]. Kurtis and Duffy (unpublished data) have found that levels of IgG2, but not of other IgG isotypes, recognizing N-terminal LSA-1 (a region that contains the T1 epitope, but not the LSA-J epitope), also predicted resistance to reinfection in their study cohort of young Kenyan men.

There is evidence from mouse models that antibodies which recognize liver stages may directly inhibit the development of liver stage malaria. Sera from mice immunized with *P. berghei* γ-spz strongly inhibited liver-stage development in cultured hepatoma cells in vitro [10]. A mAb against PyHEP17 eliminated *P. yoelii*-infected hepatocytes in vitro, and reduced the numbers of *P. yoelii* liver stage parasites in vivo [26]. However, the mechanism by which antibodies could directly affect liver stages has not been elucidated.

The studies in the *P. falciparum*-endemic areas described above, when taken together, strongly suggest that natural transmission induces a protective immune response to LSA-1 that can involve CD8+ T cells, IFN-γ, IL-10 and antibodies; hence, no clear pattern of association of protection with type-1 or type-2 immune responsiveness has emerged. The reasons for these differences at different sites

may be related to the peptides or recombinant proteins used in the assays, and studies in Ghana suggest that T1, T3 and T5 may each stimulate type-1 (IFN-γ) or type-2 (IL-4) responses, but generally not both [Hollingdale MR, Theander TG, unpublished data]. Co-stimulation with naturally occurring variant LSA-1 epitopes could induce a rapid change from IFN-γ production to that of the immunoregulatory IL-10, as reported for CSP [97]. Alternatively, IL-10 could act together with the other responses by: (a) augmenting protective humoral responses by inducing B cells to secrete antibodies [98]; (b) augmenting cell-mediated responses by attracting CD8+ T cells [99] to the site of infected hepatocytes, and/or (c) enhancing antibody-dependent cellular inhibition activity against *P. falciparum* [94].

LSA-3 and PfEXP-1

CTL responses to peptides from LSA-3 identified a new epitope restricted by HLA-B53 in studies in Gambia and Tanzania [100]. Several CTL epitopes restricted by other HLA class-I haplotypes were also identified within LSA-3. CTL were also identified specific to PfEXP-1 [100], the homologue of which is PyHEP17 in rodent malaria. These findings emphasized the diversity of *P. falciparum* antigens recognized by CD8+ T cells in humans, and supported the development of a multi-CTL epitope malaria vaccine.

SALSA

CD8+ T-cell epitopes have been identified in SALSA using lymphocytes from γ-spz-immunized chimpanzees, as well as CD4+ T-cell and B-cell epitopes defined in studies in a low transmission area in Senegal [24].

SALSA and STARP

Studies with individuals exposed to malaria and with mice and chimpanzees immunized using a recombinant protein and two synthetic peptides identified major B- and T-cell epitopes in the SALSA non-repetitive sequence [24]. Major B-cell epitopes were recognized by sera from three African areas differing in their level of endemicity; CD4-restricted proliferative epitopes, and epitopes that induced IFN-γ secretion were identified using cells from individuals in a low transmission area. CD8+ CTL epitopes were demonstrated in immunized and sporozoite-challenged chimpanzees, in which CD8+ T cells were responsible for protection. Affinity-purified anti-STARP human antibodies from people in endemic areas or from individuals immunized with γ-spz inhibited *P. falciparum* sporozoite invasion of cultured hepatic cells in vitro [101].

GLURP

A study in Senegal showed a strong correlation between protection against malaria attacks and levels of IgG2 and IgG3 against GLURP (aa 94–489; R0)

and of IgG3 against GLURP (aa 705–1178; R2) [102]. However, GLURP is expressed in both liver and red blood cell stages, and while purified anti-GLURP antibodies inhibited in vitro growth of red blood-stage parasites [103], it is not clear whether these antibodies mediate protection against liver-stage parasites.

Heat Shock Protein Pfhsp60

A γδ-T cell clone passively protected mice to sporozoite challenge [56] and recognized *Mycobacterium bovis* hsp65, which has significant sequence homology with *P. falciparum* hsp60 [104].

HLA and Genetic Resistance to Liver-Stage Antigens

There is significant evidence that the frequency with which various HLA class-I alleles are found in people resident in malaria-endemic areas has been selected by malaria infection [87, 88, 100]. The first investigation of this at a molecular and immunological level was performed in Gambia [87]. The frequency of HLA B53 among children who presented with severe malaria was significantly lower than the frequency in children with mild symptoms [87]. This was correlated with the detection of HLA-B53-restricted CTL to LSA-1 in children with mild malaria [88]. Of considerable interest was the finding that HLA-B53-restricted CTL to other pre-erythrocytic antigens, including CSP, could not be detected [88]. This 'reverse-immunogenetics' approach [105] has been applied successfully to other agents of disease, including the human immunodeficiency virus (HIV) [106]. More recently, a series of studies has identified HLA class-I-restricted epitopes in the sporozoite antigens CSP and TRAP, as well as in the liver-stage antigens LSA-1, LSA-3, PfEXP-1 [89–91, 100, 107, 108], in addition to class-II-restricted epitopes [55, 109].

It is possible to account for the predominance of all known HLA class-I alleles with only nine main functional binding specificities [110]. This gives further impetus to the concept that malaria vaccines containing HLA-restricted epitopes could induce protection in genetically diverse populations. CD8+ T cell IFN-γ responses to the T1 epitope (aa 84–107) of LSA-1 have been correlated with resistance to malaria in individuals in Papua New Guinea [89]. A sub-region within T1 (aa 94–102) has been shown to bind to members of the HLA A3 supertype [111]. Nucleotide changes that predicted three aa substitutions at the N-terminal residue 85 (encoding threonine, serine or proline) have been identified [90]. Binding of the three variant peptides spanning aa 84–92 to HLA-A*1101, which has a frequency of 67.5% in this population, was evaluated. A peptide with serine but not threonine or proline at the primary anchor position stabilized

HLA-A11 dimers in vitro. Since HLA-A11 and other members of the HLA-A3-like supertype have a preference for hydroxyl-containing aas at position 2 [49], the results with serine peptide were anticipated [90]. However, it was not clear why a similar peptide with threonine, another hydroxylaa, at position 2, did not bind. It was suggested that more sensitive assays of peptide binding, such as competition assays, may have shown significant binding [90]. Therefore, T1 contains two HLA-A3 supertype regions. HLA heterozygosity may ameliorate the course of infectious diseases in which T-cell responses contribute to resistance, presumably through a broadening of the repertoire of antigenic peptides that may be presented to T cells [112–114]. The finding that the frequency of IFN-γ responses to all three LSA-1 84–92 variant peptides was greater than expected for persons who were heterozygous at both the HLA-A and B loci is consistent with this concept.

Experimental Immunization with Liver-Stage Antigens

There is a considerable body of results from studies in mice and primates which demonstrates that immunization with liver-stage antigens both elicit CD8+ T-cell responses and IFN-γ, and confer protection. Taken together, these lay the foundation for malaria vaccine development using multiple antigens or epitopes, and/or prime-boost strategies. In many respects malaria vaccine development is best understood within the wider context of vaccine development against other infectious disease agents, particularly HIV, as many advances made in HIV vaccines have been applied with considerable success to malaria vaccines.

Recent work has demonstrated that in malaria, as in many other vaccine systems, adjuvants, delivery systems and prime-boost strategies are critical for vaccine efficacy.

Adjuvants and Delivery Systems

The importance of vaccine delivery systems and adjuvants is discussed by L. Xiao and A.A. Lal [pp 343–365], but their specific relevance to liver-stage vaccine development is detailed here. While alum is the only licensed adjuvant, adjuvant research in general is particularly exciting and relevant to malaria vaccine research. The significance of adjuvants was best demonstrated with the CSP-based RTS,S vaccine. Only a specific adjuvant, the proprietary oil-in-water SBAS2, elicited significant levels of protection in human clinical trials in the USA and Gambia [115, 116]. Besides SBAS2, several other adjuvants have been critical for the potentiation of protective anti-liver-stage immunity.

CpG

Bacterial DNA has been shown to contain immunostimulatory motifs that trigger an innate immune response characterized by the production of predominantly Th1-type cytokines, particularly IFN-γ. These motifs consist of an unmethylated CpG dinucleotide flanked by two 5′ purines and two 3′ pyrimidines [117]. Unmethylated CpG dinucleotides are more frequent in the genomes of bacteria and viruses than of vertebrates [117]. Bacterial DNA and synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotides induced murine B cells to proliferate and secrete immunoglobulin in vitro, and augmented antigen-specific serum antibody levels by up to 10-fold and IFN-γ production by up to 6-fold in vivo [117]. These effects were optimized by physically linking the CpG-containing motifs to the immunogen in vitro and in vivo. It has been suggested that these data indicate a possible evolutionary link between immune defense based on the recognition of microbial DNA and the phenomenon of 'CpG suppression' in vertebrates. Use of CpG adjuvants is being investigated for a large number of applications including immunotherapy for autoimmune diseases [118], cancer [119], and allergy [120], and vaccination against infectious diseases such as HIV [121] and parasitic diseases such as leishmaniasis [122].

CpG motifs present in these DNA bacterial plasmid vaccines contribute to the immunogenicity of DNA vaccines [123]. DNA plasmids induced production of the same cytokines stimulated by bacterial DNA both in vitro and in vivo, an effect eliminated by DNase treatment or by methylating the CpG motifs, and which was significantly increased by co-administering exogenous CpG-containing DNA [124]. These findings were interpreted as supporting the role that CpG motifs in DNA vaccines play in the induction of antigen-specific immunity [125]. In malaria, the immunogenicity of a CSP vaccine in monkeys was significantly improved using CpG [126].

Granulocyte-Macrophage Colony-Stimulating Factor

Granulocyte-macrophage colony-stimulating factor (GMCSF) is a growth factor for myeloid progenitors of monocytes and dendritic cells (DC), which upon maturation become antigen-presenting cells (APC). Injection of DNA encoding GMCSF recruits DC for immune adjuvant effects [127]. It has been widely investigated as an immunostimulatory adjuvant in infectious diseases such as tuberculosis (TB) [128], viral infections [129], and prevention of tolerance [130] which might be particularly important for overcoming tolerance in the liver. GMCSF is produced transiently during blood-stage infection in malaria, at least in mice [131]. Recently, GMCSF has been shown to increase protection by a *P. yoelii* DNA CSP vaccine in mice [132]. Moreover, a plasmid encoding murine GMCSF increased protection conferred by a malaria DNA vaccine [133].

Lipopeptides

Lipopeptide analogues of the N-terminus of bacterial lipoprotein consisting of N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-(R)-cysteine (Pam3Cys) attached to peptides were shown to be potent activators for BALB/c murine splenocytes as determined by proliferation assays [134]. In vivo, the lipopeptides were able to fully replace Freund's complete adjuvant [135]. These results demonstrated that defined lipopeptides are immunoadjuvants either combined with or covalently linked to antigens or haptens. Priming with the synthetic lipopeptide vaccine (tripalmitoyl-S-glycerylcysteinyl-seryl-serine [P3CSS] coupled to peptide) matched the priming efficiency seen with infectious influenza virus for the induction of antigen-specific CTL [136]. Lipopeptides based on four liver-stage antigens, LSA-1, LSA-3, SALSA and STARP, have successfully induced humoral and cellular responses in immunization studies in chimpanzees (see section, 'Immunization with Individual Liver-Stage Antigens').

Immunization with Individual Liver-Stage Antigens

LSA-1

Based on mouse and human studies, LSA-1 as a single component vaccine should elicit memory CD8+ T cells that home to the liver and secrete IFN-γ in order to confer protection. Other mediators targeting LSA-1, like IL-10 and antibodies, could enhance vaccine efficacy. LSA-1 is thus likely to be one element of a multi-component vaccine and LSA-1-specific responses should be additive or synergistic with those elicited by other antigens, such as those in the RTS,S vaccine. An attenuated vaccinia virus expressing seven malaria genes (PfNYVAC-7), including the N- and C-terminal non-repeat regions of LSA-1, has already been in human clinical trials in the USA, and successfully induced CTL and proliferative responses to LSA-1 [137]. Aidoo et al. [107] have identified a series of epitopes on LSA-1 and other pre-erythrocytic antigens restricted by different HLA class-I alleles [107], and a vaccine construct containing some of these epitopes is undergoing phase-I clinical safety and immunogenicity trials at the University of Oxford, UK (see section, 'DNA Prime Vaccinia Virus Boost').

MS2 bacteriophage expressing multiple copies of the T1 epitope (which contains overlapping HLA A3/11 and MHC $H2^d$ epitopes) induced specific IFN-γ responses in $H2^d$ mice [138]. Another particulate delivery system, expression of LSA-1 as a fusion protein with hepatitis B core antigen, is being developed [Taylor-Robinson AW, Rowlands D, Hollingdale MR, unpublished data]. Studies of malaria-exposed individuals consistently relate anti-LSA-1 responses with protection, and upcoming clinical trials of the various LSA-1 constructs should identify the optimal approach to adopt in order to exploit this antigen for vaccination against malaria.

LSA-3/SALSA/STARP

Structural criteria were used to select a series of 12 sequences from LSA-3, SALSA, STARP, as well as LSA-1, that were predicted to bind HLA class-II molecules. Each peptide was either used alone, with Montanide ISA-51 adjuvant, or coupled with palmitic acid [139], to immunize mice, *Aotus* monkeys and chimpanzees [140–142]. These peptides elicited strong antibody, CD4+ T-cell proliferative responses, and secretion of IFN-γ. Although the cellular source of the IFN-γ was not defined, similar viral lipopeptides have been shown to elicit CD8+ CTL [143]. It is noteworthy that these responses were consistent in all animals, particularly chimpanzees, and were thus not genetically restricted. It was reported [23, 142] that peptides from LSA-3 protected chimpanzees against *P. falciparum* sporozoite challenge. These studies support the strategy that epitopes selected from these four antigens could form the basis of a malaria vaccine.

As a result of the persuasive immunogenicity and protection data for LSA-3 lipopeptides and peptides with Montanide adjuvant, long synthetic peptide vaccines containing these protective epitopes are currently being manufactured to GMP standards for European human clinical phase-I trials projected to start in early 2001.

GLURP

A nearly full-length recombinant GLURP produced in *E. coli* adsorbed to aluminum hydroxide, $Al(OH)_3$, elicited antibodies in rabbits primarily to the C-terminal repeat R2 region that recognized authentic GLURP from *P. falciparum* grown in vitro [144]. The human immune response is primarily directed to R2, and this region of GLURP has been developed as a synthetic long peptide vaccine for testing in phase-I clinical trials in Europe that started in late 2000. While being developed primarily as a blood-stage vaccine, it is possible that protective immune responses to GLURP expressed in liver-stage parasites might also be induced in these trials.

Ty and VLP Articulate Delivery Systems: The Importance of Prime-Boost Strategies

Induction of protective cellular mechanisms against liver-stage parasites has been achieved using many different platforms, including peptides, recombinant proteins, live recombinant bacteria, viruses and DNA. However, greatly increased efficacy has been achieved using a prime-boost strategy, whereby one platform is used to prime the immune system, and a second is used to induce an enhanced boosting response. Often when used in reversed order, protection is not induced. Three basic prime-boost strategies have emerged to induce liver-stage protective immunity.

Recombinant Viruses

Whereas efficacy in mice of single recombinant viruses expressing CSP was often low or variable, priming with one virus and boosting with another significantly improved efficacy [145].

DNA Prime, Vaccinia Virus Boost

Priming with DNA followed by boosting with a recombinant vaccinia virus has induced significant protection in mice that is greater than that achieved with either DNA or virus alone. The reverse combination, vaccinia virus priming followed by a DNA boost, was ineffective [146, 147]. Much of this application to malaria derives from similar and concurrent work with HIV, where significant protective CD8+ T-cell responses have been induced by a strategy of vaccination with DNA followed by recombinant modified Ankara vaccinia virus (MVA) [147]. As discussed above (see section, 'LSA-1'), a recombinant vaccinia virus expressing LSA-1 and six other malaria genes induced CD8+ T-cell and proliferative responses to LSA-1 [137]. CD8+ T-cell-restricted epitopes from LSA-1 and other pre-erythrocytic antigens have been identified in a series of studies (see section, 'HLA and Genetic Resistance to Liver-Stage Antigens'), and incorporated into a DNA prime and MVA boost vaccine currently in phase-I clinical trials at the University of Oxford, UK. The same construct is inserted into the DNA and the MVA vaccines, and consists of a polyepitope string of pre-erythrocytic CD8+ T-cell epitopes fused to the TRAP antigen. Initial findings have established that these vaccines are safe and immunogenic. The technology of DNA vaccine delivery is developing rapidly, with use of the gene gun shown to improve immunogenicity by both conventional intradermal administration [148] and direct injection into the liver [149].

Ty Particle Prime, Vaccinia Virus Boost

As with DNA-MVA strategies, the use of Ty particle as vaccine expression systems was concurrently developed for HIV vaccines [150]. Ty particles are recombinant protein particles derived from a yeast retrotransposon (Ty) that can deliver numbers, 'strings', of epitopes or whole proteins, and which have previously been administered safely in humans [151]. A prime-boost strategy using MVA virus successfully conferred mice protection to *P. berghei* sporozoite challenge [152]. Ty particles carrying a string of pre-erythrocytic stage epitopes were highly immunogenic in mice, which were protected against *P. berghei* sporozoites [153]. Similarly, Ty particles expressing a CD8+ CTL epitope from *P. yoelii* protected mice against sporozoite challenge after boosting with recombinant vaccinia virus [154].

Conclusions

Malaria, like HIV and TB, is both a cause and consequence of poverty, particularly in Africa. Recent estimates have suggested that malaria has led directly to an accumulative loss of over 30% of the gross national product of African nations during the last 30 years. There has been recent, arguably overdue, recognition by the US Government and the European Commission, as well as by other agencies such as the World Health Organization, Wellcome Trust and the Gates Foundation, in many ways driven by African countries, that a malaria vaccine is crucial in the fight against poverty. Development of vaccines against malaria, HIV and TB is increasingly being funded either directly or indirectly, for example, by tax incentives to industry. A new partnership to develop malaria vaccines is emerging in both the industrially developed world of the USA and the EU, and particularly in malaria-endemic countries.

Malaria vaccine development has advanced dramatically since the first clinical trials in 1986. This has been boosted by the demonstration of significant and reproducible efficacy of the CSP vaccine RTS,S, and application of new vaccine technologies has rapidly taken place. All available evidence from malaria model systems and from studies in endemic areas suggest that immunity to liver-stage antigens mediate protection. The malaria liver stage is now recognized as a primary target for vaccine development. Liver-stage vaccines may be critical for prophylactic vaccination of individuals who either are malaria-naïve or who have lost their previously acquired immunity, by preventing the development of blood-stage parasitemia associated with morbidity and mortality. They are also important for enhancing the naturally acquired protective immune response of individuals resident in malaria-endemic countries that is achieved upon prolonged exposure, in order to either prevent blood-stage infection or to reduce the numbers of parasites that emerge from the liver. In that they aim to prevent or reduce the acquisition of clinical infection, vaccines that target liver-stage malaria are loosely analogous to bed nets, the use of which has been shown to significantly reduce morbidity and mortality at the population level. We anticipate significant or even dramatic advances during the next few years.

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Merozoite Antigens Involved in Invasion

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The invasion of the merozoite stage of malaria parasites into erythrocytes is a complex process requiring multiple receptor–ligand interactions, involving attachment of the merozoite to the erythrocyte surface, reorientation and binding of the apical end of the merozoite to the erythrocyte, junction formation, invagination of the erythrocyte membrane and formation of the parasitophorous vacuole [1, 2] (fig. 1). Several different antigens have been proposed to be involved in merozoite invasion, mainly on the basis of their location or being targets for invasion inhibitory antibodies (table 1). These antigens include those present on the merozoite surface as well as those present in organelles in the apical end of the merozoite (fig. 1). The former antigens are thought to be mainly involved in the merozoite recognition and binding to the erythrocyte surface, while the organellar antigens exert their functions during different phases of the actual invasion process. However, little is known about the different molecular interactions occurring during merozoite invasion and about the functions and relative importance of the different antigens involved.

As the viability of extracellular merozoites is intrinsically short, they have to find their new host cell rapidly after their release from schizonts. The initial contact between the merozoite and the erythrocyte is mediated by filamentous structures constituting the merozoite surface coat and may, thus, involve any part of the merozoite surface. By as yet undefined receptor ligand interactions, the merozoite turns its anterior end containing the apical complex of organelles, i.e. rhoptries, micronemes and dense granules, towards the erythrocyte surface. The area of the erythrocyte membrane to which the merozoite is attached shows a slight depression and becomes thickened to form a junction with the plasma membrane of the merozoite [1]. An invagination of the erythrocyte membrane is then initiated and the junction moves along the merozoite surface as a circumferential zone of attachment between the erythrocyte and merozoite at the

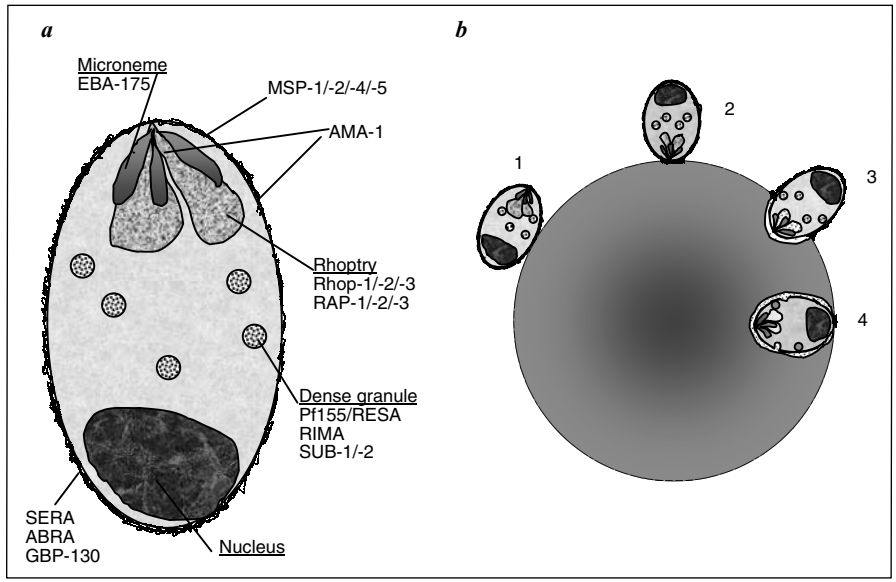

Fig. 1. ***a*** A schematic picture of a merozoite of malaria parasites. The location of the merozoite antigens discussed in the text is indicated. ***b*** A schematic view of the process of merozoite invasion into erythrocytes, depicting: (1) merozoite attachment to the erythrocyte surface; (2) merozoite reorientation and formation of a junction; (3) early phase of invasion with a sliding junction along the merozoite surface, and (4) merozoite within the parasitophorous vacuole after a completed invasion.

orifice of the invagination [1]. During merozoite entry, the surface coat is cleaved off and is absent from the portion of the merozoite within the erythrocyte invagination, while the coat appears intact outside the invagination. When the invasion is completed the orifice closes behind the parasite and the junction becomes a part of the parasitophorous vacuole membrane [1]. By means of atomic force microscopy, a lesion was demonstrated in the erythrocyte membrane at the site of merozoite entry, which is visible still at the late trophozoite stage of infection [3]. This lesion may represent a scar in the membrane as a result of the invasion, but it may also be the terminus of the parasitophorous duct, a structure proposed to form a continuity between the environment and the vacuolar space surrounding the intraerythrocytic parasite [4].

Erythrocyte Receptors

While many merozoite antigens are implicated as ligands in the merozoite–erythrocyte interactions leading to invasion, only a few erythrocyte receptors

Table 1. *P. falciparum* antigens indicated to be involved in merozoite reinvasion

Antigen	Location	Molecular weight kDa	Polymorphism	Invasion inhibition [Ref]
MSP-1	MS	195	Extensive	mAb [22] Ab [25] Peptide [21]
MSP-2	MS	45–55	Extensive	mAb [37, 38] Peptide [41]
MSP-4	MS	40	Nk	No
MSP-5	MS	40	No	No
AMA-1	Rhoptries MS	83	Yes	Ab [53] Peptide [59]
GBP130	MS	130	No	Ab [73] Peptide [74]
SERA	PV, MS-assoc.	126	Minor	mAb [67, 68] Peptide [69]
MCP-1	Moving junction	60	No	No
EBA-175	Micronemes	175	Yes	Ab [81] Peptide [82, 83]
RAP-1/-2/-3	Rhoptries	86-67/39/37	Minor	mAb [90, 92] Ab [94]
Rhop-1/-2/-3	Rhoptries	140/130/110	Nk	mAb [97]
Pf155/RESA	Dense granules	155	Minor	Ab [109, 110]
SUB-1/-2	Dense granules	47/76	Nk	No

MS = merozoite surface; PV = parasitophorous vacuole; Nk = not known.

appear to be crucial in this context. For *Plasmodium knowlesi* and *Plasmodium vivax*, the erythrocyte membrane glycoprotein carrying the Duffy blood group determinants is essential for merozoite invasion [5]. *P. vivax* merozoites only infect reticulocytes and their adhesion to the surface of these cells is independent on the Duffy phenotype [6]. As reticulocytes constitute only a minority of the total red blood cell population, all cells of which express the Duffy glycoprotein, the recognition of reticulocyte-specific receptors must be the first step in the invasion process. Probably the merozoite interacts with the Duffy-associated determinants at the time of junction formation [6].

In contrast to *P. knowlesi* and *P. vivax*, *Plasmodium falciparum* can infect Duffy-negative erythrocytes, indicating that the latter parasite uses other erythrocyte surface receptors for invasion [7]. Terminal sialic acid residues on erythrocyte surface glycoproteins, mainly glycophorin A, were identified as a major determinant of invasion of *P. falciparum* merozoites, but the merozoite–erythrocyte receptor interaction in the context of invasion appears also to

require a part of the peptide structure of glycophorin A [8]. However, studies with laboratory strains and clones of *P. falciparum* showed that the parasite may use alternative, sialic acid-independent pathways for invasion [9, 10], which also are commonly used by *P. falciparum* field isolates [11]. On the basis of invasion studies using enzymatically modified or mutant erythrocytes, at least three independent pathways of invasion may be distinguished for *P. falciparum* merozoites, defined by usage of glycophorin A, glycophorin B or an unknown 'receptor X' [9, 10, 12]. *P. falciparum* parasites may use one or more of these receptors [10], and a parasite under selective pressure may switch the invasion pathway [13].

Merozoite Surface Antigens

A large number of antigens have been identified on the merozoite surface, some of which are integral membrane proteins, either anchored in the membrane via a glycosyl-phosphatidylinositol (GPI) tail or via a hydrophobic transmembrane domain. Furthermore, several soluble antigens are associated with the merozoite surface as peripheral membrane proteins.

Integral Membrane Proteins

The merozoite surface protein 1(MSP-1) is an integral membrane protein bound to the merozoite surface via a GPI anchor and is thought to be the major constituent of the bundles of filaments forming the surface coat of merozoites. The *P. falciparum* MSP-1 is the most extensively characterized antigen of the asexual blood stages and has been implicated as a target for protective immune responses in a large number of studies, including in vitro studies in *P. falciparum* cultures, seroepidemiological studies of naturally acquired immunity and vaccination studies in non-human primates [14, 15].

PfMSP-1 is synthesized as a polypeptide of ~200 kD, which in a primary processing is cleaved into fragments of 83 (N-terminus), 30 and 38 (central portions) and 42 kD (C-terminus) [14, 16]. The latter fragment remains membrane-bound by means of a GPI anchor, while the other fragments are associated to it by non-covalent bonds, forming a complex. Furthermore, two other polypeptides of 22 and 36 kD, respectively, are non-covalently associated with the MSP-1 complex [16, 17]. The second processing of PfMSP-1 is a cleavage of the MSP-1_{42} fragment taking place just before the merozoite invades the erythrocyte, resulting in a 33- and a 19-kD fragment [18]. This processing is probably reflected by the shedding of the merozoite coat observed at the moving junction between the invading merozoite and the erythrocyte [1]. While the C-terminal fragment MSP-1_{19}, composed of two epidermal growth factor (EGF)-like

domains, remains membrane-bound and enters the erythrocyte with the invading merozoite, MSP-1_{33} is released in association with the rest of the MSP-1 complex [19].

The potential involvement of PfMSP-1 in merozoite invasion was indicated by its ability to bind to human erythrocytes via a sialic acid-dependent interaction [20]. The binding was inhibited by soluble glycophorin as well as by antibodies to the glycosylated portion of glycophorin. Importantly, PfMSP-1 bound to human and *Saimiri* erythrocytes, which are susceptible to invasion by *P. falciparum* merozoites, whereas the antigen did not bind to rabbit or rhesus erythrocytes, which are not invaded by this species of malaria parasites [20]. Furthermore, analysis of the binding to human erythrocytes of a series of 20-mer peptides spanning the entire PfMSP-1 sequence, identified several peptides as high-affinity binders in a sialic acid-independent manner [21]. Binding sequences were derived both from conserved and dimorphic sequences in the protein. Most of the peptides with high binding activity inhibited merozoite invasion in vitro [21].

An important role of MSP-1 in the invasion process is indicated by the finding that prevention of the secondary processing of MSP-1 and the shedding of the MSP-1 complex result in failure of the merozoite to invade [19]. This processing is calcium-dependent and can be inhibited by chelating agents like EDTA and EGTA, compounds also inhibiting merozoite invasion [18]. Furthermore, antibodies to MSP-1_{42}, reacting with sequences at the site of the proteolytic cleavage, inhibit the processing and merozoite invasion [22]. However, binding of these antibodies can be blocked by other antibodies that recognize adjacent or overlapping epitopes, but which themselves have no effect on MSP-1 processing or merozoite invasion [23]. Although the main target epitopes for inhibitory antibodies are located in the MSP-1_{19} fragment and are dependent on the native conformation of the EGF-like domains [19, 24], inhibition of merozoite invasion has also been obtained with antibodies directed against other regions of the antigen, including the polymorphic region of repeats in its N-terminal part [25].

In recent transfection experiments in *P. falciparum*, no parasites with a disrupted *MSP-1* gene were obtained, indicating that such disruption has a deleterious effect on parasite growth [26]. Furthermore, these experiments demonstrated that MSP-1_{19} is functionally conserved across distantly related *Plasmodium* species, as the function of PfMSP-1_{19} could be complemented by the corresponding but highly divergent sequence from *Plasmodium chabaudi.* Parasites expressing the chimeric MSP-1 invaded human erythrocytes and antibodies to *P. chabaudi* MSP-1_{19} specifically inhibited merozoite invasion in vitro of those parasites [26]. Thus, although PfMSP-1_{19} can tolerate a high degree of diversity in the primary sequence for intact function, it is remarkably conserved among

P. falciparum field isolates. This indicates that MSP-1_{19} is not under a substantial selection pressure, which is also reflected by relatively weak antibody and T-cell responses to the antigen in infected individuals [27, 28].

MSP-2 is a myristylated 35- to 52-kD glycoprotein, which is highly polymorphic, mainly due to a centrally located block of diverse sequences, including repeat and non-repeat sequences, which are flanked by highly conserved N- and C-terminal sequences [29–31]. Numerous MSP-2 alleles have been identified, which may be classified in a dimorphic pattern where the sequences fall into one of two types, exemplified by the FC27 and 3D7 alleles [32, 33]. The dimorphism is most obvious in the variable non-repetitive sequences flanking the central block of repeats. The two allele families possess different types of highly polymorphic repeats, the 3D7 type of alleles encode variable numbers of repeats of varying length, rich in alanine, glycine and serine. The FC27 types of allele in general encode one to three tandem repeats of 32 amino acids followed by one to five repeats of 12 residues [31].

The high degree of polymorphism in the central repeat region of MSP-2, indicates that it is subjected to immune pressure and that the antigen is a target for neutralizing immune responses. This is also supported by a study in Irian Jayat in which the MSP-2 gene of parasites was analyzed in consecutive samples over a period of 29 months [34]. In no individual was reinfection seen with a parasite strain containing an allele identical to the one already encountered by that individual, indicating a strong selection of emerging parasite clones by immune pressure directed to the polymorphic region of the antigen. The antibody response to MSP-2 in individuals resident in endemic areas is mainly directed against the polymorphic region of the antigen [35, 36] but, in some populations, antibodies to the conserved regions develop at later ages after prolonged exposure to parasites [35].

The involvement of MSP-2 in merozoite invasion is indicated by the inhibitory effect of monoclonal antibodies to the antigen on *P. falciparum* invasion in vitro [37, 38]. However, as MSP-2 also is a target for antibodies that inhibit merozoite dispersal [39], it is not clear to what extent the invasion inhibitory effects seen with antibodies to the antigen reflect a direct interference of the merozoite–erythrocyte interaction. In a recent study, rabbit antibodies to MSP-2 showed no significant invasion-inhibitory activity, but they promoted multiple invasion of erythrocytes, probably by cross-linking the invading merozoites [40]. The potential of MSP-2 to bind to the erythrocyte surface and thus to play a role in merozoite invasion was indicated in studies of the binding to human erythrocytes of synthetic peptides representing MSP-2 sequences [41]. Two peptides showed high specific binding to erythrocytes and inhibited merozoite invasion as well as the intraerythrocytic development of the parasite [41].

MSP-4 and MSP-5 are two recently identified small (approximately 40 kD) *P. falciparum* merozoite surface antigens [42, 43], of which the latter antigen has been reported to lack sequence variation between different *P. falciparum* isolates [44]. Both antigens are GPI-anchored integral membrane proteins, possessing a single EGF-like domain in their respective carboxyl terminus. The genes encoding MSP-4 and MSP-5 are arranged in head-to-tail orientation together with the gene for MSP-2 on chromosome 2 [43]. As yet no data on the involvement of MSP-4 or 5 in merozoite invasion have been reported.

The apical membrane antigen 1, AMA-1, of *P. falciparum* is synthesized in the late trophozoite stage and is expressed in the schizont stage as an 83-kD protein, which is rapidly post-synthetically processed by cleavage of an N-terminal peptide to a 66-kD protein [45]. Both the full-length and processed forms of the antigen are located in the neck of the rhoptries [46]. Transfection experiments with the *PfAMA-1* gene in *Plasmodium berghei* showed that the protein is correctly localized only when AMA-1 protein expression begins in late schizonts [47]. Around the time of merozoite release, the full-length antigen remains in the apical end of the merozoite, while the processed form of AMA-1 is selectively relocalized to the surface of the merozoite, where it appears to spread circumferentially [45]. The processed form of the antigen is associated with the merozoite during invasion and is present in early ring-stage parasites [45].

PfAMA-1 is a type-1 integral membrane protein with an N-terminal extracellular domain (546 amino acids), a transmembrane sequence (21 amino acids) and a C-terminal cytoplasmic domain (55 amino acids) [48]. The AMA-1 sequence contains 16 conserved cysteine residues in its extracellular domain, forming defined intramolecular disulfide bonds, creating three distinct loop-formed domains [49]. Analyses of the AMA-1 gene of different plasmodial species reveals extensive homology and conservation of the 16 cysteines [50]. In contrast to many other malaria antigens, AMA-1 does not contain any repeat sequences. The limited polymorphism of AMA-1 in different strains of *P. falciparum* reflects point mutations leading to amino acid substitutions, a high proportion of which are radical, frequently involving a change of charge [51, 52]. A comparison of 11 full-length PfAMA-1 sequences identified 53 positions where amino acid substitutions occur, most of which in domain I in the N-terminal part of the antigen [51]. In pair-wise comparisons, no two PfAMA-1 sequences differed in more than 32 residues.

Although the function of AMA-1 is not known, there is evidence, mainly from experiments in animal models, that the antigen plays an important role in the merozoite invasion process and, thus, is a target for parasite-neutralizing antibodies. Monoclonal antibodies against the *P. knowlesi* AMA-1 were shown to inhibit merozoite invasion in vitro [53]. Immunization of rhesus monkeys with a purified PkAMA-1 provided partial protection against *P. knowlesi*

challenge [54]. Using a recombinant *Plasmodium fragile* AMA-1 for immunization of *Saimiri* monkeys, partial protection was obtained against the homologous parasite [55]. Protection to *Plasmodium chabaudi* challenge was induced in mice by immunization with recombinant PcAMA-1 or passive immunization with rabbit antibodies to the antigen [56]. Furthermore, adoptive transfer of a T-cell line specific for a conserved cryptic epitope in PcAMA-1 provided help for a specific anamnestic response following in vivo challenge with *P. chabaudi*-parasitized erythrocytes, as well as afforded partial protection against *P. chabaudi* infection in nude mice [57].

In order to identify the function of AMA-1, attempts were made to knock out the *PfAMA-1* gene by targeted disruption [58]. It was not possible to generate parasites with a disrupted *PfAMA-1* gene, probably because such a disruption was severely deleterious to the growth of the parasite. The central role of AMA-1 in merozoite invasion was demonstrated by transfection of the *P. falciparum* line D10 with the *AMA-1* gene of *P. chabaudi* [58]. Although PcAMA-1 could not compensate D10 parasites for a disrupted *PfAMA-1 g*ene, expression of PcAMA-1 in D10 parasites provided a partial trans-species complementation of the function of endogenous PfAMA-1. Thus, in the presence of inhibitory anti-PfAMA-1 antibodies, parasites transfected with the *PcAMA-1 g*ene were still able to invade erythrocytes to approximately 35% of parasites in the absence of antibodies. Furthermore, expression of PcAMA-1 in *P. falciparum* allowed these parasites to invade murine erythrocytes more efficiently [58].

PfAMA-1 sequences potentially important for merozoite invasion were identified using overlapping synthetic 20-mer peptides representing AMA-1 sequences in studies of binding to human erythrocytes [59]. Three of the peptides with high specific binding were demonstrated to inhibit merozoite invasion in *P. falciparum* cultures in a dose-dependent manner [59].

Merozoite Surface-Associated Antigens

Several antigens synthesized during trophozoite development are secreted into the parasitophorous vacuole and when the schizont ruptures they are released into the culture supernatant as soluble exoantigens [60]. These antigens are usually also found associated with the merozoite surface and have thus a potential to be involved in the invasion process. However, for many of the antigens it remains to be determined if their binding to the merozoite surface is of biological significance for the parasite in vivo or rather is an artifact of the in vitro culture conditions. Antibodies to several of the antigens have been demonstrated to interfere with *P. falciparum* growth in vitro, but in most cases it is difficult to discern if this reflects a direct effect on the invasion process or if the antibodies act at an earlier stage, e.g. by preventing the dispersal of merozoites or by agglutinating the free merozoites [61].

The serine repeat antigen (SERA; SERP = serine-rich protein or p126), the acidic basic repeat antigen (ABRA) and glycophorin-binding protein (GBP)-130 are among the antigens found in the immune clusters formed by antibodies inhibiting merozoite dispersal in *P. falciparum* cultures [39]. The sequence similarity of the two former antigens with proteases, SERA with cysteine proteases [62] and ABRA with chymotrypsin [63], indicates their potential involvement in proteolytic processes essential for schizont rupture and/or reinvasion of merozoites. Both antigens show similar locations, being secreted into the parasitophorous vacuole during trophozoite development and subsequently associated with the surface of free merozoites as well as shed into the supernatant [64, 65]. Furthermore, SERA is transferred with the invading merozoite to the ring stage [65]. SERA is synthesized as a 126-kD protein and is processed at schizont rupture into fragments of 50, 47 and 18 kD, the two latter of which are linked by a disulfide bond [66]. Both monoclonal antibodies to SERA and mouse sera against recombinant antigen are efficient inhibitors of parasite growth in vitro [67, 68], but it is not clear at what stage the merozoite is targeted in this context. Further evidence for involvement of SERA in merozoite invasion comes from the identification of six 20-mer peptides, corresponding to sequences in the antigen, which showed high-affinity binding to human erythrocytes [69]. Some of these peptides inhibited merozoite invasion in vitro. Also antibodies to ABRA show a high capacity to inhibit merozoite invasion in vitro [70]. The inhibition of the proteolytic activity of ABRA by the antibodies, thus preventing the secondary processing of MSP-1, was proposed as a possible mechanism for this inhibition.

GBP-130 was identified by its binding to a matrix presenting human erythrocyte glycophorins [71]. The protein also binds to soluble glycophorins as well as to the erythrocyte plasma membrane, but shows only weak binding to intact erythrocytes, suggesting that it binds to an internal domain of glycophorin [72]. GBP-130 contains 11 highly conserved 50-amino acid repeats, which include the glycophorin-binding determinants defined by a minimum of between 3.5 and 4.5 repeats [73]. A rabbit antiserum to these repeats inhibited merozoite invasion into erythrocytes in vitro, indicating a role of GBP-130 in *P. falciparum* invasion [73]. Recently, analysis of the binding to intact human erythrocytes of overlapping 20-mer peptides identified one peptide corresponding to a sequence within the repeats of GBP-130 (amino acids 701–720) as a high-affinity binder which also inhibited merozoite invasion with high efficiency [74].

The merozoite cap protein-1 (MCP-1) is a 60-kD protein located in a cap-like pattern at the apical part of the surface membrane non-invading merozoites [75]. The protein appears to have a submembranous location and is associated with the moving junction formed between the merozoite and erythrocyte during invasion [76].

Antigens Present in the Apical Organelles of Merozoites

Antigens of Micronemes

The micronemes are several elongated tubular electron-dense structures clustered around the rhoptry ducts [77]. They are thought to discharge their granular-like contents in the early stages of invasion, releasing erythrocyte-binding proteins which are instrumental in the invasion process.

The erythrocyte-binding antigen (EBA)-175 of *P. falciparum* is a member of a family of high molecular weight erythrocyte-binding microneme antigens, also including the Duffy antigen-binding proteins (DABP) of *P. vivax* and *P. knowlesi* [78]. The proteins contain regions of cysteine-rich sequences both in the amino and carboxy terminal parts of the molecule, designated regions II and VI, respectively. Region II of EBA-175 contains a duplicated cysteine-rich region, containing 27 cysteine residues, which are conserved between different strains of *P. falciparum* [79]. Binding of EBA-175 to the erythrocyte surface appears to involve an initial lectin-like interaction with sialic acid on glycophorin A and a subsequent binding to the peptide backbone not dependent on sialic acid [8]. Erythrocyte-binding experiments to various EBA-175 fragments expressed on the surface of transfected COS cells identified region II as mediator of the sialic acid-dependent binding [8]. The secondary binding appears as a result of either a receptor/ligand-induced conformational change or the limited proteolysis of bound EBA-175 and a 65-kD processing fragment of the antigen has been shown to be involved in the binding [80].

Antibodies to a 42-amino acid-long synthetic peptide representing a conserved sequence in EBA-175 (amino acids 1062–1103) were shown to block the binding of EBA-175 to erythrocytes and to inhibit merozoite invasion in a dose-dependent manner [81]. Using overlapping synthetic peptides covering the 42-amino acid region, the erythrocyte-binding determinant was mapped to the amino acid 1076–1096 sequence of EBA-175 [82]. This peptide bound to both the native and desialylated forms of glycophorin A, and it almost completely blocked parasite multiplication in vitro. However, in a recent study a peptide comprising amino acids 1080–1099 did not show any specific binding to human erythrocytes, while several other 20-mer EBA-175 peptides were shown to bind with high affinity to the erythrocyte surface [83]. While some of the peptides were efficient inhibitors of merozoite invasion, other peptides with high binding activity showed no or only a modest invasion inhibitory activity [83]. Two of the peptides with high binding activity, representing sequences in region II of EBA-175, inhibited the binding of recombinant RII-EBA protein to erythrocytes, indicating that these are the major sequences involved in the region-II interaction with erythrocytes [83].

EBA-175 is synthesized as a 190-kD protein located in the micronemes of the merozoite and is cleaved into a 175-kD protein, which is released into the culture supernatant after schizont rupture [84]. The antigen binds both to erythrocytes and merozoites, thus bridging the two cells in an early phase of invasion [85]. Although the EBA-175 molecule contains a transmembrane domain and a cytoplasmic domain, it is not clear if the antigen acts as an integral membrane protein or if the soluble antigen is a peripheral protein binding to some structure on the merozoite surface. In any event, antibodies to EBA-175 have in some instances been observed to react with the outlining of free merozoites, suggestive of a surface localization [84].

The importance of EBA-175 in the sialic acid-dependent invasion of merozoites was demonstrated with parasites expressing a truncated form of the antigen [84]. Using targeted gene disruption, mutant forms of *P. falciparum* were created, which express a truncated form of EBA-175 (regions I–V), lacking the C-terminal cysteine-rich region VI, the transmembrane-spanning region and the cytoplasmic part of the antigen. Deletion of the C-terminal part of EBA-175 had no obvious effect on the subcellular localization, the level of expression or the release of the antigen into the culture supernatant after schizont rupture [84]. However, the disruption of the antigen was associated with a switch towards a sialic acid-independent pathway of invasion for the mutant parasite, indicating that the bridging function of EBA-175 was impaired [84].

Rhoptry Antigens

The rhoptry organelles in the apical end of the merozoite consist of two pear-shaped membrane-bound vesicles, each composed of an electron-dense basal bulb and an electron lucent duct, tapering up to the apical prominence [86]. Most of the antigens present in the rhoptries are non-covalently associated in high- and low-molecular weight protein complexes designated Rhop-H and Rhop-L, respectively [87].

The Rhop-L complex is located in the body of the rhoptries and consists of the three rhoptry-associated proteins RAP-1 (86, 82 and 67 kD), RAP-2 (39 kD) and RAP-3 (37 kD) [88]. Several alleles of RAP-1 and RAP-2 have been sequenced, revealing only a low degree of polymorphism in both antigens due to scattered point mutations [89–91]. RAP-1 undergoes two processing events, one at the time of schizont segmentation when the N-terminus of the 86-kD precursor is cleaved off, yielding an 82-kD protein [88]. The second processing is associated with merozoite release and involves only a fraction of the RAP-1_{82}, resulting in the occurrence of approximately equal amounts of 82- and 67-kD species of the protein in free merozoites. Upon invasion into erythrocytes, only RAP-1_{82} can be detected in the ring-stage parasite [92].

The function of the RAP proteins is not known, but the presence of the Rhop-L complex in association with membranous material released from the merozoite at invasion [93] indicates that the proteins are somehow involved in the invasion process. The involvement of the Rhop-L complex in merozoite invasion is also indicated by the invasion inhibitory activity of monoclonal antibodies to RAP-1 [90, 92] as well as of mouse antibodies to recombinant RAP-2 [94]. The epitopes for two inhibitory antibodies were located to the N-terminal part of RAP-1_{82}, close to the site of cleavage to generate RAP-1_{67} [90].

In an attempt to study the function of RAP-1, the *RAP-1* gene of *P. falciparum* was disrupted by means of gene-targeting technology, producing parasites expressing severely truncated forms of RAP-1 [95]. The truncated RAP-1 showed the same location as the intact protein, but it did not complex with RAP-2 and RAP-3. Furthermore, RAP-2 showed a distinct localization from RAP-1 in the mutant parasites, indicating that RAP-1 controls the targeting of RAP-2 to the rhoptries. While both RAP-1 and RAP-2 are carried with the merozoite of wild-type parasites into the erythrocyte [96], neither of the proteins were detected at the ring stage in the mutant parasites [95]. No detectable difference was observed between wild-type and mutant parasites with regard to growth rate or parasite development, indicating that neither RAP-1 nor RAP-2 play any essential role for efficient invasion.

The high molecular weight Rhop-H complex is composed of three proteins, Rhop-1 (140 kD), Rhop-2 (130 kD) and Rhop-3 (110 kD) [97], which are located in an electron-lucent compartment in the rhoptry duct [98]. Only Rhop-3 has been cloned and sequenced, revealing an apparently well-conserved protein both with regard to antigenic epitopes and overall sequence among isolates of *P. falciparum* [99]. The Rhop-H complex shows binding to lipid moieties in the inner leaflet of the human erythrocyte membrane [100], but also to the surface of mouse erythrocytes [65]. At merozoite invasion the antigen complex spreads out along the erythrocyte membrane and appears also localized to the parasitophorous vacuole membrane [101]. Involvement of the Rhop-H complex in invasion is also indicated from the low but significant invasion inhibitory activity of a monoclonal antibody [97].

Antigens of Dense Granules

The dense granules are small rounded vesicular bodies lying separately within the apical cytoplasm of the merozoite [77]. After the merozoite has entered the erythrocyte, the dense granules move to the surface of the parasite and release their granular contents into the parasitophorous vacuole [102]. This process is associated with the formation of channels from the parasitophorous vacuole. Only a few parasite antigens have been localized to the dense granules, including Pf155/ring-stage erythrocyte surface antigen (RESA) [103, 104],

ring-stage membrane antigen (RIMA) [105] and the two subtilisin-like proteins PfSUB-1 and 2 [106–108]. Although these proteins are released from the granules late during merozoite invasion, involvement of Pf155/RESA and the PfSUB proteins in the invasion process has been indicated.

Antibodies to epitopes within the repeat regions of Pf155/RESA are efficient inhibitors of merozoite invasion [109, 110] by an as yet unknown mechanism. The antibodies might enter the parasitophorous vacuole along with the merozoite or, alternatively, through a putative parasitophorous duct, and thus block the function of the released antigen. However, like many other soluble antigens present in the *P. falciparum* culture supernatants, Pf155/RESA possibly associates with the surface of merozoites and may act here as a target for inhibitory antibodies.

Although no experimental evidence has been presented on the function of the PfSUB proteins, their subcellular localization and predicted enzymatic properties indicate their potential to play a role in the invasion merozoite process. PfSUB-2 was suggested to be the MSP-1_{42} maturase, which during merozoite invasion is responsible for the cleavage of MSP-1_{42}, producing MSP-1_{19} [106].

Concluding Remarks

Research during the last decades has revealed that the merozoite invasion into erythrocytes is a very complex process involving multiple molecular interactions. The different roles in general terms of the merozoite apical organelles have been indicated, but little is still known about the specific interactions between merozoite and erythrocyte molecules during the different phases of invasion. At present, three merozoite antigens, MSP-1, AMA-1 and EBA-175, stand out as essential participants mainly in the early phases of merozoite invasion, but their relative importance in the process remains obscure. The recent availability of the sequence of the entire *P. falciparum* genome and the development of transfection methods for directed disruption of specific malarial genes, will result in a considerable speed-up in the molecular mapping of merozoite invasion. Additional antigens are expected to be identified and increased understanding of the mutual roles of the different merozoite antigens in the process will be gained.

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Asexual Blood Stages of Malaria Antigens: Cytoadherence

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A major characteristic of *Plasmodium falciparum*-infected erythrocytes is their ability to bind to various host receptors and proteins, leading to the sequestration of mature parasitized erythrocytes (PEs) in the microvasculature. Of the four species of malaria parasites that infect humans, only *P. falciparum* sequesters, a property that contributes to the special virulence of this parasite and plays a central role in the host parasite relationship [1, 2]. Several non-human malaria species including *Plasmodium chabaudi*, *Plasmodium coatneyi*, *Plasmodium fragile*, *Plasmodium yoelii*, and *Plasmodium knowlesi* may also sequester, although the degree of sequestration varies widely [3]. In *P. falciparum* and *Babesia bovis*, a related Apicomplexan parasite that can sequester, sequestration has been linked to antigenic variation and the same proteins are probably involved in both functions [4, 5]. The link between sequestration and virulence in rodent malarias is not clear and virulence can be attributed to factors other than parasite sequestration [6]. However, in *P. falciparum*, sequestration affects the survival, virulence and pathogenesis and is clearly one of the major virulence factors [2, 7].

Erythrocytes infected with trophozoite-stage parasites sequester by adhering to post-capillary endothelial cells (ECs) of various organs and to the syncytiotrophoblast of the placenta [1, 2, 8–10]. This property, first described over 100 years ago, is vital for the survival of the parasite in vivo, where mature non-adherent PEs are quickly removed by the spleen [11–13]. The molecular mechanisms of parasite adhesion are now beginning to unfold with the discovery of parasite and host molecules involved in this complex process.

Host Adhesion Receptors

Cytoadhesion is a complex process that may require binding to multiple receptors [for review see, 14]. The adhesion phenotype of PEs is not homogenous and PEs with various binding phenotypes can be found and selected [15–19]. Static adhesion assays with model cell lines, ECs and purified receptors have been used to identify and investigate molecules that support PE adhesion. PEs can interact with a surprisingly large number of host receptors including CD36, thrombospondin (TSP), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), platelet-EC adhesion molecule 1 (PECAM/CD31), P-selectin, E-selectin, chondroitin sulfate A (CSA), hyaluronic acid (HA) and integrin $\alpha_v\beta_3$ [1, 2, 14, 20–25].

One of the major host receptors is CD36, an 88-kD glycoprotein expressed on the surface of platelets, monocytes, microvascular ECs and other cell types. The PE-binding domain of CD36 resides in the immunodominant region of CD36 (amino acids 139–184) although other regions of the molecule may also contribute to the interaction [26, 27]. CD36 is clearly an important host receptor for parasite adhesion in vivo, and adhesion to CD36 is the most abundant adherence phenotype. Studies conducted with PEs from different geographical regions demonstrated that the great majority of clinical isolates and laboratory lines bind to CD36 [17, 28–33]. Although PEs with high and low binding to CD36 can be found, it is widely believed that the interaction with CD36 is the strongest and that CD36 provides the vital anchor needed for stable and long-lasting immobilization of PEs on the endothelium [17, 34, 35]. Given the wide distribution of CD36, and the low levels reported in cerebral vessels, its role in the pathogenesis of cerebral malaria has been questioned [36, 37].

The first adhesion receptor to be identified was TSP [38]. TSP is a large, trimeric protein composed of three identical 140-kD chains found in the extracellular matrix and associated with different cell types including EC [for review see, 39]. TSP is a multifunctional protein involved in cell–cell and cell–matrix attachment and can bind to a variety of proteins including CD47, CD36, *P. falciparum* erythrocyte membrane protein 1 (PfEMP1*)* and others [39, 40]. PEs with TSP-binding phenotypes are also relatively abundant [29, 32]; however, the interaction of PEs with this receptor under flow is relatively weak and its role in adhesion, in vivo, remains unclear [34, 35].

Another adhesion receptor is ICAM-1. A member of the immunoglobulin super family, ICAM-1 is composed of five immunoglobulin-like domains that are expressed on a variety of cell types. ICAM-1 has an important role in the adhesion of leukocytes to the endothelium during inflammation and is a receptor for virions of the Rhinovirus family [41]. ICAM-1 was identified as an adhesion receptor when PEs selected for increased binding to ECs were found to bind to

transiently transfected COS cells expressing ICAM-1 [42]. The binding site within ICAM-1 has been localized to a small area in the first immunoglobulin domain [43, 44] and a genetic polymorphism within this domain may affect susceptibility to severe malaria [45]. The expression of ICAM-1 is upregulated by inflammatory cytokines induced by various infections including malaria [46]. Adhesion to ICAM-1 may be associated with severe malaria [7, 17, 36], although a recent study from Malawi found lower levels of binding to ICAM-1 in severe disease [31].

PECAM-1 (CD31) is another member of the immunoglobulin super family that is constitutively expressed on most ECs. CD31 was recently described as an adherence ligand for PEs [21]. PEs from several laboratory lines bound to immobilized CD31 and to CD31-transfected cells and binding was increased after panning on human umbilical vein ECs. In one study, binding of patient isolates to immobilized CD31 was infrequent (21%) and low level [17]; however, a more recent study using CD31-transfected cells found frequent binding of clinical isolates [47].

VCAM-1, another member of the immunoglobulin super family, and two members of the selectin family, P-selectin and E-selectin, have been shown to support adhesion of a limited number of parasite isolates [17, 20, 48]. VCAM-1 and E-selectin are not expressed on resting endothelium, but expression is induced by cytokines such as tumor necrosis factor-α (TNF-α) [49]. Ockenhouse et al. [48] reported adhesion of a patient isolate to E-selectin and VCAM-1 expressed on ECs, and found high levels of binding of that isolate after sequential panning on those receptors. PEs of some clinical isolates from Thai individuals were found to roll and tether on immobilized soluble P-selectin, and to interact with the lectin domain of P-selectin [20, 50]. In several isolates, rolling was blocked by anti-P-selectin antibodies. Adhesion to these receptors is infrequent among *P. falciparum* isolates and has not been correlated with disease severity.

CSA is a glycosaminoglycan (GAG), linked via a membrane-associated protein to the cell surface. Classically, a heteropolymer of alternating glucuronic acid and 4-sulfated N-acetylgalactosamine, CSA from different sources may differ substantially in sulfation pattern. CSA has been shown to mediate adhesion of PEs to Chinese hamster ovary (CHO) cells, *Saimiri* money brain ECs [51], immobilized CSA and to thrombomodulin [22, 52]. Sulfation in the 4 position is critical for CSA-mediated adhesion [53]. Patient-based studies suggest a particular role for CSA in sequestration of PEs in the placenta [8]. Interestingly, PEs that bind to CSA typically do not bind CD36 and vice versa.

HA is a non-sulfated, high molecular weight GAG (up to several million Daltons), composed of alternating N-acetylglucosamine and glucuronic acid that was shown to support PEs adhesion [24]. Laboratory lines selected on CSA

were shown to adhere to HA, and vice versa, and placental isolates usually bound to one or both [24]. However, adhesion to the two receptors was shown to be distinct. The relatively weak adhesion under flow to HA suggests that PEs could adhere to HA in vivo only under conditions of low flow, such as those seen in the placenta.

Interactions between PE and additional receptors, such as blood group A, complement receptor 1 (CR1) and heparin-like GAG expressed on uninfected red blood cells are reviewed elsewhere. PEs also bind a variety of serum proteins like IgG and IgM, GAGs such as heparin and heparan sulfate-like molecules, and others. Interactions with these molecules may modulate and enhance both rosetting and cytoadhesion [47, 54–57].

Adhesion and Pathogenesis

Why do *P. falciparum* parasites recognize such a large number of adhesion receptors, and is there a correlation between adhesion receptors and pathogenesis? Maximal tissue tropism and lasting stable adhesion may be part of the answer. Different receptors, or combinations of receptors, may be involved in each of the steps leading to stable adhesion. Cooperation between receptors is known to enhance adhesion of PEs, which may need to tether and roll on ECs before they stably adhere, at least to some receptors [14, 34]. To be able to sequester in multiple organs the parasite may need to vary its adhesion properties, as post-capillary ECs from different organs display qualitative and quantitative differences in expression of host receptors [37]. Part of the promiscuity of the adhesion properties can be attributed to the fact that PfEMP1, the dominant *P. falciparum* adhesion receptor, is also involved in antigenic variation and has a highly diverse sequence [5, 58, 59]. The variation in sequence among *var* genes results in qualitative and quantitative differences in binding to various host receptors affecting the tissue tropism of the PEs. Alterations in receptor expression in response to inflammation may occur at different sites and stages of the infection [46, 49, 60]. The rapid switching in PfEMP1 [18] allows the parasite to quickly adapt to such changes. One example is in pregnancy, where the placenta is parasitized by PEs with adhesion phenotypes (CSA and HA) that are not commonly detected in other malaria infections [28]. This allows the parasite to adapt and utilize new opportunities to maximize its tissue tropism.

Attempts to relate severity of disease to adhesion phenotype of PEs have been disappointing. Marsh et al. [61] compared isolates from 10 Gambian children with cerebral malaria to 10 matched children with uncomplicated disease, and found no significant difference in intensity of binding to C32 melanoma cells. In a study of 59 isolates from Thai adults, Ho et al. [62] found significantly

greater adhesion of isolates from adults with biochemical evidence of severe malaria than isolates from other adults to C32 melanoma cells. Adhesion of isolates from adults in coma was similar to that of isolates from adults with uncomplicated malaria. It was suggested that pathogenesis of the two syndromes may differ, with cytokines more critically involved in the development of cerebral malaria. Ockenhouse et al. [36] studied isolates from 9 adults with severe malaria and 18 adults with uncomplicated disease. There was slightly more adhesion to immobilized CD36 for isolates from adults with severe disease but no difference in either fluid phase binding of CD36 to PEs or in adhesion of PEs to ICAM-1.

In each of the above studies, the diversity in adhesion profiles was more striking than specific differences between patient groups. Other studies conducted in Papua New Guinea [30] and Malawi [63] have also confirmed the extensive diversity of adhesion types. Cryopreserved isolates from Kenyan children with cerebral malaria, severe malarial anemia, uncomplicated disease and, most interestingly, asymptomatic parasitemic children were examined [17]. Almost all isolates bound to CD36, and most to ICAM-1, but smaller numbers bound to VCAM-1, PECAM-1 and E-selectin. Adhesion to CD36 was not higher in cerebral malaria, and was lower in severely anemic children than in isolates from other groups. Adherence to ICAM-1 was highest among isolates from children with cerebral malaria although this did not reach statistical significance. In another study in Malawi, 155 isolates from children were studied [31]. Relatively few isolates bound to CSA (27%) or thrombomodulin (16%) and binding was usually of low density. By contrast, most isolates bound to CD36 and ICAM-1 but, unexpectedly, binding densities to both CD36 and ICAM-1 were lowest for isolates from children with severe malaria. CD36 binding was lowest in severe malarial anemia, whereas ICAM-1 binding was lowest among isolates from children with cerebral malaria. Intensities of CD36 and ICAM-1 adhesion were correlated with one another, and for both there was a positive association between patient hematocrit and binding density. Another study looking at the adhesion of PEs from 49 Thai adults with uncomplicated or complicated malaria to fixed human lung ECs was recently reported [64]. PEs from 20 patients with complicated malaria were more likely to bind to human lung ECs than were PEs from 16 patients with uncomplicated disease, and binding was more commonly inhibited by antibodies to ICAM-1 or to CSA.

The most interesting results with adhesion assays using clinical isolates have come from recent studies showing associations between binding to CSA, and more recently HA, and pregnancy. Fried and Duffy [8] reported that PEs from the placenta of a small number of pregnant women at delivery bound to CSA immobilized on plastic, and also to trophoblasts from normal placenta. These isolates did not bind (or bound poorly) to CD36 and ICAM-1. Peripheral blood isolates from pregnant women had a mixed pattern of CD36 and/or CSA

binding, whereas isolates from non-pregnant women and children bound mainly to CD36 and ICAM-1 but not to CSA [28]. Isolates from the placenta also differed in antigenic type from those from children [28] and only sera from multigravid women agglutinated placental isolates and blocked PE adhesion to CSA, whereas sera from men and primigravid women frequently agglutinated isolates from young children [28, 65]. Adhesion to HA was seen in 80% of placental isolates from Malawian women, the same proportion as bound to CSA (but with different isolates showing binding to each) [24]. Thus, adhesion to CSA and HA is the only adhesion phenotype that shows direct association with a specific disease syndrome. It is likely that a combination of factors, among them the adhesion characteristics of PE, are responsible for disease complications, making clear associations between adhesion and disease hard to detect.

Antigenic Diversity and Antigenic Variation

Numerous studies have now confirmed the diversity of antigens presented on the surface of trophozoite-stage PEs, and have found that PfEMP1 is the dominant neoantigen present [66, 67]. Infections in children are caused by antigenic types not previously encountered [68]. In young children, severe disease may be caused by common (possibly more virulent) isolates while disease in older children may be associated with rare variants [69]. Antigenic variation in *P. falciparum* has been quantitated [18]. Using a clonal population, switching or emergence of novel antigenic variants was found to occur at a rate of 2% per generation. Such switches are associated with changes in PfEMP1 expression and in adhesive type [18, 70]. While figures for natural infections are not available, this mechanism may well explain the parasite's ability to evade host immune responses, and adapt to sequester in multiple sites in different hosts.

Var Genes and the *P. falciparum* Adhesion Receptor, PfEMP1

PfEMP1 was identified as a large, size diverse (200–400 kD) ^{125}I-labeled protein expressed on the surface of mature PEs and is linked to cytoadhesion [71–74]. The protein could not be extracted by neutral detergents such as Triton X-100 but is extracted with sodium dodecyl sulfate [74]. Ample observations support the role of PfEMP1 in cytoadhesion. Mild trypsinization of intact PEs cleaves the ^{125}I-labeled PfEMP1 from the PE surface and at the same time ablates agglutination and adhesion [5, 40, 72, 73]. Changes in the expressed PfEMP1 were linked to alterations in antigenicity and adhesion properties including variations in the size of PfEMP1, recognition by immune sera and selection for

particular adhesion phenotypes [15, 16, 18, 19, 58]. The strain-specific recognition of PfEMP1 by hyper-immune sera correlated with strain-specific blockade of adhesion strengthening the correlation between antigenic variation and cytoadhesion [5, 23, 72, 75]. Finally, PfEMP1 and fragments of PfEMP1 were shown to bind directly to the host receptors, CD36, TSP and ICAM-1 [40].

The cloning of members of the large, diverse family of *var* genes encoding PfEMP1 [5, 58, 59] was a major breakthrough leading to investigation of the molecular bases of binding and identification of protein domains involved in adhesion. Each parasite encodes approximately 50 *var* gene copies that vary between strains resulting in a vast number of variant genes among the parasite population [5, 58, 59, 76]. The size and sequence of *var* genes are highly diverse and antibodies to recombinant proteins derived from individual *var* genes show strain-specific agglutination, immunoprecipitation, and blockade of adhesion [5, 58, 59, 75].

Despite their extensive sequence diversity, *var* genes share several common features [23, 59, 77]. They are encoded by two exons. The first encodes the variable large extracellular domain and the membrane-spanning region and the second encodes the relatively conserved intracellular domain, the acidic terminal segment [59], which binds to knob-associated histidine rich protein (KAHRP) and host cytoskeletal proteins (fig. 1).

Two types of domain structure are found in the extracellular region of *var* genes. Duffy binding-like (DBL) domains are named for their homology to the

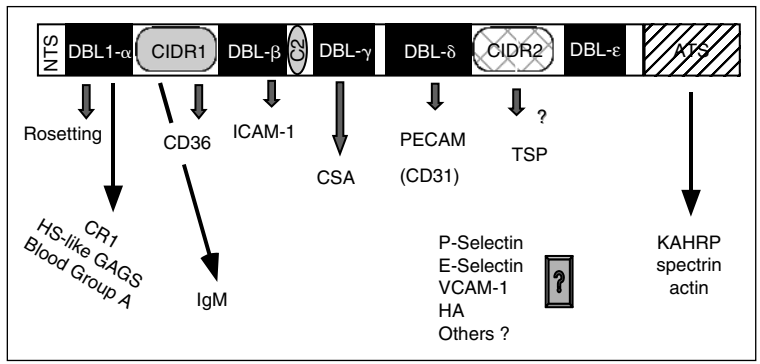

Fig. 1. Schematic representation of *var* gene domains and known interactions. The variable extracellular region of *var* genes is composed of 2–7 DBL domains and 1–2 CIDR domains. Additional regions are the N-terminal segment (NTS) before DBL-1 and the C2 region found after some of the DBL-β domains. The domains are followed by a short membrane-spanning region and an internal acidic-terminal segment (ATS). The type, number and position of DBL and CIDR domains vary among *var* genes and there are five known homology type groups (α, β, γ, δ and ε) of DBLs. The properties and binding interactions of the different domains are illustrated.

P. vivax and *P. knowlesi* Duffy-binding antigens and also share homology with the *P. falciparum* sialic acid-binding protein, EBA-175 [59]. The second domain structure is the cysteine-rich inter-domain region (CIDR) that contains a cysteine-rich motif [23, 59, 77]. The number and the location of the different domains are not fixed among different *var* genes, which may have from 2 to 7 DBL domains and 1 or 2 CIDRs [77]. Analysis of *var* gene sequences demonstrated that DBL domains can be divided into five homology group types (α, β, γ, δ and ε) and two or three CIDR domain types [77]. Only the location of the first two domains (DBL1 and CIDR1) is highly conserved in most *var* genes, and the domains c-terminal to this conserved head structure vary in number and order [for review see, 23, 77]. A new nomenclature has been proposed to help identify both the location and type of each of the domains. DBL and CIDR domains are independently numbered 1, 2 to n to identify their location in a particular *var* gene followed by a Greek letter that identifies their homology group. Thus, in the *MCvar1* gene the second DBL is DBL2-δ followed by CIDR2-γ, whereas the second DBL in *A4var* is DBL2-β [23, 77]. Examination of the sequence of 20 *var* genes identified two additional extracellular non-DBL or CIDR1 segments/domains: the N-terminal segment located at the amino terminus of all *var* genes and the C2 region that is found C-terminal to many but not all DBL-β domains [77, 78]. Besides the pairing of DBL1-α–CIDR1-α and DBL-β-C2 we also note an association between DBL-δ and CIDR2-β/γ that also appear together. The pairing of these domains may bear functional or structural consequences and may indicate that the function of *var* genes preceded antigenic variation [77].

Var genes are found on most *P. falciparum* chromosomes, primarily at or near the telomere [79], but transcription can occur in telomeric or internal chromosome locations [80]. A single dominant *var* transcript is apparent in mature PEs [19, 81] indicating that a single PfEMP1 type is expressed in each cell. The rapid switching in *var* expression (up to 2% per generation [18]) leads to appearance of new adhesion phenotypes and gives the parasite the flexibility to respond and adapt rapidly to individual variations between hosts and to changes within the host.

Adhesion Domains in PfEMP1

There are several obstacles in assigning adhesion properties to various domains of PfEMP1. The vast sequence diversity among *var* genes and the heterogeneity of PE adhesion makes it likely that a single domain may have different binding properties in different *var* genes or PfEMP1s. The actual adhesion properties of PE expressing many of the *var* genes found in sequence databases is unknown, making it difficult to verify our results. Due to the large size

of PfEMP1 we cannot express the complete protein, limiting our ability to test for structural and functional interactions between domains. We also have to bear in mind that cytoadhesion is a complex process and molecules other than PfEMP1 might participate or modulate adhesion. Despite these problems, several adhesion domains have already been mapped and precisely defined (fig. 1).

DBL1-α: A GAG-Binding Domain that Mediates Rosetting

The DBL1-α domain of PfEMP1 was shown to mediate rosetting by binding to uninfected erythrocytes [82, 83]. The recent evidence suggests that the DBL1-α domain binds a variety of molecules including CR1, blood group A antigen, heparin-like GAG, heparan sulfate and potentially other molecules [47, 54, 82, 83]. These interactions are associated with the complex phenotype of rosetting and identify this domain as the rosetting domain. The role of this domain in rosetting is reviewed elsewhere and will not be discussed here.

CIDR1-α: A Multi-Adhesive Domain that Binds to CD36

The CIDR1-α domain of PfEMP1 mediates adhesion to CD36. CIDR1s from several PE lines that bind to CD36 were expressed in CHO and COS cells shown to bind CD36. In contrast, CIDR1-α from strains that bind CSA but not CD36 did not [47, 75, 78, 84, 85; Baruch D, unpublished data]. None of the other domains of PfEMP1 tested were able to bind to CD36 [75, 78, 85].

Structure-function analysis of CIDR1 localized the minimal C36-binding domain in PfEMP1 to a 179-amino acid region of CIDR1, named rC1-2 (1–179), that contains 7 cysteine residues [75]. The adhesion motif is nonlinear and requires correct folding, reduction and alkylation of rC1-2 (1–179) ablate binding to CD36 [75].

Antibodies to recombinant rC1-2 protein blocked PEs adhesion in a sequence-specific manner [5, 75]. In contrast, rC1-2 blocked the adhesion of all CD36-adherent parasite strains tested regardless of sequence diversity [75, 86]. Importantly, rC1-2 was also able to reverse adhesion of bound PEs under flow conditions at concentrations achievable in the plasma, thus offering a possible anti-adhesion therapeutic [75, 86]. We postulate that regardless of sequence diversity, CIDR1-α of various *var* genes fold into a conserved structure that binds CD36.

Recently, several other binding properties were attributed to CIDR1-α. Reeder et al. [87, 88] found that the CIDR1 domain may be involved in adhesion to CSA. Their findings were based on antibodies to CIDR1 that blocked PE

adhesion to CSA and peptides from CIDR1 that bound to CSA [87, 88]. CIDR1-α has also been reported to be involved in binding to CD31 together with DBL-δ and in the binding of human IgM [47]. These findings highlight the promiscuous nature of the PfEMP1 adhesion domains and the multiple interactions potentially attributable to a single domain. Nonetheless, it is clear that a major role in PE adhesion of the CIDR1 domain appears to be binding to CD36.

DBL-β-C2: An ICAM-1-Binding Domain

Adhesion to ICAM-1 is found in many patient isolates and this interaction has been linked to cerebral malaria [7, 17, 36, 37]. The involvement of PfEMP1 in adhesion to ICAM-1 was suggested by a switch in the expressed *var* gene after selection of PEs on ICAM-1 and the direct binding of PfEMP1 extracted from ICAM-1-adherent PEs to immobilized ICAM-1 [15, 18, 19, 40, 89]. Recently, Smith et al. [78] identified the DBL-β-C2 region as the ICAM-1-binding domain. The tandem arrangement, DBL-β and C2, appear in many but not all genes expressing DBL-β and is not found anywhere else in the gene [77, 78]. COS-7 cells transiently transfected with the DBL2-β-C2 but not DBL2-β alone from clone A4tres bound ICAM-1 and binding was inhibited by anti-ICAM-1 antibodies [78]. Although a similar domain from another ICAM-1 binding clone failed to bind ICAM-1 [78, 85], antibodies to DBL-β of both clones blocked PEs adhesion to ICAM-1. Thus, it is apparent that a DBL-β mediates adhesion to ICAM-1 although more experimental data are needed to establish the role of the C2 domain in this interaction.

DBL-γ and Adhesion to CSA

The finding that adhesion to CSA is associated with sequestration in the placenta ignited the interest of many investigators in this interaction. CSA-adherent parasites expressed a variant PfEMP1 different from genes with CD36- or ICAM-1-adhesion phenotypes [19, 22, 90]. Antibodies to CIDR1-α and DBL2-γ domains of the *CS2var* gene blocked PEs adhesion to CSA [87]. We expressed several DBL-γ domains from mutant CHO-745 cells that lack CSA expression and found they bind to biotin-CSA [84; Baruch D, unpublished results]. The binding was inhibited by the addition of free CSA but not CSC, a chondroitin sulfate form that does not support PE adhesion [84]. However not every DBL-γ binds CSA and the DBL4-γ of *ItA4var* and DBL5-γ of *FCR3varCSA* did not bind CSA [84]. Thus, it is clear that not every DBL-γ can mediate adhesion to CSA.

DBL-δ: Role in PECAM-1/CD31 Binding

The recent indication that many PEs bind to CD31 suggests that the binding domain will appear in most *var* genes. Chen et al. [47] used fluorescence-labeled soluble CD31 to demonstrate its binding to PEs and to identify the binding domain for CD31. The receptor bound primarily to COS cells transfected with DBL2-δ and to some extent also to cells expressing CIDR1-α. CD31 also bound to recombinant proteins of these domains expressed as GST-fusion protein [47]. These results clearly demonstrated the involvement of DBL-δ in adhesion to CD31. The specific role of CIDR1-α in this interaction is still unclear.

Other Host Receptors

Some controversy exists as to whether *var* genes mediate adhesion to TSP and the domain involved with this interaction has not yet been identified [40, 89]. Eda et al. [91] suggested that *P. falciparum*-modified band 3 mediates this interaction. However, direct binding of PfEMP1 and specific fragments of PfEMP1 from several strains to TSP suggests that PfEMP1 is a receptor for TSP [40]. The sticky nature of TSP and its ability to recognize many cells and receptors makes identifying the domain a complicated task.

Var genes from PEs that bind to other host receptors, P- and E-selectin, VCAM-1, HA and others have not been reported. As there are more host receptors than *var* gene domains it is likely that the same domain type will have different binding properties in different *var* genes or that some of the interactions will require a specific combination of domains such as DBL-β and C2.

Modulation of Parasite Adhesion

Other proteins besides PfEMP1 participate in PE adhesion and modulate adhesion in various ways. The interaction between PfEMP1 and the knob structure on the surface of PEs is a good example of this. PfEMP1 is known to be clustered at knobs [5]. This localization of PfEMP1 may act to focus and concentrate PfEMP1 and facilitate adhesion of flowing PEs to the vascular endothelium [92]. Moreover, the conserved acidic terminal segment of PfEMP1 binds directly to KAHRP and to red blood cell cytoskeletal proteins spectrin and actin which may anchor PfEMP1 to the cytoskeleton of PEs at knobs [93, 94]. In fact, targeted disruption of KAHRP drastically reduced the ability of PEs to cytoadhere under flow conditions [92].

Recently, McCormick et al. [56] showed that addition of the sulfated glycoconjugates heparin, fucoidan and dextran sulfate but not chondroitin sulfate significantly increased CD36-dependent binding of PEs to human dermal microvasculature ECs [56]. Some of these GAGs are known to disrupt rosetting and their activity is consistent with binding to PfEMP1. In a similar way, formation of rosettes is largely dependent on (or increased by) binding of various serum proteins, particularly IgM [57, 95]. Thus, these molecules provide additional interaction between the PE and the host target cell and may modulate both adhesion and rosetting properties.

Other Parasite-Derived or Parasite-Induced Adhesion Receptors

It is not clear if other molecules apart from PfEMP1 are involved directly in binding. A deletion in chromosome 9 of *P. falciparum* was found to be associated with loss of cytoadherence. Disruption of *CLAG9*, a membrane protein found in the deletion region, affected cytoadhesion [96]. However, different from the CIDR1 domain of PfEMP1, there is no direct evidence for the interaction of *CLAG9* with CD36. Thus, it is possible that *CLAG9* does not have a direct effect on cytoadhesion but instead it affects the surface expression of PfEMP1. Disruption of another molecule, termed sequestrin, also believed to be involved in binding to CD36 had no effect on PE adhesion [96]. Another multi-gene family expressed on the PE surface are the *rifins* [97, 98]. The precise function of these molecules is not yet known, however, they may be linked to cytoadhesion or rosetting. Another adhesive motif is Pfalhesin, a parasite-modified sequence on the erythrocyte anion transporter, band 3. This is believed to become exposed on the PE surface by modifications induced by *P. falciparum* infection and may play a role in adhesion to CD36 and TSP [91, 99]. With the attribution of more and more interaction with host receptors to PfEMP1 it becomes clear that this protein plays the principal role in the PE interaction with most if not all known receptors and contributions from other proteins may be very limited.

Adhesion of PEs under Flow

The occurrence of cytoadhesion in infected humans is not directly amenable to study. The interaction, therefore, has been examined using a variety of in vitro, in vivo and ex vivo models [for review see, 14, 100]. Each model system attempts to simulate some aspect of the interaction between PEs and the vascular endothelium but none is perfect. Because of their relative simplicity, adhesion has been most commonly investigated in static assays where suspensions of PE

are allowed to settle onto sub-confluent monolayers of cultured cells or purified proteins in Petri dishes. Static assays ignore the shear forces exerted on adherent cells in vivo. The mechanism of PE adhesion must be studied in a system that models, as closely as possible, the dynamic nature of the circulation in vivo.

In vitro systems that attempt to mimic blood flow allow the qualitative and quantitative aspects of adhesive interactions to be studied [101]. With flow-based systems, it is possible to observe flowing cells directly and determine the range of wall shear stresses over which PEs are capable of forming an adhesive contact with host cells or a variety of immobilized proteins. Stepwise increases in shear stress, a measure of the strength of the adhesive bonds, allow the quantitative estimation of adhesive forces over the range of wall shear stresses that exist in the microvasculature. Migration of leukocytes from the circulation during an acute inflammatory response is a multi-step process mediated by different receptor-ligand combinations. The adhesive phenomena are strongly influenced by the wall shear stress operating [102]. Analogy to this process can be drawn for PEs adhesion. Each of the receptors involved in PEs adhesion form a quantitatively different interaction with the PE leading to increased interaction between the flowing PE and the endothelium until a stable stationary adhesion is achieved. As with leukocytes, many PEs first tether and roll before they firmly adhere. CD36, ICAM-1 and TSP all support PEs adhesion under static conditions. Flow-based systems reveal a possible different role and strength of interaction for each of these receptors [34]. Under flow, ICAM-1 mediates only rolling and, although stationary, adhesion to TSP is very weak. In contrast, CD36 and CSA give stable, stationary adhesion [34, 103]. Similarly, high levels of adhesion to HA occur only at shear stresses lower than those predicted to exist in post-capillary venules [24], but within the range occurring in the placenta. The role of cooperation between different receptors may also be demonstrated in these systems [20, 50, 56, 104, 105]. ICAM-1, TSP and some sulfated GAGs can significantly enhance CD36-dependent adhesion. At high wall shear stress, initial adhesion to ICAM-1 may slow down PEs, which then become immobilized via CD36 and possibly other receptors [14, 34]. At lower wall shear stresses, however, it appears that CD36 alone is capable of immobilizing flowing PEs without the assistance of ICAM-1 or other receptors. TSP and other proteins can then act to stabilize and enhance the already formed adhesion. Thus, flow-based assays reveal that cytoadhesion is a multi-step process in which different host receptors play different and complementary roles in the stable adhesion of PEs.

Cytoadhesion can be inhibited by immune serum, antibodies, soluble receptors, peptides or recombinant protein both in vitro and in vivo [2, 11, 12, 26, 73, 75, 86, 103, 106]. Administration of various agents, such as anti-adhesion antibodies, soluble adhesion receptors or rheologically active drugs could be envisioned. The in vitro flow assay is a particularly valuable method for assessing the potential efficacy of new therapies, as it provides quantitative data that are relevant

to in vivo sequestration conditions. We must remember that cytoadhesion occurs in a dynamic environment in which both the parasite and the host endothelium continuously change and develop. Because sequestration is fundamental for the survival of the *P. falciparum* parasite the dynamics of the system will lead to selection of PEs with the most suitable adhesion properties. While these dynamic properties can be assessed by flow-based systems, we still await the development of a suitable in vivo model.

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Rosetting and Autoagglutination in *Plasmodium falciparum*

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Rosette formation, in which uninfected red blood cells bind to parasitized erythrocytes, and autoagglutination, the aggregation of infected red cells in nonimmune serum, are adhesive phenotypes associated with disease severity in *Plasmodium falciparum* malaria. The purpose of this chapter is to summarize the current knowledge on the biology of these intrinsic parasite properties, as well as to discuss the existing evidence implicating rosetting and autoagglutination in the pathogenesis and disease mechanisms of severe malaria. The weight of the discussion gravitates towards rosetting, an inevitable consequence of the amount of effort spent over the last 15 years to investigate this phenotype [for review see, 1, 2], as compared to the relatively novel and less studied autoagglutination trait.

Rosetting

The formation of rosettes in vitro occurs in each cycle of intraerythrocytic growth of a parasite exhibiting the rosetting phenotype. Ring-stage parasites do not elicit rosetting, but already at the early trophozoite stage of development, i.e. 16–18 h in *P. falciparum*, uninfected erythrocytes are bound to the infected red cell (fig. 1a). Both, the number of bound uninfected erythrocytes per cell and the rosetting rate (number of trophozoite- or schizont-infected red blood cells (RBC) binding two or more uninfected erythrocytes/total number of

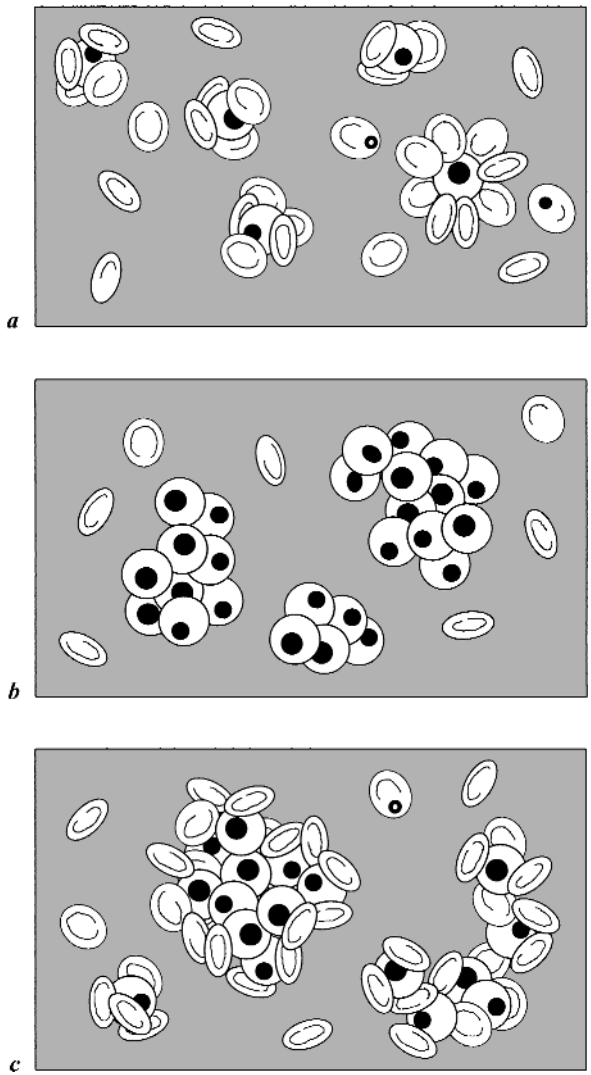

Fig. 1. Schematic diagram of the adhesive interactions of *Plasmodium falciparum*-infected erythrocytes with uninfected and infected red blood cells. ***a*** Rosetting. Distinct rosette archetypes are shown. ***b*** Autoagglutination. ***c*** Giant rosette formation.

trophozoite and schizonts × 100), reach a maximum in the late trophozoite/early schizont stage, 32–36 h after invasion [3]. The rosettes, which disappear upon bursting of the schizonts, reappear during the subsequent parasite intra-erythrocytic cycle.

Autoagglutination

Autoagglutination refers exclusively to the capacity of infected red cells to adhere to each other forming aggregates that do not include uninfected erythrocytes [4, 5] (fig. 1b). Autoagglutination, a feature of wild isolates as well as of parasites adapted to growth in the laboratory, is distinct from the agglutination mediated by malaria immune antibodies, or the stacking of normal RBC (rouleaux). No time-course analysis of autoagglutination has yet been published and, although it is clear that trophozoites and schizonts autoagglutinate, the precise window of expression of this phenotype during intraerythrocytic development is unknown. Autoagglutination may be induced by slow rotation of the assay tube but it also occurs spontaneously in the static culture, in the presence of serum from individuals with no experience of malaria.

Giant Rosetting

'Giant rosettes', consisting of several or many rosettes clustered together in which often infected RBC bind to each other, are commonly observed in clinical isolates and more rarely in in vitro propagated parasites (fig. 1c). It is not clear what triggers the formation of giant rosettes. Albeit the appearance of multi-rosette aggregates is generally associated with high parasitemias [6–9], giant rosetting may take place at various parasite densities and not be seen at all in some cases of concurrent elevated parasitemias and high rosetting rates [10; unpublished observations], suggesting the participation of additional, yet undefined factors. Giant rosettes could originate due to co-expression of rosetting and autoagglutinating phenotypes in individual parasites, or as a result of each phenotype being expressed by distinct parasite subpopulations. Giant rosette formation might also be an enhanced form of rosetting.

Prevalence of Rosetting and Autoagglutinating Malaria Parasites

The capacity to form rosettes is found in the four species of *Plasmodium* infecting humans, i.e. *P. falciparum* [6, 11], *Plasmodium vivax* [12], *Plasmodium ovale* [13], and *Plasmodium malariae* [14]. Also a property common to *Plasmodiae* of simian and other animal species, rosetting has been observed in natural host–parasite associations such as *Plasmodium fragile* in the toque monkey *Macaca sinica* [15], and in experimental models of malaria disease where parasites have been adapted to heterologous hosts, e.g. *P. falciparum*

in the squirrel monkey *Saimiri scireus* [16], *Plasmodium chabaudi* in the CBA mouse [17], and *Plasmodium coatneyi* in the rhesus monkey (*Macaca mulatta*) or the Japanese macaque (*Macaca fuscata*) [18, 19]. These and other studies have shown that erythrocyte rosette formation is widespread in the *Plasmodium* genus, existing in sequestering species, but also in species that do not sequester.

To date, in over a dozen published studies carried out in Africa, Asia, Oceania and South America, rosetting parasites have been observed in 1,259 of 1,562 clinical *P. falciparum* isolates examined, resulting in an overall rosetting prevalence of 81% (table 1). It should be noticed that this assessment of the global rosetting frequency can be an overestimate due to patient group selection in the studies, it does not reflect local variations, and it is restricted to parasites isolated from peripheral blood samples. Rosetting rates in freshly isolated *P. falciparum* range from 0 to virtually 100%, with varying overall means in different endemic regions, typically falling between 5 and 20%. However, and as it will be discussed later in this chapter, isolates from severe malaria case groups can form rosettes at significantly higher rates. Parasite genetic heterogeneity in natural infections, and clonal multiplicity in clinical isolates are likely to account, among other parasite and host factors, for the rosetting frequencies observed in wild parasites [20, 21]. Rosetting has also been found to be a common property of the genetically more homogeneous *P. falciparum* strains and lines adapted to long-term growth in the laboratory. Rosette formation was originally described and is currently studied as a trait of parasites cultured in vitro, its occurrence in vivo is still unclear. Trophozoite-containing *P. falciparum*-infected RBC (pRBC) forming rosettes have been observed upon direct examination by phase-contrast microscopy of the peripheral blood of patients with severe malaria [22]. One report describes rosettes in ocular vessels of the retina [23]. Also, clusters of sequestered pRBC and uninfected erythrocytes have been found in blood vessels from autopsy material of severe malaria victims [24, 25].

In contrast to rosetting, the few reports existing on autoagglutination of pRBC in wild and laboratory-propagated parasites are restricted to *P. falciparum* isolated from humans [4, 5, 8] or from experimentally infected *Saimiri* monkeys [26]. In a field study completed in north-eastern Kenya, autoagglutinating parasites were often found among those isolated from children with severe or mild malaria, thus establishing that the phenotype is common in natural infections [8]. Autoagglutination rates, i.e. the percentage of infected RBC which participate in autoagglutinates, ranged from 0 to 20%, sporadically higher in children with multiple severe malaria syndromes. The prevalence and intensity of the phenotype were associated with disease severity (see below).

Table 1. Rosetting in *P. falciparum* isolates: prevalence and association with severe disease

Country	Clinical group	Rosetting prevalence %	Rosetting rate[1] %	Significance	Ref.
Sri Lanka	Overall	100 (6/6)	53 (37–68)		11
Colombia, The Gambia	South American patients	38 (13/34)	16.2 (0–75 overall)		108
	African patients	55 (21/38)	11.2		
The Gambia	Uncomplicated malaria	82 (47/57)	17 (0–71)		7
	Cerebral malaria	100 (24/24)	35 (6–85)	$p < 0.001$	
The Gambia[2]	Overall	82 (14/17)	9.8 (0–44)		109
Thailand	Uncomplicated malaria	90 (9/10)	10.7 (0–47)		22
	Severe malaria	94 (15/16)	9 (0–34)	NS	
	Cerebral malaria	78 (7/9)	18.3 (0–52)	NS	
The Gambia	Uncomplicated malaria	63 (67/106)	8.5 (0–55)		10
	Cerebral malaria	100 (24/24)	28.3 (<1–70)	$p < 0.000001$	
Madagascar[2]	Uncomplicated malaria	67 (6/9)	5 (0–43 overall)		27
	Severe malaria	100 (6/6)	30.5	$p < 0.002$	
	Cerebral malaria	100 (6/6)	19.5	$p < 0.05$	
Papua New Guinea	Uncomplicated malaria	100 (151/151)	8.6 (1–56)		32
	Cerebral malaria	100 (81/81)	9 (1–71)	NS	
Kenya	Mild malaria	63 (34/54)	1 (0–82)		30
	Moderate malaria	83 (53/64)	5 (0–45)	$p < 0.02$	
	Severe malaria	89 (32/36)	7 (0–97)	$p < 0.003$	
Thailand	Uncomplicated malaria	90 (26/29)	5 (0–21)		28
	Severe malaria	100 (21/21)	8 (1–32)		
	Cerebral malaria	100 (10/10)	20 (2–71)	$p < 0.01$	

Table 1. (continued)

Country	Clinical group	Rosetting prevalence %	Rosetting rate[1] %	Significance	Ref.
Kenya[2]	Control	63 (overall)	5.4 (0–25)		31
	Anemic		9.5 (0– > 46)	$p < 0.05$	
	Nonsevere malaria		4.5 (0–26)	NS	
	Cerebral malaria		5 (0–38)	NS	
Gabon	Mild malaria	77 (44/63)	8		29
	Severe malaria	92 (68/74)	16	$p < 0.05$	
Gabon	Overall	65 (135/207)	13 (< 1–88)		67
Cameroon[2]	Uncomplicated malaria	67 (8/12)	6.8 (1–18)		103
	Maternal malaria (peripheral blood)	6 (1/16)	2	$p = 0.001$	
	Maternal malaria (placenta)	0 (0/23)	–	$p < 0.0001$	
Malawi	Uncomplicated malaria	98 (61/62)	15		33
	Severe malaria	97 (62/64)	14.6	NS	
Malawi	Uncomplicated malaria	99 (71/72)	10.6 (0–56)		104
	Moderate malaria	100 (18/18)	8.5 (1–35)	NS	
	Severe malaria	96 (46/48)	10.4 (0–47)	NS	
	Maternal malaria (peripheral blood)	61 (23/38)	2.8 (0–17)		
	Maternal malaria (placenta)	22 (5/23)	0.5 (0–8)	$p < 0.005$	
Kenya	Mild malaria	75 (18/24)	12.2 (0–63 overall)		9
	Severe malaria	86 (30/35)	22.1	$p < 0.05$	

[1] Rosetting rate expressed as percentage of pRBC infected with trophozoite- or schizont-stage parasites binding two or more uninfected RBC.
[2] Studies in which blood samples were cryopreserved upon collection.

Association of Rosetting and Autoagglutination with Severe Disease

The association between rosette-forming capacity and severe malaria, i.e. the easiness with which parasites isolated from patients with complicated disease (cerebral malaria, severe anemia) form more and larger rosettes than those from uncomplicated cases, was first shown 11 years ago in The Gambia, West Africa [7]. In that study, parasites isolated from cerebral malaria patients were found to form rosettes to significantly higher rates than those from mild disease cases (table 1). Moreover, rosette-disrupting antibodies were common in sera of children with uncomplicated malaria whereas sera of children with cerebral disease generally lacked anti-rosetting activity. Standing the proof of time and scrutiny, the correlation between rosetting and cerebral malaria, later extended to severe malaria in general, was confirmed in The Gambia [10] and has been reported from locations differing vastly from a geographical and epidemiological standpoint such as in Madagascar [27], Thailand [28], Gabon [29] and Kenya [9, 30, 31]. Moreover, giant rosette formation has been found to have an even better predictive value for disease severity [9, 10]. A similar case has been made for the autoagglutination phenotype as, in a recent study, autoagglutinates of pRBC were significantly more common (72 vs. 47% of the isolates, $p < 0.02$), and the mean autoagglutination rate higher (6.6 vs. 2.1%, $p < 0.01$) in children with severe disease than in those with mild malaria [8]. However, the reports are not unanimous. Two large studies in Papua New Guinea and Malawi failed to find a significant relationship between rosette formation and clinical outcome [32, 33]. Several factors may contribute to these discrepancies such as the geographical variation in the genetic repertoire and phenotypic characteristics of *P. falciparum*, or the complexity and diversity of pathogenic mechanisms in severe malaria. Additional factors to be considered are general difficulties in the clinical classification of patients (e.g. a proportion of the individuals diagnosed with uncomplicated malaria may have developed severe symptoms if not treated, and consequently their parasites have been wrongly assorted to a nonsevere category), and the extent of treatment prior to hospitalization which may often depend on the availability of antimalarial drugs in local markets. Capricious, unprescribed antimalarial medication could be a confounding factor in the assessment of rosettes since strong and long-lasting inhibition of rosetting, both in vivo and in vitro, has been reported for drugs such as halofantrine, quinine, and artemisin derivatives [34]. Variable frequencies of red cell polymorphisms and/or disorders among the population could also account for contradicting findings. Further, differences in methodology as, for example, the use of cryopreserved isolates in some studies whilst there is evidence that parasites may change their phenotype upon thawing and reculturing [35, 36]. Lastly, two issues should be considered,

Table 2. The molecular basis of rosetting and autoagglutination: receptors, ligands, and serum factors

Binding phenotype	Parasite	RBC receptor	Parasite ligand	Blood serum dependency	Serum factor	Ref.
Rosetting	FCR3S1.2	HS-like GAG	PfEMP1	+	IgM, fibrinogen, albumin and other proteins	40, 43, 61, 63, 68
	FCR3S1.2	Blood group A	PfEMP1	?		45, 47, 49
	TM284	Blood group B	PfEMP1?	?		45, 47
	R29	CR1/CD35	PfEMP1	+	Undefined	54, 55
	MCAMP	CD36?	?	?		57
Autoagglutination	FCR3S1.2, TM284S2	?	?	+	Undefined	5, 69

one epidemiological and another of practical nature, which together could have a decisive impact on the attempts to correlate a phenotypically variable characteristic with disease outcome. Namely, the extent of the clonal (and phenotypic) diversity of the parasite populations in individual infections, and the obligatory sampling of parasites in the peripheral circulation as opposed to the tissues where sequestration occurs. Hypothetically, the larger the number of clones in an infection, the less representative of the malignant sequestered phenotype(s) the isolate might be. In any case, there is a clear need for a better understanding of the many factors determining the development of a *P. falciparum* infection, the molecular mechanisms of pRBC adhesion to host tissues (including rosetting) resulting in sequestration, the possible geographical variation in host cell receptor and parasite ligand expression, as well as the role of the immune response in modulating virulence and disease patterns.

The Molecular Basis of Rosetting: *Multiple Host Receptors*

To date, heparan sulfate (HS)-like glycosaminoglycans (GAGs), ABO blood group antigens, and complement receptor 1 have been identified as erythrocyte receptors that mediate rosetting. Additionally, a role as rosetting receptor has been proposed for the glycoprotein CD36 (table 2).

Revealing observations were made shortly after the discovery of the rosetting phenotype in *P. falciparum*. Heparin was found to disrupt rosettes in vitro, an effect that is dose-dependent and reversible [6, 37]. Other sulfated glycoconjugates, including fucoidan, dextran sulfate, and sulfatide, can also break rosettes efficiently [38–40]. The rosette-forming capacity of the clone FCR3S1 (formerly

named PA1) was found to be sharply impaired by removal of divalent cations, or pH values over 7.2 in the assay medium [37]. By fluorescence and transmission electron microscopy it was shown that heparin binds to the surface of infected erythrocytes forming rosettes, and that rosetting and heparin binding are simultaneously abrogated upon mild protease treatment of the pRBC [41]. Taken together, these findings suggest that carbohydrates might be involved in rosetting, perhaps through a lectin-like binding mechanism. Yet, the sensitivity of rosettes from laboratory strains or freshly isolated *P. falciparum* to heparin and other glycoconjugates was found to be variable. Rosettes of only 50% of the rosetting clinical isolates collected in Gambia were disrupted by 100 IU/ml of heparin [42]. The effect of GAGs, including heparin and other sulfated glycoconjugates, is strain- and isolate-specific [39, 40]. Treatment of uninfected RBC with heparinase prevents rosette formation in some strains of parasites but not in others [40, 43], further indicating the diversity of rosetting receptors. Rosettes resistant to heparin are often efficiently disrupted by other GAGs, which suggests that *P. falciparum* can use a broad spectrum of glycan structures for pRBC–RBC interactions.

Heparin is a GAG which physiologically has been found only in granulae inside mast cells. The closely related GAG heparan sulfate is ubiquitously present on most cellular surfaces and it is composed, as heparin, of long chains of alternating variably modified uronic acid and glucosamine units. HS chains, which are covalently linked to the protein core of proteoglycans, are less sulfated and consequently are less negatively charged than heparin. A fine molecular dissection of the rosetting receptor specificity in a heparin-sensitive clone was achieved by systematically testing the rosette-inhibitory potency of modified heparin preparations, heparin oligomers, and that of di- and monosaccharides derived from heparin/HS [40, 41]. It was established in these investigations that the N-sulfated glucosamine ring is of fundamental importance for rosette-disrupting activity, or conversely rosetting binding, which heavily depends on the N-sulfation at position 2 and to a lesser degree on 6-O- and 2-O-sulfation. Furthermore, a minimal length of 12 sugar monomers in the polysaccharide chain was shown to be required for efficient inhibition of rosette formation. Overall, the data suggest that HS or an HS-like molecule is a rosetting receptor used by *P. falciparum* strains prevalent in nature. Whereas the formal demonstration of the presence of HS or HS-like molecules on the RBC surface still awaits the purification and characterization of the GAG(s) from erythrocytic preparations, HS proteoglycans have been isolated from metabolically labelled human and murine erythroid hematopoietic cell lines in which the amount produced appears to decrease in parallel with their differentiation into mature erythrocytes [44]. Furthermore, a member of the *var*/*P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family of *P. falciparum* adhesins which

specifically binds HS has been cloned and characterized in a rosetting parasite highly sensitive to heparin/HS (see below).

When studying the quantitative binding of RBC of different ABO blood groups in rosettes, it soon became obvious that almost all rosetting parasites tested, laboratory strains and field isolates have a preference for either A or B erythrocytes. This preference is reflected in larger rosettes and stronger pRBC–RBC binding forces when parasites grow in vitro in the preferred blood group [45, 46]. No parasite tested to date exhibits preference for blood group O. Importantly, it has been demonstrated that rosette sensitivity to heparin, HS, or other GAGs, drastically decreases when the parasite is cultivated in its preferred blood group (A or B) as compared to when it grows in blood group O [45, 47]. Thus, A and B blood group carbohydrates emerged as another class of glycan receptors in rosette binding.

ABO antigens are present on proteins (band 3, band 4.5) and glycolipids at the exofacial side of the RBC plasma membrane [48]. A or B antigenic specificity is conferred by the terminal hexose residue of the polysaccharide chain, which is added to the group O determinant ($Fuc\alpha_{1-2}Gal\beta$), e.g. N-acetyl-*D*-galactosamine (GalNAc) to form the A antigen [$GalNAc\alpha_{1-3}(Fuc\alpha_{1-2})Gal\beta$] and *D*-galactose (Gal) to form the B antigen [$Gal\alpha_{1-3}(Fuc\alpha_{1-2})Gal\beta$]. When tested in a rosette-inhibition assay, the A-trisaccharide dose-dependently prevents the reformation of rosettes of blood group A-preferring parasites grown in blood groups A/AB RBC, but not those in parasites propagated in O or B erythrocytes. Similarly, the B-trisaccharide inhibits rosette reformation in B-preferring strains cultivated in blood group B/AB RBC, but not in O or A red cells [45, 47]. The A-trisaccharide binds specifically to the pRBC surface of A-preferring parasites, as assessed by fluorescence [47, 49]. Enzymatic deletion of the terminal A or B sugar moiety renders the treated RBC indistinguishable from O red cells, abolishing A or B parasite preferences, and fully restoring the heparin sensitivity of rosettes [47]. Field data clearly indicate that the rosetting frequency in isolates from individuals with blood groups A/B/AB is enhanced as compared to blood group O [30, 32, 50]. Blood group A has been associated with a higher risk for the development of severe malaria [51, 52] and blood group O with relative protection against cerebral malaria [53]. Laboratory as well as epidemiological data are lacking on rosetting and blood group A_2, a subgroup of blood group A in which O and A determinants are simultaneously expressed. In considering the above findings and the well-established association between rosetting rates and severe malaria, the question inevitably arises whether the encounter of an individual carrying any of the blood groups A or B, with a parasite of the corresponding A or B preference, constitutes a factor of enhanced risk. This question has yet to be addressed in the field. In the meantime, a study recently completed in Kenya found a correlation between clinical outcome and direct binding of a

blood group A-trisaccharide probe to the patient infected RBC [9]. In this study, however, the blood group of the patients and the blood group preference of the parasites were not known.

The picture is thus gradually unfolding of rosetting *P. falciparum* able to use a system of two (or more) layers of glycan receptors on the RBC surface. One comprising few choices, i.e. ABO blood groups, and another of more diversified pRBC–RBC contacts, i.e. GAGs. In any case, the alternatives may not be exhausted since additional rosetting receptors have been reported.

Investigations on the rosetting mechanism of the *P. falciparum* clone R29, a parasite with rosettes relatively sensitive to heparin but insensitive to HS or to heparinase digestion of the RBC, led to the identification of complement receptor 1 (CR1/CD35) as a rosetting receptor. Cultures of this parasite in CR1-deficient RBC exhibit decreased rosetting, and soluble recombinant CR1 partially inhibits rosette formation [54]. The region of CR1 required for pRBC recognition has been mapped to the area of long homologous repeats involved in the binding of the activated complement component C3b [55]. The experimental evidence provided so far does not support a role for C3b or another complement component in the rosetting interaction, but rather suggests a direct receptor-ligand binding. The monoclonal antibody, J3B11, that recognizes the C3b-binding site, can reverse rosettes. This antibody disrupts rosettes of several laboratory-adapted strains and fresh isolates [55], implying that usage of a CR1-dependent rosetting mechanism is not rare in *P. falciparum*. Reportedly, CR1-mediated rosetting is not dependent on the ABO blood group of the RBC [54]. A possible function in the binding for the numerous N-linked oligosaccharides present in CR1 has not been investigated yet. While quantitatively invariant in other cells, expression of CR1 on erythrocytes varies widely, forming a polymorphic trait [56]. It has been found that erythrocytes with a CR1 polymorphism that is common in African-Americans show reduced numbers of the receptor and decreased binding to the parasite rosetting ligand PfEMP1 [55], raising the hypothesis that this polymorphism may have been selected for in malarious regions by providing protection against severe malaria.

CD36 was proposed as a rosetting receptor after experiments performed with the strain MCAMP and some other rare parasites grown in medium supplemented with fetal calf serum showed that rosettes could be reversed/blocked by anti-CD36 monoclonal antibodies or by a fairly crude preparation of soluble CD36 [57]. Enrichment of *P. falciparum* cultures for adhesion to CD36 customarily upregulates CD36-mediated cytoadherence and binding to the immobilized receptor. Rosette-forming parasites are not enriched but rather selected against by these procedures [5, 6]. Moreover, rosettes of MCAMP are not disrupted by cytoadherence-blocking fusion proteins or peptides representing the CD36-binding region of the PfEMP1 expressed by this parasite [Baruch D, personal

commun.]. How to reconcile these apparently contradictory data is not immediately evident. Cytoadherence to CD36, which is the most widely distributed binding property of *P. falciparum*, and the extremely uncommon rosetting activity sensitive to anti-CD36 antibodies could be mediated by distinct parasite ligands or different sites in the same ligand recognizing different regions or forms of the CD36 protein. Whether CD36 is present, or in which form it is expressed on the surface of normal adult RBC, is not clear. Decreasing as the erythroblast differentiates into reticulocyte, the expression of CD36 has been thought to cease upon further maturation to erythrocyte [58, 59]. In one report notwithstanding, CD36 was detected on the surface of mature RBC, although at very low levels [60]. In conclusion, an involvement of CD36 in rosetting, in particular its suggested direct role as a receptor, has yet to be firmly established.

Several Serum Proteins

The finding that immunoglobulins are bound in the fibrillar strands bridging pRBC and RBC in rosettes [24, 61], together with previous observations on the pro-coagulant activity of *P. falciparum*-infected RBC [62] and the capacity of sera from some patients with cerebral malaria to agglutinate autologous uninfected and infected erythrocytes [10], generated interest in the role of serum proteins in rosetting. Subsequent studies established that components of human serum are crucial in the formation of rosettes by laboratory-adapted as well as freshly isolated parasites [61, 63, 64] (table 2). The varying degree to which rosetting rates and rosette sizes are affected among different parasites after serum depletion in the culture medium and the finding of strains with serum-insensitive rosettes are likely to reflect the multiplicity of ligand receptors and mechanisms in pRBC–RBC adhesion. How serum factors act in rosette formation/stabilization is not yet understood but several proteins known to participate in rouleau formation of normal erythrocytes have been found to play a role, notably IgM, IgG, fibrinogen, albumin, and probably other proteins (table 2).

The occurrence of an active deposition of normal, malaria-unrelated immunoglobulins (Ig) on the surface of the *P. falciparum*-infected RBC has been known for some time [65, 66]. More recent investigations demonstrate that pRBC of a majority of rosetting strains and fresh isolates, and also those of some non-rosetting parasites, bind naturally occurring IgM and/or IgG independent of epitope specificity [24, 67]. This binding increases with parasite maturation, matching the expression of parasite antigens on the infected cell surface and the onset of adhesive phenotypes, such as rosetting and adhesion to various receptors [3]. Transmission electron microscopy (TEM) shows that the focal points for concentration of immunoglobulin bound to the pRBC surface are the knobs, where the antibodies seem to be complexed in the fibrillar structures formed between infected and uninfected RBC [24]. By indirect immunofluorescence

anti-IgM or anti-IgG antibodies label the corresponding immunoglobulin bound to the pRBC surface, and simultaneously disrupt the rosettes of parasites with the IgM- or the IgG-binding phenotype, respectively [24, 55, 68]. Rosettes of an IgM-binding strain are dose-dependently reformed by addition of human myeloma IgM to a parasite cultured in IgM-deficient medium [24]. Immunoglobulin-binding activity has been mapped to the cysteine-rich interdomain region (CIDR)1α domain of the PfEMP1 expressed by the *P. falciparum* clone FCR3S1.2, a parasite of the IgM-binding phenotype [49], and to the Duffy binding-like (DBL)2β domain of PfEMP1 in TM284S2, an IgG-binding clone [69]. Thus, the current evidence strongly suggests the participation of immunoglobulins in the rosetting molecular complex, i.e. the parasite ligand PfEMP1, the host receptor(s), and the accessory molecules maintaining the infected and uninfected RBC together.

Besides IgM and IgG, also purified fibrinogen, albumin, and other serum proteins have been found to promote rosetting [61]. As for immunoglobulin binding, the positive effect of fibrinogen on rosetting can be reversed with anti-fibrinogen antibodies, and fibrinogen can be detected on the pRBC surface of some parasite strains by TEM. Furthermore, in cultures devoid of serum proteins, near to 100% of the rosettes can be restored by the addition of IgM, fibrinogen, and albumin to the culture medium [61]. In another study, at least two unidentified serum components, besides IgM, were required for the reformation of rosettes of regular size and stability [63]. Rouleau formation of normal erythrocytes depends mainly on immunoglobulins and fibrinogen, while albumin has a synergistic action on the aggregation [70]. It is possible that rouleau formation is locally induced at knobs by enhanced exposure of yet uncharacterized erythrocytic rouleau receptors, and/or direct triggering by the initial binding of immunoglobulins to PfEMP1. The role of immunoglobulins, fibrinogen, albumin and perhaps other serum proteins in the formation of rosettes can be the direct pRBC–RBC bridging and/or the stabilization of the molecular interaction where the parasite ligand PfEMP1 plays a central function. Further studies are granted to elucidate the overall contribution of components of human serum to the various rosetting mechanisms, to establish its importance in the highly diverse natural parasite populations, and to determine whether polymorphisms in serum molecules can result in interindividual differences to support rosette formation. Such polymorphisms might affect the likelihood of developing severe *P. falciparum* malaria.

One Parasite Ligand Family

The PfEMP1 family of adherent proteins encoded by the *var* genes is thought to have a central role in malaria disease [71–73]. There are over 50 different *var* genes per haploid genome located primarily at chromosome ends and less

frequently in central clusters [74, 75]. PfEMP1 proteins are expressed in a clonally variant pattern at the pRBC surface where they are concentrated at the knobs [76]. Whereas each infected erythrocyte appears to express a single PfEMP1 variant [75, 77], the expressed copy can change in the next intraerythrocytic cycle, i.e. PfEMP1 accounts for at least part of the antigenic variation of intraerythrocytic stages of *P. falciparum* [4, 78, 79]. The extracellular segment of PfEMP1 comprises an N-terminal head structure always composed by the N-terminal segment (NTS) starting at the initiating methionine, a semiconserved DBL1 domain and a less conserved CIDR1, followed by a variable number of polymorphic DBL domains interspersed with highly diverse sequences and the domains C2 and CIDR2, not present in every variant. Five sequence classes of DBL domains (α, β, γ, δ, ε), and three CIDR domains (α, β, γ), have been identified so far [80]. PfEMP1 is anchored in the pRBC by a trans-membrane segment and a relatively conserved intracellular acidic C-terminal segment (ATS) encoded in the second exon of the *var* genes. The CIDR and the DBL domains function as adhesive modules in the PfEMP1 molecule, mediating the interaction with cytoadherence and rosetting receptors. The CIDR of most parasites binds to CD36 and less commonly to CD31, while some DBL domains may bind either to ICAM-1, chondoitin sulfate A (CSA), or probably also CD31 [49, 81–84].

Members of the *var*/PfEMP1 family have been identified as parasite ligands for rosetting (table 2). By comparing *var* transcripts from rosetting and non-rosetting R29 parasites, Rowe et al. [54] found a *var* mRNA unique to rosetting R29. Cloning, heterologous expression in COS7 cells, and functional analysis of the individual four DBL domains and the CIDR1 of this *var* gene (R29R+*var*1), demonstrated that DBL1α mediates binding to erythrocytes in a CR1-dependent fashion [54]. The DBL1α of a non-rosetting parasite, as analyzed in the same expression system, did not support RBC binding. Following a distinct approach, Chen et al. [43] used micromanipulation techniques to select individual pRBC involved in rosettes of FCR3S1.2, a parasite with rosettes highly sensitive to heparin, and single-cell RT-PCR to identify the *var* gene (FCR3S1.2*var*1) expressed by this clone. The solubilized FCR3S1.2*var*1 PfEMP1 polypeptide specifically binds heparin [41]. This binding is efficiently competed by HS but not affected by CSA. When expressed as a GST-fusion protein, the DBL1α of FCR3S1.2*var*1 disrupts rosettes of FCR3S1.2 and binds heparin and HS [41, 43]. Recombinant DBL1α also binds to uninfected RBC and to CHO cells expressing HS proteoglycans on their surface, but not to erythrocytes or CHO cells pre-treated with heparinase [41, 49]. The DBL1α, CIDR1α and DBL2δ domains of FCR3S1.2*var*1 have been transfected in COS7 cells where they are expressed on the cell surface. Only the COS7-DBL1α transfectans displayed heparinase-sensitive binding of blood group O RBC, as well as heparinase-insensitive binding

of blood group A erythrocytes [49]. However, binding of IgM immunoglobulins, which are essential for the formation of FCR3S1.2 rosettes (see above), occurs at the CIDR1α domain. Interestingly, eight consensus sequences composed of clusters of positively charged amino acids, which have been described as potential binding sites for negatively charged GAGs [85, 86], are contained in the DBL1α of FCR3S1.2*var*1. An additional five of such motifs are found in the CIDR1α, and another five in the DBL2δ domain. The predominance of putative GAG-binding sequences in the head structure of FCR3S1.2*var*1, and particularly in DBL1α, is consistent with the high sensitivity of FCR3S1.2 rosettes to heparin and with the binding of this GAG, as well as that of HS and HS-like receptors, to the DBL1α domain. Altogether, the data indicate that the rosetting ligand function of PfEMP1 resides in certain DBL1α domains, with some participation of CIDR1, the other major domain in the head structure of the molecule. A vast majority of the head structures characterized in the PfEMP1 proteins sequenced thus far comprise a DBL1α in tandem with a CIDR1α. These two domain types (DBL1α and CIDR1α) do not seem to occur anywhere else in the molecule [80]. This strong conservation of domain association in the head structure of PfEMP1 is likely to reflect functional significance, e.g. for folding, transport and/or binding activity, including rosetting pRBC–RBC interactions.

Any given rosette-forming parasite left to grow in the absence of selective pressures other than in vitro cultivation eventually reaches its characteristic level of rosetting, or rosetting rate 'at equilibrium', which may vary widely from parasite to parasite, typically from less than 5 to over 60%. The cloning of the *var* genes and the recent identification of some of their clonally variant protein products as rosetting ligands make it possible to interpret this initially puzzling observation in terms of the inherent forward- and back-switching rates of the *var*/PfEMP1 gene(s) conferring the rosetting phenotype to a particular parasite population.

A role in rosetting binding for parasite proteins other than members of the *var*/PfEMP1 family is not supported by the experimental data available at present. Polypeptides of relative low molecular mass were collectively named rosettins after their original identification on the surface of pRBC bearing parasites of the rosetting phenotype [87]. Later on, a cluster of parasite-derived clonally variant polypeptides 30–45 kD in size, which could be radio-iodinated on the pRBC surface of rosetting and non-rosetting clinical isolates and laboratory-adapted parasites, was shown to be identical with the rifins, proteins encoded by the multi-gene family *rif* of *P. falciparum* [88–90]. Rifins are transported to the surface of the host erythrocyte where they can be detected, as for PfEMP1, 14–16 h after parasite invasion, coinciding with the onset of functional and antigenic changes on the pRBC. Rifins are prominently expressed by wild parasites and are antigenic in natural infections [89]. At the time of this writing, the biological function of rifins has not been elucidated.

Autoagglutination Mechanisms

The nature of the molecular mechanisms responsible for the autoagglutination of infected erythrocytes is mostly unknown. In exploring the possible role of host molecules, experiments performed with the rosetting and autoagglutinating clones, FCR3S1.2 and TM284S2, suggest that pRBC–pRBC binding depends, at least in part, on blood serum components. Autoagglutinate formation was inhibited when serum was removed from the culture medium [unpublished observations]. Even less is known about the parasite ligands involved. In a study showing that the multiple adhesive phenotypes of the *P. falciparum* clone FCR3S1.2, including autoagglutination, immunoglobulin binding and cytoadherence to two endothelial receptors, are linked to rosetting and to the expression on the pRBC surface of a PfEMP1 with multiple binding specificities, indirect evidence was generated suggesting that PfEMP1 may participate in the formation of autoagglutinates [5]. Nevertheless, the experimental approach used, i.e. enzymatic cleavage of polypeptides on the pRBC surface, does not make it possible to exclude the involvement of other parasite molecules. The finding of co-expression of rosetting and autoagglutination in the clones FCR3S1.2 and TM284S2, as well as in other parasites, should not be generalized. Although rosetting/autoagglutinating *P. falciparum* certainly exist [5, 69], those that autoagglutinate, but do not form rosettes may be common. Upon scoring of these phenotypes in parasites isolated from 113 malaria-ill children, autoagglutination was not correlated with rosetting or giant rosette formation [8]. An element of caution must be introduced here since the involvement of pRBC in rosettes could conceivably lessen their odds to come in contact with other pRBC, leading therefore to an underrating of the double positive (rosetting/autoagglutinating) parasites. Until now, every adhesive phenotype studied in asexual stages of *P. falciparum* has proved to feature multiplicity and redundancy of mechanisms. It remains to be seen whether autoagglutination is the norm and not the exception.

Recently, evidence has been put forward showing that seeming autoagglutinates of parasite clones/strains such as ITO/C10 and MCAMP, and some clinical isolates, are indeed aggregates of pRBC and platelets where the latter form bridges between the infected erythrocytes [91]. The formation of pRBC aggregates held together by platelets, a binding phenotype described already several years ago as a characteristic of some parasites, e.g. MCAMP, but not of others, e.g. DD2 [92], has now been renamed ‘platelet-mediated clumping’ to distinguish it from pRBC–pRBC binding, i.e. autoagglutination. The CD36 glycoprotein expressed on platelets seems to play a role in the formation of clumps as these are not formed with platelets that do not express the receptor, or in the presence of an anti-CD36 mAb. Clumping of pRBC requires the expression of CD36 on platelets, but most parasites that bind to this receptor do not form clumps,

implying the existence of distinct mechanisms of binding to CD36, and/or the participation of additional host molecules. Although parasites expressing the clumping phenotype were found to occur more often (8.1 vs. 3.6%, $p < 0.01$) in isolates from children with severe malaria than in those from uncomplicated cases [91], the prevalence of platelet-mediated clumping (and autoagglutination) in natural infections remains to be investigated in depth. Clumping of infected RBC was not associated with rosetting.

RBC Polymorphisms and Rosetting

Red cells from individuals with polymorphic traits such as sickle cell or α- and β-thalassemia, which are known to protect against severe malaria, and in particular against the cerebral form of the disease [93, 94], form smaller rosettes than ordinary RBC. The binding forces between the infected and uninfected erythrocytes of these rosettes are weaker than those holding together rosettes in parasites cultured in normal red cells [46]. Moreover, low RBC volume, a common denominator of the various thalassemia syndromes, hemoglobin E disease and iron deficiency anemia, is associated with reduced rosette-forming capacity. It has been speculated that impaired erythrocyte rosetting is a general mechanism by which various red cell traits, deleterious or not, may confer protection against *P. falciparum* malaria by abating the vascular occlusion leading to severe disease [46]. A protective mechanism which in turn could explain why certain globin chain disorders such as HbS and the α- and β-thalassemias, polymorphisms of CR1 with reduced receptor expression, or the O blood group allele, reach considerable high frequencies in some regions of the world presently or formerly endemic for malaria. This provocative hypothesis can today be revisited in light of the recent finding of parasites forming large giant rosettes predominating in severely ill malaria patients [9], but should also be weighted against the fact that the occurrence of rosettes in vivo has not yet been unequivocally shown. A direct role of rosetting in vaso-occlusion is therefore yet to be proven.

Rosetting, Autoagglutination, Multiadhesion, and Severe Malaria

The term 'severity' in *P. falciparum* malaria encompasses a wide spectrum of clinical manifestations which may vary from one endemic region to another, or present differently depending on the age and immunity status of the patient. Cerebral malaria, severe anemia, and severe respiratory distress, among other complications, account for a large proportion of the mortality in malaria [95, 96; reviewed elsewhere in this issue]. Cerebral malaria is a heterogeneous clinical syndrome that has deep coma as its defining feature. Among the various existing

theories on the pathogenesis of cerebral malaria, the one that is central for the discussion on the possible role of rosetting and autoagglutination in disease causation proposes that sequestration and accumulation of infected erythrocytes in the microvasculature leads to impaired blood flow, cerebral anoxia and acute local toxic/inflammatory reactions ultimately resulting in metabolic and neurological derangement [95, 97, 98]. Postmortem histopathological studies suggest that cytoadherence of infected erythrocytes to the capillary endothelium is directly related to the triggering and development of severe malaria with cerebral involvement [16, 99, 100]. However, sequestration and binding of infected RBC to vascular endothelia is a feature of all wild *P. falciparum* isolates and, with the exception of the receptor preference for CSA and hyaluronic acid by parasites causing placental infection [101, 102], no correlation has so far been found between pRBC cytoadherence in vitro and clinical severe malaria. It has long been speculated that parasites isolated from peripheral blood may not necessarily reflect the phenotypic profile of those deeply sequestered, and it is possible that endothelial receptors critical for severe disease have been overlooked up to now. Indeed, an example of compartmentalization of phenotypically distinct parasite populations within the human host can be found in maternal malaria, as the association with in vitro receptor specificity only becomes evident when the binding phenotype is assessed in parasites collected from placental blood or flushed out from the syncytiotrophoblast cell lining, but not in parasites sampled in the peripheral circulation of the same patients [101, 102]. Compared with other vascular beds, parasitized erythrocytes infecting and sequestering in the placenta must withstand considerably slower blood flow and reduced wall shear forces. Current data suggest that rosetting may not play a role in placental sequestration since the phenotype is uncommon in *P. falciparum* recovered from this organ (table 1) [103, 104]. On the other hand, parasites forming rosettes, giant rosettes, and autoagglutinates occur more frequently in severe malaria cases as studies throughout Africa and southeast Asia show. Cytoadherent parasites with the bonus capacity to form large aggregates of infected and uninfected RBC could conceivably cause further impairment of blood flow and ultimately, vaso-occlusion. This suggestion is supported by previous ex vivo studies under flow conditions showing that rosetting/cytoadherent *P. falciparum* caused an increased blockade of the circulation as compared to parasites that bound only to endothelium [105]. A TEM study of a brain autopsy specimen from a victim of cerebral malaria revealed infected erythrocytes binding to endothelial cells also engaged in typical rosetting adherent interactions [24]. Parasites with a multi-adhesive phenotype have been generated in the laboratory by cloning and subcloning the rosetting strain FCR3S. The pRBC of some of these clones (one of them FCR3S1.2) not only bound to RBC via an HS-like GAG and the blood group A antigen, but also formed autoagglutinates, adhered

to the endothelial receptors CD31 and CD36, and bound IgM [5]. Other examples of multi-adhesiveness have also been found among nonrosetting parasites which adhere in vitro to both CD36 and ICAM-1. These two receptors, when available simultaneously on the same cell, can act synergistically to potentiate the adhesion of infected cells [106, 107]. Furthermore, using a multi-adhesion score, Heddini et al. [9] found that the ability of pRBC to simultaneously form rosettes and giant rosettes, to bind immunoglobulins to the CD31 and CD36 receptors was significantly higher ($p < 0.0005$) in *P. falciparum* isolated from children with severe symptoms than in parasites from mild cases. Thus, parasites with a broad adhesive capacity, in particular those coexpressing rosetting and/or autoagglutination and clumping with other cytoadherent and binding phenotypes, might be the ones involved in bringing about severe malaria pathology.

Closing Remarks

Progress in identifying malaria genes and antigens is continuing at a pace that no longer can be described as just increasing but rather as an explosive-like expansion. The ongoing effort to sequence and annotate the complete *P. falciparum* genome has already resulted in the discovery of a large number of new genes and open reading frames. This is expected soon to be translated into a decisive acceleration in the comprehension of the molecular background of host–parasite interaction, parasite virulence and mechanisms triggering severe disease. Major questions regarding rosetting and autoagglutination remain to be addressed. We still know very little about the parasite and host molecules operating in the formation of autoagglutinates. The data are inconclusive on the list and interplay of ligands, receptors and other factors in rosetting. Genome-scanning procedures may offer a suitable approach for the systematic identification of the molecular players in parasite cytoadhesion. A relationship between the rosetting phenotype and disease severity does exist. Similar correlations have been described for autoagglutination and platelet-mediated clumping. At present it is not clear whether these associations are causal, or a reflection of another binding phenotype. Finding out whether rosetting, autoagglutination and clumping occur in vivo, and elucidating any possible connection between these phenotypes are obvious priorities. A deeper understanding of the mechanisms involved may lead to new strategies for therapy and prevention.

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Sexual and Sporogonic Stage Antigens

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Introduction

The sexual and sporogonic stages in the lifecycle of *Plasmodia* start with gametocytogenesis in the vertebrate host and are completed in the *Anopheles* mosquito with the appearance of sporozoites in the salivary glands [1].

Knowledge of sexual stage antigens is important in understanding the biology of the *Plasmodium* lifecycle and identification of potential targets for interruption of malaria transmission. Reduction or blockade of transmission can result in malaria eradication or reduction of the disease burden depending on the transmission intensity in the geographical area [2–5].

Sexual differentiation is a multi-factorial process that involves distinct morphological stages and biochemical events triggered by innate and environmental factors [6–10]. It is initiated with the commitment of young trophozoites to become gametocytes in the vertebrate host [9]. Male and female gametocytes of *Plasmodium falciparum* will pass within the same red blood cell through a series of five distinct morphological stages until full maturity is reached (fig. 1) [8]. Subsequently, mature stage-V gametocytes will circulate in the blood stream ready for sporogony after ingestion by the mosquito. Within the mosquito midgut, a number of stimuli will trigger emergence from the red blood cell and differentiation into motile male and female gametes followed by fertilization and zygote formation. This process takes place within 2–3 h. Transition will follow into motile ookinetes which will pass through the midgut wall to become oocysts under the basal lamina of the midgut wall.

Thousands of sporozoites are formed which will at last migrate to the salivary glands of the mosquito.

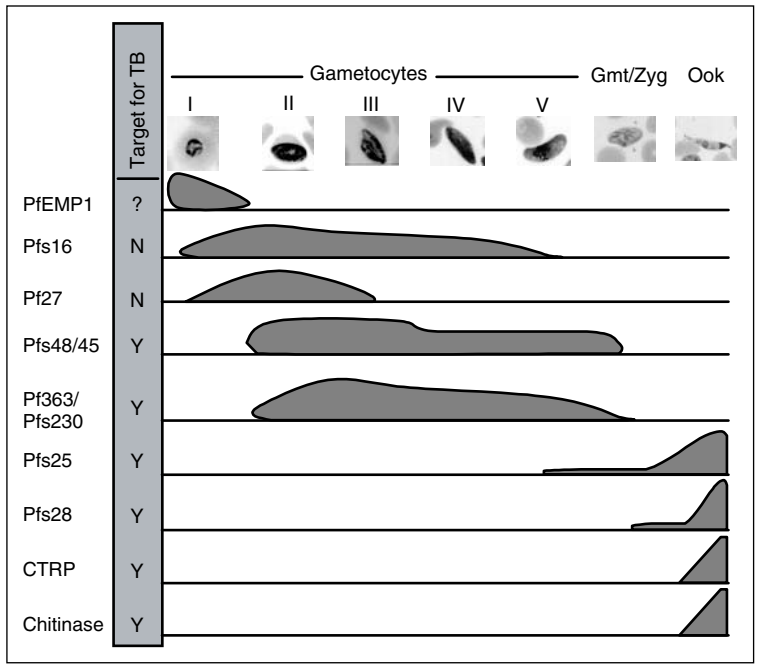

Fig. 1. Selection of *P. falciparum* antigens expressed in sexual- and sporogonic stages. Gmt = female gamete; Zyg = zygote; Ook = ookinete; TB = transmission blockade.

Sexual and sporogonic stage antigens, primarily identified in human malaria parasites (*P. falciparum*, *Plasmodium vivax*), avian malaria parasites (*Plasmodium gallinaceum*) and rodent malarias (*Plasmodium berghei*, *Plasmodium yoelii*) have been the subject of previous reviews [4–6, 11].

This chapter will primarily concentrate on sexual and sporogonic stage antigens of *P. falciparum* with some reference to *P. vivax*. Information on *P. vivax* is limited and the vast majority of studies have been carried out in *P. falciparum* because of its lethal disease impact and the technological possibility of cultivating the complete *P. falciparum* sexual cycle in vitro.

Antigens Expressed on Surface Membrane of Red Blood Cells with Gametocytes

Stage I–IV gametocytes sequester during maturation in the peripheral vasculature and re-enter the circulation as mature stage V after 8–10 days (fig. 1). Asexual parasites and gametocytes show different organ retention [12]. Spleen

and bone marrow are sites of prevalence for gametocytes but the molecular mechanisms that regulate this organ retention are little studied.

The primary mechanism for sequestration is cytoadherence as has been shown for asexual parasites involving *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) and possibly molecules of other multi-gene families [for review see, 13].

Recent evidence suggests that red blood cells with early gametocyte stages (I, IIa) do express PfEMP1 which is genetically linked to gametocytogenesis on the parasite's chromosome 9 and mediates binding to CD36 [10]. This expression is in agreement with the presence of knobs on the infected red blood cell membrane which disappear in stage IIb [14]. Other molecules are most likely expressed on later gametocyte stages. The difference in sequestration sites between asexual and sexual stages may be explained by the involvement of additional adhesion molecules. Involvement of altered band 3 has been suggested but remains controversial [10, 15].

A number of cell receptors of more mature gametocytes which are independent of CD36 and modified band 3 have been suggested in in vitro studies with transformed bone marrow cells [15]. Binding is of low avidity but may be sufficient as shear forces in the bone marrow pools are low.

Is there evidence for specific immunity that interferes with gametocyte maturation from natural infections? Production of gametocytes may be regulated by control of asexual parasitemia and be associated with the development of a repertoire of anti-PfEMP1 antibodies. Highest prevalences of gametocytes are, indeed, observed in non-immune young children and associate with the initial peak waves of asexual parasitemia [1]. The fraction of asexual forms transforming into sexual stage stages may be passively linked to the declining asexual parasitemias due to the development of strain specific antibodies. Alternatively, gametocyte maturation may be actively inhibited by interference with sequestration of early stages to the endothelial lining. Immune precipitation studies suggest that expression of *var* genes is identical in asexuals and gametocytes [16]. Waves of asexual parasitemia may therefore be linked to gametocytogenesis by expression of similar PfEMP1 variants.

Pfs16

A small fraction of asexual parasites and most likely the progeny of a single schizont commit to a high albeit not complete degree of sexual or asexual stage differentiation [6, 7, 9, 17].

Pfs16 expression immediately follows the invasion of a red blood cell in the sexually committed ring stage parasites (<24 h after infection) and is not

detectable in merozoites which are still maturing in schizonts. Expression continues from stage-I gametocytes throughout maturation in male and female gametocytes and in macrogametes although at a lower level from stage-V gametocytes onwards [18]. So far, Pfs16 expression is the earliest event in the sexual differentiation process [19].

Pfs16 has been sequenced as a repeatless and uninterrupted gene showing characteristics of an integral membrane protein [20]. Although initially described as a protein that was also detectable on sporozoites, specific anti-Pfs16 antibodies only react with gametocyte and gamete membranes [20, 21]; in fact, Pfs16 is transported over the parasitophorous vacuole and subsequently forms an intrinsic component of the parasitophorous vacuole membrane of gametocytes [20–22]. Remnants of this membrane on macrogametes are most likely responsible for the anti-Pfs16 immune reactivity with these parasites.

The position of Pfs16 on the parasitophorous membrane explains why anti-Pfs16 antibodies do not block transmission disqualifying this molecule as a transmission-blocking vaccine candidate [23]. Anti-Pfs16 antibodies, however, are highly prevalent in endemic sera from various geographic origins [23, 24]. The function of Pfs16 for gametocyte development remains elusive.

Pfg27

Pfg27 is abundantly expressed throughout the cytoplasm early after sexual commitment at about 30 h after merozoite invasion of the erythrocyte [11, 18, 25]. Almost 5–10% of the total cellular protein of stage-II gametocytes is represented by Pfg27 but the protein is not expressed on the surface membrane [18, 25].

Disruption of the Pgf27 gene results in a loss of sexual phenotype of *P. falciparum* parasites while asexual replication is unaffected [26]. Pfs16 expression of Pfg27/KO parasites is low while Pfs230 is absent; these data suggest that commitment for sexual stages occurs with abrogation of further development into both male and female gametocytes. A monoclonal antibody against a Pfg27 has been shown to reduce transmission but Pfg27 candidature as a transmission-blocking vaccine target remains highly questionable [27]. Firstly, Pfg27 is only intracellularly expressed and indeed the monoclonal antibody did not react with the parasite membrane. Secondly, the antibody is cross-reacting with sequences of Pfs48/45 and Pfs230 and thus not highly specific for Pfg27.

Specific immune responses to Pfg27 after a *P. falciparum* infection are abundantly present in malaria endemic populations which makes this antigen a potential target for sero-epidemiological studies on sexual stage exposure. A promiscuous T-cell epitope that recognizes at least five different major histocompatibility complex class-II haplotypes has been identified [28].

Table 1. The 6-cys domain superfamily of *Plasmodia*

	Family		Putative anchor sequence
	P48/45	P230	
P12	+		+
P36	+		
P41	+		
P47	+		+
P48/45	+		+
P230		+	
P230II		+	

The Six-Cys Domain Superfamily

A six-cys domain superfamily specific for *Plasmodia* has been identified which comprises of a P230 family and a P48/45 family [29] (table 1).

The characteristic cysteine-rich domain structure (approximately 120 amino acids in length) of Pf12, Pfs48/45 and Pfs230 was sufficiently conserved to reveal other members of the superfamily by genome analysis [30, 31]. Based on the expression and function of this family one can assume that the process of gamete and zygote formation is highly conserved in *Plasmodia*. Although the family members share the characteristic 6-cys domain structure, amino acid identity is low.

Pfs230 and Pf230-II genes are localized in proximity similar to Pb48/45 and Pb47 in the *P. berghei* genome [32]. Pfs230 and Pfs48/45 are expressed in the surface membrane of the gametes and form a complex (see below) [33–36]. It needs to be determined whether other members with GPI anchors are integral proteins of the surface membrane and whether they form complexes with family members that lack the anchor to provide membrane localization. A structural model was developed for Pfs230, Pfs48/45 and Pf12 with domains depending on a disulfide-binding pattern with repetitive motifs and internal and outfacing loops [30].

Pfs48/45

Surface radio-iodination of gametes has revealed the presence of a protein doublet of 48 and 45 kD molecular weight (non-reducing conditions) on the surface of gametes and zygotes [37]. Pfs48/45 is synthesized exclusively during gametocytogenesis (from day 2 onwards) and is absent in gametes [33, 38].

The Pfs48/45 gene was cloned by Kocken et al. [38] showing a deduced amino acid sequence for a 448-amino acid protein [38]. Pfs48/45 mRNA is produced immediately after invasion of the sexually committed merozoite into the

newly infected erythrocyte [39]. Two mRNA species of 2.3 and 2.8 kb, respectively, are found in gametocytes but the function of these 2 transcripts is presently unknown. Under reducing conditions only a single band of 57 kD is found while under non-reducing conditions Pfs48/45 is seen as a doublet protein of 48 and 45 kD. A corresponding doublet has also been observed in *P. gallinaceum* and *P. berghei* gametocytes [32, 40]. The doublet protein has a hydrophobic character [35]. Differences between the 45- and 48-kD protein as seen on Western immunoblots are not related to charge or glycosylation despite the presence of N-glycosylation sites [33, 41]. Interestingly all available monoclonal antibodies (mAbs) recognize Pfs48/45 as a doublet. Differences between 45 and 48 kD may relate to conformation but the true background remains to be elucidated.

Genetic polymorphism of Pfs48/45 is very limited; a single nucleotide change at position 762 dictates a lysine or asparagine at position 254 which serologically defines epitopes IIa and IIc [42, 43]. Interestingly, parasites with the IIa epitope of Pfs48/45 are predominantly found in south-east Asia while the IIc epitope is more prevalent in parasites of African origin [44].

Five distinct B-cell epitopes with a subdivision for epitope II (IIa–IIc) have been defined based on binding studies with a panel of Pfs48/45-specific mAbs [45–49]. Epitopes I–III are conformational and epitope IV is linear. For epitope V both linear- and conformation-dependent mAbs have been described [46, 49]. With the exception of epitope III, mAbs to all other epitopes are able to block transmission in the membrane-feeding assay [37, 46, 48, 50, 51]. The limited persistence of Pfs48/45 in macrogametes/zygotes and complete block of ookinete formation by anti-Pfs48/45 mAbs indicate that transmission is blocked at the level of fertilization and zygote formation [37]. Although present in male and female gametocytes and macrogametes, the presence of Pfs48/45 on the surface of *P. falciparum* microgametes is not established. There is evidence, however, that Pb48/45 is on the surface membrane of *P. berghei* microgametes [32]. Recent studies show a pivotal role in zygote formation for Pfs48/45 and Pbs48/45 [32]. P48/45 genes were replaced by disrupted genes in both *Plasmodia* species. Gametocytogenesis and gametogenesis proceeded normally but development of the mosquito stages was strongly reduced, though not completely absent. It was shown that Pbs48/45 male but not female gametes were incapacitated for zygote formation [32].

Pfs48/45 is a target for natural immune responses as demonstrated by the presence of specific antibodies in field sera where anti-Pfs48/45 antibodies are produced immediately after exposure to gametocytes [47, 52, 53]. There is no evidence that responsiveness against either Pfs48/45 or one of its epitopes is linked to particular HLA haplotypes but antibody prevalences in the population can be low [52, 54, 56]. Data from Papua New Guinea show that sero-conversion increases with age suggesting that immunological memory develops [56]. Increasing anti-Pfs48/45 antibody titers are also observed after long exposure

to gametocytes in Dutch expatriates [Roeffen WF, unpublished observation]. Antibody titers for the individual epitopes show considerable differences between and within field samples [47].

The question whether anti-Pfs48/45 antibodies in natural sera can block transmission remains unresolved. Previous studies have shown a significant or fair association between the presence of anti-Pfs48/45 antibodies and transmission blocking activity [47, 55], although this was absent or less convincing in other studies [53, 57]. Differences in methodology for antibody detection and in malaria endemicity of the study area may be responsible for this controversy. Differences have been observed in the serum capacity to cross-block transmission of *P. falciparum* isolates from the Gambia and Cameroon. Strain specificity was considered as an explanation but interpretation of the results is hampered by the low mosquito infection rates in natural infections [58]. One solution may be to dissect larger numbers of mosquitoes having higher oocyst rates and oocyst loads.

It will be difficult to resolve the question of anti-Pfs48/45-mediated transmission blockade in field sera until sufficient quantities of natural conformers of protein or fragments of Pfs48/45, which can be used for antibody absorption and purification, become available. Recombinant Pfs48/45 is immunogenic, but so far antibodies against these recombinant products neither recognize native Pfs48/45 nor block transmission [41]. Studies on correctly folded Pfs48/45 protein or fragments are in progress.

Pfs230

Pfs230 was originally identified as a surface protein by radio-iodination of the gamete surface membrane and immunoprecipitation with field sera [37]. The production of the protein starts from day 2 after gametocytogenesis onwards similar to Pfs48/45, but translation of the gene is already initiated after entrance of committed merozoites into new erythrocytes [37, 39]. The gene contains a 9.4-kb open reading frame that predicts a 363-kD protein [31]. The Pfs363-kD protein is a precursor protein that is processed upon activation of the gametocytes to a 300- and a 307-kD molecule which are membrane bound [34] (fig. 2). Thus Pfs230, which is a hydrophilic protein, refers to 300/307-kD molecules on the gamete membrane forming a stable complex with Pfs48/45 with a stoichiometry of 1:1, but Pfs48/45 and Pfs230 are also partly present in an uncomplexed form [4, 36, 59]. Only the processed derivatives of a 363-kD molecule (300 and 307 kD) are retained in the membrane (fig. 2) [34, 60]. The presence of non-complexed Pfs48/45 may depend on this processing of Pf363. Since Pfs48/45 gene knock-out parasites produce Pf363 but do not express Pfs230 on their surface, Pfs48/45 may be essential for the membrane localization of Pfs230 or even responsible for processing of the 363-kD fragment [Eling W, unpublished]. Further studies are needed to show whether

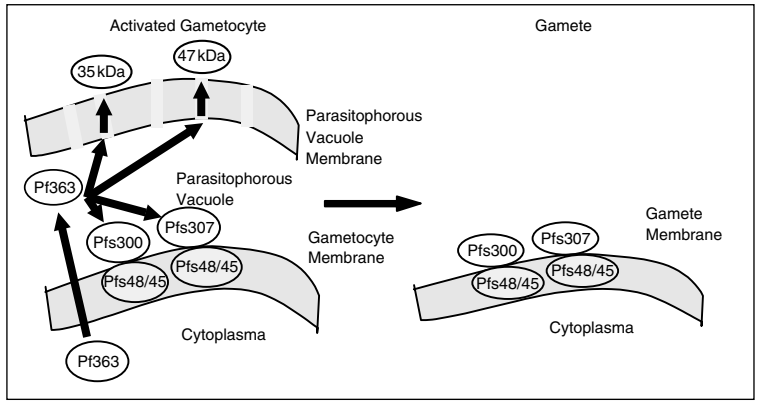

Fig. 2. Schematic diagram of the processing of Pf363 to membrane-bound Pfs300/Pfs307 (Pfs230) and interaction with Pfs48/45 during gametogenesis.

the expression, processing and localization of Pfs48/45 and Pfs230 occur in male as well as female gametocytes and gametes.

A panel of mAbs which immunoprecipitated Pfs230, recognized the processed Pf363-kD, i.e. membrane-bound Pfs230, and identified five different epitopes [59]. Subsequent studies showed that mAbs blocked transmission but only in case of complement-fixing isotypes and in the presence of active complement [61, 62]. In vitro studies showed that transmission-blocking mAbs and complement lysed *P. falciparum* macrogametes. This suggests that the mechanism of Pfs230-mediated blockade may be mere antibody binding followed by complement mediated lysis; however, mAbs against Pgs230 of *P. gallinaceum* block transmission in the absence of complement suggesting alternative mechanisms [63].

Pfs230 in gametocytes is immunogenic under field conditions but association with transmission blockade is controversial and studies are difficult to compare [53, 59, 64–68].

Antibodies have been generated against two *Escherichia coli* expressed recombinant Pfs230 peptides (regions C and F) which recognize native Pfs230 [60]. Antibodies against the C region (amino acids 443–1132) were able to reduce but not completely block transmission. In a more recent study subsections of the C region did not induce transmission-blocking antibodies, suggesting that expression of the whole region is important [68].

Pfs25 and Pfs28

Pfs25 and Pfs28 are structurally related proteins primarily expressed on the membrane of post-fertilization stages in the mosquito. They are members of

the P25/28 family which represent proteins with apparent molecular masses of 20–28 kD in a variety of *Plasmodium* species [4, 5, 9, 11]. The typical characteristics of the family include a hydrophobic leader sequence followed by epidermal growth factor (EGF)-like domains and a C-terminal GPI-anchor sequence. Pfs25 has been the most studied sexual and sporogonic stage *P. falciparum* protein to date particularly in view of its prime candidature as a transmission-blocking vaccine [see Kaslow, pp 287–307]. Levels of Pfs25 transcription are low in gametocytes and the protein is only expressed intracellularly in minute quantities; however, production peaks after onset of gametogenesis when it becomes the predominant component of the ookinete surface (fig. 1) [19, 37, 66]. The protein is shed from the membrane and surface density diminishes during ookinete penetration of the midgut and oocyst formation.

The gene of Pfs25 has a single exon that codes for a polypeptide of 217 amino acids. The protein has a structure of four tandem EGF-like domains containing 22 cysteine residues presumably anchored to the parasite surface through a GPI anchor [33, 66]. Pfs25 is glycosylated by mannose and glucosamine residues and contains palmitic and myristic acid [33].

Pfs28 was genetically cloned in the avian malaria parasite *P. gallinaceum* [69]. Amounts of mRNA in zygotes are similar for Pgs25 and Pgs28, but Pgs28 protein production starts later suggesting differential translation mechanisms [70]. Studies with genetic crosses indicated that the Pfs25 and Pfs28 genes are genetically linked [71]. Pfs28 has 14 cysteines in a similar pattern of typical EGF-like domains.

Transmission-blocking antibodies which have been induced to recombinant as well as native Pfs25 in many experimental animal species, can prevent oocyst development of both *P. falciparum* laboratory [37, 72, 73] as well as field isolates [74]. The mechanism of anti-Pfs25-mediated blockade is not entirely understood but is presumably targeted at the transition of zygotes to ookinetes and at passage of ookinetes through the wall of the mosquito midgut which becomes a hostile environment for the parasite [37, 72, 75]; because of the presence of EGF-like domains, Pfs25 may likely be involved in adherence to the midgut epithelium [11]. The second EGF domain seems to be the most important target for induction of transmission-blocking antibodies [73]. Experimental anti-P28 antibodies reduce oocyst numbers both in *P. falciparum* and *P. gallinaceum* [69, 71]. In the latter system impairment occurred at the transformation of zygote to ookinete and of ookinete to oocyst.

The combination of anti-Pfs25 and anti-Pfs28 antibodies appears to be synergistic in transmission reduction [71]. Pbs25 and Pbs21 represent the respective homologues of *P. berghei* for Pfs25 and Pfs28 [5, 76–78]. Functional studies with these proteins show that transmission in mice is only blocked when

genes for both proteins are disrupted; oocyst development is relatively intact with individual Pbs21 or Pbs25 gene knock-out parasites suggesting functional redundancy [79]. These data strengthen the case for development of a combined Pfs25/Pfs28 vaccine. Indeed, Pfs25–28 fusion recombinant proteins produced in yeast were significantly more efficient in the induction of transmission-blocking antibodies [80].

Anti-Pfs25 antibodies have so far not been found in sera from endemic countries. Although Pfs25 biosynthesis starts after 2 days of gametocyte development in vitro, production sharply increases in macrogametes/round forms in the mosquito [33]. The intracellular quantity of Pfs25 from degraded gametocytes in the human host during natural infections may not be sufficient to trigger detectable antibody production. However, T-cell responses to recombinant Pfs25 preparations are present in endemic populations which suggests sufficient processing and antigen presentation [Sauerwein RW, unpublished; Kaslow DC, personal commun.]. Pfs25 is apparently subject to limited immune pressure which is in agreement with the conserved gene sequence found in eight laboratory strains [81]. Similarly, the genetic variation of Pfs28 also appears to be limited in laboratory strains and field isolates [71, 82].

Tsuboi et al. [83] described the successful isolation of the *P. vivax* homologues of P25 and P28 genes which show the four EGF-like domains and the GPI-anchor characteristics of this family. Pvs28 has a possible N-glycosylation in the third EGF-like domain and a unique repeat in the fourth domain [84]. There is evidence for strain polymorphisms in Pvs25 and particularly Pvs28 as observed in isolates from different endemic areas [83].

Recombinant Pvs25 and Pvs28 expressed in yeast were both able to induce transmission-reducing antibodies in various strains of mice with a more effective reduction by anti-Pvs25 than anti-Pvs28 antibodies [84]. Anti-Pvs28 antibodies rather recognize the surface of mature ookinetes while anti-Pvs25 antibodies stain intracellular zygotes. This observation is in agreement with the general notion that P28 is expressed at later sporogonic stages than P25 [70]. The different staining patterns of anti-Pvs25 and ant-Pvs28 may refer to different mechanisms of blocking transmission.

Circumsporozoite and TRAP-Related Protein

This protein that is present in the micronemes of ookinetes, belongs to a family of thrombospondin-related proteins of apicomplex molecules, which play an important role in parasite invasion of cells [85]. At first, the circumsporozoite and TRAP-related protein (CTRP) of *P. berghei* origin was expressed showing a structural homologue with a previously identified *P. falciparum*

protein [86, 87]; *P. falciparum*-derived CTRP was more recently expressed as a recombinant protein [88, 89].

Gene disruption experiments show that CTRP is not critical for ookinete development, but essential for ookinete motility that drives invasion and passage through the midgut and subsequent oocyst transition [87, 89]. CTRP is not detectable on the ookinete membrane but the presence of adhesive domains in the molecule, may suggest that membrane expression is induced at least at levels to be functionally active.

Chitinase

For successful transmission, parasites have to pass through the peritrophic membrane that is formed as a membranous sac around the ingested bloodmeal in the mosquito midgut. This membrane consists of a chitinous matrix and *Plasmodia* produce an enzyme with chitinase activity for passage over this membrane into the midgut wall [90]. In the presence of allosamidin which is a potent antagonist of chitinase, sporogonic development is completely blocked showing the critical biological function of chitinase and its potential as a target for blockade of transmission [90, 91]. More recently the gene encoding a 60-kD *P. gallinaceum* chitinase, which is composed of two forms (NT1 and NT2) was sequenced [92]. The purified recombinant NT1 form of PgCHT1 shows biological activity which could be strongly enhanced by Endo-Lys-C treatment; possibly the NT1 form needs to be converted in the parasite by proteases to the more active NT2 form. In addition, evidence was presented for the presence of a second distinct chitinase [92]. Activity of both chitinases are inhibited by allosamidin.

Previously it has been suggested that midgut serine proteases may be required to activate parasite derived pro-chitinase resulting in chitinase production by ookinetes [91]. More recent data, however, show that proteolytic activity for pro-enzyme cleavage can be generated by ookinetes themselves or may not be required at all in case of *P. falciparum* chitinase [92].

Conclusions

The best characterized antigens of sexual and sporogonic stages of *P. falciparum* and *P. vivax* have been discussed but more *P. falciparum* antigens including Pfs40, Pf11.1/Pfs2400, Pf-actin genes and *P. vivax* antigens have also been identified [for review see, 4, 11]. Some proteins are expressed as sex-specific antigens including male specific α-tubulin II [6, 11]. Knowledge of the structure and biological function of these proteins is often minimal or absent. The more

recently developed transfection and gene knock-out technology, however, has been shown to be a powerful tool for unraveling biological functions of some of these proteins [26, 32, 87, 89]. Such understanding is of vital importance in defining vaccine or drug targets that interrupt malaria transmission. In addition, most antibodies against reduced sexual stage proteins show less activity and recognition, which implies that proper protein folding is critical. Definition and preparation of the right conformers with identification of target epitopes for immunity will be important not only for vaccine development but also to facilitate studies on naturally acquired (transmission-blocking) immunity in malaria endemic areas. Studies on the sexual and sporogonic stage immunity and transmission intensity have not been systematically carried out but are important for understanding the dynamics of malaria transmission.

Finally, as is the case for all life-cycle stages of the parasite, one can expect that with completion of the *Plasmodia* genome projects, a wealth of new and exciting information will become available on (novel) antigens of sexual and sporogonic stages.

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Mouse Models of Blood-Stage Malaria Infections: Immune Responses and Cytokines Involved in Protection and Pathology

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Introduction

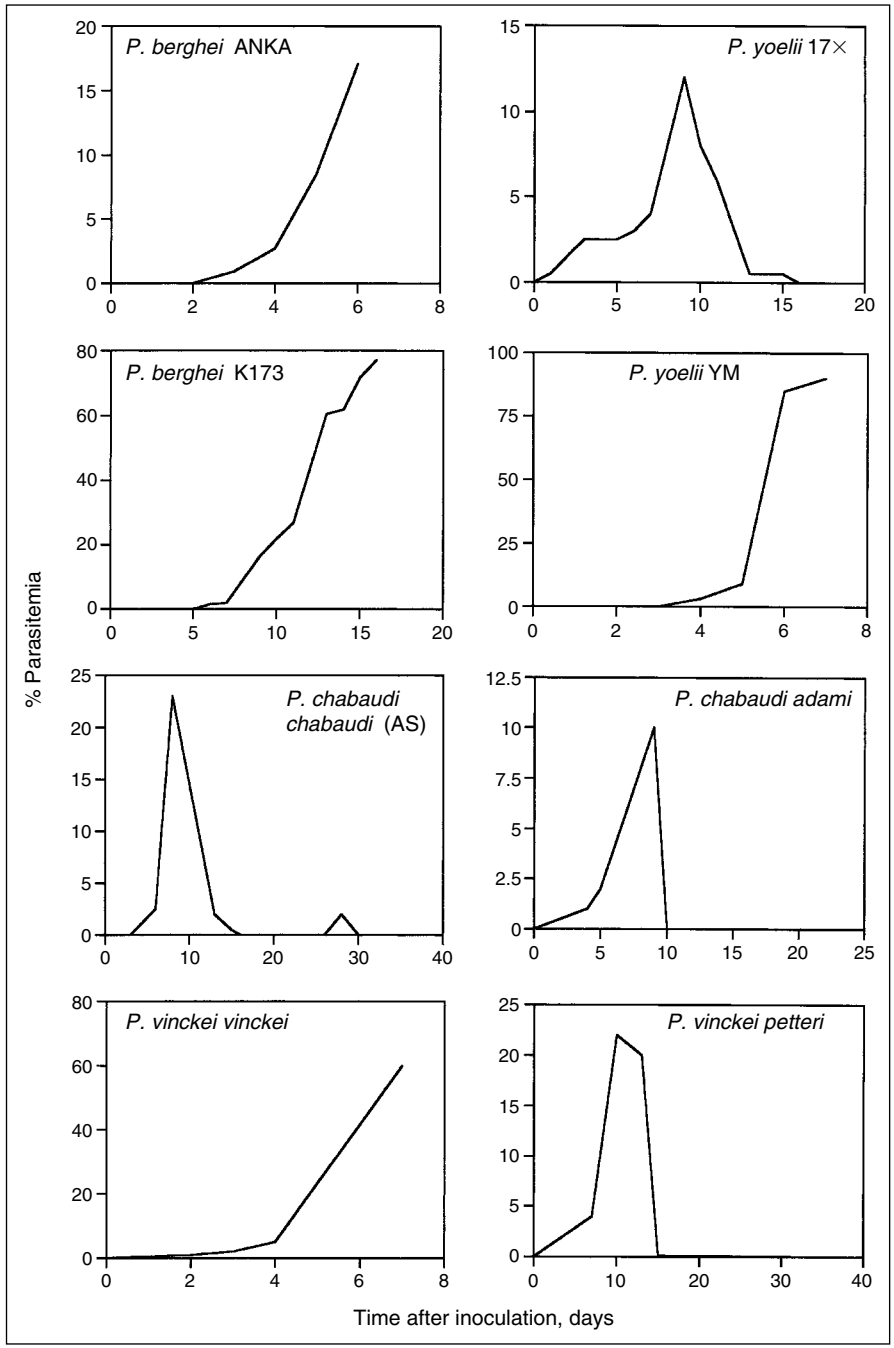
All species of the protozoan parasite, *Plasmodium*, have a complex life cycle involving both an insect and vertebrate host. The immune system of the vertebrate host is confronted with a parasite with three morphologically distinct phases of infection which are either intracellular (within hepatocytes or erythrocytes) or extracellular (sporozoites and merozoites). Both antibodies and cell-mediated immune effector mechanisms are therefore likely to be required for the elimination of parasites. Although many proteins are conserved, there are unique proteins expressed at each stage, some of which, in the case of the erythrocytic stage parasites, are variant antigens coded for by multi-gene families. The variant antigens, which are expressed on the surface of infected red cells, are thought to be a mechanism of immune evasion [1]. In natural infection of humans, acquired immunity is primarily directed towards the erythrocytic stage and takes several years to develop [2]. Before the acquisition of clinical immunity, this stage of the life cycle can be associated with considerable pathology ranging from recurrent fever, coinciding with schizont rupture, to severe and fatal complications such as anemia, renal failure, pulmonary edema and cerebral malaria [3–5]. A role for the host response in malarial pathology is indicated by a correlation of increased levels of some pro-inflammatory cytokines and the severity of disease in humans [6–8], and the amelioration of cerebral and other pathology in rodent models by removal of tumor necrosis factor-α (TNF-α) or interferon-γ (IFN-γ) [9–11]. The extent of involvement of the acquired immune response in disease is still a matter of debate.

A detailed analysis of the mechanisms of protective immunity, its broad specificity and regulation as well as a potential role in pathology is most readily approached by the use of experimental animal models. The availability of inbred and congenic mouse strains and mice with naturally occurring or genetically engineered mutations leading to defects in defined components of the immune system has allowed a fine dissection of immune mechanisms involved in protective immunity or immunopathology of malaria. However, since most laboratory strains of *Plasmodium* are not natural pathogens of the laboratory mouse, and since the different parasites can differ in virulence and in mechanisms of elimination by the host, it is argued that the rodent models are not relevant to the human infection. It is important therefore to determine how far the different rodent models exhibit features typical of human malaria before extrapolating information to human infections. The principles of immune recognition and activation of particular effector pathways are likely to be similar in the different mammalian host and parasite combinations and therefore mouse models can be used for these purposes. The more the different models resemble each other in their responses, the more readily the data can be extrapolated to humans.

This review will concentrate on immune responses elicited against erythrocytic stage parasites. For discussions of immunity to pre-erythrocytic infections readers are referred to recent reviews [12, 13]. An overall description of the major rodent parasites that have been used to investigate the mechanisms of immunity and pathogenesis is given in table 1 with the courses of primary erythrocytic infections shown in figure 1. Some parasites give rise to lethal infections in all strains of mice, others are lethal only in some strains and some parasites are nonlethal in all immunologically competent mice. Nonlethal infections are generally used to investigate mechanisms of immunity and immunoregulation. Several parasites, such as *Plasmodium berghei* (ANKA; PBA), *Plasmodium vinckei* and *Plasmodium chabaudi chabaudi* have been used extensively for studying pathology. However, so far there have been no detailed investigations in mouse models of the relationship between parasite sequestration and cerebral malaria or severe disease seen in humans. Comparisons between different rodent model infections are not always easy, as it is rare that the same immunological assays or pathological criteria have been used in each system.

Mechanisms of Protective Immunity to Blood-Stage Infections

Merozoites invade erythrocytes whereupon they undergo asexual reproduction within a parasitophorous vacuole. As erythrocytes do not have the capacity to process and present parasite antigens or may not have the major


P. berghei ANKA
P. yoelii 17×
P. berghei K173
P. yoelii YM
P. chabaudi chabaudi (AS)
P. chabaudi adami
P. vinckei vinckei
P. vinckei petteri
% Parasitemia
Time after inoculation, days

1

histocompatibility complex (MHC) molecules necessary to stimulate T cells directly, the immune response must be generated through antigen-presenting cells (APC). Furthermore, since parasite peptides within MHC class-I or class-II molecules cannot be presented on the surface of infected erythrocytes, it is unlikely that the infected erythrocyte (or the free-living merozoite) can be a direct target for specific effector CD8+ or CD4+ T cells. It is most probable therefore, that specific elimination of parasites and immunity to re-infection is via antibody-dependent mechanisms, via nonspecific parasitocidal mediators released from T cells or other cells previously activated on APC or via as yet undescribed cellular mechanisms that can recognize infected erythrocytes. On this basis therefore it is not surprising that T cells bearing αβ T-cell receptors, particularly CD4+ T cells, are required for the generation of protective immune responses against blood-stage malaria infection [14–18]. In all mouse malaria infections, mice lacking or depleted of CD4+ T cells cannot clear parasites. This is also true of immunization studies with specific malaria proteins such as merozoite surface protein-1 (MSP-1) where protection can be abrogated by depletion of CD4+ T cells prior to immunization [19]. By contrast, depletion of CD8+ or γδ T cells before infection results only in a slightly higher peak of parasitemia and a minor delay in parasitemia [14, 17, 20].

Activation of T Cells in a Malaria Infection in Mice

It is not known which APC are important in activating T cells during a malaria infection. Bone marrow-derived dendritic cells (DC), macrophages and B cells isolated from immune mice after a course of infection, all have the capability to present antigens from infected erythrocytes to T cells (table 2) [21]. Experiments from our laboratory suggest that bone marrow-derived mouse DC are rapidly activated by *P. c. chabaudi*-infected erythrocytes to produce an array of pro-inflammatory cytokines including IL-12 (fig. 2). Within 24 h MHC class II and costimulatory molecules such as CD86 and CD40 are upregulated [143]. How far this reflects the activation and maturation of DC in vivo has yet to be determined. Macrophages, which are APC specialized for the uptake of particulate antigens via a number of mechanisms [for review see, 22], have also been shown to activate malaria antigen-specific CD4+ T cells in vivo [23].

Fig. 1. Course of primary infection in different mouse models. *P. berghei* ANKA is uniformly lethal (via cerebral involvement) in all strains of mice infected. *P. berghei* K173 causes anemia in CBA mice between 15 and 22 days after inoculation; however, in C57Bl/6 mice it cause cerebral involvement and the mice died between 6 and 8 days after inoculation. *P. chabaudi chabaudi* infection is lethal in A/J and DBA/2J mice by 9 days after inoculation (table 1).

Table 1. Experimental malaria infection in different mouse strains

Plasmodium	Strain/ clone	Mouse strain	Lethal infection	Experimental study	Pathology
berghei	ANKA	CBA/T6 BALB/c C57Bl/6	Yes days 6–8	Pathogenesis of CM	CM, no anemia, no hypoglycemia
		DBA/2J	Yes days 15–22	Resolving CM model	Resolving CM, anemia
	K173	CBA/T6 BALB/c DBA/2J	Yes days 15–22	Non CM control for CM study	No cerebral involvement, anemia
		C57Bl/6	Yes days 6–8	Pathogenesis of CM	CM, no anemia
yoelii	17×	Swiss BALB/c	Yes days 7–9	Pathogenesis of of CM	CM with RBC sequestration, anemia and hypoglycemia
		CBA/Ca BALB/c C57BL/6	No	Immune mechanisms, pathogenesis	No CM, anemia, no hypoglycemia
	YM (lethal)	CBA/T6 BALB/c C57Bl/6 DBA/2J SWISS	Yes days 7–8	Vaccines	No cerebral involvement, hypoglycemia
chabaudi chabaudi	AS	C57Bl/6 CBA/Ca B10 series BALB/c	No Peak day 8–10	Immune mechanisms, pathogenesis	No cerebral involvement, anemia, hypoglycemia
		A/J DBA/2J	Yes day 9		
chabaudi adami	556 KA	BALB/c C3H C57Bl/6	No Peak day 7–11	Immune mechanisms, pathogenesis	No cerebral involvement, anemia
vinckei vinckei		BALB/c	Yes day 8	Chemotherapy, immune mechanisms, pathogenesis	No cerebral involvement, anemia, hypoglycemia
vinckei petteri	CR	C57BL/6 BALB/c	No Peak day 10	Chemotherapy, immune mechanisms	No cerebral involvement

Modified from Li et al. [142].

Table 2. Antigen-presenting cells able to present *P. c. chabaudi*-infected (pRBC) protein fragments of merozoite surface protein-1 (MSP-1) and initiate an IL-2 response from MSP-1-specific CD4+ T-cell hybridomas

Antigen	B cells		Bone marrow-derived	
	A20 lymphoma	Spleen derived	Macrophages	Dendritic cells
pRBC	+/–	+	+	+
MSP-1	+	+	+	+

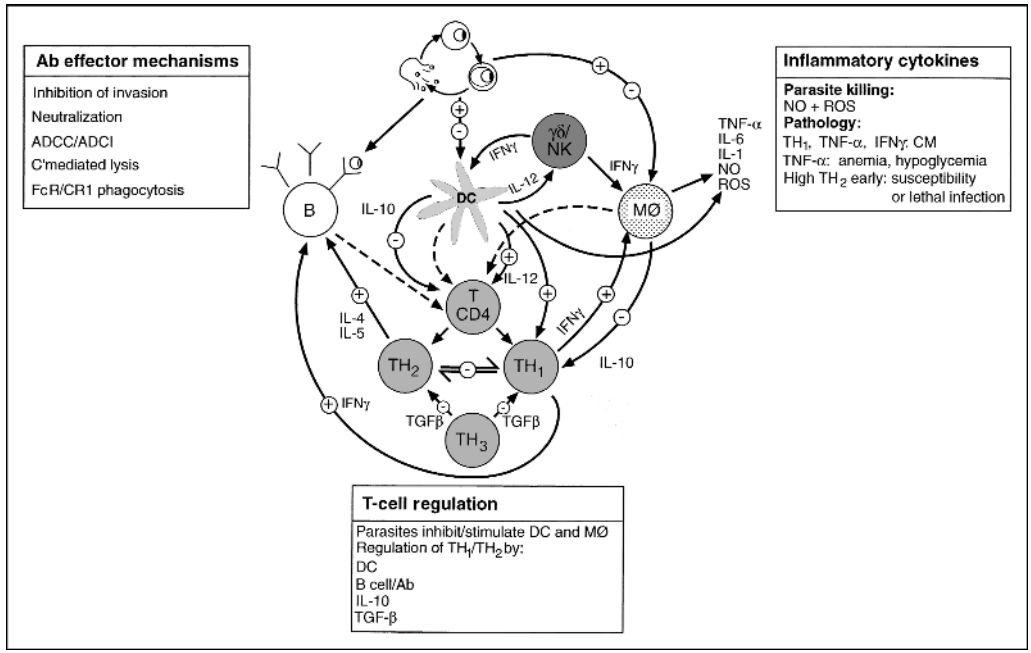


Fig. 2. An overview of immune responses and cytokine production during a blood-stage malaria infection. The solid lines represent cytokine interactions with positive (+) or negative (−) effects on cell activation or differentiation. The dashed lines represent antigen presentation and co-stimulation by antigen-presenting cells.

Parasite material is proteolytically degraded and presented on MHC class-II molecules to activate CD4+ T cells. How the antigens are presented, on which cell and the affinity of the interaction between T cell and APC determine how the T cell is instructed to become activated and divide. T cells activated in the absence

of correct costimulatory molecules may be refractory to re-stimulation or deleted. Clonal expansion and deletion following antigenic stimulus is characteristic of most infections and has been reported in *P. berghei* [24] and *Plasmodium yoelii* [Good M, personal commun.] infections, but not as yet in human malaria infection. It is certainly possible that different strains of malaria may cause deletion of CD4+ T cells to different extents. Tolerance may also be induced if parasite antigens are not presented correctly to T cells or in the absence of sufficient co-stimulatory molecules.

Heterogeneity of CD4 T Cells in Malaria

CD4+ T cells have two major effector roles: they act as helper cells for B cells, enabling the production of high affinity specific IgG antibodies and the development of B cell memory, and they are able to activate macrophages and other cells to produce TNF-α, nitric oxide (NO) and reactive oxygen species (ROS) through the release of inflammatory cytokines [25]. Both of these roles could have an impact on the control of blood-stage parasites (fig. 2). Although antibody and B cells appear to be crucial for the final elimination of parasites in mouse models, it is clear that some control of parasitemia in the acute stage of *P. c. chabaudi* and *P. vinckei* infection is possible in their absence [18, 26–28]. Therefore some antibody-independent mechanisms may be operating.

Three distinct subtypes of CD4+ T-cell responses have been identified in *P. c. chabaudi* and *P. vinckei* and their roles are summarized in figure 2. TH1-type T cells producing inflammatory cytokines such as IFN-γ and which induce macrophages and DC to secrete TNF-α, comprise the majority of activated CD4+ T cells in the early stages of a *P. c. chabaudi* or *P. vinckei* infection [18, 28–32]. This response may initiate inflammatory mechanisms that can kill parasitized erythrocytes. In order to initiate a TH1 cytokine response, TH0 precursor cells must first be given the appropriate signals in the form of IL-12 and costimulatory molecules [33]. IL-12 is one of the first cytokines initiated in infection and is probably produced early by DC and later on by macrophages and has been described in *P. c. chabaudi* infections as early as day 2 after infection [34]. Early production of IL-12 p70 in serum also correlates with resistance, as B6 mice infected with *P. c. chabaudi* (AS) produce significantly more IL-12 than susceptible A/J mice [34], and treatment of susceptible mice with rIL-12 for the first 6 days of infection will result in clearance of parasites [35]. Depletion of IFN-γ exacerbates an acute *P. c. chabaudi* (AS) infection, and IFN-γ receptor-deficient mice infected with *P. c. chabaudi* (AS) or *P. yoelii* have a prolonged acute phase parasitemia, higher recrudescent parasitemia and

greater mortality, suggesting an important role for IFN-γ and the IFN-γ receptor in the control of malaria infections in mice [11, 36–39].

T cells expressing the γδ T-cell receptor (TCR) are expanded in the period following acute infection in both human and mouse malaria infections [40–43], giving rise to the possibility that they contribute to controlling the early stages of infection. The γδ T cells involved are sub-populations with a restricted TCR usage: Vγ9 associated with Vδ2 or Vδ1 in the case of humans [44], and Vγ2 associated with Vδ4 in the case of *P. c. chabaudi* in mice [43]. CD4+ T cells are a prerequisite for γδ T-cell expansion in mice [42] and humans [45]. In *Plasmodium chabaudi adami* and *P. c. chabaudi* infections, γδ T cells are expanded only in mice controlling the primary infection, i.e. after a CD4+ T-cell response has been generated [43]. The extent to which these cells are involved in parasite clearance in mouse models seems to depend on the species of parasite and the immune status of mice. Mice fail to resolve a *P. c. adami* infection for at least 20 days when depleted of γδ T cells [20]. However, γδ T-cell knockout mice or γδ T-cell-depleted mice are able to resolve a *P. c. chabaudi* (AS) infection with only minor increases in peak parasitemia [17, 43]. The ability of γδ T cells to control parasites is best demonstrated in mice unable to make antibody responses, which would otherwise eliminate the infection. In μMT mice lacking B cells, the γδ T-cell population is dramatically expanded in the period of chronic infection following the peak parasitemia [43]. Removal of γδ T cells in these mice, either by antibody or by the generation of double knockout mice lacking both B cells and γδ T cells, resulted in a significantly elevated chronic parasitemia, clearly demonstrating the protective effect of γδ T cells in the absence of B cells. The mechanism of parasite control may be the same as TH1 CD4+ T cells as they produce large amounts of IFN-γ and, in the case of humans, TNF-α, TNF-β and IL-1 [43, 46]. Furthermore, it has been shown that human γδ T cells can kill *Plasmodium falciparum* merozoites in vitro, suggesting that they may function as cytotoxic cells recognizing an as yet, undefined target molecule on the parasite [47].

Early experiments by Clark et al. [48] demonstrated the anti-malarial effects of TNF-α in vivo in a *P. berghei* ANKA infection [48]. This has been verified in other mouse malaria infections as administration of TNF-α to susceptible strains of mice renders *P. c. chabaudi* infections nonlethal [49]. On the other hand, in the absence of a functioning TNFp55 receptor or p75 receptor or both, the primary parasitemia of *P. c. chabaudi* (AS) in mice is only slightly elevated [50, 144], suggesting that while TNF-α-dependent processes may be parasitocidal, they are not essential components of early control mechanisms.

TNF-α itself does not have direct effects on malaria parasites and it is probably the downstream events of TNF activation of cells such as macrophages, causing the release of mediators such as ROS and NO that are responsible for

parasite killing (fig. 2). Both of these mediators are known to have anti-parasitic effects in vitro in *P. falciparum* infection [51]. However, a protective function for NO in vivo is still controversial. In support of a protective role are the observations that NO levels increase during the peak of infection [31] and are reduced in infected IFN-γ-deficient mice which suffer higher parasitemias [38]. Furthermore, treatment of mice with inhibitors (*L*-NMMA) of inducible NO synthase (iNOS) results in higher acute phase parasitemias [31]. By contrast, mice deficient in iNOS do not show alteration in the course or outcome of *P. yoelii* [52] or *P. c. chabaudi* infection [53]. It appears from these data that NO could have a protective role but that is not the only antibody-independent mechanism available to control parasitemia.

After the peak of parasitemia, which is probably controlled by TH1-type T-cell mechanisms, a switch to a TH2-type T-cell cytokine response ensues which results in clearance of parasites in the blood. In *P. c. chabaudi* (AS) infections this is characterized by a functional switch approximately 28 days after infection to CD4+ T cells able to deliver co-stimulatory help and cytokines, such as IL-4, necessary for B-cell expansion and antibody production (fig. 2) [29, 54]. Despite the essential role of B cells in protective immunity, it has been difficult to show an essential role for TH2 cells. This is partly because there are no clear markers in mice that distinguish TH1 and TH2 cells and partly because of the redundant nature of the cytokine network. However, a single malaria-specific TH2 clone was shown to protect against a primary *P. c. chabaudi* infection in vivo [31]. IL-4-deficient (C57Bl/6 × 129) mice infected with *P. c. chabaudi* (AS) or *P. yoelii* can clear a primary infection with kinetics similar to wild-type mice [54, 56]. If the IL-4 defect is on the BALB/c background, the ability of the mice to control the primary acute phase and recrudescence is somewhat more impaired, and IL-4 knockout mice on either background suffer a more pronounced second *P. c. chabaudi* infection, supporting a role for this TH2 cytokine in immunity to re-infection [Langhorne and Packwood, unpublished observations]. Mice defective in other TH2 cytokines such as IL-5, IL-6 (unpublished observations) and IL-10 [11, 56, 57] show no difference in the courses of primary infection of *P. c. chabaudi*. However, IL-10-deficient mice, particularly females, suffer a more severe form of disease and a proportion succumb to a lethal infection, probably due to the overproduction of inflammatory cytokines produced in the absence of IL-10 [11]. Mice lacking B cells, and therefore antibodies, either as a result of anti-μ treatment [27, 58, 59] or in more recent experiments, by targeted deletion of the μ and J heavy chains (μMT or JHD mice, respectively) [28, 60, 61] have been used to analyze the role of B cells in malaria infections. Anti-μ-treated mice are unable to control a *P. yoelii* infection but JHD and μMT mice can control the early stages of infection with *P. c. chabaudi*, *P. c. adami* or *P. vinckei* [28, 61].

However, other studies have shown that at later time points μMT mice cannot resolve a *P. c. chabaudi* malaria infection [28, 60].

Alternative pathways of antigen presentation may also be important in anti-plasmodial immunity. IgG responses to GPI-anchored protein antigens can be initiated in part by CD4+ NK1.1+ T cells recognizing the glycolipid portion of the molecule in the context of CD1d [62]. Although this recognition pathway has not been shown to be an important component of the B-cell response to the GPI-anchored circumspozoite protein [63], it may be a mechanism for a rapid MHC-unrestricted antibody response to parasite antigen. Thus CD4+ NK1.1+ T cells could therefore play an important role in the early stages of infection and have been suggested as candidates for triggering TH2-type responses via production of IL-4.

The obvious role for B cells in protective immunity is the production of antibodies. The importance of antibody in the resolution of malaria infections was first shown in passive transfer experiments, where parasitemia could be resolved by transfer of immune sera [64, 65]; however, the mechanisms of action of antibody required to eliminate the parasite are as yet unclear. Complement fixation by antibody does not appear to be crucial for protective immunity, either in a primary infection with *P. c. chabaudi* or after rechallenge, as mice lacking the classical pathway or both pathways of complement as a result of gene targeting are able to resolve their infections [145]. In *P. yoelii* and *P. berghei* infection, only parasite-specific antibodies of the IgG2a isotype, able to bind to Fcγ receptor with high affinity have been shown to be protective upon passive transfer [66, 67]. In humans, IgG1 and IgG3 antibodies, which are functionally similar to mouse IgG2a, predominate in the sera of immune adults [64, 68, 69] and IgG1 and IgG3 antibodies from immune individuals together with monocytes were able to bring about parasite killing in vitro [68]. These observations led to the hypothesis that antibody-mediated immunity to malaria may operate by an Fc receptor-mediated event such as receptor-mediated phagocytosis or antibody-dependent cellular killing of the parasite [68]. However, more recent studies using mice lacking the Fc receptor common γ chain do not support this. FcγRIII or FcγRII mice are able to resolve a *P. yoelii* or *P. c. chabaudi* infection as normal mice [70, 71; Langhorne et al., unpublished observations].

Studies examining the antibody response against defined malarial antigens have outlined possible ways in which antibody may function. In the case of the MSP-1, mice lacking FcγRI, specific for IgG3 antibodies, could be passively immunized against a *P. yoelii* challenge with IgG3 antibodies specific for the 19-kD region of *P. yoelii* MSP-1 [72], suggesting that for this specific antigen the FcR is not necessary. In the case of human *P. falciparum* MSP-1, antibody inhibits the proteolytic processing of the final cleavage steps of *P. falciparum* MSP-1 prior to parasite invasion of the merozoite into the erythrocyte [73].

A relationship between inhibition of proteolytic cleavage and inhibition of invasion has yet to be demonstrated in mouse models.

Regulation of the Immune Response

Regulation of the different T-cell responses and hence the B-cell response in mouse models of malaria are not well understood. Factors that are able to regulate the switch from TH1 to TH2 or promote either type of response include distinct APC, cytokine environment, antigenic load or altered antigen presentation [25]. Recent studies suggest that T-cell polarization toward a certain subtype may be influenced by which subset of DC that prime the T cell for activation [74, 75]. In mice, DC originating from lymphoid precursors were shown to stimulate a TH1-type T-cell response, whereas myeloid lineage DC were better able to stimulate a TH2-type response [74]. In *P. c. chabaudi* infections, B cells or IgG from immune mice are required for the switch from a TH1 to TH2 phenotype, since μMT mice and anti-μ-treated mice are unable to switch from a predominant IFN-γ response [28, 58–60]. The switch to TH2 can be brought about by the transfer of B cells from immune mice [60] or immune immunoglobulin [Seixas and Langhorne, unpublished observation]. In this model of malaria, since antibody is required for the switch from TH1 to TH2, it is possible that antibody–antigen complexes are taken up via a route that promotes the development of TH2 cells upon antigen presentation.

In addition to TH1- and TH2-type T cells, a third subtype of cytokine producing T cells has been proposed to have a major immunomodulatory role in mouse malaria infections. TH3-type T cells secrete transforming growth factor-β (TGF-β) that is able to regulate both TH1- and TH2-type cytokine responses. Resolution of infection in *P. c. chabaudi* and *P. yoelii* infection is associated with TGF-β production [76]. Recombinant TGF-β treatment of *P. berghei* infection led to a significant decrease in serum TNF-α levels and to an increase in IL-10 production and this may be able to downregulate pathology [76] (see section on cerebral malaria).

How far this characteristic T-cell response to *P. c. chabaudi* reflects the human T-cell response has still to be demonstrated. There are no comparable studies in which antigen-specific precursor frequencies of cytokine-producing cells have been measured, and the levels of nonspecific cytokine-producing cells in peripheral blood during and after an infection have given equivocal results [77]. However, T cells of children from endemic areas, who are not yet immune, make higher IFN-γ responses to some malarial antigens than adults [78]. In immune adults there is a correlation between IL-4 production by T cells and the level of IgE in the serum [79, 80].

When examining immune responses against the malaria parasite in humans, the infection is often treated in isolation. In fact, the majority of malaria-infected individuals are also concomitantly infected with a range of protozoan, helminthic, bacterial and viral pathogens that have a significant effect on the ability to mount a protective immune response to malaria. For example, infection with gut helminths may bias the TH-phenotype towards TH2 which may lead to a delay in early IFN-γ production necessary for clearance of *Plasmodia* spp. In this regard a concomitant *Schistosoma mansoni* infection exacerbates *P. c. chabaudi* parasitemias and is associated with decreased TNF production [81].

Immune Evasion and Antigenic Variation

The impact of antigenic variation on the development of protective immunity has not yet been evaluated in mouse models. Although antigenic variation was first shown in the simian malaria *Plasmodium knowlesi* [82], the identification of multigene families coding for variant antigens of *P. falciparum* have only recently been identified. Antigenic variation has been detected at the serological level in *P. c. chabaudi* infections [83] and multigene families have been identified in *P. yoelii* [84]. It has been predicted that the rate of antigenic switching in *P. c. chabaudi* is similar to that calculated for *P. falciparum* in vitro; i.e. approximately 2% per generation [85]. However, the variant antigens have not been identified in *P. c. chabaudi* and therefore studies so far are extremely limited. Since the parasite dedicates a large number of genes to these variant antigens, it is likely that they are subject to strong selection pressure, and therefore may be major targets for the immune system. Mouse models of malaria offer an excellent opportunity to investigate this selection, the relationship between variant antigens and sequestration, and to determine whether variant antigens are the major antigens involved in inducing protective immunity.

Pathology of Mouse Malaria Infections

Depending on parasite species and on the pattern of transmission, malaria infection may present in different ways in different individuals. *Plasmodium malariae* and *Plasmodium ovale* infections cause little morbidity and almost no mortality [86], whereas *Plasmodium vivax* infections are more severe and debilitating though usually self-limiting. *P. falciparum* infections are most often life-threatening in nonimmune individuals; in highly endemic areas, severe

anemia, hypoglycemia, renal failure, and the more fulminant cerebral malaria (CM) [87, 88] are the most pernicious manifestations and usually the cause of mortality in children. In regions where entomological inoculation rates are lower and transmission less stable or highly seasonal, CM is typically seen in both children and adults. Nonimmune visitors to endemic areas are also at risk of developing fulminant CM [87].

Anemia, hypoglycemia, loss of body temperature and loss of body weight are general features of most mouse malaria infections. Cerebral complications have been observed only in a limited number of infections. Thus, mouse models may contribute to understanding the pathogenesis of the complications of malaria infection in humans, but they may not replicate the human disease in full. Therefore, extrapolating data from these models to human disease must be done with caution. Some of these manifestations seem to be directly linked to the level of parasitemia [36]. However, in others there is a clear causal link with the cytokines induced in the host either directly by the parasite or via the immune response. Removal or depletion of cytokines involved in downregulating inflammatory responses, such as IL-10, clearly exacerbates the severity of disease and can lead to death [11, 57]. In the absence of IL-10, IFN-γ and TNF-α are upregulated. Treatment of infected IL-10 gene KO mice with anti-IFN-γ antibodies reduces mortality [11]. Treatment with anti-TNF-α reduces mortality, and partially ameliorates hypoglycemia, and anemia [Li and Langhorne, unpublished observation], suggesting a link between inflammatory cytokines and disease in mouse malaria.

Cerebral Malaria

CM is a clinical syndrome characterized by diffuse, potentially reversible encephalopathy associated with fitting and loss of consciousness [89, 90] that cannot be explained by any other cause. The main rodent model for CM is PbA infection of CBA or C57Bl/6 mice. Reports from some laboratories [91] suggest that BALB/c mice are resistant to the development of CM induced by PbA. PbA-infected CBA and C57Bl/6 mice die between 6 and 8 days after infection with a low level of parasitemia (i.e. 10–15% parasitized red blood cells, RBC) [92]. This leads to CM with the mice developing neurological symptoms such as fitting, ataxia, hemiplegia and coma [93, 94], which are also prominent clinical signs of human CM [88, 89]. Another mouse model of CM which is very similar to PbA infection is *P. berghei* K173 (PbK) infection in C57Bl/6 mice [95]. However, PbK infection in CBA mice does not lead to the development of neurological complications and has therefore, been employed as a control when studying the pathogenesis of CM in CBA mice infected with PbA [92].

An important difference in mouse CM is that the sequestered cells are monocytes [96] rather than infected RBC as in the human disease. This questions the relevance of the PbA- and PbK-mouse CM model to the human condition. For this reason, attempts have been made to develop other mouse models of CM that reproduce the pathology of human CM better. One such infection is *Plasmodium yoelli* 17× lethal strain infection in Swiss or BALB/c mice. This infection shows cerebral involvement, with petechial hemorrhages and sequestration of infected RBC [97–99]. Hence, this model may be more closely related to human CM than PbA infection; however, it is not as popular as PbA infection for the study of the pathogenesis of CM because of the problems associated with reproducibility [94].

The pathogenesis of CM, in all the mice tested so far, is dependent on T cells and IFN-γ. Thus, CM does not develop in PbA-infected CBA mice depleted of CD4+ T cells [100] and mice suffering from murine AIDS have a low incidence of cerebral complications upon PbA [101]. Furthermore, both IFN-γ and IFN-γ-receptor gene knockout mice do not develop CM [9, 10] when infected with PbA. However, neither B cells, their products, nor B cell antigen presentation appear important for the development CM in mice, as B cell-deficient JHD and μMT mice developed this clinical syndrome upon infection with PbA [10].

In contrast to mouse models, the role of T or B lymphocytes in the pathogenesis of CM in humans is not clear-cut, although indirect evidence supports a role for the immune system. CM is more commonly seen between the ages of 1 and 4 years, suggesting that maternal immunity protects the new-born during the first year of life. However, as maternal immunity wanes, CM starts to be seen. Later, with gradual exposure to malaria and the development of immunity the incidence of CM drops.

IFN-γ probably mediates its effects during the development of CM in mice by priming monocytes to produce TNF-α. There is increasing evidence that TNF-α plays an important role in the pathogenesis of mouse CM. The treatment of PbA-infected mice with anti-TNF-α antibody is reported to prevent CM and its associated pathology [102]. Studies by Lucas et al. [103], using TNF-α-receptor gene knockout mice, also indicate that TNF-α is important for the development of CM in experimental mice. Contrary evidence has been reported where higher plasma levels of TNF-α occur in mouse malaria with no cerebral complications than in mice with CM [104]. This has led to the suggestion that TNF-α produced locally within the central nervous system, or its vasculature, may be important in the pathogenesis of CM in experimental mice.

TNF-α has also been implicated in the pathogenesis of human CM. A single nucleotide polymorphism (TNF_{-376}) affecting the binding of the transcription factor, OCT-1, which subsequently alters gene expression in human

monocytes has been associated with susceptibility to CM in Africans [105]. Others have also demonstrated high plasma levels of TNF-α in severe cases of CM in children [6, 7]. However, in *P. vivax* malaria where CM is absent and in noncerebral *P. falciparum* malaria, high systemic cytokine levels have also been reported [106, 107], suggesting that systemic TNF-α production is neither specific to nor solely responsible for the development of CM in humans either. Hence, locally produced TNF-α in the brain or its vasculature might be the critical factor in the pathogenesis of CM in both mice and humans.

The reason why some malaria parasites should predispose to cerebral involvement in certain strains of mice while others do not has been investigated by many authors. An imbalance in favor of inflammatory cytokine production over the anti-inflammatory or protective cytokines has been employed to explain this. IL-10 and TGF-β are thought to play a protective role against experimental murine CM because susceptible mice are significantly protected from developing CM when injected with recombinant IL-10 [108]. A decrease in the expression of TGF-β genes has also been reported in mice suffering from CM [91]. Hence, an increase in the production TNF-α and IFN-γ and a reduced expression of IL-10 and TGF-β has been associated with the development of CM in mice. These data are consistent with an imbalance towards a TH1 response in the host immune response, in mice developing the cerebral complications of malaria (fig. 2). Autopsy studies of human CM patients using immunohistochemistry showed that TGF-β1, β2 and β3 are differentially expressed in astrocytes adjacent to brain vessels, macrophages/microglial cells in the gliosis of ring hemorrhages and Durcks granuloma, and in smooth muscle and endothelial cells of brain vessels with sequestration, respectively [109]. The authors concluded that TGF-β may therefore play a role in the pathogenesis of CM in humans. However, no causative effect was demonstrated.

Anemia in Malaria Infection

Previous studies indicate that severe anemia is a common clinical presentation in human malaria infection [88, 110, 111]. Anemia could in fact contribute to the cerebral pathology seen during malaria infection because severe anemia has been reported to occur in 94% of humans who develop CM [110]. However, the nature of its pathogenesis is not fully understood, although hemolysis [112] and ineffective erythropoiesis [113, 114] may be partly responsible. Hemolysis may occur during malaria infection by the following mechanisms: (1) direct lysis of RBC by the parasite; (2) autoimmune RBC destruction, and (3) clearance of infected RBC by the spleen. The factors that contribute to ineffective erythropoiesis during malaria infection have been the subject of investigation

in many laboratories. As in CM, animal models of anemia in malaria infection have also been used in some of the studies.

Virtually all the laboratory malaria parasites produce anemia in many of the mouse strains available. *P. yoelii* infection causes anemia in CBA, BALB/c and C57Bl/6 mice [115]. *P. c. chabaudi* (AS) infection causes anemia in all mouse strains examined [36, 116]. *P. vinckei vinckei* infection causes anemia in BALB/c and CBA mice [117, 118]. PbK infection causes anemia in CBA mice [9] and PbA infection causes anemia in DBA/2J mice [93]. One of the findings in many of these studies is that TNF-α is likely to play a very significant role [113, 118–120] in the pathogenic process of ineffective erythropoiesis during malaria infection. Chronic exposure of nude mice to TNF-α has been demonstrated to inhibit erythropoiesis [121] and TNF-α enhances erythrophagocytosis in a transgenic mouse model in which TNF-α is overexpressed [122]. These support the notion that increased systemic TNF-α concentrations, as can occur during malarial infections, may contribute to the development of malarial anemia in mice.

A role for TNF-α in the pathogenesis of malarial anemia has also been suggested in humans as well. TNF-α was shown to inhibit the proliferation of human erythroid progenitors in bone marrow cultures [119], suggesting that excessive TNF-α production might contribute to anemia in an infectious disease like malaria by predisposing to dyserythropoiesis. Furthermore, an association has been observed between TNF_{-238} A allele, which is located in the TNF promoter region and malarial anemia in children from The Gambia [123].

An imbalance between the pro-inflammatory and the anti-inflammatory cytokines may be the crucial factor in the development of malarial anemia. Studies carried out on African children [124, 125] have reported a lower IL-10 concentration in children with malarial anemia. Hence, an imbalance in favor of IL-10 might prevent the development of malarial anemia by suppressing the inflammatory effects of TNF-α (fig. 2).

Hypoglycemia in Malaria

Hypoglycemia is defined as a blood glucose concentration below 2.2 mmol/l. It has been associated with a bad prognosis during malaria infection in humans, especially when it occurs together with CM [88, 126]. During malaria infection in human hyperinsulinemia [127], excessive TNF-α production [128] and quinine administration [129, 130] have been reported to contribute to the development of hypoglycemia. Quinine administration is thought to predispose to malarial hypoglycemia by inducing hyperinsulinemia [127, 129]. A defect in the glucose counter-regulatory hormone (cortisol, growth hormone, catecholamines and glucagon) response is not a plausible contributing factor to malarial

hypoglycemia in human as the counter-regulatory response was shown to be intact in a study on Thai patients with *P. falciparum* infection [127].

Mouse malaria studies on the pathophysiology of hypoglycemia suggest that hyperinsulinemia is the dominant factor in the development of hypoglycemia. CD1 and (C57Bl/6 × BALB/c) F1 mice infected with *P. c. chabaudi* (AS) or the lethal strain of *P. yoelii* YM develop hypoglycemia. Inoculation of these mice with the nonlethal strain of *P. yoelli* does not induce hypoglycemia. The pathophysiology of hypoglycemia during *P. c. chabaudi* (AS) or the lethal strain of *P. yoelii* YM infection has been thoroughly investigated [131–133]. The development of hypoglycemia in these mice can be inhibited by diazoxide, an inhibitor of insulin secretion [131], whereas inhibition of TNF-α activity or its production had no effect on the development of hypoglycemia during *P. c. chabaudi* and *P. yoelii* YM infection. These data suggest that hyperinsulinemia and not TNF-α contribute to the development of hypoglycemia in CD1 and (C57Bl/6 × BALB/c) F1 mice infected with *P. c. chabaudi* and *P. yoelii*. Indeed, infection of (C57Bl/6 × BALB/c) F1 mice with *P. c. chabaudi* and *P. yoelii* YM normalized the hyperglycemia and restored insulin secretion in mice made diabetic with streptozotocin [133], providing a direct causal link between *P. c. chabaudi* and *P. yoelii* YM infection, and increased insulin secretion.

There is a lot of controversy surrounding the role of TNF-α in the pathogenesis of malarial hypoglycemia. Mice transgenic for human TNF-α did not develop hypoglycemia when infected with the lethal strain of *P. yoelii* YM, and administration of human recombinant TNF-α to mice did not produce a fall in blood glucose concentration. In (CBA × C57Bl/6) F1 mice infected with PbA where hypoglycemia does not develop, the blood glucose concentration was reported to increase concomitantly with serum TNF-α concentration [132]. All these suggest that TNF-α does not cause hypoglycemia in *P. yoelii* and PbA infection. Rather, an increase in systemic TNF-α concentration during PbA infection appears to predispose to hyperglycemia. The work of Clark et al. [104] reporting induction of hypoglycemia in PbA-primed mice by the administration of human recombinant TNF refutes the argument that TNF-α does not contribute to the development of hypoglycemia during malaria infection in mice. This group also provided evidence suggesting that TNF-α may contribute to the development of hypoglycemia during *P. vinckei vinckei* infection in BALB/c and CBA mice [48, 134].

Taken together, a role for TNF-α in the pathogenesis of malarial hypoglycemia appears to depend upon the mouse and parasite combination. The effect of a systemic increase in TNF-α on glucose metabolism during malaria infection in mice is not well understood at present. Both hypoglycemia [48, 134] and hyperglycemia [132] have been reported to occur. Nevertheless data suggesting that TNF-α is a potent inducer of insulin resistance in both mice and humans [135] tilt the weight in favor of the argument that increased systemic

TNF-α concentration does not contribute to the pathogenesis of hypoglycemia during malaria infection in mice. Indeed, the blood lactate concentration is reduced during PbA infection in CBA mice [136], where hyperglycemia rather than hypoglycemia has been reported [132]. This is consistent with the idea that the effect of systemic increase in TNF-α concentration during malaria infection in mice is to induce insulin resistance and therefore reduce the metabolism of glucose via the glycolytic pathway.

Susceptibility and Resistance to Lethal Infections

An important feature of mouse malaria infections is that different strains of mice are differentially susceptible to lethal infections. Similarly, closely related strains of parasites exist which are differentially virulent in the same strains of mice. While there are no immediate correlates of these host–pathogen relationships in human malaria infections, studies on resistance, susceptibility and virulence in mouse models may pinpoint components of the host or parasite important in determining the outcome of an infection. Thus lethal and nonlethal variants of *P. yoelii* [137] and *P. c. chabaudi* (AS) give rise to lethal infections in some strains of mice (e.g. A/J [116] and DBA/2 [36]) but not others (C57Bl/6, B10 series, 129, CBA [36, 116]). Mortality in the susceptible strains is highest in male mice and is not related directly to the H-2 [36, 116], although later parasite clearance in resistant mice may be linked to genes coded for in the MHC [140]. In genetic studies, susceptibility to a lethal infection has been mapped to regions on two different chromosomes; 8 and 9 [138, 139]. The actual genes involved and the molecules they code for, have not yet been identified. A feature common to lethal infections seems to be a slow or lower initial inflammatory response as evinced by low macrophage activation, delayed TNF-α response [141] and the presence of high levels of TH2 cytokines such as IL-5 early in the response [54]. Therefore, similar to several of the other severe manifestations of malaria in mice, lethality seems to be related to the nature or the regulation of cytokines. However, in contrast to the pattern of cytokines in CM, hypoglycemia and anemia, it appears that insufficient inflammatory responses will also lead to severe infection and death. Immunity without pathology depends on a fine balance (fig. 2).

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Malaria and the Immune System in Humans

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Introduction

Malaria infection gives rise to host responses which are regulated by both the innate and adaptive immune system as well as by environmental factors. Acquired immunity is both species- and stage-specific. It is rarely sterile, but rather associated with low-grade parasitemia and episodes of clinical disease throughout life [1, 2]. In endemic areas, children born to immune mothers are protected against disease during their first half year of life by maternal antibodies. This passive immunity is followed by 1or 2 years of increased susceptibility before acquisition of active immunity [1]. In general, acquisition of active immunity to malaria is slow and requires repeated parasite exposure to be maintained. Genetic variability of both the human host and the parasite, parasite-induced immunosuppression and other reasons account for this instability [3].

In this chapter we will discuss the immune regulation of malaria blood-stage infection in humans, focussing on *Plasmodium falciparum*, the most widely spread and dangerous of the human parasites.

Innate Immunity

Innate mechanisms of parasite growth inhibition by the human host are probably the reason for the low parasitemias seen in acute *P. falciparum* infection [3]. The humoral and cellular mechanisms of this ‘nonspecific’ defense are poorly defined. Recent studies in nonparasitic systems have demonstrated that a family

of germ line encoded receptor proteins are important for innate host defense in both invertebrates and vertebrates. In mammals, activation of macrophages through such 'toll-like receptors' leads to the induction of effector genes whose products control and execute this innate defense in a large variety of bacterial and viral systems [4]. Although not as yet as extensively investigated for parasitic infections, it is likely that this system is of equal importance for the innate defense against malaria.

Malaria infection gives rise to strongly elevated blood concentrations of non-malaria-specific immunoglobulin [5], but the importance of the underlying polyclonal B-cell activation for innate immunity is not known. This is also true for the CD4+ T cells from malaria-naïve donors responding by in vitro proliferation and cytokine production upon exposure to malaria antigens [6]. In contrast, neutrophils, mononuclear phagocytes and natural killer (NK) cells appear to play a role in innate immunity seen early in malaria infections. In particular, NK cells have been shown to increase in numbers and to be able to lyse *Plasmodium falciparum*-infected erythrocytes in vitro [7]. However, NK cells are also potent producers of cytokines such as interferon-γ (IFNγ) and this capacity, leading to parasiticidal macrophage activation, may be of greater importance for innate malaria immunity than their potential to lyse infected host erythrocytes [8].

Related cell types probably playing a role in innate malaria immunity are the NKT cells which in mice carry both the NK1.1 surface marker and αβ T-cell receptors (TCR) [9]. These cells are potent inhibitors of liver-stage parasite replication in mouse malaria systems in vitro [10]. Furthermore, NK1.1+ CD4+ murine T cells have also recently been reported to regulate IgG antibody responses to glycosylphosphatidyl inositol-anchored *P. falciparum* protein, a response which may be important for a rapid, specific but major histocompatibility complex (MHC) unrestricted parasite control [11]. Human NKT cells express TCR homologous with those of murine NKT cells as well as other NK cell markers. Both murine and human NKT cells are activated via their invariant TCR when confronted with lipid antigen in association with the MHC class I like CD1 molecules [12]. This activation does not require immunization and may, therefore, be important for regulating innate malaria immunity.

T cells bearing the γδ TCR are also strongly expanded during the early phases of malaria infection and may contribute to innate parasite control [13]. In support of this, γδ T cells but not αβ T cells from malaria-naïve donors inhibit parasite replication in vitro [14, 15]. This difference might be related to differences in antigen recognition by the two types of TCR or, alternatively, to the presence on γδ T cells of NK receptors [16, 17], the non-antigen-specific ligation of which results in rapid secretion of proinflammatory cytokines. For further details regarding γδ T cells see the section on cell-mediated immunity below.

Humoral Immunity

In residents of endemic areas, malaria infection induces strong humoral immune responses, involving production of predominately IgM and IgG but also of other immunoglobulin isotypes. While a large proportion of this immunoglobulin is non-malaria-specific, reflecting polyclonal B-cell activation, up to 5% or more represent species- as well as stage-specific antibodies reacting with a wide variety of parasite antigens. Passive transfer of IgG from immune donors already suggested long ago that antibodies may be protective [7, 18] by reducing parasitemia and clinical disease. These early studies also established that some of the important antigens inducing such protective responses were shared by *P. falciparum* parasites worldwide regardless of geographical origin [19].

Some Important Malaria Antigens

Of major importance for the development of humoral immunity to the malaria blood stages are the parasite antigens expressed on the surface of infected erythrocytes. The predominant antigens involved are members of highly variant families. This variability enables the parasites to evade the immune response and, therefore, constitutes an important virulence factor [20]. In line with this, antibody-mediated inhibition of merozoite invasion of erythrocytes is less effective with parasites from the antibody donor than with those from other donors [21]. Similarly, culture of parasites in the presence of anti-malarial antibodies reduces their susceptibility to antibody-mediated growth inhibition as compared to that of those previously cultured without antibodies [22].

The predominant variant parasite antigens on the surface of *P. falciparum*-infected erythrocytes are encoded by the multi-gene family *var* [23, 24]. The gene products, called *P. falciparum* erythrocyte membrane protein 1 (PfEMP-1), are highly variant polypeptides of 200–350 kD [25] equipped with several binding sites mediating adhesion of infected erythrocytes to the vascular endothelium of capillaries and post-capillary venules [26, 27]. This 'cytoadherence' in small peripheral vessels is believed to protect the parasites from being destroyed in the spleen. Although the *var* genes occur in 40–50 copies/haploid genome, only one gene product is expressed in infected erythrocytes containing late-stage parasites [28, 29]. Another multi-gene family encoding parasite antigens on the erythrocyte surface are the *rif* genes, occurring in at least 200 copies, mostly located subtelomerically on several parasite chromosomes [30, 31]. The *rifins* have an accessory role in the binding of uninfected to infected erythrocytes giving rise to rosetting [32; for further references and discussion of these variant parasite antigens see, 20, 33, 34].

Many additional parasite encoded molecules in infected erythrocytes display a high degree of antigenic diversity, reflecting expression of allelic genes or of

alternative genes belonging to multi-gene families [20, 33]. Candidate antigens for the induction of protective antibodies may be located in apical organelles or on the surface of merozoites as well as on the surface of infected erythrocytes. Important examples are merozoite surface proteins (MSP)-1–5 [35]. The most thoroughly investigated of these is MSP-1, containing both a conserved C-terminal amino acid sequence (19 kD) which is carried over by the parasite when it invades uninfected erythrocytes and antigenically variable sequences which are released [20, 33].

Antibodies

Malaria infection induces both polyclonal and specific immunoglobulin production. Although antibodies of different isotypes may have protective functions, IgG is most important in this respect. In protected individuals, cytophilic antibodies of IgG1 and IgG3 isotype have frequently been found to prevail [36, 37]. The ratio of IgG1 to IgG3 antibodies appears to be highest in subjects whose antibodies are also most efficient in parasite neutralization in vitro, supporting the functional relevance of these findings [38]. Significant elevations of IgG3 antibodies in certain populations and associated with disease episodes have been reported [39, 40]. However, elevated concentrations of IgG2 antibodies may also be associated with decreased risk of *P. falciparum* infection: this has been seen in certain individuals whose monocytes carry a special allelic variant of a Fcγ receptor (RIIA) having the capacity to bind this normally not cytophilic immunoglobulin subclass [41].

Malaria infections of both humans and experimental animals are also associated with elevations in total IgE and IgE anti-malarial antibodies [42, 43]. Induction of this immunoglobulin isotype reflects a switch of regulatory T cell activities from Th1 to Th2 due to repeated exposure of the immune system to the parasites. However, IgE elevation is also under genetic control as demonstrated by comparison of mono- and dizygotic twins from malaria endemic areas [44]. IgE elevation appears to be associated with malaria pathogenesis as the blood concentrations of this isotype are significantly higher in patients with cerebral or other forms of severe disease than in those with uncomplicated malaria [42, 45]. A pathogenic effect of IgE is probably due to local overproduction in microvessels of tumor necrosis factor (TNF) and nitric oxide (NO) caused by IgE-containing immune complexes. Such complexes may induce and cross-link CD23, the low-affinity receptor for IgE on monocytes and perhaps endothelial cells, resulting in their activation [46]. However, these results do not exclude that IgE antibodies also may be protective.

Antibody-Dependent Protection

Antibodies may protect against malaria by a variety of mechanisms. Thus, they may inhibit merozoite invasion of erythrocytes [47] and intra-erythrocytic

growth or enhance clearance of infected erythrocytes from the circulation by binding to their surface, thereby preventing sequestration in small vessels and promoting elimination by the spleen [48, 49]. In particular, opsonization of infected erythrocytes significantly increases their susceptibility to phagocytosis, cytotoxicity and parasite inhibition by various effector cells such as neutrophils and monocytes/macrophages [50, 51]. Interaction of opsonized erythrocytes with these effector cells induces release of factors such as TNF which may cause tissue lesions but which are also toxic for the parasites [52].

Obviously, antigenic diversity and variation of the parasites will greatly affect the protective efficiency of antibodies [20]. Thus, exposure of the immune system to an infecting parasite gives rise to variant-specific anti-PfEMP-1 antibodies which will inhibit cytoadherence and reduce the risk of renewed infection by parasites expressing the same PfEMP-1 as the originally infecting one [53]. However, the presence of such antibodies will also contribute to the selection of different variants against which these antibodies do not protect [54, 55]. Similarly, natural infection also induces strain-specific antibodies against the highly variable *rifins* [32]. However, the possible protective function of anti-*rifin* antibodies remains to be established.

Cell-Mediated Immunity

Cell-mediated immune responses induced by malaria infection may protect against both pre-erythrocytic and erythrocytic parasite stages [56].

CD4+ and CD8+ T Cells

Of these major T-cell subpopulations, CD4+ T cells are essential for immune protection against asexual blood stages in both murine and human malaria systems. For CD8+ T cells which have important effector functions in pre-erythrocytic immunity [57] and which contribute to protection against severe malaria [58, 59], this role is less clear. It has been proposed that CD8+ T cells may regulate immunosuppression in acute malaria and downmodulate inflammatory responses [60]. In any event, as human erythrocytes do not express MHC antigens, lysis of infected erythrocytes by CD8+ cytotoxic T lymphocytes has no role in the defense against blood-stage parasites.

In contrast to the CD8+ T cells, the regulatory and effector functions of CD4+ T cells are well established for both experimental and human malaria. For experimental malaria, evidence for this is based on adoptive transfer of protection by such cells and on increased susceptibility to infection of CD4+ T-cell-depleted mice [for references and discussion see, Langhorne et al., pp 204–228; 3]. For *P. falciparum* malaria in humans, the existence of

functionally different CD4+ T cells in naturally exposed donors has also been established experimentally. These cells respond to malaria antigen by in vitro proliferation and/or secretion of cytokines, e.g. IFNγ or IL4 [61]. In general, these in vitro responses are poorly correlated with protection [3, 60]. Nevertheless, in vitro stimulation of CD4+ T cells from malaria-exposed donors may result in the production of IL4 in concordance with the serum concentrations of antibodies specific for the antigens used for lymphocyte stimulation [62, 63]. Furthermore, enhanced IFNγ production and proliferation have been reported for T cells from donors recovering from a malaria attack [64].

γδ T Cells

Cells expressing the γδ TCR normally represent less than 5% of all T cells in the peripheral blood of healthy adults. The TCR of approximately 75% of these cells is made up of Vγ9 and Vδ2 chains while a minor fraction expresses Vδ1 with no preferential Vγ association [65]. In healthy West Africans, the frequency of γδ T cells in the blood is about twice that of Caucasians, mainly due to an increase in the Vδ1 subset [66]. In vitro stimulation with *P. falciparum* extracts of blood mononuclear cells not previously exposed to malaria also results in γδ T-cell activation, with a majority of the responding cells expressing Vγ9/Vδ2 [67, 68] and a minority Vδ1 [69]. Activated γδ T cells but not αβ T cells from malaria-naïve donors inhibit parasite replication in erythrocytes in vitro, supporting their protective function and, in particular, their role in innate defense against the malaria parasites [14, 15]. γδ T-cell activation is associated with IL-2 receptor (IL2R) signaling, initiated by cytokines such as II-2, IL-4 and IL-15 [70, 71]. Malaria antigen-activated γδ T cells produce primarily but not exclusively pro-inflammatory cytokines [15], suggesting that protection against the parasites by these cells involves both regulatory and cytotoxic functions. However, it should be emphasized that these cellular activities also may be implicated in malaria pathogenesis [for references see, 3, 15].

Antigens from plasmodial schizonts potently stimulate γδ T cells [72, 73]. These cells recognize certain antigens conventionally in association with MHC class-I or II molecules [68, 71]. However, γδ T cells also recognize non-peptide antigens, with no need of MHC presentation [74]. These activating ligands are relatively small (molecular weight <500 kD) and mostly contain phosphoesters [75]. Such phosphoantigens were first described for *Mycobacterium tuberculosis* and also for *P. falciparum* [72, 76]. These ligands bind directly and specifically to the γδ TCR.

The antigens seen by Vδ1 T cells are less well known although it has been reported that intra-epithelial Vδ1 T cells may react with the stress-induced proteins MICA and MICB [77], suggesting that they might recognize epithelial cells damaged by infection [75].

The Cytokine Network

Protective anti-malarial immunity reflects cellular activities such as antibody production, phagocytosis, cellular cytotoxicity and parasite inhibition exerted by lymphocytes, neutrophils and mononuclear phagocytes. However, some of these cellular activities may also cause tissue damage and the course of a malaria infection is highly dependent on the balance between the cytokines secreted by the various cells when activated [60]. In any event, proinflammatory cytokines such as IFNγ, IL-1, IL-6 and others may be protective by inducing parasite killing by monocytes/macrophages and neutrophils [60, 78]. IL-12, produced by mononuclear phagocytes and other cells, contributes to protection against pre-erythrocytic and blood infection by initiating a Th1 anti-malaria response in mice as well as in monkeys [79, 80]. In contrast, anti-inflammatory cytokines such as IL-10 counteract the production and possible cytopathic effects of the proinflammatory cytokines [81, 82]. Recent studies of human *P. falciparum* malaria emphasize the importance of the balance between pro- and anti-inflammatory cytokines. Thus, elevated IL-6/IL-10 ratios in plasma due to relative IL-10 deficiencies predict a fatal outcome of severe malaria [83]. Moreover, anemic children from certain holoendemic areas have lower IL-10/TNF ratios than those with uncomplicated disease, suggesting that IL-10 may inhibit induction of anemia by TNF [84]. Malaria-induced IL-10 has also been found to predict resistance to *P. falciparum* infection, supporting the balancing role of anti-inflammatory cytokines [85].

A cytokine which has a central role for both protection and malaria pathogenesis is TNF. TNF does not kill parasites directly but exerts protection by activating the anti-parasitic effects of the various leukocytic effector cells [for references see, 86]. With regard to pathogenesis, TNF levels are positively correlated with disease severity as well as with malaria fever [87–90]. The primary source of this TNF are monocytes/macrophages activated by various parasite products [60]. However, as described in a previous section, IgE containing immune complexes also contribute to local overproduction of TNF in severe malaria [46]. Variation in the amounts of TNF produced by these cells has a genetic basis and is decisive for the outcome of an infection. Thus a single nucleotide polymorphism in the TNF-promoter region –308 is associated with elevated TNF production and an increased risk of cerebral *P. falciparum* malaria [91–93]. In contrast, children with low plasma levels of TNF due to a single nucleotide polymorphism at the TNF promoter allele –238A are susceptible to severe malarial anemia [94]. The mechanisms governing the underlying regulations may involve altered gene transcription due to changes in transcription factor binding to the corresponding TNF promoter region [91].

Nitric Oxide

As discussed above, anti-malarial antibodies may control blood-stage parasites both on their own and in cooperation with different effector cells [48, 49, 52].

Cell-mediated parasite neutralization with or without antibody participation involves both phagocytosis or other cellular activities [3, 79] including the release of mediators such as cytokines and reactive oxygen intermediates [95, 96]. More recently, interest has been focussed on the role of NO in anti-parasitic immunity. In both mice and human malaria, production of proinflammatory cytokines also gives rise to NO production through induction of NO synthase in various leukocytes, endothelial cells and probably other cells [97, 98]. NO is inhibitory to different life cycle stages of the malaria parasites, including the disease-producing asexual blood stages [86]. However, NO has also been reported to have some immunosuppressive effects in experimental malaria, resulting in enhancement of these infections [99]. Several findings also indicate the involvement of NO in the pathogenesis of human cerebral malaria [86, 99]. Furthermore, its chronic overproduction in association with subclinical infection in malaria-exposed children may contribute to anemia [100]. Nevertheless, peripheral blood mononuclear cells of children with prior mild malaria express higher levels of inducible NO synthase than those with prior severe malaria [101, 102]. The results of these two recent studies also support the protective role of NO in malaria.

Malaria and Pregnancy

Pregnant women exhibit elevated susceptibility to *P. falciparum* infection, commonly associated with premature delivery, increased perinatal mortality and reduced birth weight of the newborn. The prevalence of infection and parasite densities are highest in the first half of pregnancy and are also accompanied by transient depression of cell-mediated immunity.

During pregnancy there is a pronounced sequestration of *Plasmodium*-infected erythrocytes in the intervillous spaces of the placenta, reflecting their preferred cytoadherence to placental syncytiotrophoblasts. One major placental host receptor for infected erythrocytes is chondroitin sulfate [103, 104], interacting with distinct binding structures in the DBL3 domain of PfEMP-1 [105]. Chondroitin sulfate-binding parasites are variants only found in pregnant women [106]. Another more recently described placental receptor for *P. falciparum*-infected erythrocytes is hyaluronic acid [107].

Chronic plasmodial infection of the placenta is associated with intervillous inflammation which is especially severe in primiparae. In contrast, multigravidae are less susceptible to placental malaria, at least in part due to their production of antibodies inhibiting placental cytoadherence [108].

Maintenance of successful pregnancy is also associated with Th2-biased immune responses within the maternal uterus and the fetoplacental unit [109].

This bias probably contributes to the severity of placental infection which may be more efficiently contained by a Th1 type of immune response [110].

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Genetic Regulation of Malaria Infection in Humans

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Introduction

As more and more genomic sequences from both humans and *Plasmodium falciparum* become available, functional genomics will lead to a greater understanding of host-parasite interactions. The information generated from this post-genomic area will advance knowledge of parasite and human genes which influence the pathogenesis and manifestations of malaria and will have a major impact on drug and vaccine development [1, 2]. This chapter will deal with the genetic aspects involved in susceptibility or resistance to *P. falciparum* malaria, with major focus on the genetic regulation of the immune response to the parasite.

Genomics of the *P. falciparum* Parasite

Experimental approaches have uncovered adaptations of the human *P. falciparum* parasite to the host defense system including sequestrations and antigenic variation of parasitized red blood cells and gene amplification in response to drugs. The 25- to 30-megabase genome of *P. falciparum* contains 14 nuclear chromosomes, bounded by telomeres and organized in nucleosomes [3–5]. Molecular karyotyping of different *P. falciparum* isolates demonstrated frequent and considerable size polymorphism among homologous chromosomes. A genetic linkage map using microsatellite markers has been used to construct a map of more than 900 markers that fall into 14 interfering linkage groups corresponding to the 14 nuclear chromosomes [6]. In addition several

full-length restriction maps and gene assembly have been determined by using shotgun optical mapping [7, 8]. Through differential hybridization and sequencing, large differences in gene expression were observed between the different stages of the parasite [7]. For a high level of resolution, allelic association studies including establishment of the occurrence of single nucleotide polymorphisms may be required as pointed out in recent studies of recombination rates in a 5-kb region containing the *msp-1* gene [9, 10].

The sequencing of the *P. falciparum* genome is well under way, with chromosomes 2 and 3 now finished and many of the other 12 chromosomes nearing completion [11, 12]. Once identified, specific *P. falciparum* genes can be manipulated inside the parasite. Functional complementations, gene knock-outs, and allelic modifications have all been utilized for this purpose [13–16].

Another level of analysis seeks to clarify the complex interplay of gene expression levels determining the developmental stages of the parasite life cycle. Important results have already come forth using DNA microarrays and oligonucleotide chips allowing genome-wide screenings [2, 7]. Karyotypes and chromosomes of the other *Plasmodium* spp that infect humans have also been determined [17].

In addition to a nuclear and mitochondrial genome, plasmodia harbor extra-chromosomal DNA which is phylogenetically related to a prokaryotic plastid DNA [18]. This plastid is localized in an organelle surrounded by four membranes and is referred to as the 'apicoplast'. This nonphotosynthetic plastid has been shown to be the basis for the parasite's susceptibility to certain antibiotics with anti-malarial activity [18, 19] and may be a good target for the development of new anti-malaria drugs.

Genetics of Malaria Infection

Malaria gives rise to a broad spectrum of disease phenotypes, varying from asymptomatic infection to several severe and frequently fatal syndromes such as cerebral malaria, severe anemia and multiorgan failure [20]. About 50% of *P. falciparum*-infected children become symptomatic but of these only a small percentage develop severe malaria [21]. The reasons for this are multifactorial and include parasite virulence, transmission intensity, host susceptibility and other factors. All these factors are likely to be involved in the genome-shaping interactions that reflect ongoing evolutionary forces affecting host–parasite relationships.

Host Genetic Contributions to Malaria Susceptibility

The influence of the genetic make-up of the host on susceptibility to infections has been established in numerous animal studies [22] and there is also

much evidence for the role of genetic factors in human infectious diseases [23–26]. Genetic resistance factors for malaria were first discovered more than half a century ago when malaria therapy in the management of syphilis suggested some marked interindividual differences in susceptibility to malaria in non-immune subjects [27]. However, systematic attempts to identify malaria susceptibility loci by linkage and association analysis in both human populations and animal models are relatively recent. For some of the genes encoding hemoglobin and red cell variants, the overlapping geographic distribution with *P. falciparum* is thought to have resulted from evolutionary selection by malaria [28].

Red Blood Cell Polymorphisms

Several red blood cell polymorphisms have been described which affect susceptibility to malaria. The best known are the hemoglobin abnormalities (hemoglobinopathies). Epidemiological evidence for thalassemias [28, 29], sickle cell hemoglobin and glucose-6-phosphate dehydrogenase deficiency have provided evidence for their protective effect against *P. falciparum* malaria [30, 31]. More recent evidence has suggested that hemoglobin E also protects against malaria [32, 33], and that the erythrocyte band 3 is responsible for ovalocytosis in South Asians [34, 35]. However, the mechanisms whereby these diverse polymorphisms protect against malaria are unknown. It has been suggested that some of the protection may be immune-mediated and that interactions between susceptibility to *P. falciparum* and *Plasmodium vivax* may be relevant for protection against *P. falciparum* in populations where both are prevalent. This interpretation was based on the findings that children with α-thalassemia have a significantly higher incidence of malaria than non-thalassemic children. In South East Asia *P. falciparum* is rare and the higher incidence of *P. vivax* infections may confer some cross protection against subsequent exposure to the *P. falciparum* parasite [36]. These two parasite species coexist in many parts of the world but *P. vivax* is rather rare in West Africa. One reason for this is that the erythrocytes of Duffy blood group-negative donors, who predominate in these regions, are resistant to invasion by *P. vivax*. The Duffy blood group antigen is an example of how genetic epidemiology may give insight into the molecular basis of susceptibility to infectious diseases. Today we know that the Duffy antigen is a widely expressed chemokine receptor, known as DARC [37]. Interestingly a single nucleotide polymorphism disrupts a GATA-1-binding site in the *DARC*-promoter region and thereby inhibits its expression on erythrocytes but not on other cells [38].The parasite molecules that bind DARC are now well defined and this has led to the identification of a family of parasite-binding proteins of great biological importance for parasite invasion into red blood cells [39].

Genetic Regulation of the Immune Response

Improved knowledge of gene expression and gene regulation has provided a basis for identifying fundamental immune processes, and for interpreting the impact of genetic polymorphisms on the regulation of host immune responses to malaria [40].

Linkage of MHC (and Non-MHC) Genes with Immunity and Pathogenesis in Malaria

The best studied immune response genes belong to the major histocompatibility complex (MHC). The MHC is located on chromosome 6 in humans and contains at least 50 genes. The MHC is polygenic (several HLA class-I, class-II and class-III genes) and is very polymorphic as reflected by multiple alleles at each locus. HLA class-I and II products play a major role in the presentation of foreign antigens to T cells, and MHC class-III genes encode for polypeptides of the complement system as well as for molecules involved in inflammation. HLA class-I molecules present antigens from pathogens living in the cytosol to $CD8^+$ T cells, resulting in killing of the infected cells. In contrast, HLA class-II molecules present exogenously delivered antigens to $CD4^+$ T cells which will activate them to produce cytokines and initiate an immune response.

The association between HLA and human malaria infections was first demonstrated in a study conducted in Sardinia [41]. Since then evidence has come forth for both MHC-independent and MHC-dependent control of malaria infection in rodent and human malaria. In mice, different variants of *Plasmodium yoelii* and *Plasmodium chabaudi* may give rise to lethal infections in some strains of mice but not in others [42, 43]. Mortality in the susceptible strains is highest in male mice and thus not related to the H-2 system but parasite clearance in resistant mice appears to be linked to H-2 [44]. However, the actual genes involved remain to be determined. In humans a study in The Gambia reported that HLA-B53 was associated with resistance to both cerebral malaria and severe malarial anemia, whereas the class-II allele HLA-DRB1* 1302 was associated with resistance to severe anemia only [45]. The mechanism behind the protective effect of HLA-B53 was suggested to be due to a more effective antigen-presenting capacity of the specific HLA antigens resulting in increased killing of the parasites. In favor of this was the identification of HLA-B53-restricted $CD8^+$ T cells in the same study population [46] and the finding of specific cytotoxic T lymphocytes to other conserved motifs of the liver-stage antigen-1 [47, 48]. Linkage analysis in The Gambia revealed a pronounced effect of MHC genes on the risk of complicated malaria [49].

The various MHC associations with specific anti-malarial T- and B-cell responses in experimental malaria systems also gave rise to concerns that widespread and genetically determined human non-responsiveness to defined

malaria antigens could have serious consequences for malaria vaccine development [50]. However, only weak or no associations of poor lymphocyte responses to a variety of defined *P. falciparum* antigens and MHC alleles have been reported [51, 52]. Nevertheless despite the difficulties in demonstrating HLA-restricted malaria-specific T- and B-cell responses, several lines of evidence suggest that these responses are genetically regulated. Thus, monozygous twins are more concordant in their anti-malarial immune responses than dizygous twins or their siblings regardless of HLA [53–55].

Other Genes Associated with Immune Responsiveness

A factor influencing responsiveness to specific malaria antigens is the T-cell receptor (TCR). The TCR-Vβ gene repertoire is partly regulated by MHC genes although other factors are also involved in shaping it [56]. In a small twin study conducted in The Gambia neither MHC nor the malaria parasite seemed to be the dominant factors in the shaping of the TCR Vβ repertoire [57].

In recent years, population-based association studies have highlighted the potential importance of a number of candidate genes. Tumor necrosis factor (TNF) has attracted great interest because of its dual role in host defense and in the pathogenesis of cerebral and severe malaria. The gene encoding TNF-α is located in the MHC class-III region [58]. Three different TNF-promoter polymorphisms appear to be independently associated with different forms of severe malaria [59, 60]. Gambian children who are homozygous for the *TNF-308A* allele have increased susceptibility to cerebral malaria [59] and a similar trend was observed in Kenyan children [61]. The same allele has been associated with various other infections like leishmaniasis, lepromatous leprosy, trachoma and meningococcal septicemia [61]. In a study conducted in Sri Lanka carriers of this allele had increased risk of severe infectious diseases in general [62]. DNA foot-printing of the TNF promoter region has identified another variant allele, *TNF-376A*, which affects the binding to the TNF promoter of the transcription factor OCT-1 [63]. The *TNF-376A* allele is associated with increased susceptibility to cerebral malaria in both Kenyan and Gambian children. A third allele, *TNF-238A*, is associated with susceptibility to severe anemia in The Gambia [64]. Whether this association is a direct effect of TNF or just a determinant of malaria susceptibility linked to other genes remains to be determined [60].

In Gabon, a single nucleotide polymorphism of the inducible nitric oxide synthase (*iNOS2*) promoter has been associated with protection from severe malarial anemia, and in The Gambia a *NOS2* microsatellite polymorphism has been associated with susceptibility to fatal malaria [65, 66]; however, neither of these associations have been found in Tanzania [67].

A polymorphism in the endothelial receptor, the intercellular adhesion molecule-1 involved in the sequestration of parasitized erythrocytes, has also been

suggested to contribute to differences in malaria morbidity. However, results obtained from three different case-control studies have yielded conflicting results. In Kenya it appeared to predispose to cerebral malaria [68], while it protected against severe malaria in Gabon [69] and had no effect at all in The Gambia [70].

Alternative approaches to study the genetics of malaria in humans are segregation and linkage analysis. Among these methods, linkage studies (parametric and nonparametric) search to locate a chromosomal region showing a non-random segregation with the phenotype within families, whereas association studies are typically case-control studies elucidating a particular marker allele between unrelated affected or unaffected subjects.

In one segregation analysis, a predominant recessive gene controlling blood infection levels was detected [71]. More recent analysis by the same group showed the existence of complex genetic factors controlling blood infections [72, 73]. Interestingly, these studies revealed a strong interaction between genetic factors and age, the younger the children the more prominent the genetically related differences. In a recent candidate-region approach a sib-pair linkage between the chromosome region 5q31–q33 and *P. falciparum* blood infection levels was seen [73]. This region contains numerous candidate genes encoding immunologically important molecules such as cytokines, growth factors and growth factor receptors, all involved in the control of immunity to *P. falciparum* blood-stage infections [74]. The region is also linked to plasma IgE levels [75, 76], bronchial hyperresponsiveness [77] and schistosomiasis infection [78, 79].

A recent comparison of the response to *P. falciparum* malaria in three West African ethnic groups, Fulani, Mossi and Rimaibé, living in the same area, provided new evidence for the importance of genetic factors [80]. In this study clear interethnic differences in infection rates, febrile malaria and antibody responses to several malaria antigens were demonstrated, with significantly lower susceptibility and higher anti-malaria antibody levels in the Fulanis [80–82]. The results could not be accounted for by differences in malaria protective measures, sociocultural or environmental factors or previously known genetic factors of malaria resistance. Most recently, it was shown that one interethnic variability possibly explaining the higher anti-malaria antibody levels in Fulani was an association with the –524 T allele in the IL-4 promoter [83]. However, this polymorphism was not associated with higher anti-malaria antibody levels in the other ethnic groups indicating that the polymorphism itself is nonfunctional.

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Pre-Erythrocytic Malaria Vaccines to Prevent *Plasmodium falciparum* Malaria

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Imperative for a Malaria Vaccine

A safe and effective vaccine to prevent malaria due to *Plasmodium falciparum* is an urgently needed tool for reducing the global burden of the disease. Drug-resistant strains of the parasite and insecticide-resistant mosquito vectors continue to spread, making the identification of promising vaccine candidates more important than ever. Encouraging progress towards the fielding of a vaccine to prevent the disease is being made, however, and this chapter will provide both a background and an update of the most recent studies in this exciting research area.

Design Considerations for a Malaria Vaccine

The design of candidate malaria vaccines involves the selection of antigens that target the parasite at vulnerable portions of its life cycle. Malaria species that have been adapted to robust rodent models, such as *Plasmodium berghei* and *Plasmodium yoelii*, have been powerful tools for identifying both candidate antigens and immunization strategies. Sporozoites transmitted by the bite of the female *Anopheles* mosquitoes can be targeted by antibodies directed against their surface antigens as they travel through the bloodstream [1]. These antibodies block the ability of sporozoites to invade liver cells. A variety of cell-mediated responses, including lymphokines and cellular cytotoxicity, are able to kill infected liver schizonts, and thus prevent the maturation and release of mature *P. falciparum* exo-erythrocytic forms into the bloodstream [2]. To date, the most promising vaccine candidates tested in humans have been based on a small number of

sporozoite surface antigens which have been extensively investigated using these rodent systems.

The design of a candidate vaccine is influenced greatly by the proposed labelling indication. In the case of pre-erythrocytic vaccines, the intent is to actually prevent infection. An ideal pre-erythrocytic vaccine would therefore induce immune responses capable of eliminating sporozoites from the circulation or killing all infected hepatocytes, thereby conferring 'sterile immunity'. It would be especially important that such a vaccine be highly effective in nonimmune subjects since these individuals would have no effective immunity against blood-stage parasites. The theoretical feasibility of developing such a vaccine was established more than 30 years ago by experimental immunization of human subjects with large numbers of irradiation-attenuated sporozoites [3]. This impractical but effective process rendered immunized subjects completely protected from heterologous sporozoite challenge and this model has been crucial to the further development of a pre-erythrocytic vaccine.

The Circumsporozoite Protein as the Basis of a Malaria Vaccine

One of the first malaria genes to be identified through this model was the circumsporozoite (CS) protein [4]. The CS proteins of all malaria species contain a central segment of species-specific amino acid repeat sequences flanked by nonrepeat regions. In the case of *P. falciparum*, the central portion contains approximately 40 repeats of the amino acid sequence asparagine–alanine–asparagine proline (NANP) and several asparagine–valine-aspartic acid–proline (NVDP) repeats [5]. The central repeating unit is immunodominant relative to the flanking regions and is the portion of the molecule to which protective antibody responses are principally directed [6].

Two alum-adjuvanted candidate vaccines based on the CS repeat sequences were developed in the mid 1980s, tested in humans and found to be safe, but both suffered from generally poor immunogenicity [7, 8]. In most of the volunteers, repeated immunization induced relatively low titers of antibodies against the CS repeats. Nevertheless, a small number of volunteers who did respond well were shown to be protected against homologous experimental sporozoite challenge. These pivotal challenge data confirmed that sterile immunity against malaria could be induced in humans by immunization with a synthetic subunit vaccine. Since protection seemed to correlate with high antibody levels, it was felt that the development of more immunogenic versions of these vaccines would lead to better efficacy.

Subsequent sporozoite vaccines, also based on the NANP repeat, contained modifications conducive to the enhancement of immune responses, such as

conjugation of recombinant CS repeat protein with *Pseudomonas aeruginosa* exotoxin A (R32ToxA) [9] and formulation of a similar antigen (R32NS1) in liposomes containing monophosphoryl lipid A (MPL) [10] or an emulsion of MPL, mycobacterial cell wall skeleton and squalene (Detox®, Ribi Immunochem) [11, 12]. Each of these strategies resulted in generally higher antibody levels, but surprisingly they did not significantly enhance efficacy.

While these clinical studies were in progress, the role of protective T-cell responses directed against the liver-stage form of the parasite was elucidated in greater detail. Studies in rodents and humans immunized with irradiated sporozoites revealed that not only antibodies, but CD4+, CD8+ T cytotoxic (CTL) T-cell responses, and cytokines such as interferon-γ (IFN-γ) were important components of the protective immune responses against the pre-erythrocytic stage [13]. Target epitopes for these T cells have been localized to the C-terminal region of the CS protein [14–19]. Region II, a charged highly conserved sequence which lies in this portion of the molecule, was implicated in the binding of sporozoites to hepatocytes [20]. The recognition that peptide sequences lying outside the CS repeat were important for induction of humoral and cellular responses against the hepatic stage led to the inclusion of nonrepeat portions of the molecule in the next generation of CS-based vaccines. One such candidate (NS1RLF) was constructed so as to be completely devoid of repeat epitopes and contained both N-terminal and C-terminal flanking regions. This vaccine was formulated as a recombinant protein encapsulated in liposomes and adjuvanted with MPL. The vaccine was found to stimulate a potent CTL response in mice and induced a brisk immune response in humans that included antibodies that recognized sporozoites [21]. Unfortunately, no protection from experimental sporozoite challenge was observed and this strategy has been abandoned.

Initial Development of Candidate Malaria Vaccine 'RTS,S'

In collaboration with SmithKline Beecham Biologicals (SBBIO) our group at the Walter Reed Army Institute of Research turned to what has become a more promising approach. This strategy was based upon SBBIO's development of an expression system in which the CS repeats as well as the C-terminal region were incorporated into highly immunogenic HBsAg particles. The components of this new vaccine are two polypeptides (RTS and S) that are synthesized simultaneously in *Saccharomyces cerevisiae*. During purification they spontaneously form composite particulate structures (RTS,S) that constitute the vaccine antigen. RTS is a single polypeptide chain corresponding to amino acids 207–395 of the CS gene in the 3D7 strain of *P. falciparum* fused to the amino terminus of the hepatitis B surface antigen (HbsAg; adw serotype). S is a polypeptide of

226 amino acids that corresponds to HBsAg. RTS includes 19 copies of the tetrapeptide repeat motif (NANP) plus the C-terminal region of the protein minus the hydrophobic anchor sequence [22]. Several formulations of the vaccine were manufactured and preclinical studies included a series of increasingly potent adjuvants. The initial clinical studies involved one formulation in which RTS,S was adsorbed to alum, and a second in which it was adsorbed to alum to which was added the immunostimulant 3-deacyl-MPL. This second adjuvant formulation is known as SmithKline Beecham Adjuvant System 4 (SBAS4). Although antibody responses were modest, most volunteers developed cellular responses against the CS protein. More importantly, following sporozoite challenge, 2 of 8 volunteers in the RTS,S/SBAS4 group were protected whereas all 6 volunteers that received the alum formulation developed malaria [22].

Encouraged by these results which suggested that greater immunogenicity would be associated with greater efficacy, two additional formulations were studied in a phase I/IIa trial. In that study, the SBAS4 formulation was tested along with a new formulation (RTS,S/SBAS3) containing a proprietary oil-in-water emulsion, and another (RTS,S/SBAS2) containing the same emulsion plus the immunostimulants MPL and QS21 [23]. These new adjuvant systems were chosen on the basis of preclinical studies in Rhesus monkeys that demonstrated their safety and immunogenicity. The data showed that SBAS3 promoted strong antibody responses while SBAS2 induced both humoral and cellular responses [Heppner DG, personal commun.]. Volunteers immunized with either RTS, S/SBAS3 or RTS,S/SBAS2 developed some of the highest anti-repeat antibody responses induced by any sporozoite-based vaccine, but both the SBAS4 and SBAS3 formulations were poorly protective. In contrast, an unprecedented 6 of 7 volunteers who received the SBAS2 formulation were protected [23]. Type-1 Th cellular responses, especially the production of IFN-γ upon stimulation with CS peptides were promoted by the SBAS2 adjuvant formulation and were presumed to be an important component of the protection. Subsequently, expanded safety and immunogenicity trials of RTS,S SBAS2 in over 200 malaria-naïve and malaria-immune subjects have revealed the vaccine to be safe and highly immunogenic, and recent experimental challenge studies involving more than 50 volunteers indicate that the vaccine consistently induces significant protection when given on two- or three-dose schedules [24].

Initial Field Trials of Candidate Malaria Vaccine 'RTS,S'

These encouraging results were followed up recently with SBBIO-sponsored clinical trials involving malaria-immune adult male residents of The Gambia. A phase-I safety and immunogenicity study was conducted by investigators at

Great Britain's Medical Research Council laboratories in Banjul, at a study site where there was minimal ongoing malaria transmission. This phase-I study indicated that the vaccine was safe, mildly reactogenic and immunogenic in otherwise healthy malaria-immune adults [25]. A follow-up phase-IIb pilot efficacy study was conducted in malaria-immune adult Gambian men who were immunized during a period of low transmission and followed for the occurrence of new malaria infections during a period of active malaria transmission [26]. In this study, conducted at the MRC Field Station in Basse, Upper River Division, The Gambia, 360 men were randomized to receive three doses of either RTS,S/SBAS2 or human diploid rabies vaccine, given intramuscularly at 0, 1 and 6 months. The vaccination schedule was timed to coincide with the dry season during which transmission of *P. falciparum* was low. The final dose was administered approximately 1 month before the expected onset of the rainy season. Volunteers were treated with sulfadoxine/pyrimethamine to clear asexual-stage parasites 1 month before the beginning of the 15-week efficacy follow-up period. The vaccine was found to be safe, mildly reactogenic and immunogenic. Of these, 250 men received all three doses and had adequate follow-up data. Vaccine efficacy was estimated to be 71% (95% CI 46–85%) during the first 9 weeks of follow-up, but decreased to 0% during the last 6 weeks of the follow-up period. The reasons for the short duration of efficacy are likely to be multiple. Subset analysis revealed that younger, less malaria-experienced men became infected in the control group earlier than did the older, more highly immune members of their cohort. As vaccine efficacy waned, the incidence in the RTS,S/SBAS2 group was being compared to the control group from which the most susceptible subjects had been removed. Antibody responses also declined during the period of follow-up such that the mean levels at the end of the follow-up period were equivalent to those after a single dose of vaccine. As has been seen in other studies of sporozoite vaccines, no boosting of antibody responses was seen following sporozoite exposure. Finally, it is likely that the entomologic inoculation rate was typical of that previously described for this region and was higher at the end of the transmission season than it was at the beginning.

This important field study extended important data previously obtained in studies of malaria-naive volunteers immunized with RTS,S/SBAS2 and exposed to sporozoites using the WRAIR standard challenge model. First, it confirmed the predictive value of the model – efficacy in the field was similar in magnitude and duration to that observed in the laboratory [27]. Second, there was no evidence of strain-specific immunity, as polymerase chain reaction analysis of CS genes from isolates collected from subjects who became infected during the study revealed that the frequency of polymorphic sequence variants at the Th2R and Th3R regions characteristic of the NF54 clone (from which the vaccine was

derived) were similar for the RTS,S/SBAS2 and rabies vaccine groups. Third, it demonstrated that natural boosting through exposure to sporozoites was unlikely to enhance or sustain vaccine-induced immunity. Finally, the data strengthen the importance of high levels of antibodies against CS repeat epitopes as a potential correlate of immunity, though a specific protective cutoff value could not be identified.

The results obtained with RTS,S/SBAS2 in The Gambia must be viewed cautiously. Clearly they mark an important milestone – the first time that vaccine-induced protection from infection with *P. falciparum* has ever been observed in a field setting, and the capacity building and experience gained with African co-investigators were invaluable. It is hoped that these encouraging initial efforts will be followed up with additional studies that expand the study populations to younger age groups and to regions with different malariametrics. Strategies that incorporate prime-boosting or combination vaccine regimens involving RTS,S/SBAS2 are also an important area for future research.

Platform Technologies for a Multicomponent, Multistage Vaccine

The feasibility of developing malaria vaccines that target multiple stages of the parasite life cycle is being pursued in parallel. Several approaches to multi-component immunization have been explored: (i) immunization with an attenuated vector capable of expressing cloned parasite antigens; (ii) immunization with a mixture of plasmid DNA constructs that are able to express different parasite antigens in vivo, and (iii) immunization with novel recombinant or peptide-based constructs in which target epitopes have been strung together to create neo-antigens.

While a number of these strategies show promise in rodent models, there is limited clinical data to date. One such vaccine was based on an attenuated vaccinia virus (NYVAC). In collaboration with Pasteur Merieux Connaught and Virogenetics Corporation, investigators at WRAIR expressed seven malaria genes representing all three principal stages of the life cycle (CS, TRAP, LSA-1, SERA, MSP-1, AMA-1 and Pfs25) in a construct called NYVAC-Pf7 [28]. Preclinical studies with a version expressing the *P. berghei* CS gene showed that the vaccine was highly protective [29]. However, in a large phase-I/IIa trial, the vaccine was found to be safe but only moderately immunogenic, especially with regard to antibody responses [28]. T-cell responses were more pronounced, and a significant proportion of subjects developed CTL responses against CS, TRAP or LSA-1. Disappointingly, at challenge only 1 volunteer was protected though a statistically significant delay in the number of days to patent infection was observed in the non-protected volunteers. Strategies for enhancing the

immunogenicity of this vaccine including, in particular, use of prime immunizations with vaccinia followed by boosts with recombinant products are currently under consideration.

An alternative strategy involves the use of plasmid DNAs to express target genes in vivo [30, 31]. Although the mechanisms by which DNA immunizations work are not completely understood, it is believed that DNA injected directly into dermis or muscle cells is taken up and the encoded proteins expressed by the normal cell machinery. The subsequent fate of the antigen is not clear, but may be processed by professional antigen-presenting cells and transported to regional lymph nodes where the primary immune response takes place. Recently, a phase-I clinical trial of a DNA plasmid vaccine expressing the full-length CS protein was conducted [32, 33]. CTL responses were observed, but no antibody responses were detected, even at very high doses. No adverse events were reported, though many issues about the long-term safety of DNA vaccines remain unresolved [34]. Nevertheless, DNA vaccines have important potential advantages that may outweigh these difficulties: DNA is relatively inexpensive to produce and purify, and DNA vaccines should be quite stable. Once solutions to the problem of poor immunogenicity are found and concerns about long-term safety are resolved, DNA vaccines may yet be one of the most powerful strategies for developing a multicomponent malaria vaccine.

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Vaccines against Asexual Stage Malaria Parasites

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Introduction

More than 100 years after the first identification of the malaria parasite by Dr. Lavern in 1861, the number of reported cases and deaths caused by malaria parasites are on the rise. According to a recent estimate about 2 billion people are living in areas where malaria is transmitted, with 300–500 million clinical cases, and over 1 million deaths each year [1]. Although a proportion of these increases can be accounted for by the growing human population living in tropical areas, the fact remains that the parasite is endemic in most parts of the tropics where *Anopheles* mosquitoes are present, despite decades of effort aimed at control or eradication. The exceptions are major cities and a few rural areas where a high standard of living is associated with adequate vector control and rapid case identification and treatment, or where pollution and other environmental changes have rendered conditions unsuitable for the vector. Drug resistance and in some cases insecticide resistance have aggravated the problem.

Many believe that the ideal solution would be to develop a vaccine that halts the parasite during the asymptomatic pre-erythrocytic stages. The concern over this approach is that the successful transition of even a single parasite to the blood stages (due to a 'leaky' pre-erythrocytic vaccine) will result in a viable erythrocytic stage infection that carries the potential for severe disease and death, as if the individual had never been vaccinated. This is because the majority of malarial antigens expressed during the early pre-erythrocytic stages are not expressed during erythrocytic stage infection, leaving individuals immunized with a pre-erythrocytic vaccine still susceptible to erythrocytic stage infection. Particularly in areas of intense transmission, where entomological inoculation rates may exceed 300 infective bites per year, it seems unlikely that

pre-erythrocytic stage vaccines will be able to prevent all blood stage infections. For this reason, it is imperative to develop a vaccine that incorporates components that target parasites developing within erythrocytes. It could be argued, in fact, that protecting against erythrocytic stage infections is the most important goal for malaria vaccines designed for residents of endemic areas, since the pre-erythrocytic stages are essentially harmless to the host and since there are several lines of evidence that erythrocytic stage immunity does indeed prevent the morbidity and mortality associated with malaria infection.

The first of these is naturally acquired immunity (NAI), a state of partial immunity acquired over several years by persons surviving multiple malaria infections, that limits both the level of parasitemia and also the symptoms associated with parasitemia [2]. NAI explains why older children and adults living in endemic areas tolerate blood-stage infection without clinical symptoms. NAI does not constitute resistance to pre-erythrocytic stages, as demonstrated by the rapidity with which persons clinically immune to malaria redevelop parasitemia in cleared-cohort studies [3]. The fact that NAI is achieved in essentially 100% of individuals, including older children, after sufficient exposure, suggests that it should be possible to mimic the process by an appropriately designed vaccine.

A second line of evidence that erythrocytic stage immunity protects against morbidity and mortality is that the passive transfer of antibodies from adults with NAI to parasitemic children with clinical disease markedly depresses their parasite levels [4, 5]. This antibody-mediated suppression transcends parasite strain (but not species) and suggests that antibodies against parasite antigens expressed during the erythrocytic stages constitute the mechanism underlying the protection afforded by NAI.

A third line of evidence is the finding in several studies that the use of insecticide-impregnated bednets reduces morbidity and mortality without affecting the prevalence of parasitemia [6, 7]. One hypothesis to explain this finding is that effectively reducing the entomological inoculation rate causes a corresponding reduction in the number of parasites developing through the liver, and this in turn increases the incubation period in the erythrocytes (i.e., the period between release from the liver and developing the first symptoms). This extra time allows additional maturation of the immune response against that particular blood-stage parasite (possibly mediated by antibodies recognizing its variant antigens). Because parasite numbers in a naïve host grow about tenfold every 2 days, and assuming that a certain density of parasites is needed to surpass the ‘clinical threshold’ and trigger the onset of symptoms, each log reduction in pre-erythrocytic stage parasites achieved by bednets (or by a pre-erythrocytic vaccine) adds 2 more days for the erythrocytic stage immune response to mature. One or two extra days may be sufficient to prevent severe

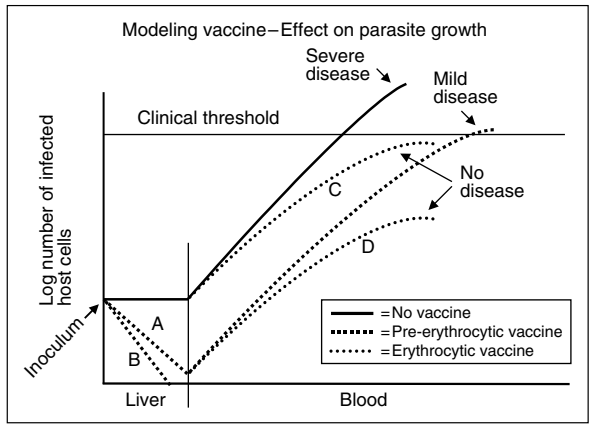

Fig. 1. The development of a malaria infection in an unvaccinated, malaria naïve host is depicted by the solid line. In this scenario, there is no parasite mortality in the liver. Merozoite release into the blood results in logarithmic growth until the clinical threshold is surpassed. A slight degree of erythrocytic stage immunity develops (represented by the diminishing slope of the line at the apex) but is not sufficient to prevent severe disease. The development of a malaria infection in a host with partial (A) or complete (B) pre-erythrocytic immunity is depicted by the dashed line. In the case of partial immunity (A), logarithmic growth in the blood begins at smaller parasite numbers than in the unvaccinated host, allowing more effective erythrocytic stage immunity to develop by the time the clinical threshold is surpassed, resulting in mild disease. The development of malaria in a host with erythrocytic (C) or both pre-erythrocytic and erythrocytic (D) immunity is depicted by the dotted lines. In both cases, parasitemia is suppressed before the clinical threshold is surpassed, avoiding disease altogether. Vaccine development strategies incorporating the use of pre-erythrocytic and/or erythrocytic stage antigens can be expected to achieve these effects. An additional strategy, an anti-toxic vaccine, would serve to raise the clinical threshold.

disease, or even symptomatic disease in those who are partially immune. An alternative explanation, however, is that insecticide-impregnated bed nets may reduce illness by eliminating a proportion of infections with virulent malaria strains, rather than by increasing the time available for the induction of immune responses targeting blood stage parasites.

These concepts are illustrated in figure 1. The underlying assumption is that the length of the period between release from the liver and onset of symptoms (represented as surpassing a 'clinical threshold') is inversely related to the effectiveness of the erythrocytic stage immune responses. Erythrocytic stage immunity could act either by reducing the slope of this curve (e.g., antibodies interfering with parasite growth and development), or by raising the clinical threshold (e.g., antibodies neutralizing malaria toxins that induce cytokine release and associated symptoms). If the parasite-suppressing component of the

immunity is sufficient, presumably the clinical threshold will never be crossed, and the vaccine recipient will be protected against malaria-induced disease, even though not protected against parasitemia. Such a vaccine would be ideal for persons living in an endemic area. For travelers returning to malaria-free areas, prevention of clinical illness would of course be highly desirable, but at the same time it would be preferable to diagnose such asymptomatic infections and treat with anti-malarial drugs.

This review describes various approaches to developing a vaccine that targets erythrocytic stage parasites, and key candidate antigens that induce immune responses that inhibit merozoite invasion or, interfere with parasite development inside erythrocytes. The current status of the anti-erythrocytic vaccine effort and future directions are also discussed.

Approaches to Development of an Asexual Stage Vaccine

Preventing Clinical Malaria by Attacking Erythrocytic Stage Parasites

The goal of an anti-asexual stage vaccine is to reduce the numbers of or totally eliminate erythrocytic forms of the parasite. Malaria researchers around the world are working to develop a vaccine that would induce immune responses that recognize and destroy extracellular merozoites or parasites developing within erythrocytes. Proteins present on the surface of merozoites, and proteins involved in the process of invasion (e.g., antigens present in apical organelles), or parasite proteins anchored on the surface membrane of infected erythrocytes are the obvious targets of such a vaccine. This could be accomplished by one of the following mechanisms.

Inhibition of Merozoite Invasion into Erythrocytes. One of the potential targets of an asexual stage vaccine is the extracellular merozoites released from infected hepatocytes or infected erythrocytes. These merozoites remain extracellular for a brief period (a few seconds) before their attachment and invasion into erythrocytes. Specific parasite molecules located on the merozoite surface or on apical organelles (micronemes and rhoptries) serve as ligand(s) that interact with receptors on erythrocytes [8]. Antibodies to these parasite ligands are known to prevent such interactions, and thereby inhibit merozoite invasion. Likewise, agglutinating antibodies directed against the merozoite surface could also render merozoites incapable of invading erythrocytes. Complement-fixing antibodies directed against merozoite surface proteins could also cause the lysis of malaria merozoites.

Attacking Parasites Developing within Erythrocytes. Immediately after merozoite entry, parasites begin to undergo maturation inside the erythrocyte. Depending upon the human *Plasmodium* species, the erythrocytic stage developmental cycle takes 48–72 h. The uninucleate merozoite develops through

the ring, trophozoite, and schizont stages. The schizont stage is characterized by nuclear division, producing 18–36 progeny merozoites. The intra-erythrocytic parasite is surrounded by a parasitophorous vacuole. During the parasite developmental process, some parasite proteins are released into the erythrocyte cytoplasm while others are transported to and anchored into the erythrocyte membrane. It has been demonstrated that antibodies can destroy intraerythrocytic parasites through a process known as antibody-dependent cellular inhibition (ADCI) [5]. In this mechanism of parasite killing, antigen-specific cytophilic antibodies destroy intraerythrocytic parasites in the presence of monocytes. Two *Plasmodium falciparum* erythrocytic stage antigens, merozoite surface protein 3 (MSP3) [9] and glutamine-rich protein [10], have been identified as targets of ADCI and are being developed as vaccine candidates.

Work performed in murine malaria models has demonstrated that antibody-independent cellular mechanisms alone can also eliminate erythrocytic stage parasites [11]. This mechanism of immunity is thought to operate through the induction of antigen-specific effector T cells that release cytokines which activate macrophages and other immune cells in proximity to infected erythrocytes to generate free oxygen radicals, ultimately resulting in the destruction of intraerythrocytic parasites. Whether such cellular mechanisms are effective against human *P. falciparum* parasites is not yet known. Nonetheless, generation of memory CD4+ T cells is important to inducing long-lasting protective immunity against erythrocytic stage parasites [12].

Preventing Clinical Malaria by Blocking Pathogenesis

Another approach to the reduction of malaria-related morbidity and mortality is to prevent the pathogenic effects of *P. falciparum* infection without directly attacking the parasites themselves. Studies performed over the last several years suggest that the pathogenesis of *P. falciparum* malaria is associated with parasite sequestration in postcapillary venules in the infected host [13]. This circulatory obstruction is thought to be caused by adherence of infected erythrocytes to endothelial cells (cytoadherence) and, by binding of infected erythrocytes to other erythrocytes or to noninfected erythrocytes (rosetting). These factors cause circulating parasites to sequester in localized tissues, and may have evolved as a mechanism to escape destruction by the host immune system, and in particular splenic clearance. Strategies for preventing severe malaria by disrupting sequestration by reversing cytoadherence or by neutralizing malaria toxins are discussed below.

Blocking/Reversing Cytoadherence. Blocking cytoadherence would reduce sequestration and at the same time increase the rate of splenic clearance, since maturing schizonts would remain in circulation. Malaria parasites adhere to host endothelial cells via specific receptor-ligand interaction [14]. Research

conducted over the last several years has led to the identification of several receptors on the host endothelial cells, and of corresponding parasite ligand molecules expressed on the surface of infected erythrocytes. The following molecules on host endothelial cells have been identified as receptors: CD36, thrombospondin, ICAM-1, E-selectin, VCAM-1, and CSA [14–16]. *Pf*EMP1, a highly variant parasite protein (var) expressed on the surface of parasitized erythrocytes has been shown to contain domains that serve as parasite ligands that bind to host receptors [17]. Scientists around the world are working to develop vaccines using regions on var to induce antibodies that would block parasite cytoadherence and thus prevent the clinical symptoms of severe malaria.

Blocking Malaria Toxins. The pathologic effects of toxins released during malaria infection have been known for a long period of time. Studies performed over the last decade have shown that toxins produced by rupturing schizonts stimulate macrophages to produce tumor necrosis factor-α (TNF-α) in vitro [18]; at the same time, it is known that levels of circulating TNF-α are associated with clinical disease [19, 20]. One parasite material identified as a malaria toxin is glycosylphosphatidylinositol (GPI), a membrane anchor moiety present on several parasite surface proteins [21]. Purified GPI isolated from *P. falciparum* parasites has been shown to induce the production of TNF-α and nitric oxide from macrophages and vasculoendotheial cells, and to regulate iNOS expression in macrophages [22]. In a recent study in children, the presence of anti-GPI antibodies was associated with resistance to severe malaria [23]. Experiments are underway to determine whether antibodies induced by immunizing with a GPI moiety would prevent or reduce the clinical manifestations of severe malaria.

Candidate Antigens for Vaccine Development

A number of proteins expressed during the erythrocytic stage of the parasite life cycle are being developed as candidate vaccine antigens. In regard to the many promising vaccine candidates, the decision to further develop an antigen for human testing depends in part on the capacity of the antigen to elicit antibodies that block parasite growth in vitro and the demonstration of protective efficacy in in vivo challenge models. For some of the *P. falciparum* vaccine candidate antigens, no orthologue genes have been identified for the murine or simian malarias. In these situations, the vaccine potential of the antigen derives from field studies suggesting an association between antibody and/or T-cell responses and protection from clinical malaria. The following proteins from erythrocytic stage parasites are the major focus of research for the development of a vaccine against the human malarias.

MSP1

MSP1, a 190- to 230-kD protein, was the first protein identified on the surface of erythrocytic stage merozoites [24]. During or at the time of the schizont rupture and release of merozoites, the precursor MSP1 is processed into at least four distinct fragments [25]. Among these fragments, the C-terminus 42-kD fragment ($MSP1_{42}$) is of particular interest and has been extensively studied. $MSP1_{42}$ is further processed into a soluble 33-kD fragment and a 19-kD fragment ($MSP1_{19}$) that remains attached to the merozoite membrane through a GPI-anchor. $MSP1_{19}$ contains two epidermal growth factor-like domains that are rich in cysteines residues [25].

Data from several epidemiological and laboratory studies demonstrate that MSP1 is a target of protective immunity. It has been suggested that the presence of antibodies against $MSP1_{19}$ is associated with protection from clinical *P. falciparum* malaria [26]. A recent study has demonstrated that IgG antibodies against two major allelic forms of block 2 of MSP1 are strongly associated with protection against *P. falciparum* malaria [27]. MSP1 gene sequences obtained by nucleotide sequencing of the gene from various field isolates show extensive polymorphism suggesting that the molecule is under intense immune pressure [28]. In vitro studies show that monoclonal antibodies and polyclonal antibodies specific to the 19- or 42-kD fragments block the entry of merozites into erythrocytes [25, 29].

The most convincing evidence for involvement of MSP1 in protective immunity comes from in vivo challenge experiments performed in mice and monkeys. Immunization with purified parasite-derived MSP1 protected mice against lethal *Plasmodium yoelii* challenge [24]. More recently several laboratories have demonstrated that immunization with recombinant $MSP1_{19}$ expressed in *Escherichia coli* as a fusion protein with glutathione S-transferase [30–32] or as a nonfusion protein in *Saccharomyces cerevisiae* [33] induced protection against lethal *P. yoelii*. Furthermore, *E. coli* produced $MSP1_{42}$ (that includes $MSP1_{19}$), and $MSP1_{19}$ induced a comparable degree of immunity against *P. yoelii* parasites [34]. The precise mechanism of $MSP1_{19}$ vaccine-induced immunity in mice is not clear, however, available data suggest that immunity is antibody-mediated [32, 33, 35], and mouse H-2 locus-dependent [32].

As in murine studies, immunization with parasite-purified MSP1 conferred protection in *Aotus* monkeys against *P. falciparum* challenge [36]. In subsequent years, a recombinant-produced carboxy-terminal region of MSP1 has been the subject of major vaccine development efforts. Immunization of monkeys with yeast-produced $MSP1_{19}$ or baculovirus-produced $MSP1_{42}$ has produced a high degree of protection against lethal *P. falciparum* [37–39]. In a more recent approach, $MSP1_{42}$ was expressed as a transgene in mice, and

recombinant protein was purified from mouse milk. In *Aotus* monkeys, the mouse milk-produced $MSP1_{42}$ induced immunity that was comparable to baculovirus-produced $MSP1_{42}$ [Stowers A, unpublished].

MSP2

MSP2 of *P. falciparum* has a predicted molecular mass of 28 kD, although the actual size of the protein varies between 45 and 55 kD [40]. The carboxy-terminal region of the protein contains sequences that resemble the GPI anchor that is thought to attach the protein to the merozoite surface [41]. MSP2 is extensively polymorphic with domains of conserved and highly variable regions. The variable region of the molecule falls within two major allelic forms [40, 42] although the existence of hybrid genes resulting from intragenic recombination has also been reported [43]. The extent of sequence polymorphism in MSP2 is further illustrated by a recent study in the Oksibil region in Irian Jaya [44]. In this study, no MSP2 alleles were found to be the same at the beginning and end of the 29-month study period when MSP2 alleles were assayed either in the population as a whole or on an individual level. The highly polymorphic nature of MSP2 suggests that this molecule may be involved in protective immunity.

Further evidence for the involvement of MSP2 in protective immunity comes from several studies. Monoclonal antibodies that recognize epitopes from the variable region of MSP2 inhibit parasite growth in vitro [45]. In The Gambia and in Papua New Guinea, the presence of IgG_3 antibodies to the 3D7 allele of MSP2 has been associated with protection against clinical malaria [46, 47]. No orthologue genes for MSP-2 from other *Plasmodia* have yet been identified. However, in one study immunization with synthetic peptides from the conserved region of the *P. falciparum* MSP2 protected mice against challenge with the murine malaria *Plasmodium chabaudi*, suggesting the presence of protective cross-reactive epitopes between the two malaria parasites [48].

MSP3

The MSP3 of *P. falciparum*, also known as secreted polymorphic antigen associated with merozoites, is a 48-kD protein that is localized in the lumen of the parasitophorous vacuole and on the merozoite surface [9]. Evidence for the involvement of MSP3 in protective immunity comes from the following study. Cytophilic IgG_1, and IgG_3 antibodies isolated from protective human sera recognize MSP3. These sera mediate in vitro killing of *P. falciparum* parasites through an ADCI mechanism. The target epitope responsible for ADCI activity on MSP3 has been identified [9].

MSP4/MSP5

MSP4 and MSP5 of *P. falciparum* are proteins of 272 amino acids. The genes encoding these two proteins are located in tandem on chromosome 2, next to the gene encoding for MSP2 [49]. Both these proteins are anchored to the merozoite surface through GPI-attachment sequences, contain hydrophobic signal sequences, and each protein has a single EGF-like domain in the carboxy-terminal region of the molecule [49]. In murine malarias *P. yoelii*, *P. berghei*, and *P. chabaudi*, MSP4 and MSP5 are represented by a single gene designated MSP4/5 [50, 51]. These MSP4/5 genes bear close structural similarities to the *P. falciparum* MSP4/MSP5 genes, and contain hydrophobic signal sequences, predicted GPI-anchor sequences, and a single EGF-like domain [51]. The vaccine potential of these proteins is provided by an in vivo immunization challenge experiment performed with *P. yoelii* MSP4/5. Immunization with *E. coli*-produced MSP4/5 emulsified in complete Freund's adjuvant (CFA) protected mice against challenge with lethal *P. yoelii* [52].

AMA1

This molecule was first identified as a 66-kD protein in *Plasmodium knowlesi*, referred to as Pk66 [53]. The *P. falciparum* orthologue of this protein is called the apical membrane antigen 1 (AMA1), and is a 83-kD protein that is processed into a 63-kD polypeptide [54, 55]. Following synthesis, AMA1 is first localized in the neck of the rhoptries but after schizont rupture the protein is also detected on the surface of merozoite [56]. Results obtained by comparing the AMA1 nucleotide sequence of 5 laboratory isolates suggested only a limited variability [57]. However, nucleotide sequencing of AMA1 gene from 11 field isolates revealed a significant degree of diversity [58]. The genes encoding the orthologue of AMA1 have been cloned and sequenced for other *Plasmodium* species including *P. knowlesi* [59], *P. chabaudi* [60], *P. fragile* [61], *P. vivax* [62], *P. yoelii* and *P. berghei* [63].

Several in vivo passive and active immunization experiments suggest that AMA1 is involved in eliciting protective immune responses against erythrocytic stage parasites. The first evidence for the vaccine potential of AMA1 came from studies performed with Pk66 (*P. knowlesi* AMA1 orthologue). A monoclonal antibody directed against Pk66 prevented the invasion of *P. knowlesi* merozoites into rhesus erythrocytes in vitro [64]. Vaccination with affinity-purified Pk66 induced protection in 4/6 monkeys following challenge with erythrocytic stage *P. knowlesi* parasites. Furthermore, sera from protected monkeys inhibited parasite growth in vitro, suggesting that Pk66 vaccine induced inhibitory antibodies [65]. In another study, immunization of *Saimiri* monkeys with baculovirus produced AMA1 of *P. fragile* delivered in Montanide ISA 720 induced protective immune responses that allowed vaccinated monkeys to self-clear infection

against erythrocytic stage *P. fragile* parasites [66]. Upon rechallenge with heterologous *P. falciparum*, vaccinated monkeys did not develop any detectable parasitemia suggesting the presence of conserved protective epitopes on the AMA1 molecule from the two parasites. More recent studies further establish the protective role of AMA1 against erythrocytic stage parasites [66]. In murine malaria *P. chabaudi adami*, immunization with *E. coli*-produced, refolded AMA1 ectodomain vaccine delivered in Montanide ISA720 protected mice against erythrocytic stage parasite challenge [67]. This immunity was antibody-independent and T-cell-dependent. In another study, anti-AMA1 antibodies generated by either immunization of rabbits with an *E. coli*-produced, refolded *P. falciparum* 3D7 AMA1 ectodomain (rAMA1), or antibodies obtained from immune human sera affinity purified against rAMA1 strongly inhibited merozoite invasion against homologous and two heterologous strains of *P. falciparum* parasites [68].

EBA-175

The erythrocyte-binding antigen (EBA) of *P. falciparum* was identified as a 175-kD protein (EBA-175) in the supernatants of cultured parasites [69]. This protein serves as a merozoite ligand that binds to the erythrocyte receptor, glycophorin A, through a receptor-ligand interaction [70]. The gene encoding EBA-175 [71] and the orthologue genes for merozoite ligand proteins of *P. knowlesi* [72], and *P. vivax* [73], known as Duffy-binding proteins, have been cloned and sequenced. These molecules are localized in the microneme of the parasites and are classified as belonging to a family of erythrocyte-binding proteins [74]. A cysteine-rich domain (termed RII domain in EBA-175) has been identified as the erythrocyte-binding domain in all three proteins [75]. Recently, the RII domain has been the focus of intense vaccine development efforts. RII has been produced as recombinant protein in baculovirus and *Pichia pastoris* and as DNA plasmid. Immunization of *Aotus* monkeys with recombinant RII when delivered in a heterologous prime boost regimen (DNA immunization followed by protein boost) protected 3 of 4 monkeys against *P. falciparum* challenge. However, immunization with either DNA alone or recombinant protein alone gave no protection [76].

SERA

The serine repeat antigen (SERA) of *P. falciparum*, a protein of approximately 130 kD, contains 985 amino acids with 37 consecutive serine residues [77]. SERA is expressed as a soluble protein in the parasitophorous vacuole surrounding the trophozoites and early schizonts [78]. Based on the structural resemblance of SERA to cysteine proteases, it has been postulated that during merozoite rupture it may function as a protease [79]. Several in vitro and in vivo studies suggest the vaccine potential of SERA. Monoclonal antibodies to SERA

block the entry of *P. falciparum* merozoites into erythrocytes [80]. In in vivo challenge studies, immunization of *Saimiri* monkeys with affinity-purified SERA from parasites induced partial protection against *P. falciparum* challenge [81]. In subsequent years, various regions of SERA were produced as recombinant proteins in yeast. Immunization of *Aotus* monkeys with two fragments of recombinant SERA (SERA-1, amino acids 24–285 and SERA-N, amino acids 24–506), delivered in CFA induced low levels of protection against *P. falciparum* parasites [82]. In two other studies, immunization with SERA-1 delivered in CFA or MF75.2 adjuvants induced partial protection [83, 84]. However, adjuvant MF75.2, delivered without vaccine, also had some non-specific protective effect.

Recently the immunogenicity of several subunit SERA vaccines has been assessed in mice. IgG (predominantly IgG2a, IgG2b and IgG3) purified from sera of mice immunized with the recombinant N-terminal domain of SERA had a parasite growth-inhibitory effect in a complement-dependent manner [85]. In another study, SERA was produced as DNA vaccine plasmids encoding the 47-kD domain (amino acids 17–110) or the N-terminal domain (amino acids 17–110). Immunization of mice by co-injection with SERA plasmids with a DNA plasmid encoding the hepatitis B surface antigen by gene gun induced high levels of anti-SERA titers. In comparison, immunization with the SERA DNA plasmid alone gave lower antibody titers [86].

RESA

Ring-infected erythrocyte surface antigen (RESA), a protein of 155 kD, was first described by two independent groups [87, 88]. In the freshly invaded merozoite, the protein is located in the lumen of the parasitophorous vacuole and then translocated to the cytoplasmic side of the erythrocyte membrane [89]. RESA is comprised of two tandem repeat sequences that contain immunodominant B- and T-cell epitopes [90, 91]. A number of studies point towards the vaccine potential of RESA. Polyclonal and monoclonal antibodies directed against the epitopes in the repeat regions inhibit merozoite invasion in vitro [92, 93]. A recombinant *E. coli*-produced vaccine encoding for the repeat units of RESA when delivered in CFA gave only minimal protection in *Aotus* monkeys against *P. falciparum* challenge [94]. A synthetic RESA vaccine consisting of repeat units and conjugated to diphtheria toxoid induced no protection in *Aotus* monkeys [95]. In a passive transfer study, affinity-purified anti-RESA antibodies reduced parasite burden in *Aotus* monkeys [96].

RAP1 and RAP2

Rhoptry-associated proteins 1 and 2 (RAP1 and RAP2) of *P. falciparum* are located in the rhoptry organelles of the parasites [97]. The genes encoding

for RAP1 and RAP2 have been cloned and sequenced [98, 99]. Several lines of evidence suggest that RAP1 and RAP2 are important vaccine candidate antigens. Monoclonal antibodies against RAP1 block the invasion of merozoites into red cells [100, 101]. Vaccination with affinity-purified RAP1/RAP2 protected *Saimiri* monkeys against lethal *P. falciparum* challenge [102]. Recently the vaccine efficacy of *E. coli*-produced RAP1 and RAP2 (rRAP1 and rRAP2) delivered in three different adjuvant formulations was tested in the *falciparum-Saimiri* challenge model. Following challenge infection, the highest degree of protection was observed in monkeys immunized with rRAP2 emulsified in ISA 720 [103].

PfEMP1

P. falciparum erythrocyte membrane protein 1 (PfEMP1), a variant molecule of 200–350 kD present on the surface of infected erythrocytes, is encoded by members of the *var* gene family [104–106]. The recombinant forms of this molecule elicit homologous, but not heterologous, agglutinating antibodies [17]. Each infected erythrocyte expresses on its surface a single form of PfEMP1. Each haploid genome contains about 50 copies of *var*, primarily but not wholly in subtelomeric regions. Recombination between *var* genes apparently occurs at a high frequency, and may occur during meiosis or mitosis [107]. Despite the highly variant nature of the molecule, the extracellular domains can be identified through regions of homology: Duffy binding-like (DBL) domains, so-named by their homology with other DBL antigens, cysteine-rich interdomain region (CIDR), C2 domains, and the N-terminal sequence [108]. PfEMP1 has been implicated in adhesion: among other adhesive phenomena demonstrated in vitro, CIDR1α binds to CD36, DBL-γ to chondroitin sulfate A, and DBL1α has been implicated in rosetting [108].

Strategies to develop a *var* gene vaccine based on PfEMP1 will need to overcome the problem of antigenic variation. Malaria vaccines for use in pregnancy currently in development are designed to elicit anti-adhesion antibodies against CSA-binding parasites. Over successive pregnancies, women in endemic areas naturally develop anti-adhesion antibodies that are associated with protection, and are globally cross-reactive, suggesting that DBL-γ may contain conserved epitopes recognized by anti-adhesion antibodies.

Other asexual stage PfEMP1 vaccines are being developed that will elicit antibodies 'pan-reactive' with field isolates, or reactive with many or common isolates. Evidence exists that specific isolates within endemic areas are commonly recognized, and that these commonly recognized isolates are associated with clinical disease [109], raising the possibility that PfEMP1 vaccines targeting a finite number of common PfEMP1 variants might confer broad protection against malaria.

Clinical Trials: Current Status

SPf66 Vaccine

The SPf66 vaccine, developed at the Institute of Immunology in Bogota, is comprised of *P. falciparum* sequences from three putative erythrocytic stage antigens and the 4 amino acid repeats of the pre-erythrocytic stage circumsporozoite protein (CSP) assembled as a synthetic polyvalent vaccine [110]. This vaccine was based upon an earlier synthetic vaccine formulation in which immunization with a combination of three synthetic peptides induced protection in *Aotus* monkeys against *P. falciparum* challenge [111]. SPf66 has undergone the most extensive clinical testing conducted for any malaria vaccine; over the last decade five major field trials have been conducted on three continents [112–116]. Earlier trials conducted in young children and adults showed moderate efficacy. In two South American trials in adults, the vaccine gave a protective efficacy of 34% (95% CI 18.8–45.7) [112], and 35% (95% CI 8.4–54.2) [115]. SPf66 vaccine also showed promise in a clinical trial in Tanzania conducted in children aged between 1 and 5 years. The vaccine gave an efficacy of 31% (95% CI 0–52), reducing the incidence of clinical infections [113]. However, no significant efficacy has been demonstrated in subsequent trials in children and adults in Thailand (2- to 15-year olds) [116] or Brazil (7- to 70-year olds) [117] or in infants in The Gambia (6- to 12-month olds) [114] or in Tanzanian infants as administered through the EPI schedule [118]. There is no clear explanation for the variability seen in different clinical trials and at present there are no further trials planned with SPf66.

Recombinant Protein and Synthetic Peptide Based Vaccines

A number of trials have been conducted or are planned based upon the erythrocytic stage antigens alone or in combination with other antigens. A phase-I trial conducted between 1992 and 1993 evaluated the safety and immunogenicity of two recombinant proteins administered in combination, Ro 46-2717 (a circumsporozoite protein) and Ro 46-2924 (a MSP2 protein) adsorbed into alum [119]. The vaccine was given to 33 healthy Swiss volunteers. The vaccine components appeared to be safe and immunogenic, with peak antibody titers occurring 4 weeks after the second of three doses. Five volunteers were challenged without evidence of protection [119]. A subsequent phase-I trial of safety and immunogenicity of two recombinant malaria vaccines based on $MSP1_{19}$ was conducted in 40 healthy young American adults [120]. Volunteers were given 2 or 3 doses of alum-adsorbed vaccine containing $MSP1_{19}$ derived from the 3D7 or the FVO strain of the parasite fused to T-helper epitopes of tetanus toxoid. Three volunteers developed hypersensitivity reactions after the third dose of vaccine and one volunteer had apparent histamine-associated hypotension [120].

It was concluded that both $MSP1_{19}$ vaccines were immunogenic, but that it would be necessary to alter the formulation to improve safety and immunogenicity profiles.

The Walter Reed Army Institute of Research (WRAIR) has completed a phase-I dose-escalation trial of an *E. coli*-produced $MSP1_{42}$. The same group has also completed a phase I/IIa trial of a combination vaccine, comprised of $MSP1_{42}$, and R,TSS (based on CSP), in healthy volunteers in the USA. In addition, in early 2002, WRAIR has planned a phase I trial with $MSP1_{42}$ vaccine in adult residents in Kenya. The NIAID Malaria Vaccine Development Unit is currently developing a C-terminus MSP1 vaccine and research is ongoing for development of candidate MSP3, MSP4, and MSP5 vaccines [121].

Phase-I trials have been conducted in both Papua New Guinea and Australia with a mixture of three recombinant blood-stage antigens. The vaccine contains recombinant MSP2 (3D7 allele), and parts of MSP1 (190Cs.T3) and RESA (C terminal 771 amino acids) formulated in adjuvant Montanide ISA 720. The vaccine has been shown to be safe and the three antigens appeared to be as immunogenic when given separately as when given in combination to nonimmune volunteers [122]. The combination vaccine has induced low antibodies, but strong T-cell responses to the MSP1 and RESA portions. Given the previous evidence that these proteins diminish the multiplication rate in vitro of *P. falciparum*, nonimmune volunteers in a phase-IIa study were challenged 4 weeks after the second dose of vaccine and the growth rate of parasites was measured using a sensitive polymerase chain reaction assay. There was no apparent reduction in parasite growth rate in these volunteers [123]. A field trial with this vaccine in 5- to 9-year olds has been conducted and has shown more promising results [Genton B, unpublished]. The Australian malaria vaccine program is also developing recombinant vaccines based on the erythrocytic stage antigens, AMA1 and RAP2. Recently, the first phase-I trial with AMA1 was initiated by this group and further trials with AMA1 and RAP2 are planned. There are several new synthetic peptide vaccines under development. Investigators at the Institut Pasteur, and the University Hospital of Lausanne, under the sponsorship of the European Malaria Vaccine Initiative (EMVI), have tested a synthetic MSP3 vaccine. This vaccine, delivered in Alum or Montanide was tested in 36 healthy European volunteers. The EMVI in collaboration with scientists from the Netherlands has initiated another trial with synthetic glutamate rich protein.

DNA Vaccines

At the Naval Medical Research Center in Silver Spring, Md., USA, we are currently working on the development of a combined pre-erythrocytic stage and erythrocytic stage vaccine. The rationale for this vaccine is based on the hypothesis that a two-tiered approach will be necessary for an effective malaria

vaccine, with the erythrocytic stage component attacking any erythrocytic stage parasites which break through the pre-erythrocytic stage defense. The major reason for our focus on this technology has been the documented capacity of DNA vaccines to induce CD8+ T-cell responses that appear to be critical for the destruction of pre-erythrocytic stage parasites [124, 125].

In addition, DNA vaccines provide a flexibility of design in that plasmids can be added and subtracted with relative ease. The current multi-stage vaccine in development will incorporate several pre-erythrocytic stage antigens (chosen from *Pf*CSP, *Pf*SSP2, *Pf*EXP1, *Pf*LSA1, and *Pf*LSA3) and several erythrocytic stage antigens (chosen from *Pf*MSP1$_{42}$ FVO, *Pf*MSP1$_{42}$ 3D7, *Pf*EBA-175 RII, *Pf*AMA1, *Pf*MSP3, *Pf*MSP4, *Pf*MSP5, *Pf*SERA and *Pf*RAP2).

Prime-Boost Strategy for Optimization of Vaccine-Induced Immune Responses

Heterologous 'prime-boost' strategies have demonstrated significant promise in animal models of a number of diseases [126–128] including malaria [129–132]. In the malaria model, heterologous strategy with DNA immunization (with or without cytokine coexpression) followed by immunization with either recombinant protein or pox virus has resulted in superior immune responses when compared to DNA alone. Priming with DNA and boosting with recombinant attenuated vaccinia virus expressing the same antigen resulted in enhanced CD8+ T-cell immunogenicity and protective efficacy against pre-erythrocytic stage *P. berghei* [131] and *P. yoelii* [132].

Malaria vaccines will be most successful in protecting people residing in a malaria endemic area if they induce immune responses that are boosted by natural infection. DNA vaccines appear to have this characteristic. We have found that in the *P. falciparum-Aotus* challenge model, immunization with a three DNA plasmid combination vaccine, *Pf*MSP1$_{42}$, *Pf*EBA-175 and *Pf*AMA-1, induced modest levels of antigen-specific antibody responses. However, following *P. falciparum* challenge, antigen-specific antibody titers increased 10- to 100-fold, suggesting that DNA immunization primed the immune system for subsequent boosting with parasite infection [133].

There is further evidence to support the advantages of a heterologous prime-boost approach for *P. falciparum* erythrocytic stage antigens. In rhesus monkeys, compared to a single-dose immunization with a recombinant *Pf*MSP1$_{19}$ alone, priming with a *Pf*MSP1$_{42}$ DNA plasmid followed by boosting with the recombinant protein gave significantly higher antibody responses [134]. In *Aotus* monkeys, priming with DNA encoding *Pf*EBA-175 RII (a *P. falciparum* erythrocytic stage antigen) and boosting with a single dose of recombinant *Pf*EBA-175 RII in

adjuvant was shown to elicit antibody responses comparable to those elicited by immunization with 4 doses of recombinant *Pf*EBA-175 RII in adjuvant. Following *P. falciparum* challenge, 3 of 4 *Aotus* immunized with the DNA-protein immunization regimen were protected, while no protection was seen in *Aotus* that received only recombinant *Pf*EBA-175 RII [76]. Furthermore, there was excellent boosting of antibody responses after *P. falciparum* challenge in the DNA-primed monkeys, but not in those primed with recombinant protein. These findings indicate the importance of pursuing prime-boost strategies for vaccine design.

Future Direction: Antigen Discovery through Genomics Research

At present, there are three basic approaches to the development of asexual stage vaccines. The first approach, optimizing the immune responses to one or a few key antigens is the approach adapted by most groups. The multiple efforts of a number of groups to develop an MSP1-based vaccine exemplify this approach. The second approach has been to optimize the immune responses to a combination of a number of well-defined asexual stage antigens alone or in combination with pre-erythrocytic stage antigens. This approach is illustrated by current efforts by our group at the Naval Medical Research Center to develop a DNA-based vaccine with up to 5 pre-erythrocytic plasmids and up to 4–10 erythrocytic stage antigens, followed by boosting with recombinant proteins and/or poxvirus. The third approach to vaccine development is that of duplicating the immunity which is induced by exposure to the entire parasite. The models of natural immunity in the field and irradiated sporozoite-induced protection are based upon such whole parasite-induced immunity. It is indeed possible that protective immunity will require the induction of immune responses to hundreds of target proteins, and not only a few proteins as used in the first two approaches. The sequencing of one isolate of *P. falciparum* by the Malaria Genome Project and the current plans to supplement the database by sequencing a variety of clinical isolates of *P. falciparum* provide the foundation for this approach [135, 136].

The effort to sequence the entire genome of *P. falciparum* began in mid 1996. Since that time, we have seen the publication of the complete sequences of chromosome 2 and chromosome 3 [137, 138]. It is expected that by the beginning of 2002, the sequencing of the entire 14 chromosomes of *P. falciparum* will be complete. The project's success has been possible because of the well-orchestrated efforts of a consortium of centers and funding agencies. With the availability of full-sequence data from *P. falciparum*, scientists should make rapid progress in the identification of additional targets of protective immunity.

However, the development of new vaccines based upon data gleaned from genomic sequence will require a complex series of steps [139]. First, the proteins expressed from the targeted parasite stages must be identified. Second, the cellular and subcellular locations of each of the expressed proteins need to be determined. Third, the protective epitopes within the target proteins will need to be identified. Finally, vaccine delivery systems to optimally deliver these antigens will need to be identified. A number of new technologies are becoming available to aid in the functional analyses of the *P. falciparum* genome, including DNA microarrays for serial analysis of gene expressions and high-throughput proteomics to identify expressed proteins. The future vaccine(s) might consist of 100–1,000 antigens from different stages of the parasite life cycle delivered as a plasmic DNA cocktail encoding entire genes or multi-epitope strings.

Conclusions

The limited numbers of clinical trials conducted to date with erythrocytic stage vaccines produced as recombinant proteins or synthetic peptides have produced only moderate success. At present, clinical trials using a few key erythrocytic stage proteins are underway and several more trials are being planned. An important challenge remains that of finding the optimal adjuvant formulation for delivery of these vaccines. At the same time, efforts are being made to identify new target antigens and to find ways to deliver them in a multiple antigen format. With the completion of *P. falciparum* genome sequencing, and with sequencing of other important *Plasmodia* underway, it is anticipated that within the next few years hundreds of new target antigens will be identified. Thus, antigen discovery by genomics research will probably set the direction of future vaccine development. DNA vaccine technology provides an easily manipulated system for delivery of such a multi-antigen vaccine, and work is ongoing to optimize such DNA delivery vehicles. It is also possible that the currently available delivery systems such as recombinant proteins, synthetic peptides, naked DNA plasmids or other recombinant vectors such as poxviruses or adenoviruses will meet the need. Recent studies suggest that the induction of protective immune responses against malaria parasites may require a heterologous prime-boost strategy such as DNA priming followed by recombinant virus or recombinant protein boost.

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Transmission-Blocking Vaccines

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Despite identification of the etiologic agent a century ago and proof-of-principle nearly 50 years ago that acquired humoral immunity can prevent infection in mosquitoes feeding on a malaria parasite-infected vertebrate, only two human phase-I trials of transmission-blocking vaccines have been undertaken to date. This chapter briefly reviews the extensive body of evidence indicating the biological feasibility of eliciting antibodies that block mosquito infectivity and the variety of mechanisms by which sporogonic development is disrupted. The more mundane but practically important aspects of transitional research required for transmission-blocking vaccine development are also briefly summarized. A more detailed review has been recently published elsewhere [1]. The hurdles to be overcome in the development of effective transmission-blocking vaccines are now primarily those of vaccine composition, formulation, administration, deployment and most importantly, willpower.

The Goals of Attacking Sexual-Stage Parasites with Transmission-Blocking Vaccines

In addition to achieving the primary goal of reducing morbidity and mortality, highly effective anti-sporozoite, anti-liver-stage and/or anti-asexual-parasite-stage (protective not anti-disease) vaccines may, as an added consequence of reducing parasite loads, substantially reduce malaria transmission. In contrast, the primary goal of anti-sexual vaccines is to prevent or substantially reduce mosquito infectivity. When achieved, the result will be to reduce parasite transmission. Although not accepted by all, one could argue that substantial reductions in transmission rates will eventually lead to a reduction in morbidity and mortality and might even in some cases lead to elimination of the parasite in geographically isolated areas, particularly when used with other control measures such as bednets, vector control, drugs and other vaccines.

Addition of 'transmission-blocking vaccines' could increase the effective product life of protective vaccines or chemotherapeutic agents by blocking the spread of escape mutants resistant to the protective vaccine components or the anti-malarial medication, respectively. A transmission-blocking vaccine might also convert a marginally effective protective anti-sporozoite vaccine to a fully effective one by reducing the sporozoite inoculation rate in a geographical area. In addition, transmission-blocking vaccines might be useful in preventing the reintroduction of malaria into areas where a successful parasite elimination program has been deployed; by requiring all visitors and returnees to be fully immunized to sexual stages or sporogonic stages, the maintenance of parasite elimination would be made easier.

The Path Leading to and the Biological Basis for Transmission-Blocking Vaccine Development

The cumulative observations of Laveran [2], Ross [3] and MacCallum [4] at the turn of the 20th century established the etiologic agent of malaria, the mode of transmission by mosquitoes, and the sexual development of the parasite in the mosquito midgut. However, it was not for another 60 years, in 1958, before vaccination with malaria parasites was shown to reduce infectivity of parasites in mosquitoes fed directly on vaccinated, parasitemic chickens [5]. Using the facile avian malaria model, Huff et al. [5] showed that chickens developed humoral immunity that blocked parasite infectivity when vaccinated with a mixed population of asexual and sexual-stage *Plasmodium gallinaceum* parasites. In the 1970s, using the same species of malaria parasites but this time highly purified sexual-stage parasites as immunogens, Gwadz [6] and Carter and Chen [7] showed that the target antigens of transmission-blocking immunity were contained within the sexual-stage parasites. The 1980s and the advent of monoclonal antibody (mAb) technology and the development of a membrane-based mosquito-feeding apparatus lead to the identification of a series of surface proteins which were targets of transmission-blocking antibodies [8, 9]. The first gene encoding one of these targets, Pfs25, was isolated in 1988 [10]. In the early 1990s recombinant protein was expressed that elicited transmission-blocking activity in sera from vaccinated rodents and non-human primates [11]. The first clinical trials were initiated in 1994.

Although much remains to be described, the biology of sporogonic development (from gametogenesis and fertilization, to zygote transformation and ookinete penetration of the mosquito midgut, to oocyst maturation and sporozoite migration from the midgut to salivary glands) is sufficiently well known to provide a rational design for developing and testing transmission-blocking vaccine candidates. Other than progress on specific vaccines against P25 and P28 antigens,

the specific sexual-stage antigens that are targets of immune responses are discussed by R.W. Sauerwein and W.M.C Eling (pp 188–203). A brief review of sporogonic development is presented here to provide the conceptual approaches being considered in the development of transmission-blocking vaccines; detailed reviews can be found elsewhere.

Gametocyte Development in the Vertebrate Host

Initiation of Gametocytogenesis. The mechanism triggering gametocytogenesis (differentiation of asexual blood-stage parasites to male (microgametocytes) and female (macrogametocytes) gametocytes) remain poorly understood. Recently the contribution of erythropoiesis and in particular the kinase pathways that are triggered by the hormone erythropoietin have been implicated [12]. The rare schizont stage that does yield gametocytes appears to do so by committing most but not necessarily all progeny to sexual-stage parasites. The ratio of male to female gametocytes from a single schizont is an area of active research: a recent report by Smith et al. [13] suggests that the merozoites released from a single committed schizont become either all male or all female gametocytes. If the ratio is not fixed by the parent schizont, then some humoral mechanism may be evoked to preserve the characteristic female-biased gametocyte sex ratio. As these mechanisms are elucidated, any parasite-produced factors will provide the basis for anti-gametocytogenesis vaccines.

Early Gametocytes. The early stages of gametocyte development are morphologically similar to trophozoites and are rarely observed in the circulation because they are likely sequestered. On their surface early gametocytes (stages I–IIA) express cytoadherence molecules, such as PfEMP1 [14]. Immune responses, particularly IgG, to adherence molecules such as PfEMP1 may regulate the production of early gametocytes and suggest that vaccines directed at the adherence molecules used by asexual blood stages may have a direct impact on gametocyte maturation as well [15].

Late Gametocytes. Stage-IIB gametocytes begin to lose the classic cytoadherence molecules that asexual blood-stage parasites express [16]. The parasites may undergo a transition from high-avidity binding to a lower-avidity adhesion, particularly in stages II and IV [17]. This transition suggests that the late gametocyte may express surface adhesion molecules unique to sexual stages. Immune responses to these antigens provide yet another attack point for anti-gametocyte vaccines. The surfaces of the circulating late gametocyte-infected erythrocytes appear to be devoid of unique antigenic markers. The absence of such markers result in an immunologically invisible state for the late mature gametocytes [18]. The mechanism by which male and female parasites clear the surface of the infected erythrocyte of parasite markers during gametocytogenesis is not presently known.

Gametogenesis and Fertilization – The First Golden Hour in the Midgut

The Trigger. Within seconds of ingestion by mosquitoes, both male and female gametocytes undergo gametogenesis. The process is triggered by at least three factors: a decrease in temperature in the poikilothermic mosquito vector of at least 5°C below that of the vertebrate host; an increase in pH; and, a mosquito midgut-derived, gametocyte-activating factor, which recently has been identified as xanthurenic acid [19]. Although the latter may represent a rational target for drug therapy or development of refractory mosquitoes, it is unclear if a vaccine approach could be exploited that blocked these triggers of gametogenesis.

Exflagellation of Male Gametocytes. In a process called exflagellation, male gametocytes dramatically emerge from within the erythrocyte to yield approximately eight male gametes from a single male gametocyte. As the gametes emerge they avidly bind to neighboring uninfected and infected erythrocytes, creating 'exflagellation centers'. The adherence appears to be to a specific male gamete receptor that involves either the sialic acid moiety or the peptide backbone of glycophorin(s) [20]. Whether this is a member of the family of other malaria parasite proteins that bind glycophorins or a candidate for vaccine development remains to be determined.

Emergence of Female Gametocytes and Fertilization. Female gametocytes emerge from erythrocytes, likely as a result of a parasite-driven disruption in the integrity of the erythrocyte membrane. Once released, each female gametocyte forms a single non-motile female gamete which is the target of the motile male. Clearly, antibodies directed toward the presumptive fertilization receptors involved in male recognition of female gametes are attractive targets of transmission-blocking vaccines. In addition, the theoretical 'pheromones' involved in the non-random attraction of males to females and interference with the cell membrane fusion that occurs after initial recognition might also be attractive targets as well.

Development of the Zygote within the Bloodmeal. During the 20–24 h after fertilization, the round non-motile zygote undergoes a morphologic transformation into the elongated, motile ookinete. An intermediate form, the retort, extends a cytoplasmic protrusion containing the apical complex. In addition to the new surface proteins on the retort and ookinete, the apical complex is a reservoir of other potential vaccine targets, many of which are just now being identified and described. Antibodies to all of these proteins may inhibit the morphologic transformation of the parasite, forming the basis of an in vitro assay of vaccine efficacy (see next section). Unfortunately, this transformation is not easily reproduced in vitro.

Invasion of the Mosquito

By 24 h after fertilization (depending on the species of malaria parasite), the parasite is equipped to egress to the periphery of the bloodmeal, engage the

chitinous peritrophic matrix (PM) that encapsulates the bloodmeal and traverse both the PM and the midgut epithelium [21]. The potential vaccine targets include molecules involved with motility, initial recognition of the PM and midgut epithelium, and enzymes that contribute to the disruption of the PM (e.g., chitinases) and the integrity of the midgut epithelium. Other targets are mosquito midgut antigens. Carbohydrate epitopes on the midgut surface and proteins, both on the midgut surface and secreted (e.g., proteases essential to parasite development), all represent potential vaccine targets [22, 23]. The distinct advantage of developing vaccines targetted at eliciting antibodies that recognize mosquito epitopes is that they may result in blocking transmission of more than one species of malaria parasite. Care must be used in developing such vaccines so that either the vaccine has no (or perhaps a positive) effect on the fecundity or life span of the mosquito, or alternatively, is uniformly fatal to mosquitoes. Because mosquito populations are so large in many of the areas where such a vaccine will be deployed, vaccines that fall between completely neutral and completely fatal might rapidly select for resistant mosquitoes.

Mechanisms of Transmission-Blocking Immunity and Immune Evasion: The Basis for Clinical Assays in Support of Vaccine Development

Understanding the mechanisms associated with acquired and vaccine-elicited transmission-blocking immunity, although not necessary, greatly assists in the identification of new vaccine targets and the validation of established ones, respectively. The membrane-feeding assay is the gold standard that has been used extensively by several laboratories to validate new antigens as targets of transmission-blocking antibodies [8, 9]. The assay combines in vitro-cultured parasites (mature gametocytes, gametes, zygotes or ookinetes can be used in the assay) with laboratory-reared or wild-caught mosquitoes to determine the effect of antibodies on mosquito infectivity. Briefly, in the standard membrane-feeding assay, in vitro-cultured mature gametocytes are re-suspended with medium containing blood and the test antibodies. Serum complement is added when complement-dependent transmission-blocking activity is being determined. The suspension is placed in a water-jacketed glass cylinder, on the bottom of which a thin membrane, such as Parafilm™, has been applied. Starved female mosquitoes probe through the membrane and are permitted to feed on the blood for minutes to hours. The mosquitoes are maintained in an insectary for 6–8 days after blood ingestion and then are dissected. Only mercurochrome-stained midguts from mosquitoes that have developed a full clutch of eggs (an indication that a bloodmeal was successfully ingested and digested) are scored for parasites.

Based on the discussion in the previous section above and using the membrane-feeding assay, target antigens of transmission-blocking antibodies can be placed into one of three general categories: (i) pre-fertilization target antigens which are expressed either solely or predominately in gametocytes and elicit antibodies that disrupt parasite development either by mediating complement-dependent lysis of gametes or by somehow interfering with parasite emergence or fertilization; (ii) post-fertilization target antigens, which are expressed solely or predominately on zygotes or ookinetes and elicit antibodies that block the morphological transformation of the round, non-motile zygote into the oblong motile ookinete and/or block the egress of the ookinete from the bloodmeal to the mosquito midgut epithelial basal lamina, and (iii) late-midgut-stage target antigens, such as parasite-produced chitinase which is required for penetration of the chitin-containing PM that the midgut epithelium secretes, are the bloodmeal, and a mosquito-produced protease that appears to activate the parasite-produced chitinase [1].

At this time in transmission-blocking vaccine development, the effort to analytically and clinically validate surrogates of efficacy are of high priority. Defining the operational characteristics [24] of such assays do not require clinical trials; whereas the clinical validation can only be initiated after one or more phase-IIb clinical trials or appropriately designed clinical trails of acquired immunity have been completed. Until such trials and clinical validation of assays are completed, in vitro or ex vivo assays of transmission-blocking activity can only be presumptive evidence of vaccine efficacy.

The risk in using such presumptive data in making pivotal decisions in vaccine development are both that a good vaccine is discarded because the clinical assay is too stringent or that resources are wasted on an ineffective vaccine because the clinical assay is not stringent enough. The mechanisms of transmission-blocking immunity are briefly reviewed below from the perspective of development of clinical assays.

Immune Response to Pre-Fertilization Target Antigens – The Basis for in vitro Surrogate Assays of Vaccine Efficacy

The number of pre-fertilization target antigens identified and at least partially characterized has grown from two (Pfs230 and Pfs48/45) [8, 9, 25–27] in the mid-1980s to almost a dozen or more. Still the two lead pre-fertilization candidate antigens have not changed, in part, because Pfs230 and Pfs48/45 are the best characterized of the bunch and, in part, because both have been 'validated' as targets by the standard membrane-feeding assay. The major attractiveness of these antigens are also their major weakness: these antigens are expressed in gametocytes as they circulate in the human host. As such, humans vaccinated with these antigens may boost an immune response to Pfs230 or Pfs48/45 during

any subsequent natural infection that results in appreciable mature gametocyte production. Evidence that this will likely occur comes from the observation that some humans known to have been exposed to gametocytes have anti-Pfs230 and/or anti-Pfs48/45 immune responses [28]. The flip side of the same coin is that these antigens clearly have been under immune selection and appear to have evolved to have limited immunogenicity and antigenic diversity. Furthermore, whether boosting after a natural infection will boost transmission-blocking antibodies or only antibodies that do not block or, worse yet, inhibit binding of transmission-blocking antibodies has not been adequately addressed.

In addition to simple steric hindrance blocking parasite emergence, motility or fertilization, pre-fertilization target antigens may bind isotype-specific antibodies that disrupt normal parasite development by complement fixation or antibody-dependent cellular inhibition. The data supporting these latter two mechanisms of immunity are summarized in the subsequent two sections.

Complement-Mediated Lysis

Pfs260 is the precursor to the gamete target antigen Pfs230. During emergence, the N-terminus of Pfs260 is shed, leaving the C-terminal portion, Pfs230, associated with the surface of gametes [29, 30]. Using an in vitro complement-mediated lysis model of *Plasmodium falciparum* gametes, Healer et al. [31] found a positive association of complement-mediated lysis in human sera with antibodies to Pfs230 but not with antibodies solely to Pfs260. Similar to the N-terminus of Pfs260, antibodies to two other gametocyte-specific proteins, Pfs48/45 and Pfg27/25, were not associated with gamete lysis. Because the ability of an antibody to effectively fix complement is isotype-dependent, the association of complement-mediated lysis should also be associated with the specific anti-Pfs230 antibody isotype. Consistent with this notion, all human sera in this study that mediated gamete lysis had anti-gamete surface antibodies of either IgG1 and/or IgG3 isotypes.

In an elegant study using isotype switching of a single mAb, Roeffen et al. [32] clearly demonstrated this isotype-dependence of anti-Pfs230 transmission-blocking activity. The parental anti-Pfs230 mAb 63F2A2 (IgG_1) did not reduce the *P. falciparum* transmission in a bioassay regardless of the presence of complement; whereas isotype-switched clones, mAbs 63F2A2.2b (IgG_{2b}) and 63F2A2.2a (IgG_{2a}) reduced transmission by 91% and greater than 99%, respectively [32]. Other investigators have also shown that the presence of complement is necessary for anti-Pfs230 antibodies to effectively block transmission in membrane feeding assays [30].

Thus, the preponderance of data strongly support the hypothesis that Pfs230 is a major target of complement-fixing antibodies and that this activity may be important for antibody-mediated transmission-blocking immunity.

This well-established fact provides a basis for using an in vitro complement-mediated gamete lysis assay as a surrogate assay for vaccine efficacy with one caveat – it may prove reliable only for assessing Pfs230-based transmission-blocking vaccines. It may not be appropriate to use such an assay for evaluating the efficacy of other transmission-blocking vaccines. In addition, refinements in the current assay's format and throughput will certainly be necessary for its use in support of human clinical trials.

Phagocytosis

Clearly a multitude of humoral and cellular components, including monocytes and polymorphonuclear neutrophils, comprise the ingested bloodmeal. In the absence of immune responses, including opsonic antibodies, to surface molecules of mature gametocyte (see discussion above) responses, the intra-erythrocytic gametocyte is essentially not susceptible to phagocytosis. Unique surface antigens expressed starting hours after the parasite is ingested by the mosquito (i.e., those expressed by late gametes, ookinetes, oocysts and sporozoites) would not be targets of opsonic antibodies either, as by the time the target antigen is expressed on the surface, the cellular components of the bloodmeal would be inactive. The question remains whether the early gamete target antigens are targets of opsonic antibodies. In vitro data suggest that phagocytosis is contributory. In vivo data are a bit less clear: addition of leukocytes to a bloodmeal seems to accentuate transmission-blocking activity; however, phagocytosis has recently been shown to proceed less efficiently in the lower temperature and altered milieu of the mosquito midgut [33]. The contribution of monocytes and polymorphonuclear neutrophils to reduction of infectivity in nature is not clear. The utility of their inclusion in clinical assays to be used as surrogates of efficacy remains to be determined.

Antigenic Diversity – The Need for Polyvalent Transmission-Blocking Vaccines

As with other infectious agents, parasites evade the human immune response through natural selection of parasite populations that express target antigens that have variant or completely lack B- or helper-T-cell epitopes. It is, therefore, quite expected that antigenic diversity would be present in some of the target antigens of pre-fertilization transmission-blocking immunity. Most if not all of these antigens are expressed while gametocytes circulate in the human host and antibodies to these targets are ingested along with the infectious bloodmeal. Those antigens expressed solely in the mosquito vector presumably would not be under similar immune selection. The nucleotide diversity present in these post-fertilization target antigens presumably has arisen by some other selective mechanism.

Nucleotide sequences for Pfs230 and Pfs48/45 are now available [25, 27]. Limited studies of nucleotide diversity have been undertaken, and nucleotide

sequence differences have been described [34, 35]. Until the B- and helper T-cell epitopes for these candidate antigens have been fine-mapped, studies of antigenic diversity by immunofluorescence assay (IFA) reactivity of mAbs with laboratory and field isolates may be more informative than nucleotide sequence alignment. Graves et al. [36] found that Pfs48/45 has at least one variant epitope and that the transmission-blocking effects of anti-Pfs48/45 mAbs are strain-specific. Subsequent studies of laboratory and field isolates have essentially confirmed these findings. Foo et al. [37] also reported limited antigenic diversity in a minor population of gametocytes at one of two Pfs230 B-cell epitopes. Unfortunately studies that simultaneously evaluated parasite isolates by nucleotide sequence analysis and IFA have not been published, so the exact structural bases of the observed antigenic diversity are not known.

Antigenic diversity has at least two practical considerations when developing transmission-blocking vaccines: (1) if efficacious, how long will the current vaccine remain efficacious, and (2) how many of the existing immunogen variants (i.e., the equivalent of serotypes) need to be included in the current version of the vaccine for the vaccine to be of sufficient utility to be used. With respect to consideration of the selective pressure for vaccine-resistant parasites that will result from the use of transmission-blocking vaccines, it is important to remember that sporogonic development represents a significant bottleneck in the parasite's lifecycle. A detailed discussion of the vaccine implications of this bottleneck can be found elsewhere [1]. Suffice it to say that only a small portion of circulating gametocytes are ever ingested by mosquitoes and that of those ingested less than a percent develop into oocysts. Thus, the selective pressure of transmission-blocking vaccines is on a relatively small number of parasites. Perhaps the more immediate, important practical consideration, which is whether multiple variant forms of each target antigen will need to be included in a vaccine to elicit broadly effective transmission-blocking immune responses, has not yet been adequately addressed to reach a firm conclusion.

Boosting after a Natural Infection

Because the pre-fertilization target antigens are expressed while gametocytes circulate in the human host, boosting of the immune response may occur after natural infection. This could provide a durable transmission-blocking immune response after a single or just a few vaccinations. Unfortunately the evidence from field studies to support this 'boosting hypothesis' is scant. The limited studies of natural boosting that have been done suggest that boosting is only effective if natural re-infection occurs within about 4 months, at least in human *Plasmodium vivax* infections [38]. Laboratory studies that have demonstrated a boosting response after natural infection have been limited to vaccination of naïve animals [39]. A prior natural infection may irreversibly perturb the

immune response to a subsequent subunit vaccination such that an ineffective immune response results – this needs to be adequately studied.

Whether the whole issue of boosting subsequent to natural re-infection is irrelevant may depend upon two factors: (1) the advent of novel delivery systems, such as slow release particles or vectored vaccines that provide for immunogen persistence, and (2) the ability to maintain circulating antibody levels sufficient to block transmission. Microspheres of polylactide polyglycolide [40] or transgenes delivered by viral vectors that continue to express subunit immunogens for prolonged periods of time provide new possibilities of maintaining effective immune responses. Such maintenance of the immune response may not be adequate: many vaccines actually 'work' by priming the immune system so that, upon subsequent inoculation by the pathogen, the kinetics of the immune response results in an clinically aborted infection. Whether novel delivery systems will work by simply maintaining an immune response remains to be determined.

Immune Responses to Post-Fertilization Target Antigens – The Basis for Early Clinical Field Trials

With the exception of parasite-produced chitinase [41, 42] and mosquito-produced protease [43, 44], the biological function of post-fertilization target antigens are currently not known. Thus, other than in vitro assays to detect antibody- mediated inhibition of chitinase or protease activity, rationally designed in vitro surrogates of efficacy do not currently exist for the lead post-fertilization target antigens. This is not to say that insights into the mode of action of antibodies to post-fertilization target antigens have not been made. In fact studies demonstrating that the block in parasite development can occur at multiple stages of sporogony in the mosquito midgut. Polyclonal antiserum to Pfs25 and Pfs28 block zygote to ookinete transformation and in animal model systems, inhibition of ookinete invasion has been observed [45, 46].

Because it has been known for sometime that for almost all target antigens of transmission-blocking antibodies, the biologically relevant B-cell epitopes are disulfide bond-dependent, it is unlikely that measurements of total antigen-specific antibody response in humans will be highly predictive of transmission-blocking activity. ELISAs that only detect antibodies that recognize the relevant conformational epitopes may prove useful; such assays have been tested and show imperfect correlation to transmission-blocking activity as assayed by membrane-feeding mosquitoes (unpublished data). Avidity as well as fine specificity and the presence of interfering antibodies may all need to be determined to strengthen the correlation with the accepted 'gold-standard', the membrane-feeding assay.

The more pressing issue is how predictive is the membrane-feeding assay of in vivo transmission-blocking activity and, even more importantly, what is the

association between a reduction in morbidity and mortality as a result of inhibition of malaria parasite transmission and transmission-blocking activity measured by the standard membrane-feeding assay. Of course, such an association cannot be determined until sufficient studies have been done in parallel in humans. In the meantime, a first approximation of the in vivo utility of transmission-blocking vaccines may be extrapolated from membrane-feeding assays. Whether the failure to elicit potent transmission-blocking activity in this clinically unvalidated assay should be used to make a no-go decision to proceed to phase-IIb studies is controversial. Again, the risk in using such presumptive data in making pivotal decisions in vaccine development are both that a good vaccine is discarded because the clinical assay is too stringent or that resources are wasted on an ineffective vaccine because the clinical assay is not stringent enough.

Critical Path for Transmission-Blocking Vaccines

Until recently, most if not all vaccines in use were either whole organisms (attenuated or killed bacteria or viruses) or the toxins (detoxified, of course) they produced. Whole malaria parasites, in the case of transmission-blocking vaccines – sexual stages, would make potent vaccines that likely would not require additional adjuvants to elicit potent transmission-blocking activity. However, manufacturing large quantities of parasites for vaccine use in humans is currently not feasible; therefore, the only viable approach is to produce subunit immunogens as vaccines.

Immunogen Production

With few, if any, exceptions, the sole use of transmission-blocking vaccines is eliciting a potent humoral response in the form of transmission-blocking antibodies. Other than stimulating helper T-cells, transmission-blocking immunogens do not require elicitation of cellular responses. Thus, the primary goal of immunogen production is to faithfully recreate the relevant B- and helper T-cell epitopes. Although several linear B-cell epitopes have been identified that appear to be recognized by antibodies having transmission-blocking activity [47], to date synthetic linear peptides alone have not effectively elicited transmission-blocking antibodies. Furthermore, all of the current lead vaccine candidates (Pfs230, Pfs48/45, Pfs28, Pfs25, and Pvs28 and Pvs25) are all relatively cysteine-rich and have multiple disulfide bond-dependent conformational B-cell epitopes [10, 25, 27, 48, 49]. In the absence of compelling data to support the production of transmission-blocking vaccines comprised solely of linear epitopes, the current focus of the developmental process of a transmission-blocking vaccine is and should be on the faithful recreation of disulfide bond-dependent conformational epitopes.

Recombinant protein expression in bacteria provide one of the least expensive, least complex vaccine production systems available. For organisms, such as malaria parasites, that do not typically N-glycosylate their surface proteins, bacterial expression systems have an added appeal because potential N-linked glycosylation sites do not need to be perturbed. Although bacterial expression has been used successfully to produce immunogens with disulfide bond-dependent B-cell epitopes, including other malaria parasite immunogens, they have not been universally useful in the production of transmission-blocking vaccine candidates [45]. The appeal of bacterial expression systems is significantly reduced once protein refolding must be added to the purification process.

Eukaryotic expression systems, including naked DNA and viral vectors, have been used much more successfully [50, 51]. As the goal is to elicit antibodies and not cell-mediated immunity, immunogen delivery that traffics via major histocompatibility complex class II is certainly preferred. For this reason, a great deal of effort has been placed on the approach of producing subunit recombinant proteins. A frequently encountered complication in the development of expression constructs expressing subunit immunogens is instability or poor levels of transcription and/or translation due, in part, to the aberrantly high A–T content of the malaria parasite genome. The remedy – creating a synthetic gene based on preferred codon usage of the heterologous expression system selected – is quite straightforward, and it is also quite laborious [52]. Nevertheless, such an approach in a recombinant yeast expression has proven a convenient production system for several of the target antigens.

Even when such an approach has been successful for bench-top production of research-grade material, not infrequently scaling up for manufacturing clinical-grade material has been problematic [53]. Scale-up from shaker flask to mid-scale fermentation (50–300 liters) frequently takes a nonlinear course. In part the problems have been in technology transfer (changes in personnel, equipment, and raw materials), and, in part, they have been a consequence of inadequate access to scale equipment for adequate process development. There is often the tendency to underestimate the time-consuming and unpredictable nature of developing controlled, reproducible processes, particularly product recovery (protein purification). Unfortunately the rule rather than the exception has been a dramatic decrease in biological activity during the transition from research-grade to clinical-grade material. This problem is made nearly unmanageable when rapid and relevant in-process analytic tools are not available. These all-important analytic assays of identity, purity, potency and stability of the vaccine immunogen are often inadequately developed and validated (both analytically and clinically) before applied. In their absence, lot-to-lot variation and vaccine suitability for use in safety and pivotal proof-of-principle studies will come into question.

Vaccine Formulation and Delivery

The most powerful adjuvant or best optimized delivery schedule cannot overcome a poor selection of immunogen. Nevertheless, the proper choice of adjuvants, carriers, route and schedule of vaccine administration are often required to elicit the desired immune response, even for some of the best immunogens. Even if the exact immune response desired could be well defined, the state-of-the-art of immunology and vaccinology have not yet progressed to the point where rational selection of the immunogen(s), adjuvant, delivery system, route and schedule of administration can be made. The art is still largely empiric.

Nevertheless, some good guesses can be made specifically for transmission-blocking vaccines because the goal is almost certainly to elicit antibodies that recognize macromolecules on the surface or secreted from sexual-stage parasites. Delivery systems known to elicit primarily cell-mediated immune responses are unlikely to provide anything but a good priming response. That many of the B-cell epitopes are conformational and disulfide bond-dependent suggests that care be given to avoid reducing or pH environments conducive to disrupting cystine residues. Soluble monomeric antigens have for the most part been poorer immunogens than those adsorbed to carriers or made as multimeric aggregates or particles – so although simply adding immunomodulators such as ODNs or QS21 to soluble antigen seems most expedient, empirically they have performed less well than when immunogen adsorbed to alum is formulated with these adjuvants (unpublished data). Unfortunately, what has been found to work with one immunogen, may not work with another quite similar one [53].

Safety Studies and Interpretation of Early Phase-I Immunogenicity Data

In academia and less so in private industry, much of the pre-clinical research and development effort is focused on enhancing efficacy. Oftentimes minimal pre-clinical safety studies are performed to detect or diminish acute toxicity prior to the first human trial. To be sure, multiple safeguards are in place that mitigate the potential for life-threatening adverse events from occurring during the first trials in humans; nevertheless, more subtle acute toxicity is often first detected in phase-I trials. As it should be, the primary goal of early phase-I trials is detecting safety concerns with the dose of immunogen, adjuvant and delivery system. Preliminary evaluation of immunogenicity can and should be made but with caution.

A case in point: the first human phase-I clinical trial of a malaria transmission-blocking vaccine occurred in 1994. In that randomized, controlled, double-masked study of 16 volunteers, 1 of 8 volunteers that received the third dose of the test article, TBV25H adsorbed to aluminum hydroxide, experienced an atypical hypersensitivity reaction. The reaction was not dissimilar to that observed in other human trials of subunit malaria vaccines in which alum was used as an immunogen carrier [54, 55]. To accommodate the pH-dependent stability of a

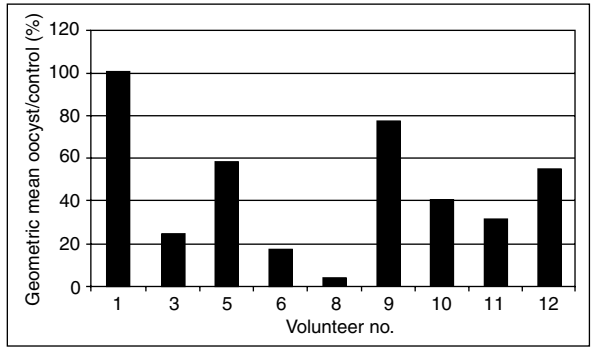

Fig. 1. *Transmission-blocking activity in human volunteers 2 weeks after a boost with TBV25H/alum*. Thirteen human volunteers were enrolled in a human phase-I trial. Nine volunteers had been previously vaccinated with NYVAC-Pf7 and 4 volunteers served as controls. All volunteers received a single intramuscular dose of 250 μg of TBV25H adsorbed to aluminum hydroxide. Serum collected just before the booster vaccination and 2 weeks thereafter was fed to 18–25 starved female *Anopheles freeborni* mosquitoes, which are dissected 7–8 days thereafter. The mercurochrome-stained midguts are scored for oocysts and the geometric mean of Williams is calculated. Pre- and post-vaccination ratios of geometric means are shown above for the volunteers primed with NYVAC-Pf7; the naïve controls had no detectable transmission-blocking activity.

new adjuvant, QS21, added to the formulation to enhance immunogenicity, the pH of the 1× PBS buffer used during alum adsorption was decreased from pH 7.4 to 6.8 in the formulation of clinical-grade TBV25H. This change in pH increased the percentage of TBV25H bound to alum without compromising stability. In probe studies in mice, these lower pH conditions for the formulation of TBV25H appeared to be better tolerated, as evidenced by fewer animals experiencing acute distress after three injections of high doses of TBV25H [53]. Had the formulation been optimized for safety as well as immunogenicity, the immunogenicity observed in the first human trial would more likely have been similar to the second human trial, in which a viral vector prime (NYVAC-Pf7) was boosted with TBV25H/alum (fig. 1). The latter study documented that it is biologically feasible to elicit transmission-blocking antibodies in humans with a Pfs25-based vaccine.

Initial Proof of Concept: Phase-IIb Studies

Clues as to which formulation is most potent in eliciting transmission-blocking activity may emerge from initial phase-I studies of previously non-exposed adult volunteers. Unlike the development of sporozoite and hepatic-stage vaccines where use of a challenge model in previously naïve individuals is safe and ethical, there currently exists no ethical artificial challenge

model for transmission-blocking vaccines. Furthermore, even if there were, it is prudent to always remember that the indication for use of transmission-blocking vaccines will likely be solely for populations residing in malaria-endemic or epidemic regions. The lack of potent transmission-blocking activity in studies performed in adults residing in developed countries where there is no prior history of malaria parasite exposure should be interpreted with extreme caution.

Genetic differences in immune responses and differences in the immune response based on prior infectious disease exposures make it likely that immunogenicity in naïve human volunteers studied in these initial phase-I studies may not at all be predictive of the responses obtained in previously infected humans from malaria-endemic regions. Until there is solid documentation that the immune responses in naïve individuals is highly predictive of previously infected individuals, the pivotal decision as to whether to proceed with a vaccine formulation to phase-I testing in malaria-endemic regions should be based primarily on safety data.

Clearly, for the same reasons, vaccine formulations that appear 'safe' in malaria-naïve populations must be retested for safety in phase-I trials in previously infected volunteers before a final decision is taken to proceed to phase-II studies in the same population. The design of proof-of-principle phase-IIb studies have been described previously. Selecting and preparing sites for field testing of transmission-blocking vaccines have begun. The resources required to test such vaccines are unique, particularly with respect to the need to handle infected mosquitoes.

Key elements in the design of phase IIb are the selection of the testing sites [56]. In addition to requiring the infrastructure to rear or at the very least dissect mosquitoes, the phase-IIb site-selection process requires accurate assessment of the transmissibility of malaria parasites at the site. The need for such an assessment, which incorporates the rate of infection, rate of gametocytemia, and prevalence of pre-existing transmission-blocking immunity in the target population, is driven by the need for these data in determining the sample size. Modest sample sizes can be used to prove principle in placebo-controlled studies, particularly if a one-sided test of significance is used. Because the practical consequence of the test vaccine being equal to or worse than placebo is most likely the same, a one-sided test of significance may be appropriate [1].

A particularly attractive, although somewhat complex phase-IIb design, the so-called three-pronged mosquito feed, is described in the next section.

Analytical and Clinical Validation of Clinical Surrogate Assays

Arguably one of the most important goals of the first phase-IIb studies should be to establish that a subunit vaccine will elicit an immune response in humans that is efficacious in vivo and that the preferred surrogate of efficacy correlates or is at least associated with in vivo efficacy. One design being

considered for proof-of-principle studies and clinical validation of the membrane-feeding assay is the so-called three-pronged mosquito feed test: (1) laboratory-reared mosquitoes purposely fed or trapped wild mosquitoes fortuitously fed on naturally infected study volunteers are quantitatively assayed for infectivity; (2) at about the same time that mosquitoes are fed directly on the study volunteer, blood collected from the volunteers is washed free of any serum antibodies and then fed via a membrane-feeding apparatus to laboratory-reared mosquitoes to establish the inherent infectivity of the circulating gametocytes in that donor at the time of the direct feed, and (3) serum antibodies collected from the volunteer are mixed with known infectious gametocytes and fed via a membrane-feeding apparatus to laboratory-reared mosquitoes to establish that blocking activity is in fact due to antibodies [1]. Ideally such a clinical validation of the membrane-feeding assay would be: (1) preceded by a rigorous analytical validation of the assay to establish the operational characteristics of the assay at the field site, and (2) incorporated into a randomized, placebo-controlled, double-masked trial performed at several sites that differed in the rate and seasonality of transmission and in the prevalence and incidence of parasitemia.

Because the mechanism by which antibodies mediate transmission-blocking activity may differ according to the target antigen, ultimately the membrane-feeding assay or any other in vitro assays need to be validated (or at least undergo an impact analysis) as predictive tests for each class of specific target antigens. For example, assays that measure complement-mediated lysis of gametes may be appropriate for quantifying transmission-blocking antibodies to Pfs230, but may be irrelevant for measuring transmission-blocking antibodies to Pfs25 or Pfs28. Likewise, the addition of complement or polymorphonuclear neutrophils to membrane-feeding assays for late sexual-stage target antigens may increase assay variability without providing improved specificity or sensitivity. Once verified for a specific class of immunogen (e.g., complement-dependent, pre-fertilization, post-fertilization, enzymatic, etc.), formal validation for use with alternate formulations is unlikely to be necessary.

Paths Forward

The largest obstacles remaining for the development of transmission-blocking vaccines include: (1) incomplete/inadequate process development for manufacturing bulk immunogen and for analytics used in the bulk immunogen production; (2) lack of clinically validated in vitro surrogates of efficacy; (3) absence of proven delivery systems and adjuvants suitable for use in humans, and (4) impediments to rapidly testing lead formulations in proof-of-principle human trials.

Bulk Immunogen Production

The near absence of industrial interest in bringing the full power of biotechnology to bear on the bulk immunogen production of transmission-blocking vaccine candidates has certainly slowed the pace of process development. Similarly, industrial expertise in applying state-of-the-art quantitative analysis to immunogen identity and potency assays has not been adequately applied to transmission-blocking vaccines. Although most pharmaceutical companies have opted not to vigorously pursue malaria transmission-blocking vaccine research and development, the scientists in these companies are usually eager to assist. A persistent effort to recruit and retain the interest of industrial scientists, particularly process engineers, should be of high priority.

Significant effort needs to be placed on the development of biologically relevant analytic assays. Presumably the faithful recreation of conformational B-cell epitopes is essential for eliciting an effective immune response. Validated immunological reagents, such as a panel of mAbs that recognizes properly folded as well as a panel that recognizes misfolded recombinant protein, are a good start; however, simple immunoblots and ELISA are often not sharp enough razors to dissect potent from inactive immunogens. More sensitive yet rapid and high throughput assays are needed for real-time, in-process evaluation to guide process development. A clear, solid link to in vivo efficacy substantially reduces the risk that such analytic assays actually provide added value.

Clinical Validation of the Membrane-Feeding Assay

Once a surrogate of efficacy assay has been analytically and clinically validated and accepted by appropriate regulatory agencies, subsequent clinical trials designed to determine optimal immunogen dose, route and schedule, adjuvant and delivery systems may proceed at a significantly quicker pace and at substantially lower cost. The final formulation for pivotal phase-III trials can be selected with much lower risk. Equally important, if the immune responses in naïve adults residing in developing countries as assessed by the surrogate efficacy assay are found to be predictive of immune responses in adults and children of the target population, then clinical development studies of late-stage vaccine, such as clinical lot consistency studies, and manufacturing processes and/or site changes become much less costly and are much quicker to complete. The substantial reduction in risk provided by a regulatory agency-approved surrogate of efficacy represents a powerful pull to industrial involvement.

Vaccine Formulations

With over 100 potential immunomodulators and delivery systems for subunit immunogen from which to chose, how best to proceed? No doubt well-characterized, stable immunogens, carefully formulated in clinical-grade

adjuvants and delivery systems, administered by route and schedule similar to that thought feasible in the clinic, and judiciously tested in non-primate and primate models will continue to be essential. Knowing that the desired immune response is solely antibody-mediated and directed at either secreted or surface-associated macromolecules on sexual-stage parasites, and having a rational, albeit unvalidated, assay that mimics the biology of malaria parasite transmission, transmission-blocking vaccine development can proceed in a reasonably logical manner. If nothing else, these pre-clinical studies help to winnow the potential variables to a more manageable number to be tested in the clinic.

Stability testing is one important aspect of vaccine formulation that is often overlooked in the early stages of vaccine development. Extensive analytic testing, done at the time the material is purified or formulated and filled, yield essential information required to have a well-characterized biopharmaceutical. However, the biologically relevant data for immunogenicity is not the potency of the vaccine product on the date it was filled but rather on the date it was administered to volunteers. Probe stability studies done on front-run products should be initiated as soon as possible to maximize stability information. The hook, of course, is that the data are only meaningful if a relevant, reliable and sensitive analytical potency assay is available.

Roadblocks to Essential Clinical Trials

Perhaps the biggest roadblock to proceeding into essential clinical trials (the initial phase-IIb study) is the notion that the formulation must elicit complete transmission-blocking activity in non-human primates or in the initial phase-I human trials conducted in the country in which the clinical-grade immunogen is manufactured before it should be taken forward. There are no data to refute this premise, but there are no data to support it either. The empiric approach in which the best current guess for formulation, dose, route and schedule is made on pre-clinical studies and early phase-I trials, and for which the only no-go decision is made based on safety, is no doubt more expensive than the conservative approach of exhaustively exploring different recombinant expression systems, a myriad of formulations, and using an unvalidated assay to make 'rational' choices. Until a formulation is taken to phase-IIb studies and the membrane-feeding assay is clinically validated (for example by using the three-pronged approach described above), we risk making the more costly mistake of delaying deployment of an important tool in malaria control – transmission-blocking vaccines.

The prospects for development of effective transmission-blocking vaccines are as good if not better than vaccines for other stages of the malaria parasite life-cycle. Perseverance, willpower, and a multidisciplinary, multinational, private/public sector, coordinated effort is now what is needed to add transmission-blocking vaccine to our armament of malaria control measures.

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Nucleic Acid Vaccines against Malaria

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Rationale and Strategy for Development of a Malaria Vaccine

It is estimated that there are 300–500 million new infections and 2–4 million deaths annually, and that approximately 40% of the world's population are at risk of malaria [1]. The profile of increasing resistance of the *Plasmodium* spp. parasite to chemoprophylactic and chemotherapeutic agents highlight the urgency for development of an effective malaria vaccine. The long-term objective of malaria vaccine research is to develop and field a vaccine that prevents the majority of naïve recipients from developing any clinical manifestations of disease after exposure to *Plasmodium falciparum*, and to prevent the development of severe disease and death in those individuals who do become ill by limiting the effects of blood-stage infection. Preventing the clinical manifestations of malaria will require completely preventing parasites from ever invading an erythrocyte. Accordingly, the primary goal of our program is the development of a vaccine targeted against the infected hepatocyte, since complete efficacy of such a vaccine would prevent both the clinical symptoms of malaria (which manifest in the erythrocytic stage) and the transmission of malaria (which occurs during the sexual stage). Preventing severe disease and death will require limiting the effects of blood-stage infection either by reducing parasite replication, reducing cytoadherence, and/or inhibiting the effects of toxic materials released by the parasite. Therefore, a secondary focus of our work is on the erythrocytic (blood) stage of the life cycle.

To achieve our primary and secondary goals, we have created a clearly defined and integrated program of basic research, preclinical research and development, product development, and clinical trials. Core components of this

program include identification of the antigenic targets of protective immunity against malaria, characterization of the mechanisms of protective immunity, development and refinement of vaccine delivery systems that induce the required immune responses against the identified antigenic targets, and clinical testing of candidate vaccines. This is an iterative, recursive process in which results from clinical trials of first-generation vaccines redirect and focus work on antigen discovery, immune mechanisms, and vaccine delivery systems, and in which promising results with new vaccine approaches in animal models are rapidly integrated into ongoing and planned clinical trials. In this review, we summarize the current status of our programmatic efforts to develop a DNA-based malaria vaccine, and illustrate the transition of promising developments in the laboratory to clinical assessment in humans.

Multistage Malaria DNA Vaccine Operation

Our efforts to date have been aimed at the induction of good or optimal immune responses to 15 well-characterized *P. falciparum* antigens: five pre-erythrocytic stage proteins that are expressed by irradiated sporozoites in infected hepatocytes, and 10 proteins expressed in the extracellular phase of the erythrocytic stage of the life cycle, on the surface or in the apical organelles. We have formed an international consortium, including scientists from 6 continents, to work on this project, and have named the project 'MuStDO' (Multistage Malaria DNA Vaccine Operation). The goal of the MuStDO-5 vaccines is to elicit protective T-cell responses against the five target antigens expressed in the infected hepatocyte (CSP, SSP2/TRAP. LSA-1, LSA-3, and Exp-1), thereby preventing the development of the parasite in the host hepatocyte. The goal of the MuStDO-10 vaccines is to elicit protective antibody responses against 10 parasite antigens expressed on the surface of the merozoite (MSP1, MSP2, MSP3, MSP4, MSP5, AMA1) or infected erythrocyte, or in the apical organelles (EBA-175, AMA1, RAP2). The overall goal is to elicit CD8+ and CD4+ T cell as well as antibody responses. Our hypothesis is that by reducing the numbers of parasites emerging from the liver (T-cell immune responses directed against those antigens expressed by irradiated sporozoites in hepatocytes) and priming the immune system to erythrocytic stage antigens that will be boosted by infection from natural exposure (antibody responses directed against parasite proteins expressed on the surface of merozoites or infected erythrocytes or apical organelles), one will reduce the severity and mortality of *P. falciparum* malaria. Non-immune travelers will require a malaria vaccine that provides immunity comparable to that elicited by the irradiated sporozoite vaccine. However, reducing the morbidity and mortality of malaria in the field

may require the induction of different immune responses against multiple targets expressed at distinct stages of the parasite's life cycle (multivalent, multistage, multi-immune response vaccine).

We are focusing on DNA vaccines as our core technology because of their demonstrated ability to induce preferentially CD8+ T-cell and Th1 type immune responses which have been difficult to induce by the more traditional vaccines, and because of their simplicity of design, modification and large-scale production, ease of mixing, stability, and lack of requirement for a cold-chain. The simplicity of the DNA approach implies that it should be possible to combine many DNA sequences, each encoding different antigens (multivalent) from one or more stages of the life cycle (multistage), and thereby broaden the immune response (multi-immune response).

Preclinical Studies of Malaria DNA Vaccines in Animal Models

In 1993, we provided the first demonstration that immunization of mice with plasmid DNA encoding a pre-erythrocytic stage *Plasmodium yoelii* antigen, PyCSP, could induce antigen-specific CD8+ cytotoxic T lymphocyte (CTL) and antibody responses and confer protection against sporozoite challenge [2]. Moreover, the levels of CD8+ CTL induced by DNA immunization were found to be significantly greater than the levels induced by protective immunization with irradiated sporozoites [2]. More recently, we have shown that the frequency and magnitude of antigen-specific interferon-γ (IFN-γ) responses are also significantly greater than those induced by protective immunization with irradiated sporozoites [Dobano C, unpublished data]. In 1994, we established that immunization of mice with plasmid DNA encoding another antigen, PyHEP17, could also protect against sporozoite challenge, and that immunization with a mixture of plasmid DNA vaccines could circumvent the genetic restriction of protection seen following immunization with each vaccine alone [3]. These data provided experimental support for our concept of a multivalent vaccine against malaria, and the foundation for our DNA-based approach to malaria vaccine development.

We subsequently established the immunogenicity of four different *P. falciparum* pre-erythrocytic stage DNA vaccines in mice [4] and rhesus monkeys [5], alone and in combination, and showed that there was no major effect of mixing on the induction of either CTL or antibody responses. The immunogenicity of *P. yoelii* erythrocytic stage DNA vaccines in mice [6], *Plasmodium knowlesi* pre-erythrocytic and erythrocytic stage vaccines in mice [7] and rhesus monkeys [8], *P. vivax* pre-erythrocytic and erythrocytic stage vaccines in mice [9], and *P. falciparum* pre-erythrocytic and erythrocytic stage DNA

vaccines in rabbits [10], rhesus monkeys [5], and *Aotus* monkeys [11, 12] has now been also established. These data in animal models provided the foundation for transitioning to a series of clinical trials in humans.

Phase-I Clinical Trial of a PfCSP DNA Vaccine Administered Intramuscularly

The first trial, conducted in 1997, was a phase-I dose-escalating safety and immunogenicity study, designed to determine if a single gene DNA vaccine encoding the *P. falciparum* circumsporozoite protein (CSP, PfCSP) was safe, well tolerated, and immunogenic in normal, healthy humans. Preclinical safety was established in mice and rabbit models, with particular emphasis on tissue distribution and the potential for genomic integration [13, 14]. Groups of 5 volunteers received three doses of 20, 100, 500, or 2,500 μg of PfCSP DNA in a 1-ml volume via conventional needle injection in alternate deltoid muscles at 4-week intervals. The vaccine was shown to be safe and well tolerated in all volunteers [15]. Antigen-specific, genetically restricted, CD8+ T-cell-dependent CTL were induced by DNA vaccination in 11 of the 17 individuals who were evaluable (that is, who expressed HLA alleles known to present one or more of the peptides for in vitro evaluation of immune responses) [16]. Specifically, peptide-specific, genetically restricted and CD8+ T-cell-dependent CTL responses which met our strict criteria for positivity were detected in 2 of 4 volunteers immunized with 20 or 100 μg DNA, in 3 of 4 volunteers immunized with 500 μg DNA and in 4 of 5 volunteers immunized with 2,500 μg DNA. The frequency and magnitude of CTL responses were greatest in the 500- and 2,500-μg groups, as compared with the 20- and 100-μg groups ($p \leq 0.04$), and there was no significant difference between the 2 higher dose groups after the third immunization. Responses were induced by as little as 2 doses of 20 μg DNA (induction of CTL after a single immunization was not assessed). The DNA-induced CTL responses were dependent on CD8+ T cells, but not CD4+ T cells, and were genetically restricted by multiple HLA alleles (10 defined epitopes restricted by HLA-A1, A2, A3/A11, B7, B35 or B53 alleles). Disappointingly, however, antibodies to PfCSP could not be detected in any of the individuals at any of the time points studied [15].

Preclinical Studies Investigating Route of Administration in Animal Models

Previously, in the *Aotus* monkey model with a *P. yoelii* CSP (PyCSP) DNA vaccine, we had shown that no detectable antibodies were elicited when the

vaccine was administered intramuscularly (IM), as in our clinical trial, but that excellent antibody responses were elicited when the PyCSP DNA was administered intradermally (ID) [11]. Therefore, we decided to look at alternative administration strategies in humans. In particular, we considered the needleless jet injection device, the Biojector™ 2000 (Bioject Inc., Portland, Oreg.). Initially, we assessed the suitability of the Biojector™ route of administration for plasmid DNA in rabbits and rhesus monkeys, specifically comparing the response to DNA vaccines administered IM by needle, IM by Biojector™, and IM (70% of dose) and ID (30% of dose) by Biojector™. In rabbits immunized with 500 μg of the same PfCSP DNA vaccine that had been used in the first clinical trial, 25- to 50-fold higher sporozoite-specific antibody responses were induced by IM/ID injection using the Biojector™, and 5- to 8-fold higher antibody responses were induced by Biojector™ IM, as compared with the responses induced by immunization with the same DNA administered IM by needle [10]. Similarly, in rhesus monkeys immunized with a mixture of 500 μg tetravalent Pk DNA vaccines (encoding two *P. knowlesi* pre-erythrocytic stage antigens and two *P. knowlesi* erythrocytic stage antigens), 10- to 20-fold higher antibody responses against both sporozoite and blood-stage parasites were induced by Biojector™ IM and Biojector™ IM/ID immunization as compared with IM needle [8]. These data in rabbits and rhesus monkeys supported clinical assessment of the Biojector™ route of administration for plasmid DNA.

Phase-I Clinical Trial of a PfCSP DNA Vaccine: Route of Administration

A second phase-I clinical trial of the PfCSP DNA vaccine was therefore designed, using the same immunization regimens assessed in the rabbit and rhesus monkey models [Epstein JE, unpublished]. The primary goal of that study was to determine if DNA immunization of humans by alternate routes of administration would elicit PfCSP-specific antibody responses. Secondary goals were to determine if DNA immunization of humans could induce the class-I restricted IFN-γ responses considered important for protection against malaria, and if altering the route or method of administration of a DNA plasmid would enhance immunogenicity.

Groups of 5 volunteers received 3 doses of 2,500 μg PfCSP DNA at 4-week intervals IM by needle, IM by Biojector™, or IM (70%) plus ID (30%) by Biojector™. Vaccine administration by all 3 routes, including Biojector™ IM and Biojector™ IM/ID, was shown to be safe and well tolerated [Epstein JE, manuscript submitted]. Further, antigen-specific and genetically restricted

CD8+ CTL responses were induced by all 3 routes of administration [17]. Overall, antigen-specific, CD8+ T-cell-dependent and genetically restricted CTL responses were detected in 8 of 14 individuals following DNA immunization; 3 of 5 immunized with Biojector™ IM/ID, or with Biojector™ IM, and 2 of 4 immunized with needle IM [17]. In that study, we also evaluated the capacity of plasmid DNA vaccination to induce antigen-specific IFN-γ responses, since murine studies have established that class-I-restricted IFN-γ responses are the primary immune effectors contributing to the sterile protection induced by immunization with irradiated sporozoites. IFN-γ responses were assessed by ELIspot using peripheral blood mononuclear cells (PBMCs) incubated with up to 15 defined PfCSP-specific peptides known to bind to HLA class-I or class-II molecules expressed by the volunteers. Overall, IFN-γ immune responses to multiple class-I and/or class-II epitopes were detected in all 14 individuals following DNA immunization (range 10–543 spot forming cells (SFCs)/10^6 PBMCs) [17]. In the needle IM group, responses were detected in 4 of 4 volunteers to 7 of 9 peptides in 17.6% of assays; Biojector IM, in 5 of 5 volunteers to 9 of 9 peptides in 26.5% of assays; and Biojector IM/ID, in 4 of 5 volunteers to 7 of 9 peptides in 17.3% of assays. Responses to the 9-mer class-I peptides were detected in 13 of 14 volunteers, responses to the longer 15- to 23-mer peptides were detected in 12 of 14 volunteers, and 11 of 14 volunteers produced IFN-γ in response to both short and long peptides. In all cases, the production of IFN-γ was dependent on CD8+ T cells [17]. IFN-γ and CTL responses to multiple peptides were detected simultaneously in the same volunteer. These data therefore established that CD8+ CTL and CD8+ IFN-γ responses could be induced by all three routes of administration. However, as noted in the first clinical trial, antigen-specific antibodies could not be detected in any of the volunteers [Epstein J, manuscript submitted].

The comparative immunogenicity of the different routes of administration was determined by comparing the frequency and magnitude of responses. Overall, the data suggested that Biojector™ IM was the most effective route of those assessed for the induction of antigen-specific IFN-γ responses, and that Biojector™ (IM and/or IM/ID) was more effective than needle IM for induction of antigen-specific CTL responses [17].

First-Generation DNA Vaccines Are Suboptimal

These phase-I single gene clinical trials have established that DNA vaccines encoding malaria antigens are safe and well tolerated, and can induce antigen-specific, CD8+ T-cell-dependent and genetically restricted CTL and IFN-γ responses in normal healthy humans. However, despite the induction of

IFN-γ responses and CTL by each of 3 different routes of DNA administration, we believe that the frequency and magnitude of these responses are suboptimal, probably not adequate for protection, and can be significantly improved. Moreover, antibodies could not be induced in humans by any of the different routes of DNA administration, despite the same DNA vaccine having been shown to induce antibody responses in mice [4], rabbits [Aguiar JC, manuscript submitted], *Aotus* monkeys [18] and rhesus monkeys [5].

Even in animal models, our data and those of others clearly demonstrate that the immunogenicity of these first-generation DNA vaccines is not optimal. Specifically, in mice *P. yoelii* DNA vaccines can protect against sporozoite challenge, but not all strains are protected, and not all mice within a given inbred strain are protected [2, 19–21]. Further, this DNA-induced protection does not withstand high challenge doses and is not sustained for a long period [Sedegah M, unpublished data; Doolan DL, unpublished data]. PyCSP DNA protects only 1 of 5 inbred strains of mice [20]; in the protected strain, DNA-induced CD8+ CTL responses are good, antibody responses are modest, and CD4+ T-cell responses are poor [2, 19, 21; Weiss WR, unpublished]; PyHEP17 DNA protects only 3 of 5 inbred strains [20], in all strains, CD8+ CTL and CD4+ T-cell responses are poor [Doolan DL, unpublished data; Weiss WR, unpublished data] and antibody responses are negligible [20]. In non-human primates, *P. falciparum* DNA vaccines (PfCSP, PfSSP2, PfExp-1, PfLSA-1) induce only modest CD8+ CTL, IFN-γ, and antibody responses (CD4+ T-cell responses not assessed) [5]. In rhesus monkeys, recent studies of *P. knowlesi* DNA vaccines, in the absence of heterologous boosting have shown evidence of immunogenicity but not of protection [8]. These suboptimal responses have led us to devote significant efforts to immune enhancement strategies for DNA vaccination, with particular emphasis on specific immunization strategies (prime/boost) or modifications of the inserted gene (codon optimization), plasmid backbone (inclusion of immunostimulatory CpG sequences), vaccine formulations or vaccine delivery systems.

Heterologous Prime/Boost Immune Enhancement Strategies in Animal Models

An increasing body of data in animal models of malaria [22] as well as other diseases show that heterologous prime/boost strategies using plasmid DNA, recombinant attenuated vaccinia virus (NYVAC), COPAK, canarypox (ALVAC) virus, modified vaccinia virus Ankara (MVA), fowlpox virus, cowpox virus, adenovirus, influenza virus, yeast retrotransposon (Ty particles), recombinant protein in adjuvant, and synthetic peptide are far more effective with regard to immunogenicity and protective efficacy than homologous immunization with

DNA recombinant virus or recombinant protein in adjuvant. In general, data indicate that plasmid DNA (with or without cytokine coexpression) represents the best modality for priming an immune response, but that heterologous boosting with recombinant poxviruses generates the most robust CD8+ T-cell responses (but sometimes poor antibody responses), and heterologous boosting with recombinant protein in adjuvant generates the most robust antibody responses (but sometimes poor CD8+ T-cell responses). Robust Th1 CD4+ T-cell responses are induced by either method of heterologous boost. In all cases, the order of immunization is crucial.

In our laboratory, in the *P. yoelii* rodent model, we demonstrated that boosting with recombinant vaccinia virus increased CD8+ T-cell immunogenicity (CTL and IFN-γ responses), antibody responses and protective efficacy of a PyCSP DNA vaccine [19], that co-immunization of PyCSP plus a plasmid encoding murine GM-CSF resulted in a significant increase in CD8+ and CD4+ T-cell immunogenicity and protective efficacy [21], and that priming with the combination of DNA plasmids encoding PyCSP and murine GM-CSF [21] and boosting with recombinant poxvirus expressing the same antigen induced a 30-fold increase in antigen-specific antibodies, a 100-fold increase in antigen-specific IFN-γ spot-forming cells, a significant ($p < 0.05$) increase in protection, and the capacity to reduce the dosage of DNA by 10–100 fold, as compared to immunizing with DNA alone [23]. The relative magnitude and quality of this vaccine-induced protection, as assessed by the capacity to induce sterile protection in all immunized mice and to withstand high dose sporozoite challenge, was such that irradiated sporozoite > PyCSP DNA + GMCSF prime/PyCSP poxvirus boost > PyCSP DNA prime/PyCSP poxvirus boost > PyCSP DNA. Recently, we have also demonstrated enhanced immunogenicity (CD8+ and CD4+ T-cell responses) and protection with heterologous PyCSP DNA prime/PyCSP adenovirus boost, as compared with homologous DNA immunization [Brice GT, manuscript in preparation]. We have also demonstrated significantly enhanced antibody responses (but similar T-cell responses) with a Venezuelan equine encephalitis (VEE) replicon (an alphavirus replicon) encoding PyHEP17, as compared with DNA alone [Luke TR and Weiss WR, unpublished data], suggesting the potential for these constructs in prime/boost regimens. Finally, studies in our laboratory have established that although immunization with irradiated sporozoites remains the gold standard for long-term protection in mice and humans, the heterologous DNA prime/recombinant poxvirus boost immunization strategy represents the optimal strategy for inducing antigen-specific immune responses [23; Brice GT, unpublished; Dobano C, unpublished].

In the *P. falciparum/Aotus* monkey model, priming with DNA encoding PfEBA-175 (an erythrocytic stage antigen) and boosting with recombinant EBA-175 region-II protein in adjuvant (Montanide plus CpG oligonucleotide) was

shown to elicit antibody responses comparable to those elicited by immunization with 4 doses of recombinant protein in adjuvant, and was associated with increased protective immunity as compared to that achieved after homologous immunization [12]. Furthermore, data indicated that homologous immunization with DNA alone induced moderate antibody responses that were significantly boosted by exposure to the native parasite upon challenge, providing experimental support for our MuStDO concept.

In the *P. knowlesi*/rhesus macaque model, a series of three studies have shown that heterologous boosting with a recombinant tetravalent poxvirus (canarypox or recombinant attenuated vaccinia, COPAK) resulted in enhanced T-cell immunogenicity and protection [7, 8]. Data suggested that the DNA prime/vaccinia boost regimen induced both cellular responses able to eliminate infected hepatocytes and antibodies able to control blood-stage infection in those monkeys which did develop parasitemia.

DNA-based clinical trials against malaria are now underway or planned using these heterologous immunization strategies, among others. However, because of the complexity and cost of these heterologous regimens, and the possibility of side effects associated with the use of attenuated viruses in immunocompromised individuals, we are working to make DNA vaccination alone as immunogenic and protective as prime/boost approaches. Moreover, it should be recognized that even with the high level of immunogenicity and protection achieved by priming mice with PyCSP DNA plus GM-CSF DNA and boosting with recombinant pox virus expressing PyCSP, the protection is almost never 100%, and while it is excellent against a modest sporozoite challenge of 50–100 sporozoites (50 ID_{50}s), it does not withstand challenges with tens of thousands of sporozoites. In contrast immunization with radiation-attenuated sporozoites induces protection against challenge with greater than 100,000 sporozoites in essentially all recipients [Sedegah M, unpublished]. Thus, we believe that there is great room for improvement even with the prime/boost approach.

Immune Enhancement Strategies: Coadministration of Immunomodulatory Molecules

Based on data in the *P. yoelii* rodent model demonstrating that coimmunization of PyCSP plus a plasmid encoding murine GM-CSF resulted in a significant increase in CD8+ and CD4+ T-cell immunogenicity and protective efficacy [21], we designed a study to evaluate the effect of coadministration with plasmid GM-CSF in humans. In this open-labeled, phase-IIa trial, termed MuStDO-5.1, a mixture of five plasmids encoding the MuStDO-5 antigens,

PfCSP, PfSSP2/TRAP, PfEXP-1, PfLSA1, and PfLSA-3, administered to volunteers either with or without an additional plasmid that expresses human GM-CSF, is currently being evaluated for safety, immunogenicity, and protective efficacy in volunteers in the United States [Richie TL, Wang R, Charoenvit Y, unpublished]. Specifically, we are assessing the effect of mixing 0, 20, 100, or 500 μg of a plasmid expressing human GM-CSF with a mixture of 500 μg each of the five liver-stage DNA vaccines. The primary goal is to determine whether coadministration of plasmid encoding human GM-CSF is well tolerated and safe, and if it has any positive impact on immunogenicity or protective efficacy. However, while the GMP manufacturing for MuStDO-5.1 has been in progress, several developments have taken place in the laboratory, some of which have shown that we can markedly improve on the current generation of vaccines included in MuStDO-5.1.

Immune Enhancement Strategies: Modification of Target Gene for Optimal Expression in Mammalian Cells

One promising development has been that of codon optimization of the *P. falciparum* genes. Codon bias has been observed in many species (see codon usage database, http://www.kazusa.or.jp/codon/), and the usage of selective codons in a given gene has been positively correlated with its expression efficiency. We have recognized that the genome of *P. falciparum* is extremely A+T rich with a content of 80% AT overall and 76% within coding regions. Consequently, codon usage in *P. falciparum* is markedly different from that seen in highly expressed mammalian genes, and expression of DNA-encoded *P. falciparum* antigens in mammalian cells may be inefficient. We have therefore designed synthetic *P. falciparum* genes which utilize the codons most frequently used by an ensemble of highly expressed human genes; in most cases the G+C content of the genes has been changed from approximately 26% to approximately 56% [24]. Studies with the synthetic codon-optimized plasmids have shown that changing the codon usage resulted in at least a 5- to 40-fold enhancement of in vitro expression of the encoded antigen in mammalian cells in standard in vitro transient transfection assays, as compared with that of a plasmid expressing the corresponding native gene sequence [24, 25]. Moreover, in vivo, the synthetic gene-based plasmids showed a similar positive immune enhancement effect afforded by changing the codon usage, with approximately 5- to 100-fold higher antibody titers being induced in outbred mice [24, 25]. Studies regarding the effect of changed codon usage on T-cell immunogenicity are ongoing [Sedegah M, unpublished data; Dobano C, unpublished data]. Plasmids expressing the synthetic genes of 9 of the MuStDO

antigens are now under construction under GMP conditions for future evaluation in the clinic [26, 27].

Genomes to Vaccines

Here, we have outlined our strategy and current status with regard to developing a multivalent, multistage, multi-immune response DNA-based vaccine against malaria, based on a limited number of well-characterized *P. falciparum* antigens. However, data generated to date suggest that the current generation of subunit vaccines based on a limited number of antigens may provide neither optimal protection nor protection on genetically diverse backgrounds. The protective immunity in our two human models, the irradiated sporozoite vaccine and naturally acquired immunity, is elicited by exposure to the whole parasite not just a single or few proteins. Duplicating and sustaining the protection that is induced by whole organism vaccination therefore may require a vaccine as complex as the whole organism, which incorporates hundreds or thousands of antigenic epitopes in a vaccine formulation capable of inducing the appropriate immune response in the context of diverse host genetics. Therefore, in parallel to our MuStDO series, an alternate approach to vaccine development is being pursued in our laboratory. This is based on the assumption that the sustained, strain-transcending immunity achieved after vaccination with irradiated sporozoites is multi-antigenic, potentially directed against thousands of proteins expressed by irradiated sporozoites in hepatocytes, and that the naturally acquired immunity seen in endemic areas is likely due to immunity to the hundreds or thousands of proteins expressed on the surface of merozoites and the surface of infected erythrocytes. Therefore, our future efforts are aimed at reproducing the breadth and multiplicity of the whole organism induced protective immunity by vaccination, by capitalizing on the data obtained from the malaria genome project and advances in gene expression, proteomic and molecular immunology technologies to develop and refine a vaccine based on an unprecedented number of parasite-derived proteins and the T- and B-cell epitopes from these proteins. We propose that a vaccine based on an expanded number of sequences encoding those minimal epitopes considered important in protection, and eliminating sequences with no apparent role in protection, will be more immunogenic and protective in a broader population range than vaccines based on a limited number of complete antigens. It is possible that only this 'whole genome' approach may be adequate to combat a parasite as complex as *Plasmodium*. The current challenge is to adequately test the more simple vaccines in the field, while developing improved and perhaps more complex malaria vaccines.

Conclusion

The introduction of DNA vaccine technology has facilitated an unprecedented multi-antigen approach to developing an effective vaccine against complex pathogens, such as the *Plasmodium* spp. parasites that cause malaria. However, our recent demonstrations and those of others have shown that first- or second-generation DNA vaccines on their own are not optimal, and that heterologous prime/boost immunization strategies, involving priming with DNA and boosting with poxvirus or recombinant protein in adjuvant, offer enormous promise for the development of an effective malaria vaccine. Therefore, efforts are directed towards determining why heterologous prime/boost approaches are so much more immunogenic than DNA vaccines on their own, and to developing strategies for making DNA alone equal to or better than heterologous prime/boost regimens and for decreasing the amount of DNA required for induction of optimal immunogenicity and protective efficacy. Our focus remains on DNA as a core technology because of the enormous advantages conferred over the more traditional vaccines, not the least of which is the opportunity to create an entirely new generation of vaccines by capitalizing on the vast information deriving from microbial and human genome-sequencing projects.

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Antidisease Vaccines

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Introduction

Whilst eradication of malaria by the induction of population-wide sterilizing immunity might be desirable, it is not generally considered to be a feasible objective at least for the current generation of antimalarial vaccine candidates, and the reasonable view is that transmission of the parasite and infection of the host is likely to continue in the face of vaccination. Thus, prevention or reduction in morbidity and mortality is the realistic objective of most current malaria vaccine candidates. Traditional approaches to antimalarial vaccines seek to provide this clinical protection indirectly, by killing the parasite or by reducing parasite multiplication. To this end, they have as their target antigenic proteins expressed either on the surface of sporozoites, infected hepatocytes, merozoites or schizonts or in specialized invasion organelles such as rhoptries. Even the putative transmission-blocking vaccines aimed at the surface antigens of parasite sexual stages seek to increase public health by lowering the overall burden of parasites in the population as a whole. Clearly, unrestrained parasite multiplication and sequestration of excessive numbers of parasites in vascular beds are important in malarial pathogenesis, and a broad correlation does exist between parasite burden and disease manifestations. It may be that by reducing the inoculum or the rate of parasite multiplication in the host, a longer period is required to reach clinical thresholds, allowing more time for the development of protective immune responses. Nonetheless, because of the complexity of the host/parasite relationship in different groups, parasite levels and host disease are to some degree and in certain instances dissociable, and a reduction in parasitemia will not necessarily lead to a reduction in malaria-specific morbidity or mortality. Indeed, as discussed below, in areas where acquired immunity serves to reduce

disease burden in older age groups, reduction of transmission may have the paradoxical effect of raising malaria-specific morbidity and mortality rates.

A number of problems are associated with attempts to reduce morbidity and mortality by a reduction in parasite burden. The nature of the parasiticidal immune mechanisms that must be conferred upon the host still remains unclear. In the case of asexual stage vaccines, several approaches are predicated upon inhibition of merozoite invasion of red blood cells, although it remains to be established that this can provide a long-term mechanism for limiting parasite multiplication. Indeed, inhibition of parasite invasion by antibody is not a predictor of immune status in endemic areas [1]. Converging evidence suggests considerable redundancy in invasion pathways, immune evasion strategies and problems of major histocompatibility complex (MHC)-linked genetic restriction in the immune response to these antigens. Genetic liability, antigenic diversity, antigenic variation and immunosuppression may also enhance evasion of immunity and breakthrough parasites. Furthermore, where vaccines kill the parasite, vaccine-induced selective pressure is inevitable. For these reasons, multicomponent vaccines are considered desirable, and a great deal of effort is currently devoted to the expression of recombinant proteins, appropriately folded, or to the generation of T-cell effector vaccines against hepatic stages of the parasite.

A technically and conceptually distinct approach is offered by the possibility of developing 'antidisease vaccines'. These would aim to reduce malarial morbidity and mortality directly by immunizing against those parasite products which are the chief cause of host pathology. In these cases, the death of the parasite is not a necessary objective in fulfilling of the aims of the vaccine. Precedences for antidisease vaccines are to be found in the highly effective tetanus and diphtheria toxoid vaccines which both protect the host against the most injurious consequences of infection by targeting the bacterial toxins which cause disease [2]. The enormous public health value of these vaccines is demonstrated by their inclusion as two of the six vaccines currently given to children world-wide in WHO's Expanded Program of Immunization (tetanus, diphtheria, pertussis, measles, mumps, rubella). In outlining the problems facing parasiticidal vaccines, it is not intended to give the impression that potential problems do not also apply to the development of antidisease vaccines. They do, not least being the fact that few targets are yet validated, and at present the approach rests upon untested assumptions about preventing pathology by either antibody-based or cellular mechanisms. However, the approach is a novel one in the context of malaria vaccines, and is sufficiently different to other approaches to ensure that a distinct set of problems is likely to be presented, which may or may not be insuperable. This chapter therefore will seek to outline arguments for and against the development of an antidisease vaccine, highlighting those areas

where such approaches may differ from more traditional attempts to confer immunity to malaria by immunization with antiparasite vaccines. As there are few data so far concerning specific antitoxic vaccine candidates, their application to human disease remains largely conjectural.

Arguments from Acquired Immunity

Humans can and do develop functional immunity to malaria. The targets and mechanisms of this immunity remain far from clear. At the heart of the rational (as opposed to empirical) approach to malaria vaccine design lies the proposition that vaccines should be developed based on our knowledge of the targets and determinants of this naturally acquired immunity. The empirical approach asserts that all vaccines represent departures from the natural order and that we should not be limited to mimicking naturally acquired immunity, especially as this takes many years to develop. The distinction between rational and empirical approaches is not necessarily a great one in all cases, as most vaccine candidates currently under development have at least a minimal amount of supportive evidence to warrant their candidature, and all candidates should have to satisfy empirical criteria of efficacy for continued development. Nonetheless, the evidence adduced in support of vaccine candidates does not rely heavily on information pertaining to naturally acquired immunity. In certain cases, responses to various single malarial antigens, including the vaccine candidates merozoite surface protein-1 [3, 4], liver-stage-specific antigen-1 [5, 6], and circumsporozoite surface protein [7] have been associated with a reduced risk of malaria. However, some of these findings have not been reproducible, and thus there is little consensus regarding their significance. Our paucity of knowledge about the targets and mechanisms of naturally acquired immunity in humans is no doubt a reflection of the difficulty in executing the appropriate epidemiological studies, and constitutes a major limitation in rationalization of malaria vaccine design. Longitudinal prospective cohort studies measuring immune responses to defined candidates in a comparative manner and correlating these with risk of disease would appear to represent the best way to provide answers to some of these questions in the future.

Notwithstanding uncertainty about the role of specific antigens as targets of functional immunity, investigations of the natural history of malaria in endemic areas by clinical and epidemiological studies has over the years generated important and useful insights [8]. In areas of high transmission, immunity to malaria is acquired in two stages: an initial phase of clinical immunity which operates despite persistent high parasitemias, followed after some years by an antiparasite immunity which limits parasite numbers, replication and burden

within the host. Infants born of immune mothers appear to be relatively resistant to infection and severe clinical episodes, which is thought to result from passively acquired IgG [9]. Patent parasitemia and severe clinical illness develop after 6 months of age, when passively acquired IgG is lost. This susceptibility to malarial disease lasts until the end of the 3rd year, and is associated with the classical picture of clinically severe disease in young children, and high mortality. After this stage, a clinical immunity becomes apparent, manifest as lower rates of disease despite persistent high parasitemias continuing into primary school years. Parasite densities and prevalence may remain high throughout childhood as disease manifestations progressively wane. It is noticeable that the acquisition of antiparasite immunity, manifest as the ability to reduce parasite densities, is delayed and only comes to maximal fruition significantly later. Furthermore, antiparasite immunity is of the concomitant type, rather than sterile immunity. The reasons for this are likely to be manifold and include antigenic diversity [10], antigenic variation [11], redundancy in invasion and cytoadherence strategies, epitopic suppression [12], active immune suppression [13] and immune dysregulation. The implications for the development of antiparasite vaccines would appear to be that for any vaccine to be effective as a public health tool, it must in a cost-effective way incorporate multiple components to deal with these diverse parasite strategies, and that antiparasite vaccines are unlikely to impart sterile immunity to recipients.

The relatively early-onset clinical immunity outlined above is sometimes referred to as ‘clinical tolerance’, defined as the ability to remain asymptomatic despite relatively dense parasitemia. The use of the word ‘relatively’ indicates that such parasite burdens would normally induce disease. In other words, the threshold parasitemia for onset of clinical symptoms becomes elevated. In a comparative study of semi-immune and immune individuals, it was found that the threshold of clinical symptoms for primary school children was 11,000 parasites/μl, compared with the lower level of 30–450/μl for adults [14]. Clinical tolerance applies not only to the non-life-threatening symptoms of acute malaria, but also coincides with a reduced incidence of severe disease. However, that the severity of symptoms is proportional to parasite density appears true in non-immunes. These patients develop symptoms at lower parasite densities than semi-immunes.

A number of mechanisms may account for clinical immunity or tolerance. Christophers [15] was the first to observe the phenomenon in 1929 and proposed that it reflects the acquisition of an antitoxic immunity. Sinton [16] subsequently endorsed this idea. McGregor et al. [17] also observed that the development of tolerance to circulating parasites and their products was one of the first signs of immunity, and considered that this phenomenon could reflect the development of antitoxic immunity. A recent study suggests that immunity to certain aspects

of severe malaria is acquired after one or two infections [18]. However, no immunological correlate of 'antitoxic immunity' has yet been described, and indeed it is not yet established that clinical tolerance results from an immune response sensu stricto. Indeed, passive transfer of immunoglobulin from adults into febrile malarious children did not appear to confer an antitoxic effect and clinical amelioration appeared to follow from a reduction in parasite density [19, 20]. Alternative explanations for the phenomenon of clinical tolerance include the acquisition of a state of physiological nonresponsiveness to malaria toxins. For example, exposure to lipopolysaccharide (LPS) can be followed by downregulation of LPS-responsive signaling pathways in macrophages [21]. Alternatively, exposure to malaria may result in temporary low responsiveness or anergy among T lymphocytes which might contribute to disease manifestations. The main argument against these latter possibilities and in favor of an acquired immune mechanism mediating tolerance is that physiological desensitization or lymphoid anergy is unlikely to operate over very long time scales, whereas clinical tolerance to malaria appears to be a relatively robust phenomenon. Another possibility, discussed more fully below, is that severe disease requires a contribution by T lymphocytes of the T_H1 type, and that tolerance is associated with a switch to a T_H2-dominated helper response, which although preventing severe disease is less efficient in controlling parasitemia until a range of appropriate antibody specificities is achieved (see below).

Therefore, notwithstanding the current uncertainty surrounding the mechanisms of 'tolerance', the observation that children develop clinical immunity to the toxic aspects of malarial pathology long before their parasitemias start to fall provides some evidence in support of the concept of antidisease vaccination. Compared with this putative 'antitoxic' immunity, naturally acquired antiparasite immunity appears hard-won, serving to underscore the potential limitations of antiparasite vaccines. Therefore, establishing whether or not tolerance or clinical immunity results from an active immunological function as opposed to physiological or desensitization processes is of high priority for this area of research.

Reducing Malaria Transmission May Increase Mortality and Morbidity

Although mosquito bionomics, encapsulated in the concept of vectorial capacity, determine the broad level of malaria transmission, naturally acquired immunity in humans is the controlling force that determines the relative incidence and prevalence of malaria in the population in areas of holo- or hyperendemicity. As clearly established during the era of the global malaria eradication campaign, in such areas where acquired immunity operates at the population

level, a reduction in malarial transmission may have the unwanted result of increasing malaria-specific morbidity and mortality as immunity wanes [22]. This reflects the basic processes of concomitant immunity or premunition, where continual exposure to the parasite is required to build and maintain immunity, and removal of the parasite can allow naturally acquired antiparasite immunity to be lost. This has been recently reviewed [23, 24]. One objection raised against sporozoite, liver-stage and sexual-stage vaccines is that by interruption of transmission and prevention of blood-stage infection they may paradoxically prevent the acquisition of immunity in naïve groups or allow the waning of acquired immunity or premunition in adults. This can lead to more severe malaria once breakthrough blood-stage infections establish. This is more than an academic concern as the negative impact on herd immunity of mass prophylaxis or vector control campaigns has been well documented [22]. Indeed, even blood-stage vaccines which may serve to suppress parasitemias by targeting a specific pathway, may leave recipients relatively more susceptible to clinically severe episodes should breakthrough infections occur in the longer term.

A significant argument against these considerations is that independent of immune status, infants may be at greater risk of death due to malaria than slightly older age groups. If so, reducing transmission may reduce the death rate simply by moving the burden of disease to age groups constitutively more able to withstand the impact of infection. The major concern would then be whether, if applied on a population-wide basis, interventions which reduce infection and transmission rates could change a pattern of stable transmission into an unstable one and therefore predispose to epidemics.

Clearly, conferral of clinical immunity upon target populations by an antidisease vaccine would obviate these concerns. Reduction in early childhood risk of mortality without suppression of parasitemia would allow the normal acquisition of immunity following infection. In this regard, few approaches have quite the same potential as antidisease vaccines. Such an intervention could of course be of benefit to every population group at risk of disease, whether nonimmune travelers, migrants, military, or residents of endemic areas. However, it would be of special benefit to infants and pregnant women in disease-endemic areas. Because it would probably not impede the natural development of immunity, of all antimalarial immunoprophylactic measures under consideration, a safe effective antidisease vaccine would arguably be the best suited for integration into horizontal maternal and child health services to be administered along with other childhood vaccines. These considerations suggest that an antidisease vaccine would be suitable as a stand-alone agent, and there is no particular reason to believe that an antidisease vaccine will necessarily require administration along with antiparasite vaccines. It could of course be integrated into other control measures. As noted above, however, as there are few data so far concerning specific antitoxic vaccine candidates, and indeed antiparasite vaccines,

these considerations in application to human disease remain speculative at this point.

In contrast to these considerations which are generally supportive of the antitoxic vaccine approach, it has been argued that antitoxic vaccines run the risk of exacerbating disease if they inhibit the expression of normal acute-phase responses which serve to limit parasite replication [25]. In this view, the typical pathophysiological host response to malaria is adaptive, helping to limit parasite replication. As severe disease is manifest in only a small proportion of those infected, the majority of those receiving an antitoxic vaccine may be prevented from mounting an adequate febrile defense. Fever is proposed to be a density-dependent regulator of parasite growth, acting to protect the host against high parasitemia regardless of immune status [25]. Creative mathematical modeling of these concepts confirms that density-dependent feedback effects do result in sine waves [26]. Indeed, the malaria toxin has been described as a 'calling card', the function of which is to elicit a host response that limits parasite population density [25]. However, 'suicide pill' explanations of biological function in unicellular organisms sit uneasily with Darwinian logic. Furthermore, although ingenious, these considerations do not account for the tolerance phenomenon where semi-immunes with the highest parasite burdens are least likely to fall ill. Tolerance clearly demonstrates that far from being a problem, a rise in the clinical threshold is associated with clinical immunity and there is no evidence that this is bad for the host. Nonetheless, these speculations help to underscore the importance for antitoxic vaccine development of carefully conducted preclinical trials in various model systems, as well as phase-I and phase-II clinical trials, which should measure clinical threshold and the rate of parasite replication in adult volunteers. Indeed, as the impact of any particular vaccine on pathogenesis remains to be determined, careful preclinical and clinical trials in volunteers should be sought for all malaria vaccine candidates.

Mechanisms of Malarial Pathogenesis

As the aim of antidisease vaccines is to block malarial pathology, an understanding of the molecular basis of malarial pathophysiology is essential for rational development of the field. Currently three basic processes are thought to contribute to varying degrees to the range of syndromes associated with clinically severe malaria: site-specific localization of parasites via cytoadherence (sequestration), the local and systemic action of malaria toxin, and the production of regulatory cytokines by the innate and acquired immune systems. The latter two processes are closely related as cytokines can influence the response to toxin and malarial toxins can regulate T-cell responses (unpublished data)

as well as directly induce the expression of cytokines from nonlymphoid tissues [27]. Furthermore, both cytokines and malarial toxins can each directly induce the expression of other proinflammatory loci such as inducible nitric oxide (NO) synthase (iNOS) [28], thereby raising levels of NO, which may be a regulator of pathogenesis [29]. The syndrome seen in severe falciparum malaria in African children typically consists of fever, metabolic acidosis, hypoglycemia, and cerebral involvement including seizures and coma. Other disease manifestations in African children include severe malarial anemia, and the relationship of the three processes outlined above to the etiology of this condition remains unclear. Furthermore, the clinical picture may be somewhat different in adults and in other parts of the world. For these reasons, severe malaria in South-East Asia often includes multiorgan and system disturbances, including renal failure, pulmonary edema, shock, jaundice, etc. As considerations of cytoadherence-targeted vaccines are dealt with elsewhere in this volume, we will focus briefly here on the role of toxins and cytokines in pathogenesis.

The first truly modern proposition of the toxin theory of malarial pathogenesis can be ascribed to Camillo Golgi, in 1886. Following his demonstration that malarial fever is synchronous with the developmental cycle of the blood-stage parasite [30, 31], he and other turn-of-the-century 'Italian school' malariologists (including Bignami, Bastianelli, Celli, Grassi and Marchiafava, among others) hypothesized that the proximal cause of the febrile paroxysm was a released toxin of parasite origin [32]. The toxic basis of disease processes in malaria was widely recognized during the early years of the 20th century. Maegraith [33] specifically proposed that not only fever but also other pathological processes in malaria were the result of a parasite toxin and suggested that this agent exerted systemic effects through the induction of endogenous mediators of host origin. Clark [34] and Clark et al. [35] subsequently identified tumor necrosis factor (TNF) as a major host mediator of disease. Consequently, the production of this and related pyrogenic cytokines, such as interleukin-1 (IL-1) and interleukin-6 (IL-6) from monocyte/macrophages, is often taken as a useful surrogate marker for the initiation of pathological processes in malaria infection. Clark [34] and Clark et al. [35] proposed that TNF output resulted from a functional malarial endotoxin, and showed that macrophages from malarious mice produced excessive levels of TNF when stimulated with various agonists. Bate et al. [36, 37] extended this work to show that crude extracts of rodent malaria parasites could induce macrophages to secrete TNF in vitro. It is now appreciated that a wide range of pathology in clinically severe malaria infection results from the action on host tissue of toxin-induced TNF, IL-1 and IL-6, leading to a systemic inflammatory cascade resulting in multiorgan involvement with renal failure and pulmonary edema as well as shock, etc. Cytokines (and the toxin itself) directly activate vascular endothelial cells and upregulate expression of adhesins such

as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), which may mediate binding of malaria-infected erythrocytes to the vascular endothelium, thereby contributing to organ-specific and cerebral disease syndromes. Indeed, organ-specific disease may in part result from the local release of toxin from sequestered parasites, leading to enhanced activation of the vascular endothelium, further parasite binding, and excess local production of NO and other mediators.

T-cell products, particularly interferon-γ (IFN-γ) and interleukin-12 (IL-12), clearly contribute to macrophage and vascular activation in a variety of conditions. This reflects a basic process whereby the T_H1 side of the immune system upregulates inflammatory responses involving multiple target tissues. IFN-γ exerts these effects by signaling and transcriptional activities focussed on the regulatory and promoter elements controlling the expression of diverse loci such as iNOS, adhesins, tissue factor and other cytokines etc. Indeed, IFN-γ synergizes with malaria toxin to promote iNOS and TNF expression [28, 38]. The data from clinical and epidemiological studies implicating T_H1 cytokines in human cerebral malaria are strongest for TNF [39, 40], probably derived from macrophages, and less clear-cut for the various predominantly T-cell-derived cytokines such as IFN-γ and IL-12. In severe malaria, elevated plasma cytokines tend to be associated with systemic pathologic abnormalities, not cerebral involvement [41]. Cerebral malaria may well reflect a more localized production of mediators in the brain, but to date the contribution of T-cell-derived cytokines to human cerebral malaria remains unclear. An absolute requirement for IFN-γ is established in the *Plasmodium berghei* ANKA cerebral malaria syndrome [42]. IFN-γ and T_H1 responses are also implicated in other systemic inflammations such as endotoxic shock [43].

The anti-inflammatory cytokines IL-4 and IL-10 produced by the T_H2 lymphocyte subset are well recognized as important negative regulators of pro-inflammatory gene expression in mononuclear phagocytes. The fact that these elaborate regulatory mechanisms are so widely implicated in the homeostatic control of inflammation in multiple disease states strongly suggests that malaria will prove no different. An intriguing possibility is that the progression in human populations from disease susceptibility through tolerance/clinical immunity and on to antiparasite immunity, mirrors the progression noted in rodent malaria infections from T_H1 to T_H2-dominated responses [44].

In both human and murine severe malaria, the lymphoid source of T_H1 polarized cytokines is unknown, as is the specific provenance of counterregulatory IL-4 and IL-10, but may include conventional CD4+ or CD8+ T cells, γ/δ T cells [45], natural killer (NK) cells or CD1-restricted NK T cells [46]. However, the onset of clinically severe malaria happens rapidly, particularly in rodent models, where the responsible wave of IFN-γ production occurs around day 3 of

infection. For these reasons, conventional CD4+ or CD8+ T cells may not be responsible for the initial T_H1 response because of their markedly slower kinetics, and attention has focussed on those T-cell subsets capable of producing rapidly large amounts of T_H1 polarized cytokines, such as γ/δ T cells. To date there has been little focus on CD1-restricted NK T cells despite this population fulfilling these criteria [47]. If postmortem studies establish an association of locally active T_H1 polarized cytokines with human cerebral malaria, identification of the responsible population becomes a priority, because T cells may be influenced towards a T_H2 polarized cytokine profile by appropriate immunization. This possibility becomes more likely if the critical T_H1/T_H2 balance is a feature of a particular lymphoid subset such as γ/δ T cells or CD1-restricted NK T cells, as the restricted T-cell receptor chain usage of these populations [48, 49] indicates that they recognize a limited range of ligands, and they may therefore be amenable to manipulation through immunization with defined antigens. Although at present a somewhat remote possibility, these considerations raise the possibility of devising T-cell-based antidisease vaccines.

In summary, the generation and maintenance of the inflammatory cascade in severe malaria, as well as the more general pathophysiology of the infection, are complex processes involving multiple factors acting in parallel and in concert. Variables which contribute to the outcome of disease include host and parasite genetics, the expression of diverse agonists and antagonists, environmental variables such as nutrition, concurrent/prior infection, and acquired immunity mediated through multiple mechanisms. An important parameter of disease is the organ-specific sequestration of parasites according to expression of parasite ligands and host receptors. Although malaria toxins appear to play a central role in the initiation and maintenance of the inflammatory cascade, the overall outcome will depend upon the contributions of many other variables. In highlighting the role of malarial toxins in pathogenesis, it is not intended to argue against a role for other factors in disease promotion. Put simply, as a defined parasite product, the malaria toxin is at present the most amenable target for the development of an antidisease vaccine.

Identity of the Malarial Toxin

Because TNF is believed to play a central role in the etiology of severe malaria, the production of this cytokine in vitro is often taken as a surrogate marker of parasite toxicity, and as an endpoint for tracking the purification of toxin activity. Bate et al. [36, 37] showed that crude extracts of rodent malaria parasites induce macrophages to secrete TNF in vitro, and suggested that the toxin is a phospholipid [50]. However, these crude parasite extracts contain multiple

activities derived from the host as well as parasite, and their activities show considerable batch-to-batch variation [51]. For these reasons, the specific biochemical identity of the toxin has long remained obscure. We identified a molecule of *Plasmodium falciparum* origin with the properties of such a toxin, which appears to be the major agent responsible for induction of TNF [27, 28, 38, 52–55]. It belongs to the class of glycolipids known as glycosylphosphatidylinositol (GPI). This structure represents at least 95% (a conservative estimate) of the total carbohydrate modification of *P. falciparum* schizonts [56]. This doubtless reflects the very low levels (indeed, virtual absence) of N- and O-linked glycosylation in these parasites [57]. The comprehensive structure and biosynthetic pathway of the malarial GPI toxin has now been elucidated [58–60], and comprises the structure $NH\text{-}CH_2\text{-}CH_2\text{-}PO_4\text{-}(Man\alpha1–2)6Man\alpha1–2Man\alpha1–6Man\ \alpha1–4GlcNH_2\alpha1–6(acyl)$ *myo*-inositol-1-PO_4-diacylglycerol. Recent evidence indicates the existence of an unsaturated fatty acyl chain (*cis*-vaccenic acid) in the *sn*-2 position [61]. The GPI structure is completely conserved among more than 20 geographically distinct isolates examined to date [62].

Biological Activities of Parasite GPIs

It is clear that GPIs retaining the fatty acid moiety intact can leave the membrane and re-associate with other cells. GPI-linked complement restriction factors freely transfer in vivo from erythrocytes to the endothelium [63]. The membrane-form variant surface glycoprotein of *Trypanosoma brucei* with an intact GPI anchor can transfer from parasite to erythrocytes in vivo [64], and human GPI-anchored CD55 can transfer from erythrocytes into schistosomes in vivo [65]. When *Leishmania* parasites are grown in vitro, the GPI-anchored lipophosphoglycan is shed into the culture medium and retains the fatty acid-phosphatidylinositol (PI) moiety intact, consistent with a nonenzymatic mechanism of release [66]. Furthermore, GPI-anchored proteins are not always associated with membranes: they can also be found under natural conditions in solution (with GPI lipid intact), where they exchange among cells in a normal physiological process [67, 68]. Thus there is little reason to doubt that intact GPI may be released upon schizont rupture to interact with host tissues.

Our studies to date establish that the GPI toxin of malaria has major effects on diverse host tissues:

(i) In human and murine macrophages, GPI is a major TNF and IL-1 inducer, and is sufficient to kill mice when inoculated in a standard assay of TNF-driven lethality [27]. It is also able to induce iNOS expression and NO output [28]. The GPI of *T. brucei* variant surface glycoprotein has similar properties [53].

(ii) In human vascular endothelial cells GPI is able to regulate ICAM-1, VCAM-1 and E-selectin expression and induce NO output [28, 55].

(iii) GPI is alone sufficient to induce profound hypoglycemia by a TNF-independent route when inoculated into recipients [27].

(iv) GPIs appear to be suppressive of conventional MHC-restricted T cells (unpublished). This, rather than toxin activity on macrophages, may provide the selective (Darwinian) basis for the high levels of GPI expression in parasites.

(v) Although as yet there are no data in this regard, GPI may exert additional effects either directly or indirectly through cytokines and NO, e.g. an influence on erythropoiesis. GPI may therefore conceivably contribute to malarial anemia.

GPI Appears to Be the Dominant Proinflammatory Agent of Parasite Origin

We wished to determine whether malarial GPI is a major or minor contributor to the parasite-induced TNF response. Because many GPI-anchored antigens are essential for parasite survival, we reasoned that a GPI knock out might be a lethal mutation, and therefore sought to answer this question using neutralizing monoclonals as probes of GPI function. Monoclonals specific to malarial GPI were produced and shown to neutralize 100% the production of TNF and NO in response to crude parasite extracts [28, 52]. These data provide the best evidence to date that GPI is an essential component of parasite-induced proinflammatory gene expression.

These observations concerning macrophage activation by GPIs of *Plasmodium* and *T. brucei* have now been validated by independent laboratories, particularly in *T. brucei* and *Trypanosoma cruzi* [69–71]. These early observations have been more recently confirmed in *T. cruzi* [72] and *P. falciparum* [61]. Based on enrichment tabulation, these studies concur that GPIs constitute the dominant inflammatory (TNF, IL-1, NO-inducing) molecules in these diverse parasite systems.

Although there are no published studies substantially at odds with our proposition that GPI is the dominant toxin of parasite origin, some other workers in this field have argued in reviews for the existence of one or more alternative non-GPI glycolipid toxins [73]. This apparent discrepancy may be explained by the recent demonstration that many cultured lines of *P. falciparum* are contaminated with *Mycoplasma* species [74]. Mycoplasmas produce various agents with potent macrophage activation including GGPLs and low molecular weight proteoglycolipids. GPI is solely a eukaryotic product. *Mycoplasma* contamination may well account for various biological properties ascribed to crude parasite

extracts in the literature where screening for this contaminant has not been expressly reported [75, 76].

Signal Transduction by Parasite GPIs

Malaria GPI exerts a wide range of effects in diverse host tissues by activation of common signaling and transcriptional pathways in each target cell type. Nanomolar GPI induces rapid activation of the protein tyrosine kinases (PTKs) p53/56^{lyn} and p59/62^{hck} in macrophages [38]. *Hck* is particularly implicated in the regulation of TNF expression as overexpression potentiates the TNF response to agonists and blockade of expression inhibits TNF synthesis [77]. The minimal structural requirement for PTK activation is the evolutionarily conserved core glycan sequence Manα1–2Manα1–6Manα1–4GlcN1-6*myo*-inositol. These data suggest that binding of the GPI glycan alone to an as yet unidentified cell surface receptor is sufficient to initiate activation of *src*-family PTKs. GPI also induces PTK-dependent signaling in the vascular endothelium. GPI-associated diacylglycerols independently activate the novel Ca^{2+}-independent ε isoform of protein kinase C (PKC) [38].

Thus GPI imparts at least two separate signals through the structurally distinct glycan and fatty acid domains [38], leading to activation of two great classes of kinase (PTK tyrosine kinases and PKC serine/threonine kinases). This leads to the downstream activation of extracellular signal-regulated kinase (ERK) and other mitogen-activated protein (MAP) kinases (unpublished). The activation of the MAP kinase cascade by GPIs of *T. cruzi* has recently been reported [78]. Clearly, GPI is a distinctive signaling mechanism in that it acts as both an agonist and second messenger substrate. The glycan alone synergizes with PKC-activating agonists such as phorbol esters, IFN-γ, and of course GPI-derived diacylglycerols, as measured by TNF output. TNF, IL-1, ICAM-1 and iNOS gene expression require PTK, PKC and ERK (MAP kinase) signals. PTK and PKC-specific antagonists (Herbimycin A and Calphostin C) at low concentrations each block gene expression and toxin action [38].

TNF, IL-1, iNOS, VCAM-1 and ICAM-1 are host loci implicated to varying degrees in the pathophysiology of severe malaria. Expression of these loci is regulated by members of the NFκB/c-*rel* family of transcription factors, which are intimately involved in the regulation of cell adhesion, inflammatory and acute phase responses. NFκB/c-*rel* family members are also both positive and negative regulators of lymphocytes [79]. NFκB/c-*rel* members are activated by a complex cascade involving PTKs, PKCs and MAP kinases [80, 81]. GPI and crude parasite extracts activate identical signaling pathways (p59^{hck}, nPKCε), with identical kinetics, the same NFκB isoforms, in the same target cells and

with the same gene expression endpoints. To determine whether GPI-induced signaling pathways were involved in malaria-induced pathogenesis, we made use of the *P. berghei* ANKA murine cerebral malaria model. A diverse range of pharmacologically distinct PTK, PKC and NFκB specific antagonists, all of which antagonize the signaling, transcriptional and inflammatory cascade initiated by GPI, blocked TNF output induced by crude parasite extracts and completely or partially prevented murine cerebral malaria. Parasitemias were identical in test and control groups, indicating that these agents do not affect pathology through an effect on parasite growth rates (unpublished data).

A consideration of these basic processes in toxin action provides insight into the many diverse activities of the molecule. In summary, it appears that malarial GPI initiates basic signaling processes in multiple host tissues, leading through common transcriptional pathways to the expression of those proinflammatory host loci most implicated in malarial pathogenesis. GPI furthermore acts cooperatively with IFN-γ to induce many of these loci. In addition, these components synergistically induce secretion of TNF, which itself also synergizes strongly with IFN-γ and GPI. The level of cooperative activity of these proinflammatory agents is likely profoundly to influence the development of malarial pathophysiology. Counterregulatory negative feedback loops are also evident, as seen in the redox inhibition of NFκB by NO, itself induced by GPI and TNF. Overall, these data therefore provide evidence in support of the proposition that GPI plays a role in the initiation and maintenance of the lethal inflammatory cascade in malaria.

Human Immune Responses to the GPI Toxin of Malaria

A recent study has provided preliminary evidence for immunological recognition of parasite GPI by humans living in disease-endemic areas [61]. Parasite GPIs were enriched by HPLC and used as capture antigens in ELISA. Low levels of reactivity were detected with control sera from nonendemic areas, whereas adults from areas of holoendemic transmission were highly positive. When the GPIs were treated with nitrous acid to deaminate the samples (thereby cleaving the glycan from the PI moiety), the reactivity of a limited number of sera shifted to a more hydrophobic region of the TLC plates, suggestive of reactivity to the PI portion of the GPI. Sera were not, however, widely reactive with commercially available PI species. These data raised the possibility that the target of these human antibody responses are found within the inositol acylation, a surprising result given that this is a 'self' structure is found within human tissues [82, 83]. The acquisition of these anti-GPI antibodies increased with age in parallel with other antiparasite responses. The same study provided evidence that anti-GPI antibodies may be associated with reduced risk of fever and with

raised levels of hemoglobin [61]. These findings are supportive of the concept that anti-GPI antibody levels may be associated with reduced risk of clinical disease but also indicate that much needs to be done to validate GPI as a target antigen, especially as the compositional purity of HPLC-enriched GPI preparations remains to be confirmed.

Malarial GPI was thus shown to elicit an immune response in both rodents and humans. Antibodies raised against the GPI are capable of neutralizing TNF output from macrophages elicited by crude parasite extracts, indicating that GPI is indeed the dominant toxin of parasite origin. An important area for investigation concerns the epitopic specificity of these responses. Why is the malarial GPI immunologically non-self, given that the malarial GPI consists of little more than the evolutionarily conserved core glycan modified by an addition mannose residue, and three acyl chains? As all GPIs to date from higher eukaryotes bear extensive side chain modifications to the glycan, the conserved core glycan may not normally be presented to the immune system for clonal deletion of self-reactive B cells. In other words, the core glycan may occur as an internal component within all mammalian GPIs, but sterically hidden, and may not exist as an independent recognizable moiety. The possibility that the GPI lipids elicit IgG responses from the host [61] requires further validation.

GPI as a Target for Immunotherapy

Mortality from clinically severe malaria remains high, even in the best intensive care settings, and treatment relies upon regimen little changed in over 50 years, namely antiparasite drugs and supportive therapy. This is also true of sepsis. However, clinical trials in sepsis aimed at downregulating the inflammatory cascade using antibodies against LPS or TNF have proved to be disappointing. Monoclonal antibodies to LPS appear too strain- and species-specific for wide applicability. In malaria, passive transfer of anti-TNF antibodies has been shown to increase significantly the severity of neurological deficit [84]. It is likely that in both malaria and sepsis most damage is already done to the host before therapeutic intervention and the inflammatory cascade may be irreversible under the time scales required. However, although this can be true for some malaria patients, others experience a more attenuated battle against systemic inflammation, and may deteriorate after admission following release of toxin upon schizont rupture. Thus prompt administration of an antitoxin may conceivably be of clinical benefit. This approach warrants caution given the risks associated with use of anti-TNF antibodies [84]. Among other considerations, there is insufficient data at present as to non-self discrimination by anti-GPI antibodies to determine whether they could be administered

immunoprophylactically. Along with the potential for GPI as an antidisease vaccine, these areas are currently under investigation.

GPI as an Antidisease Vaccine Candidate

The data reviewed above suggest malarial GPI has several properties to qualify as an antidisease vaccine candidate. There is evidence that an antitoxic immunity is the first and possibly most important actively acquired step in reducing childhood mortality from malaria. Whether this is an acquired immunity or reflects a downregulation of physiological responses to malaria remains an important issue. The GPI appears sufficiently non-self to elicit antibodies in murine and human systems, although much needs to be done to determine the provenance and epitopic specificity of these antibody responses. As GPI is a well-defined structure these studies are quite feasible. Antibodies directed towards GPI are able to neutralize comprehensively the induction of proinflammatory gene expression by crude parasite extracts. Therefore, to date GPI appears to be the dominant toxin of *P. falciparum* origin and is likely to play an essential role in the initiation and maintenance of the inflammatory cascade.

An additional consideration is that GPI is 100% conserved (invariant). Although not yet proven, loss of GPI is very likely to be a lethal mutation. GPI structure is furthermore extremely difficult for the parasite to change, involving wholesale loss or gain of function for one or more glycosyltransferases or phosphoethanolamine transferases functioning as one of a tightly coordinated series. Preliminary data (unpublished) suggest that blockade of GPI toxicity may protect the host without killing the parasite. Therefore, direct selective pressure may be limited, and vaccine-selected variability relatively less likely to arise.

Although the data in support of GPI as a vaccine candidate are reviewed here, it is clear that the next stage requires proof-of-principle testing in animal models with defined GPI structures. Until these data are forthcoming, considerations of GPI as an antidisease vaccine candidate remain speculative. These experiments are ongoing, and it is hoped that the issue is resolved shortly.

Finally, it is worth noting that should proof-of-principle testing in animal models warrant further development of GPI as an antidisease vaccine candidate, the chemical nature of the target ensures that very different considerations will apply to the mass production of a defined antigen. The *n*-pentenyl glucoside strategy pioneered by Campbell and Fraser-Reid [85] has succeeded in generating an intact GPI, and this and alternative routes such as Seeberger's glycal assembly [86, 87] provide attractive possibilities for the generation of an experimental synthetic vaccine. The native material is a six-residue inositolpentasaccharide,

with lipid modifications. Once minimal immunogenic structures are established it may be possible to rationalize the mass synthesis to GMP grade by a number of methods. Although it is clearly premature to speculate about per dose costs at this stage, there are grounds for believing that a chemically synthetic oligosaccharide vaccine could be mass produced at low cost as compared with appropriately folded recombinant proteins.

Note Added in Proof

In collaboration with Dr. Peter Seeberger, MIT, we have recently shown that immunization of mice with a chemically synthetic version of the core GPI glycan of *P. falciparum* provides significant clinical protection against the lethal *P. berghei* ANKA cerebral malaria syndrome (unpublished). These data provide proof-of-principle in an initial model for the further development of anti-toxic vaccination against malaria.

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Adjuvants and Malaria Vaccine Development

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Introduction

Despite decades of intense research and significant advances in the field of immunology and molecular biology, the development of an effective vaccine for the control and prevention of malaria has been difficult. Development of an effective vaccine for malaria is challenging because: (1) the pathogen's antigenic composition during different stages of the life cycle is complex; (2) there is multiplicity of epidemiology and clinical end points; (3) the candidate vaccine antigens exhibit genetic diversity and variation; (4) we lack a complete understanding of the characteristics of naturally acquired immunity and the mechanism of protection, and (5) alum, the adjuvant approved for human use has not worked well in human trials conducted so far.

There is ample evidence suggesting that a malaria vaccine for prevention and control of malaria is feasible, and there is a consensus that an effective malaria vaccine would contain antigens/antigenic determinants from different stages of the parasite. Such a multistage, multicomponent vaccine would induce 'multiple layers' of immune response that would be capable of intervening at the level of circulating sporozoites, infected hepatocytes, infected red blood cells, and mosquitoes. The first evidence for an efficacious vaccine was provided in 1960s by immunizing mice with radiation-attenuated sporozoites [1]. Several studies have shown that natural immunity confers protection against high-density parasitemia and clinical manifestations of illness [2, 3]. Similarly, several studies have revealed that antibodies against specific parasite antigens can block the development of the parasite in mosquitoes [4]. These observations have

provided the proof-of-the-principle that a vaccine can be developed for the control and prevention of malaria. The challenge facing vaccine researchers is to develop an easy-to-produce recombinant vaccine that can generate long-lasting protective immune responses that are: (1) directed against different stages of the parasite; (2) strain-transcending, and (3) boosted by natural infection.

Studies of natural immunity and those with the irradiated sporozoite model have shown that the protection against malarial parasites is mediated by certain types of immune responses. In the case of the irradiated sporozoite model, protection is largely mediated by the generation of cytotoxic T lymphocyte (CTL) responses and production of interferon-γ (IFN-γ) and its effector molecules such as nitric oxide (NO) in the liver [5, 6]. Th-1 immune responses also appear to be important in protection against blood-stage parasites. In the case of natural immunity in humans, protective immunity is frequently associated with high levels of NO expression and malarial antigen-specific, cytophilic IgG_3, which is the human equivalent of murine IgG_{2a} [7–12]. Similar observations have also been made with rodent malarial vaccine models, in which protection against malarial parasitemia is concurrent with the generation of a Th-1 response, with an increased expression of IFN-γ and IL-12 and high levels of IgG_{2a}, even though the protection may or may not be NO-independent [13–19]. In studies using transmission-blocking vaccines, antibodies mediate the inhibition of parasite development in the mosquito midgut [4]. Thus, an effective malarial vaccine would need to elicit immune responses comparable to those elicited in the irradiated sporozoite and transmission-blocking models, as well as natural malaria immunity. Therefore, the vaccine would need to contain antigenic determinants from different stage-specific antigens to induce cytophilic as well as neutralizing antibodies, Th-1 cytokine responses, and in the case of pre-erythrocytic vaccines, malaria antigen-specific CTL responses. A schematic representation of the effects of such a multistage, multivalent malaria vaccine are shown in figure 1.

In addition to the use of the 'right antigen', the efficacy of a recombinant vaccine depends on the use of an appropriate adjuvant to mediate the induction of multiple layers of protective immune responses. In the absence of a 'right response' the outcome of the immunization will always be negative, i.e. failure of the vaccine to neutralize the pathogen and/or protect against the clinical manifestations of the illness. Adjuvants play a very important role in the augmentation and modulation of immune responses, and need to be considered as an essential step in the critical pathway for vaccine development (fig. 2). Adjuvants hold the key for the success of a vaccine formulation, and the same adjuvant may not work for all antigens. For instance, hepatitis B vaccine formulated in alum is effective, but studies have shown that several candidate malaria vaccines formulated in alum are poorly immunogenic and confer no significant protection.

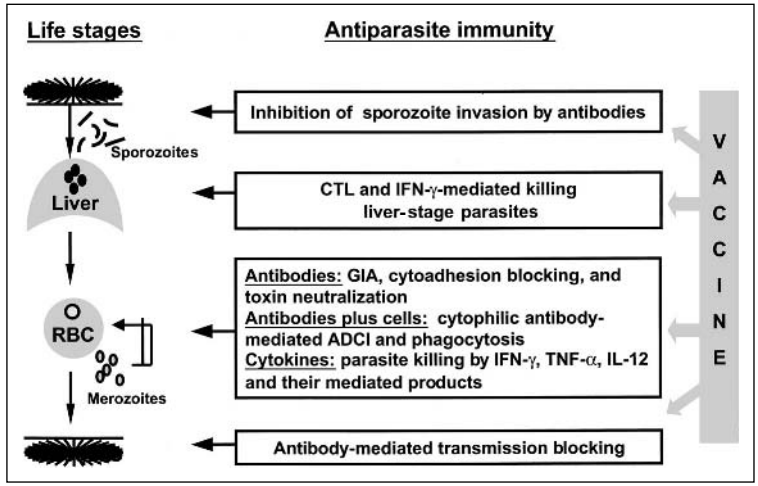

Fig. 1. Immunity against various stages of malarial parasites and the desired immune responses induced by candidate multistage, multivalent malarial vaccines.

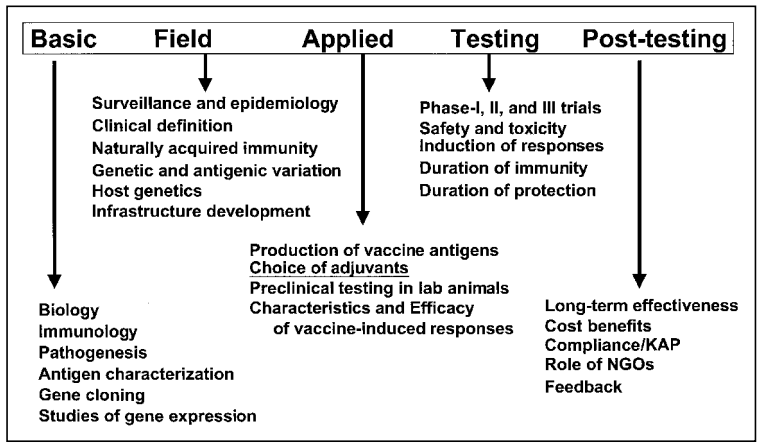

Fig. 2. The paradigm of malaria vaccine development.

Although adjuvants increase the immunogenicity of the vaccine antigen, different adjuvants preferentially activate different arms of the immune system, which may lead to either Th-1 or Th-2 responses alone or both Th-1 and Th-2 responses. As a result, the choice of adjuvants depends on the type of immune responses desired for maximum effects of the vaccine. For example, a

pre-erythrocytic malaria vaccine that uses alum as an adjuvant can be ineffective, because alum is known to generate a Th-2 response, which can suppresses Th-1 and CTL responses [20]. In this situation, the choice of an adjuvant that elicits Th-1 and CTL responses, or the inclusion of other adjuvants (such as QS21) or immunostimulants (such as monophosphoryl lipid A, MPL) may be necessary.

In this chapter, we will review the status of adjuvant work in malarial vaccine development, and will discuss the property and mechanism of action of adjuvants. We will concentrate on adjuvants that have been commonly used in malarial vaccine development and testing to date, and those that are usable and potentially usable in humans.

Definitions and Properties of Adjuvants

The word adjuvant was coined from the Latin word *adjuvare*, which means to help or aid. Adjuvants can improve immune responses to vaccine antigens in several different ways. For instance, they can improve the immunogenicity of weak antigens; accelerate the speed and duration of immune responses; modulate antibody isotype, affinity, and specificity (against antigens and pathogens); stimulate cell-mediated immunity, including induction of select cytokines; promote the induction of mucosal immunity; enhance immune responses in immunologically immature, or senescent individuals; decrease the quantity of vaccine antigen, which lowers the cost of the vaccine; and they can help overcome antigenic competition in combination vaccines or genetic restriction on immune responses.

A vaccine formulation often contains ingredients other than antigen and adjuvant. A carrier is often used for peptide-based antigens. A carrier is an immunogenic molecule which, when conjugated to a small molecule (such as a peptide) augments the immune response of the latter. Examples of carriers are bovine serum albumin, keyhole limpet hemocyanin, and tetanus toxoid. A vehicle is sometimes used to facilitate the uptake of antigens by antigen-presenting cells (APCs) and transports antigens from injection site to lymphoid tissues. Examples of vehicles are liposomes, immunostimulatory complexes (ISCOMs), and microfluidized squalene emulsions. These vehicles also function as particulate adjuvants. The formulation may also contain immunomodulators or immunostimulants, which induce the production of cytokines and enhance immune responses. This is in contrast to depot-type adjuvants, such as alum or oil-in-water emulsions which function by creating a steadily releasing reservoir of immunogen. Examples of immunomodulators are muramyl peptides, lipopolysaccharides (LPSs) and derivatives (such as MPL), cytokines, and some

cationic detergents. These immunomodulators can be used alone as adjuvants but are often used in combination with a vehicle or other adjuvants.

Delivery systems assist in the interaction between vaccine epitopes, APCs, and effector cells. It is well recognized that soluble proteins are generally targeted to the class-II antigen-presenting pathway and they do not go to the class-I pathway. Novel adjuvant delivery systems that deliver the antigen to the APCs for the intracellular transport of these antigens to the cytosol are needed for the induction of CTLs. Examples of delivery systems include particulate carriers, live vehicles, microspheres, encoded vaccines and adjuvants. Particulate carriers, including emulsions, liposomes, microspheres and ISCOMs function to enhance antigen targeting, uptake, and APC activity. These particulate systems allow presentation of a monomeric antigen in a multimeric particulate form, improve targeting to the lymphatic system, and reduce the amount of antigen required to induce an effective immune response. Several studies have been conducted to investigate liposomes as a delivery system for antigen and/or adjuvant [21]. The advantage of using these lipid spheres include protection of protein or DNA from degradation and an increase in circulation time. ISCOMs are a stable complex composed of cholesterol, phospholipid, saponin and antigen [22]. Studies have shown that ISCOMs generate both Th-1 and Th-2 as well as CTL responses [22].

In malaria vaccine development, liposomes have been used successfully in several candidate vaccines, especially in the circumsporozoite protein (CSP)-based vaccine RTS,S [21, 23–25]. An attenuated strain of vaccinia has been used in the production of a CSP-based vaccine against *Plasmodium berghei* and a multistage, multivalent malaria vaccine, NYVAC-Pf7, against *Plasmodium falciparum* [26–28]. The attachment of lipid tails such as monopalmitic acid to peptides or proteins can induce CTL responses specific to these antigens [29].

Mechanisms of Adjuvants

The mechanisms of adjuvants is not well understood and very few studies have been conducted to elucidate their mechanism of action. Consequently, the use of adjuvants for human use has been hampered. Some adjuvants, such as alum and oil adjuvants, cannot be readily used in in vitro studies. Similarly, in vivo studies in laboratory animals with knockout genes are also difficult due to the compensation by alternative immune mechanisms. It is clear, however, that adjuvants have multiple mechanisms of action. They may influence the immune response at several levels, including the mobilization of appropriate APCs to the injected site, enhancing efficient antigen processing and presentation, and influencing cytokines and costimulatory signals necessary for optimal

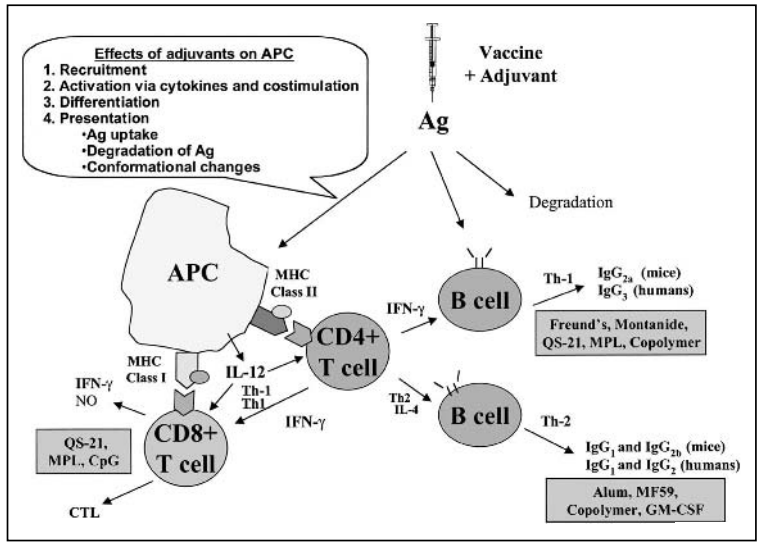

Fig. 3. Role of adjuvants in the induction of immune responses. Mechanisms of adjuvant action.

immune response. Adjuvants influence antigen uptake either by formation of an antigen depot as demonstrated by alum and emulsions, or by formation of microparticles, which facilitate the entry of antigens into APCs. Vaccine adjuvants also help antigens elicit rapid, potent, long-lasting immune responses and have been shown to influence antigen-processing, attachment of the antigen to the APC and its internalization. T-cell activation requires exposure to processed antigen and signaling by cytokines and costimulatory ligands and adjuvants are thought to enhance immunity primarily through upregulation of these molecules.

Some possible effects of adjuvants on the immune system are shown in figure 3. After immunization, antigens may follow three possible pathways: undergo degradation, be taken up by APCs, or bind to B cells. The pathway antigens follow can be influenced by the adjuvant used. Adjuvants can: (1) protect the conformational integrity of an antigen and present it to the appropriate immune effector cells; (2) deliver the antigen directly to the cytosol for presentation with major histocompatibility complex (MHC) class-I molecules; (3) enhance antigen uptake by affecting the recruitment, activation, differentiation and/or presentation of APCs (macrophages, B lymphocytes, and dendritic cells), and (4) direct the immune responses to either Th-1 or Th-2 pathway by modulating the cytokine network and costimulation signals.

Different adjuvants have been shown to induce different types of immune responses. For example, alum and MF59 mainly induce a Th-2 immune response (associated with humoral immunity and production of IL-4, IL-5 and IL-10) against the antigen, whereas QS-21 and Freund's complete adjuvant (FCA) favor a Th-1 immune response (associated with cell-mediated immunity, CMI, and production of IL-2, IFN-γ and IL-12). As a result, adjuvant may affect different aspects of the immune responses including titers, affinity, IgG subclasses, T-cell responses, and cytokine responses. Conventionally, the efficacy of an adjuvant has been measured by its ability to mount and boost antibody titers and CMI to a given antigen.

The characteristics of an 'ideal' vaccine adjuvant are: (1) biodegradability and biocompatibility; (2) not toxic, carcinogenic, teratogenic or abortogenic; (3) non-antigenic and not immunologically cross-reactive with tissue antigens; (4) induce a minimum of injection site reactogenicity; (5) a well-defined chemical structure; (6) induce a minimum of nonspecific effects on the immune system; (7) acceptable for administration to humans, and be safe to administer to young children and immunocompromised individuals; (8) effective for peptides, proteins, polysaccharides and DNA; (9) effective after a single dose with least amount of adjuvant and antigen; (10) induce long-lasting and stable humoral and cellular arms of the immune system; (11) capable of being administered orally; (12) induce systemic and mucosal immunity; (13) promote antigen uptake by lymphoid tissues; (14) easy and inexpensive to produce in large quantities in a reproducible fashion, and (15) a long shelf-life, preferably at room temperature, and easy to mix with antigens.

Currently Available Adjuvants

Freund's Adjuvants

Freund's adjuvants are water-in-oil emulsions of mannide monooleate and mineral oil, with (FCA) or without (Freund's incomplete adjuvant, FIA) heat-killed mycobacteria added. Developed over 60 years ago, FCA is now considered the gold standard for adjuvants, largely due to its immunostimulatory potency. It is, however, too toxic for use in humans. The most common adverse reactions to FCA and FIA are cystic swelling and muscle induration which largely result from the delayed type of hypersensitive responses.

It has been hypothesized that Freund's adjuvants exert their affects through at least 3 mechanisms: (1) the establishment of a depot of vaccine antigen at the injection site allowing a gradual and stable release of the antigen; (2) providing a vehicle for transporting the emulsified antigen through the lymphatic systems to draining lymph nodes and the spleen, which creates multiple sites for the

generation of immune responses, and (3) interactions with APCs and other mononuclear cells. As a result, FCA and FIA stimulate the production of high titers of antibodies against the vaccine antigen. Although a broad subclasses of antibodies are generally generated by Freund's adjuvants, the immune response induced by Freund's adjuvant is predominantly Th-1. Induction of CTL responses against soluble antigens by Freund's adjuvants has been reported, but additional studies are needed to confirm the observation.

Almost all malaria vaccine candidate antigens have gone through initial immunization testing with FCA and FIA as adjuvants, probably due to the assumption that only antigens that have shown efficacy against parasite challenges can qualify as vaccine candidates. The protection induced with Freund's adjuvants, however, is not necessarily malarial antigen-specific, because monkeys or mice receiving only FCA and FIA have lower parasitemia than nonimmunized control animals, indicating that some efficacy of malaria vaccines formulated in Freund's adjuvants is due to nonspecific protection conferred by FCA and FIA [30, 31]. Because of the adverse effects associated with the use of Freund's adjuvant, Animal Care and Use Committees are limiting its use in laboratory animal studies. This can be potentially circumvented by the use of less toxic Freund's vaccine formulations, because FCA and FIA from different suppliers vary in the mycobacterium species and mineral oil composition used, some of which do not generate as severe adverse reactions as others [32].

Alum

Aluminum-based mineral salts, alum, are the only adjuvant currently approved for human use by the US Food and Drug Administration. Alum is a safe, well-tolerated adjuvant that has been used for the last 70 years. It is composed of insoluble gel-like precipitates of aluminum hydroxide or aluminum phosphate, and bind the antigen by electrostatic interactions. Studies have shown that alum generates efficient Th-2 responses, but poor cell-mediated and CTL responses [33, 34]. The binding capacity of alum differs with proteins and between aluminum hydroxide or aluminum phosphate, and is affected by temperature, pH, ionic concentration, etc. Therefore, not all antigens are adsorbed efficiently by aluminum hydroxide and/or aluminum phosphate, which can lead to poor outcome of immunization.

Alum has been widely used in malarial vaccine development studies. Poor immunogenicity, however, is seen with most malaria vaccine candidate antigens [20, 35–39]. Phase-III trials of SPf66 formulated in alum have been conducted in South America, Africa, and Asia. Although initial results from South American SPf66 trials showed promise, subsequent randomized, double-blind, placebo-controlled field studies in Gambia, Tanzania, and Thailand failed to

show significant protection against malaria in immunized individuals [40–42]. Despite the lack of high adjuvanticity with malaria antigens, alum has become the benchmark or reference for evaluating new adjuvants or candidate malarial vaccines in laboratory animal systems.

Adjuvants Potentially Usable in Humans

Because of the reactogenicity of Freund's adjuvants and the poor adjuvanticity of alum, several new adjuvants usable in humans have been developed. Although progress in this area is slow, a few new adjuvants have shown promise and have been used in clinical trials. A few of these new adjuvants have been used in malarial vaccine development, such as QS21, Montanide ISA 720, MPL, and block copolymer.

QS-21

QS-21 is a purified fraction isolated from saponin, which is a complex mixture of triterpenoids derived from *Quillaja saponaria* (soapbark tree) responsible for the adjuvant activities. QS-21 has been shown to induce both humoral and cell-mediated immune responses. Compared to alum, QS-21 stimulates strong Th-1 and CTL responses [43–46]. QS-21 has been used in clinical trials for at least 29 different vaccines. More than 1,600 volunteers have received QS-21, and results have shown that QS-21 has minimal side effects that last for a brief period [47, 48]. QS-21 and its parental products have been widely used in veterinary medicine and are simple to formulate.

QS-21 has been used in the clinical trails of 2 candidate malarial vaccines. An initial phase-I/IIa trial of RTS,S, a CSP-based malaria vaccine candidate, in an oil-in-water emulsion plus the immunostimulants MPL and QS-21 (the new adjuvant formulation is called ASO2, which was formerly known as SBAS2) showed complete protection against sporozoite challenge in 6 of 7 immunized individuals [49]. Immunized individuals developed strong lymphoproliferative, IFN-γ, and antibody responses. However, there was no CSP-specific CTL response, and the immune responses did not correlate with protection [49, 50]. The protection was reduced 6 months after the last immunization, when only 2 of 7 previously protected individuals were completely protected against rechallenge; others had delayed appearance of parasitemia [51]. A more recent study has shown the protection efficacy of RTS,S/SBAS2 to be 41% [52]. A phase-I trial in Gambia also demonstrated that RTS,S/SBAS2 is safe, and there was an increase in the antibody titers to CSP after vaccination [53].

QS-21 has also been used successfully in other candidate malarial vaccines. QS-21 was shown to increase the immunogenicity of a synthetic multiple antigen peptide malaria vaccine containing T and B epitopes in *P. falciparum* CSP repeat region in both mice and *Aotus* monkeys [54]. A recent phase-I human

trial demonstrated that antibody titers elicited by this vaccine were comparable to multiple exposures to irradiated *P. falciparum*-infected mosquitoes [55]. The use of the QS-21-containing adjuvant SBAS2 has also produced strong antibody responses in rhesus monkeys immunized with *Plasmodium vivax* AMA-1 [56]. Mice vaccinated with two MSP-1 EGF domains in SBAS2 were protected against a lethal *Plasmodium yoelii* infection in an antibody-dependent mechanism [57].

Montanide ISA 720

Montanide ISA 720 is a water-in-oil emulsion of metabolizable oil and mannide monooleate. It is similar to FIA with the mineral oil replaced by a metabolizable oil. Studies in various laboratory animals have shown Montanide ISA 720 to be a safe and strong adjuvant [58]. Phase-I studies in humans have shown Montanide ISA 720 to be well-tolerated with minor local effects [59, 60].

Most of the preclinical and clinical tests on Montanide ISA 720 have been conducted with malarial vaccine development. Peptides from several pre-erythrocytic malarial antigens formulated in Montanide ISA 720 were used to immunize *Aotus* monkeys and chimpanzees. The formulation generated strong proliferative, IFN-γ and antibody responses against the peptides in the immunized animals [61, 62]. The usefulness of Montanide ISA 720 as an effective adjuvant in malarial vaccines was also demonstrated in blood-stage malarial candidate vaccines in *Saimiri* monkeys. In this study, 4 of 5 *Saimiri boliviensis* monkeys immunized with AMA-1 in Montanid ISA 720 were partially protected against homologous challenge with *Plasmodium fragile* blood-stage parasites and completely protected against heterologous rechallenge with *P. falciparum* blood-stage parasites [63]. In a more recent study, *Saimiri* monkeys immunized with RAP1 and RAP2 or RAP2 in Montanide ISA 720 had antibody levels and protection against challenge comparable to those immunized with RAP1 and RAP2 and FCA/FIA. This protection was associated with the generation of antibodies against RAP2, and protected monkeys were also protected against a rechallenge 126 days after the first challenge [32].

Several phase-I clinical trails of a three-component blood-stage vaccine of *P. falciparum* have been conducted with Montanide ISA 720 as an adjuvant. The vaccine was shown to be safe and well tolerated. Although vaccinees developed strong proliferative responses to the vaccine antigens, the antibody production was very low [64–66]. Results of challenge of vaccinated individuals with low inoculum of blood-stage parasites (140 infected erythrocytes) showed that the vaccine was ineffective in conferring protection against malarial infection at the two-dose regime tested [65].

Block Copolymer

Another adjuvant under investigation for human use is nonionic block copolymer, which consists of a simple linear chain of the hydrophobic polyoxypropylene flanked by two chains of the hydrophilic polyoxyethylene [67]. The antigen binds to the hydrophobic surface of copolymers by hydrophobic and hydrogen bond interactions. Nonionic block copolymer adjuvants have been shown to induce high-titer, long-lasting antibody responses and to modulate the antibody isotype and specificity [68]. They induce both Th-1, Th-2 and possibly CTL responses. Studies in various laboratory animals have shown that the aqueous formulation of CRL1004 (P1004) and 1005 (P1005) is safe to use, and the use of CRL1005 in humans has been tested in a phase-I clinical trail [69].

Copolymer adjuvants have been used by us over the last several years. Earlier studies in mice with whole blood-stage antigens showed that copolymer formulations protected against virulent challenge with *P. yoelii* malaria and predominantly induced antibodies of the IgG_{2a} isotype, suggesting that in this model block copolymer mainly induces Th-1 responses [67]. This was supported by the recent observations of prolonged induction of IFN-γ by this vaccine, although a weak Th-2 response was also seen [70]. Mice immunized with recombinant *P. vivax* MSP-1_{19kD} and P1005 developed both Th-1 and Th-2 responses against the immunogen [71].

The potency of P1005 as an adjuvant has also been demonstrated in nonhuman primates. Studies in *Saimiri* monkeys using a multiple antigen construct (MAC) containing *P. vivax* CSP repeats and P1005 showed that immunized animals had high titers of antibodies against the CSP repeats and the protective epitope AGDR for at least 7 months after the last immunization. Five of 11 monkeys that received MAC in P1005 were completely protected against challenge with 10,000 sporozoites. Twenty-two weeks after the last immunization, 3 of the 11 monkeys immunized with P1005 as the adjuvant were completely protected when animals were rechallenged with 30,000 sporozoites. The protection was directly related to the anti-AGDR antibody titer [72, 73].

In another study, we found that *Saimiri* monkeys immunized with a recombinant MSP-1_{19kD} in P1005 were partially protected (longer prepatent period and lower parasitemia) against *P. vivax* blood-stage infection [74, 75]. Monkeys immunized three times with MSP-1_{19kD} in P1005 had significantly higher prechallenge IgG antibody titers against the immunogen, lower asexual blood-stage parasites, and higher proliferative responses than those immunized with the vaccine formulated in alum. *Saimiri* monkeys immunized with *P. falciparum* RAP1 and RAP2 in P1005 also had protection (2 of 6 immunized animals) against blood-stage infection comparable to those immunized with the antigens formulated in FCA or Montanide ISA 720, even though the antibody titers

generated by P1005 were much lower [32]. No clinical trial of malarial vaccines has been conducted with block copolymer adjuvants.

MPL

One class of adjuvants currently under investigation is the immunostimulatory adjuvants which modulate the immune response at the cytokine level through activation of MHC molecules or through costimulatory molecules. MPL, derived from the LPS of Gram-negative bacteria *Salmonella minnesota*, has been shown to have similar modes of action as LPS [76, 77]. Because MPL usually exists in an aggregated form and is amphiphilic, it forms a particulate structure together with the associated antigen, which can be more efficiently processed by APC. MPL can be used directly as an adjuvant in aqueous phase, or in an oil-in-water emulsion together with Tween-80 and oil (mineral oil, squalane, or squalene). Studies have shown MPL to induce Th-1 cytokines such as IFN-γ and CTL responses [78–80]. However, MPL does not generate strong antibody responses, thus it is frequently used together with other adjuvants such as alum and QS21. MPL has been used extensively in clinical trials for cancer and infectious disease vaccines and has been shown to be well tolerated with minimum side effects.

MPL has been used extensively in the development of CSP-based vaccines against *P. falciparum*. Earlier studies showed that humans immunized with CSP administered with an adjuvant containing mycobacterial cell-wall skeleton and MPL in squalane had significantly higher antibody titers, avidity, and inhibition of sporozoite invasion of hepatoma cells than those that received CSP in alum [81]. Two of the 11 individuals immunized with the MPL vaccine formulation were protected against sporozoite challenge [82]. The addition of MPL to the carboxy and amino terminal of the CSP formulated in liposomes and alum increased antibody titers against both the vaccine antigen and sporozoites in humans. One high-dose MPL vaccine formulation recipient developed a CSP-specific CTL responses [20]. Similar results were also obtained with humans immunized with RTS,S: addition of MPL increased the immunogenicity of the vaccine antigen formulated in alum, and 2 of 8 persons that received MPL-alum were completely protected against sporozoite challenge, 1 of whom had CTL against CSP [38]. A more recent study with RTS,S has shown that mice developed CTL against RTS,S encapsulated in liposomes only after MPL was added to the adjuvant formulation. These mice also had high levels of NANP-specific antibodies of the IgG_1 and IgG_{2a} subclasses [25]. The immunogenicity and protection conferred by RTS,S, however, were significantly higher when MPL was formulated together with QS-21 (SBAS2 adjuvant) as compared with MPL formulated together with alum [49]. MPL in humans in all of these trials was well tolerated.

MF59

MF59 is a water-in-oil emulsion vehicle formulated with metabolizable oil (squalene) and stabilized with an emulsifying agent (Span 85). It has been used as an adjuvant in various vaccines against viral pathogens, such as influenza, hepatitis B and C, Herpes simplex, cytomegalovirus and HIV-1. Overall, MF59 has been used in nearly 20,000 humans, and minimal reactogenicity was seen. These studies have revealed that MF59 is safe and well tolerated in humans. As a result, MF59 has been included in a licensed vaccine product (Fluad) in Europe, marking the first European approval of a non-alum adjuvant for human use [83].

Mechanism studies show that MF59 increases the recruitment of macrophages and dendritic cells thus increasing the efficiency of antigen presentation to T cells [84]. Preclinical studies have shown MF59 to produce mostly Th-2 immune responses. Clinical studies using MF59 with the influenza vaccine have shown enhancement of humoral as well as mucosal immune response [85]. Studies with subunit cytomegalovirus vaccine have shown that the vaccine antigen with MF59 is more immunogenic than vaccine antigen with alum [86].

Aotus monkeys immunized with RESA in MF59.2 mixture produced only low anti-SERA titers and were not protected against blood-stage parasite challenge. In contrast, monkeys immunized with RESA formulated in Freund's adjuvant or MF75 (a formulation similar to MF59 but also containing pluronic block polymer and muramyl tripeptide-phosphatidylethanolamine) were protected against the challenge [87]. This protection, however, could be non-specific because animals immunized with MF75 alone were also protected. Partial protection against *P. vivax* was also achieved in *Saimiri* monkeys immunized with a multiple antigen construct containing CSP repeats formulated in MF75 [72, 73]. In the earlier development of MF59, muramyl tripeptide-phosphatidylethanolamine was frequently added to the formulation. However, this mixture was reactogenic in humans [88].

CpG

Other immunostimulatory adjuvants include cytosine-phosphorothiolated or phosphodiestered guanine (CpG) motifs, which are immunostimulating oligodeoxynucleotide (ODN) consist of a central nonmethylated CG dinucleotide flanked by less highly conserved sequences. Their sequence is rare in vertebrates and relatively common in many lower organisms including bacteria and viruses [89]. The human immune system is able to distinguish microbial DNA from its own based on the differences in frequency and methylation of CpG dinucleotides in particular base contexts. Immune effector cells such as B cells, macrophages, dendritic cells, and natural killer cells appear to have

developed recognition receptors that bind the microbe-restricted structure of CpG motifs. Studies have shown bacterial DNA to have immunostimulatory effects on the immune system [90] and to be mitogenic for B cells and to activate macrophages to generate a proinflammatory response releasing TNF-α, IFN-γ, IL-6, or IL-12 [89]. Synthetic CpG ODN that contain unmethylated CpG motifs have been shown to induce Th-1 and CTL responses in mice immunized with peptides or soluble protein [91, 92]. CpG ODN have also been shown to upregulate the expression of antigen-presenting molecules, costimulatory molecules, cytokine receptors and adhesion molecules [89]. The exact mechanism of CpG ODN action is not known, but it is thought to involve nonspecific endocytosis and endosomal maturation [93]. CpGs have also been shown to convert immature dendritic cells into mature APCs [94]. CpG has undergone limited testing in humans.

A study of *Aotus* monkeys immunized with a synthetic peptide (PADRE 45) containing amino acid sequences derived from *P. falciparum* CSP formulated in Montanide ISA 720 has shown that animals that received ODN-containing CpG motifs produced significantly higher levels of antibodies to CSP and sporozoites than those that received the ODN without CpG motifs [61]. The effect of vaccine formulated in CpG could be nonspecific. CpG ODN administration in mice in the absence of antigen 1 or 2 days prior to challenge with *P. yoelii* sporozoites conferred sterile protection against infection. The protective effects of CpG ODNs were dependent on interleukin (IL)-12, as well as IFN-γ. Moreover, CD8+ T cells, NK cells, and nitric oxide were implicated in one CpG ODN-induced protection [95]. These data indicate that oligodeoxynucleotides containing CpG motifs improve immunogenicity of vaccine antigen specifically and nonspecifically and may potentially be effective adjuvants in malaria vaccine development.

Cytokines

Because cytokines are known to regulate and modulate immune responses, they are also used as adjuvants. Some of the cytokines used as adjuvants include IL-2, which increases T-cell proliferation and MHC class-II expression, IL-12, which induces a Th-1 response, and granulocyte-macrophage colony stimulating factor (GM-CSF), which attracts immune cells to the site of immunization. In the murine system, when used as adjuvants, IL-12 promotes a Th-1 response with predominant production of IgG_{2a} whereas GM-CSF promotes a Th-2 response with predominant production of IgG_1 [96]. Although cytokines are being studied for use as adjuvants in human vaccines, there are many issues to consider including short half-life in vivo, dose-related toxicity, species specificity, and stability.

Injection of recombinant IL-12 in mice and rhesus monkeys resulted in complete protection against *P. yoelii* and *Plasmodium cynomolgi* challenge

[97, 98]. Partial protection against *Plasmodium chabaudi* AS was also achieved by injection of IL-12 before parasite challenge. The IL-12-induced protection required IFN-γ and TNF-α and occurred via a NO-dependent mechanism [99]. Use of a malaria DNA vaccine consisting of the *P. berghei* CSP and the mouse IL-12 genes directly into mouse liver resulted in protection in more than 71% of mice. This protection was associated with a Th-1 immune response and high expression of iNOS in the hepatic lymphocytes [100].

With the increased use of DNA vaccines in malarial vaccine development, GM-CSF becomes a popular cytokine adjuvant to boost the immunogenicity of naked DNA. Intramuscular immunization with a plasmid expressing the *P. yoelii* CSP protected mice against challenge with *P. yoelii* sporozoites. This protection was improved either by coadministration of a plasmid expressing murine GM-CSF and/or by boosting with recombinant poxvirus expressing the CSP. These mice also had higher titers of antibodies against sporozoites, more CTL activity, and more IL-2 and IFN-γ secreting cells. GM-CSF plasmid alone did not protect, and control plasmid expressing inactive GM-CSF did not enhance protection [101, 102].

Concluding Remarks

Development of a vaccine is a process that starts with the investigations of biology, pathogenesis, gene cloning, protein expression and goes through steps involving characterization of the candidate antigens, tests of immunogenicity, and determination of the nature and extent of genetic diversity. Candidates that meet the 'Go' criterion move forward in the clinical development pathway (fig. 2). Adjuvants are key in this process (fig. 3). In the field of malarial vaccine development, efforts have mostly focused on the characterization of natural immunity, the identification of protective antigens, the elucidation of effector mechanisms, and the construction/expression of candidate antigens/multicomponent vaccines. Very few studies have focused on the mechanisms of adjuvant action, which has limited our capacity to predict which adjuvant or adjuvant combination would be most useful. There is an urgent need for a concerted effort to characterize the mechanisms of adjuvants known to be usable in humans, and to develop new adjuvants usable in humans. These developments in adjuvant research will in turn expedite the development of vaccines for malaria and other infectious diseases.

There is evidence suggesting that different adjuvants promote different types of immune responses to the same malarial vaccine antigen by different mechanisms [103]. Understanding the characteristics of various adjuvants will allow researchers to choose the appropriate adjuvant based on the desired

immune response. Currently, most candidate malarial vaccines have gone through preclinical tests with only a few adjuvants, usually FCA and alum, which are then switched in clinical trials to other adjuvants that are potentially usable in humans. This can be a costly practice, because the immune responses generated by the adjuvants used in clinical trials may not be the most desired type, which can lead to poor performance of a good vaccine candidate [104]. On the other hand, most vaccine tests in laboratory animals and clinical trials do not have appropriate adjuvant controls. This can also be problematic because some adjuvants such as FCA, IL-12, MF75 and CpG have been shown to induce non-specific, short-lasting protection against malaria, which can lead to the over-estimation of the efficacy of candidate vaccines.

Another factor contributing to the poor understanding of the mechanisms of adjuvants is the lack of extensive characterization of immune responses in human clinical trials. Most malaria vaccine clinical trials conducted so far emphasize the safety and protection efficacy aspects of candidate malarial vaccines, relying on the use of laboratory animals for the characterization of immune responses elicited by the vaccine. This makes it hard to evaluate the quality of immune responses in clinical trials, because animal species and genetic background are known to affect immune responses elicited by adjuvants [103, 104]. Adjuvant research can benefit greatly if immune responses elicited by adjuvants are fully investigated in human vaccine studies, regardless of the outcome of vaccines. Very useful information on adjuvants can be generated from unsuccessful vaccine trails.

Even the limited number of adjuvants potentially usable in humans have not been used to their fullest extent in malarial vaccine development. Most candidate malarial vaccines have only been tested with one or two adjuvants that are usable or potentially usable in humans, and there is a tendency of preferential use of certain adjuvants without extensive comparison of the performance of various adjuvants, largely due to the high cost associated with adjuvant comparison. Because the types of immune responses elicited by adjuvants are also affected by the nature of the antigens used in the vaccine formulation, it is difficult to generalize findings with a specific vaccine formulated in a particular adjuvant to other vaccine formulations. The spectrum of immune responses can frequently be broadened by the use of multiple adjuvants. Therefore, combined use of several adjuvants can probably maximize the utility of the current adjuvants that are usable or potentially usable in humans. This is exemplified by the recent success with the CSP-based RTS,S vaccine. Even though soluble recombinant CSP has been disappointing in inducing protective immunity in various vaccine trials, particulate CSP in a particulate vehicle (oil-in-water emulsion or liposomes) formulated in a potent adjuvant, QS-21, with a strong immunostimulant, MPL, has proven to induce protection against sporozoite challenge

[25, 38, 49, 51]. Extensive research in this area is needed to generate other safe and potent adjuvant formulations that are usable in humans.

In summary, adjuvants regulate the spectrum and quality of immune responses to candidate malarial vaccines, thus are an essential part of the malarial vaccine development process. Despite a lack of understanding of adjuvanticity and the limited availability of potent adjuvants that are usable or potentially usable in humans, successes have been achieved recently with a few vaccine antigens and adjuvant formulations. With better understanding of the nature and development of protective immunity against different life stages of malarial parasites and the availability of various vaccine candidate antigens, the development of an effective malarial vaccine is no longer a fantasy. The challenge is to direct the immune responses generated by the candidate vaccine to the type shown to be protective, by using the right adjuvant formulations. In this regard, the elucidation of adjuvant mechanisms, the use of current adjuvants that are usable or potentially usable in humans, and the development of new adjuvants for human use should be a priority area for malarial vaccine researchers and funding agencies.

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Malaria Vaccine Trials

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Introduction

Although no malaria vaccine has yet gone into routine use, considerable progress has been made in the development of malaria vaccines during the past 20 years. A number of parasite proteins have been identified as candidate vaccines and the optimum method of their delivery has been characterised, as described earlier in this book. The first vaccine trial in man which involved artificial challenge of adult volunteers with infected mosquitoes was carried out by Clyde et al. [1] at the University of Maryland in 1972. Subsequently, several further challenge studies have been reported [2–16] (table 1) and many more trials of the safety and immunogenicity of malaria vaccines in volunteers have been undertaken. The first report of a field trial of a malaria vaccine in an endemic area, a study of a pre-erythrocytic vaccine undertaken in Burkina Faso, was made in 1990 [17]. Fourteen field trials have been reported subsequently [18–33] and there are several others, including trials in Brazil and Kenya, which have never been fully described (table 2). Much has been learnt during the course of these studies which have become increasingly professional.

In this chapter, we discuss the requirements for an effective malaria vaccine, some of the factors that are likely to influence whether a malaria antigen is taken into clinical trials or not, the different phases of vaccine evaluation and some of the lessons that have been learnt from the trials done so far. We have concentrated on trials in man and make reference to animal studies only when these are relevant to human trials. Our review concentrates on *Plasmodium falciparum* as most clinical trials done so far have been with vaccines directed at infection with this parasite.

Table 1. Vaccine trials in man utilising artificial challenge with infected mosquitoes

Year of report	Antigen	Subjects	Efficacy	Comments	References
Irradiated sporozoites					
1973	*P.f.* sporozoites	Adult volunteers	1/3 protected	Protection species – but not strain-specific; protection sustained for 7 months	1, 2
1975	*P.f.* + *P.v.* sporozoites	Adult volunteer	1 volunteer protected against *P.f.* and *P.v.*	Protection short lasting	3
1976	*P.f.* sporozoites	Gambian children	No protection against infection	Only 2 sporozoite innocula given	4
1979	*P.f.* sporozoite	Prison volunteers	3/7 protected	Protection achieved only in those receiving 400 or more bites; protection only short lasting	5, 6
1991	*P.f.* sporozoites	Volunteers	3/5 protected	Protection in 3/3 volunteers who were exposed to large numbers of irradiated mosquitoes. Protection persisted for 9 months in 1 volunteer	7–9
Recombinant CSP vaccines					
1987	$(NANP)_3$ TT toxoid	Adult volunteers	1/3 protected	Protection seen in the volunteer with the highest antibody level. Delayed pre-patent period in infected subjects	10

Table 1. (continued)

Year of report	Antigen	Subjects	Efficacy	Comments	References
1987	R32 tet_{32}	Adult volunteers	1/6 protected	Delayed pre-patent period in two non-protected volunteers	11
1994	R32 NSI_{81} + monophosphoryl lipid A	Adult volunteers	2/4 protected	Challenge restricted to volunteers with the highest antibody concentration	12
1997	RTS,S (CSP/HbsAg) + monophosphoryl lipid A +QS21	Adult volunteers	6/7 protected	Protection lasted a few months	13
Erythrocytic vaccines					
1988	SPf66[1]	Adult volunteers	4/5 partially protected	Reduction in level of parasitaemia observed	14
Combination vaccines					
1995	CSP [Ro 46-2717] + MSA 2 [Ro 46-2924]	Adult volunteers	No protection in 5 volunteers	Vaccine immunogenic but not protective	15
1998	NYVAC-Pf7 (7 antigen vaccine)	Adult volunteers	1/34 volunteers protected	Delayed pre-patent period in the infected volunteers	16

[1]SPf66 contains NANP so could be considered a combined vaccination vaccine although it is usually considered as a blood-stage vaccine.

Table 2. Field trials of malaria vaccines

Date of report	Vaccine	Comparator	Country	Study group		Efficacy[1] % (95% CI)	Comments	References
				age, years	n			
Pre-erythrocytic								
1990	$(NANP)_3$ TT	Tetanus toxoid	Burkina Faso	3–5 months	123	None	Vaccine immunogenic; small study groups	17
1994	R32 Tox-A	Tetanus/ diphtheria toxoid	Thailand	18–45	199	None	Only 26 cases of *P.f.* malaria	18
1999	RTS,S/ASO2	Rabies	The Gambia	18–45	306	Short-term protection	End-point infection and not clinical malaria	19
Erythrocytic								
1992	SPf66	Alum	Colombia	18–21	399	82	Army recruits; not randomised; only 11 cases of *P.f.* malaria	20
1993	SPf66	Tetanus toxoid, alum[2]	Colombia	>1	1,548	34 (19, 46)	Highest efficacy (77%) in children	21
1994	SPf66	Tetanus toxoid alum[2]	Ecuador	>1	585	67 (–3, 89)	Only 16 cases of *P.f.* malaria	22
1994	SPf66	None	Venezuela	>1	2,439	55 (21, 75)	Efficacy against *P.v.* malaria 41% (95% CI 19, 57); not randomised	23
1994	SPf66	Tetanus toxoid, alum[2]	Tanzania	1–5	586	31 (0, 52)	Efficacy at 18 months follow-up 25% (95% CI 1, 44)	24, 25

Table 2. (continued)

Date of report	Vaccine	Comparator	Country	Study group		Efficacy[1] % (95% CI)	Comments	References
				age, years	n			
1995	SPf66	IPV	The Gambia	1–5	630	8 (–18, 29)	Efficacy at 2-year follow-up 8% (95% CI –20, 30)	26, 27
1996	SPf66	Tetanus toxoid, alum[2]	Colombia	>1	1,257	35 (8, 54)	Protection persisted for 2 years; no protection against *P.v.*	28
1996	SPf66	Hepatitis B	Thailand	2–15	1,221	–9 (–33, 14)	Study in refugees	29
1998	SPf66	Tetanus toxoid alum	Brazil	7–60	800	–2 (–33, 22)	Efficacy against *P.v.* malaria 20% (95% CI 45, 1); study in migrants	30
1999	SPf66	Alum	Tanzania	<1	1,091	2 (–16, 16)	No interference with response to EPI vaccines	31
2000	MSP1, MSP2, RESA	Montanide ISA 720	Papua New Guinea	5–9	120	62 (13–84)[3]	Reduction in parasite density not seen in children treated with sulphadoxine/pyrimethamine	32
Combination vaccines								
1995	$[NANP]_{19}$ –5.1	Placebo	Nigeria	6–12	194	None	No reduction in the incidence of fever or of parasitaemia	33

[1] Indicates protection against first clinical attack of falciparum malaria unless stated otherwise. Both active and passive case protection were used in most studies.

[2] Tetanus toxoid given for the first injection and alum alone for subsequent injection.

[3] End point was parasite density and not the incidence of infection.

Table 3. Requirements for a malaria vaccine targeted specifically for travellers or for the population of malaria-endemic countries

Characteristic	Travellers vaccine	Endemic area vaccine
Efficacy	Very high	Moderate to high
Safety	Very high	High
Duration of protection	Short-term useful	Long-term needed
Boostability by		
Vaccination	Highly desirable	Highly desirable
Infection	Not necessary	Highly desirable
Cost	Moderate cost acceptable	Must be cheap

What is Needed from a Malaria Vaccine?

Malaria vaccine development has followed a rather haphazard course so far, being influenced by chance discoveries of particular malaria antigens and/or the enthusiasm of individual investigators. However, as many more potential vaccine antigens are identified through the genome project and as the resources available for malaria vaccine development increase, it is important that this process is conducted in a more rational way than has been the case to date. An essential first step in this process is definition of the characteristics required for a successful vaccine.

The perfect malaria vaccine would induce sterilising immunity that was lifelong, provide cross-species immunity, be protective in the very young and be compatible with the expanded programme on immunisation (EPI) so that it could be administered as part of the routine immunisation programme. Malaria vaccines of the first generation are very unlikely to meet all of these very stringent requirements and they will almost certainly have one or more weaknesses. The relative importance of individual shortcomings will depend upon the population for whom the vaccine is intended. Two major groups of potential recipients of malaria vaccines are short-term, non-immune travellers to a malaria-endemic area and long-term residents of a malaria-endemic region. Malaria vaccines targeted at each of these groups have different requirements (table 3).

Vaccines for Travellers

Vaccines for short-term travellers to endemic areas must be highly effective at preventing infection and very safe. Because any blood-stage infection in a non-immune traveller is likely to be associated with symptoms, vaccines for travellers must be able to prevent patent blood-stage infections. A highly efficacious pre-erythrocytic stage vaccine that induced an immune response

which resulted in the destruction of all parasites before invasion of red blood cells occurred would be the best way of achieving this objective. However, a highly effective blood-stage vaccine could also provide a satisfactory level of protection in a manner analogous to that provided by schizontocidal drugs taken for chemoprophylaxis. Because short-term travellers usually have the option of chemoprophylaxis, vaccines for travellers must be at least as safe and as effective as the chemoprophylactic regimen recommended for the area to be visited. Vaccination, even when not totally protective, may be an attractive option for travellers to areas where parasites are highly drug-resistant and where chemoprophylaxis is ineffective or has serious side effects. The duration of protection provided by vaccination is less critical for travellers than for the residents of malaria-endemic areas, although it would be helpful if, after a primary course of immunisation, immunity could be boosted quickly by a single dose of vaccine given shortly before a further visit to an endemic area.

Vaccines for the Population of Malaria-Endemic Areas

Residents of malaria-endemic areas are likely to remain at risk from malaria for most of their lives, so an important requisite for a vaccine intended primarily for this population is that it should provide long-lasting protection. Booster immunisation as a result of naturally acquired infections might help to sustain this immunity but this effect could be lost if the vaccine is effective enough to lower the overall level of transmission in the community where it is used. In highly endemic areas, young children are the group most at risk from severe malaria so that a vaccine targeted at the population of highly endemic areas must provide protective immunity in the very young and its administration should, if possible, be compatible with the immunisation schedule of the routine EPI programme. Because older children and adults resident in a malaria-endemic area already have some background immunity to clinical malaria, acquired as a result of natural infections, a vaccine which provided a lower level of protection than a 'travellers' vaccine could still be useful, especially if it is able to boost naturally acquired immunity. For example a blood-stage vaccine which lowered parasite density might prove to be clinically useful, even if it has little effect on the incidence of infection. Similarly, a partially effective pre-erythrocytic vaccine might also protect against death from malaria and from severe infections in a manner analogous to that achieved by insecticide-treated materials. Whether or not an imperfect malaria vaccine will be a useful method of control in a particular situation will depend upon its level of efficacy relative to other available control tools, its acceptability and its cost.

Pregnant women resident in a malaria-endemic area are in particular need of protection against the infection because of the deleterious effects of malaria on the outcome of pregnancy. In an increasing number of geographical areas,

it is becoming difficult to provide this protection through chemoprophylaxis or intermittent treatment. Thus, pregnant women, especially primigravidae, are a priority group for vaccination. Whilst long-term protection would be desirable, a 'travellers' type of vaccine that provided a high degree of protection over a short period could also be useful. A vaccine that prevented sequestration of *P. falciparum* parasites in the placenta would protect pregnant women against some of the deleterious effects of malaria during pregnancy, for example production of a low birth weight baby. However, it would not necessarily protect the mother from anaemia, another serious complication of malaria in pregnancy. The safety of any malaria vaccine given during pregnancy would require stringent evaluation and vaccination prior to pregnancy, for example on graduation from school or on marriage, would be preferable if protection could be sustained for several years.

Justification for Taking a Candidate Antigen into Human Vaccine Trials

Deciding which malaria antigens should be taken into clinical trials has not been a major problem so far, as there have been relatively few candidates to choose from. However, the parasite genome project will change this radically by identifying hundreds of parasite proteins that could potentially become vaccines. Determining which antigens justify the substantial investment that vaccine development requires will not be an easy task. Factors likely to be taken into consideration in making this decision include the following.

(1) Evidence that an antigen plays an important role in the survival and/or pathogenicity of the parasite. If it can be shown that an antigen plays an important role in some essential biochemical or functional activity of the parasite, then this provides strong grounds for its candidacy as a vaccine. For example it may be possible to show that the antigen plays a critical role in invasion of a liver cell by a sporozoite or invasion of a red cell by a merozoite. Recently developed gene knock-out techniques provide a valuable way of obtaining this kind of information although it cannot be assumed that an immune response against a particular antigen will necessarily mimic the effect of disruption of its gene by molecular techniques. Antigens which have been shown to have an important biological function that are being developed as vaccines include circumsporozoite protein (CSP)1, CSP2 and erythrocyte-binding protein-175.

(2) Evidence from animal experiments that immunisation induces protective immunity. Animal studies may involve either vaccination of a natural host with an antigen homologous to the human one under consideration or immunisation of an unnatural but permissive host, such as the *Aotus* monkey, with an antigen derived from a human parasite. Which of these models is the most

relevant to the development of malaria vaccines for man is uncertain. Vaccination and challenge of chimpanzees with a human malaria parasite is probably the animal model of most relevance to human studies but one that can be used only sparsely because of ethical and economic considerations. Animal immunisation studies can provide useful information on immunogenicity and safety as well as on efficacy. Unfortunately, demonstration of immunogenicity in an animal model is not always a predictor of a similar degree of immunogenicity in man. Safety studies in animals may be especially important for vaccine candidates which have some homology with human proteins and which could theoretically induce autoimmunity. Animal experiments can be helpful in encouraging development of a vaccine based on a particular antigen but most malariologists now agree that they are not an essential step on the pathway of development.

(3) Evidence from studies of naturally acquired immunity that immune responses to the antigen are associated with protection. Study of immune responses to the antigen in question resulting from naturally acquired infections may show some association between these responses and protection against either malaria infection or clinical malaria. This kind of evidence usually comes from studies in which immune responses at the start of the study are related to the incidence of re-infection after treatment. Unfortunately, none of the antibodies used in these studies can be linked unequivocally to clinical protection as discussed elsewhere in this book.

The way in which treatment/re-infection studies are done has been refined in order to allow the effect of potential confounding factors such as age, the level of exposure to malaria and bednet use to be minimised. However, such studies can measure only association and not causation and it is impossible to exclude the possibility that demonstration of an association between protection against infection and an immune response to antigen A is seen only because responses to antigen A are strongly linked to those to antigen X, responses which were not measured. If evidence that protection is linked to a response to a particular antigen is found in several different epidemiological situations, then the probability that the association is valid and not a chance finding is increased, although the possibility of association with another immune response is still not excluded. Furthermore, it is possible that highly effective vaccines will be developed that incorporate antigens to which the host does not usually respond, perhaps as a result of modulation by the parasite of a response to an essential antigen. Despite these caveats, evidence that naturally acquired immune responses to CSP, merozoite surface protein (MSP)1, glutamate-rich protein (GLURP) and MSP3 are associated with protection against malaria in some situations has strengthened the candidacy of these antigens as vaccine candidates.

How to balance the pieces of evidence obtained from the different types of studies described above to give an informed judgement on whether to take

Table 4. A summary of the advantages and disadvantages of a 'top-down' or 'bottom-up' approach to the development of combination vaccines

	Top-down	Bottom-up
Advantages	Rapid development	Each component validated No interference between antigens Savings in cost
Disadvantages	May include unnecessary components Cost Side effects Interference between antigens	Slow development

a particular candidate antigen into clinical trials is difficult and, in the end, is likely to depend upon the intuition of the investigators and their financial supporters.

Single Candidates or Combinations

It is unlikely that any single antigen will be found that meets all of the criteria for a perfect vaccine. Thus, the most effective malaria vaccines are likely to contain a combination of antigens from the same stage of the parasite's life cycle or a combination of antigens from different stages. Researchers usually assume that vaccines which induce a response to antigens present in each stage of the parasite's lifecycle will be superior to single-stage vaccines. This is likely to be the case but the knowledge needed to predict the short- and long-term effects of such vaccines in different epidemiological settings is lacking. How the components of a combination vaccine should be determined is open to question. There are two main possibilities (table 4). One approach is to use knowledge gained from animal experiments, previous vaccine trials and intuition to define a vaccine 'cocktail' [34]. The advantage of this approach is speed, for the first 'cocktail' produced may be highly effective. However, if this is the case, it may never be known which components of the combination are essential, unless a series of depletion experiments is done subsequently. Inclusion of unnecessary components increases the potential for side effects and cost. Furthermore, if this approach is followed, negative interactions between components of the vaccine may lead to a potentially protective component being rejected from further evaluation. The alternative approach is to test each component of a potential combination vaccine individually and then to gradually

Table 5. Phases of malaria vaccine trial development

Phase	Objectives	Numbers	Design
I	Safety and immunogenicity	Small (tens)	Randomised, placebo-controlled
II[1]	Efficacy–artificial challenge (safety and immunogenicity	Small to medium (tens)	Randomised, placebo-controlled
III	Efficacy–natural challenge (safety and immunogenicity)	Medium (hundreds)	Randomised, placebo-controlled[2]
IV	Effectiveness (safety)	Large (thousands)	'Stepped-wedge' or case-control

[1] Some authorities separate phase-II trials into phase-IIa (artificial challenge) and phase-IIb (natural challenge) but we believe that it is more logical to consider the latter type of study as a phase-III trial.

[2] It may be more appropriate to use a control vaccine rather than a placebo.

build up a combination of individual components shown to be effective, the approach used in the development of SPf66. The disadvantage of this approach is that, in the absence of an effective laboratory test for protective immunity, a large number of clinical trials will be needed and the development process will be slow. It is not obvious which of these two approaches is the best and so it is fortunate that different research groups have adopted each approach. Thus, the American Navy research group is working towards a 15 valent vaccine (MustDo15) without field testing of every component [34] whilst other groups are investigating the combination of pre-erythrocytic stage vaccines with the blood-stage vaccine MSP1 using a 'bottom-up' approach.

The Developmental Pathway

Malaria vaccines must go through a complex developmental process that will produce sufficient data to persuade the regulatory authorities that the vaccine is both effective and safe. Registration in the United States or in Europe is likely to be needed for any malaria vaccines intended for global use. Malaria vaccine trials can, like trials of other vaccines, be classified as phase-I, phase-II, phase-III or phase-IV trials (table 5). Phase-II trials are sometimes divided into

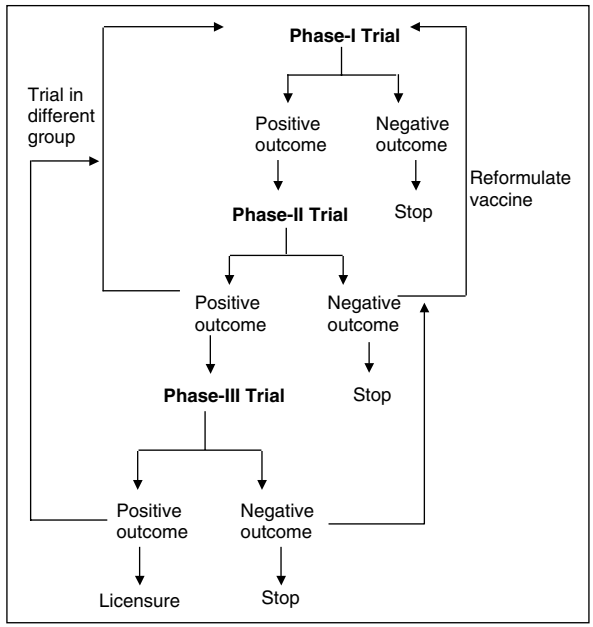

Fig. 1. The pathway of development for malaria vaccines.

phase-IIa studies which involve the collection of immunogenicity and safety data and phase-IIb trials which involve artificial challenge with infected mosquitoes. Several malaria vaccines have reached the stage of phase-III trials (table 2) but none has yet reached the stage of registration for routine use.

Although it is convenient to consider vaccine development in terms of an orderly progression from phase-I to phase-IV studies, development of a promising vaccine is likely to involve several iterative loops (fig. 1). For example encouraging results from a phase-II or III trial in adults might lead to additional phase-I or II studies in young children or phase-I studies with a modified vaccine.

Is it necessary for all malaria vaccines to go through each phase of the developmental process? Phase-I trials are essential for safety reasons and it may be necessary to undertake phase-I trials in several different populations (see below). Phase-II trials with artificial challenge in a limited number of non-immune volunteers can give valuable information about the efficacy of pre-erythrocytic vaccines and such studies are likely to be on the development pathway for vaccines of this type. However, in the case of blood-stage vaccines designed to reduce the clinical severity of malaria, artificial challenge studies are not appropriate for ethical reasons and development may proceed directly from extensive safety and immunogenicity studies to a phase-III field trial in an endemic area.

Phase-I Trials

Phase-I trials provide important information on the immunogenicity and safety of a vaccine and allow factors such as the optimum dose, immunisation schedule and optimum adjuvant to be determined. Initial phase-I trials are likely to involve only small numbers of subjects, perhaps 10 or 20, but if these show that the vaccine is safe then larger phase-I studies may be done involving 50 or more volunteers. Because both the safety and immunogenicity of a vaccine may be influenced by prior exposure to malaria, it is essential that phase-I studies are undertaken in malaria-exposed subjects as well as in non-exposed volunteers, if the former are the long-term target group for the vaccine. Similarly, if the vaccine is to be used in young children, phase-I studies must be undertaken in this group of subjects after preliminary studies have been done in adults. Opinion is divided as to how rapidly age de-escalation studies should be done. Some advocate stepwise progression from adults to adolescents to young children to infants, a slow process. Others argue that if a vaccine is intended primarily for use in young children it is appropriate to progress rapidly to this group missing out adolescents on the grounds that, in a highly endemic area, adolescents have relatively little to gain from possible protection against malaria to balance against the risks of receiving an experimental vaccine. Vaccination of very young children should be delayed until the safety and immunogenicity of the vaccine in slightly older children has been shown because of the complicating factors of maternally acquired antibody and of potential adverse interactions with EPI vaccines. However, if the vaccine is ultimately intended for use in infants, then the consequences of simultaneous administration of the malaria vaccine and routine EPI vaccines should be an important component of phase-I and later studies [35].

Regular follow-up of volunteers entered into phase-I or phase-II trials provides information on the incidence of common side effects such as fever and local reactions to injection with the vaccine and its adjuvant, but they are not usually large enough to provide information on less frequent, but potentially serious, side effects such as anaphylaxis. Thus, further safety data must be collected during phase-III trials and during post-licensure surveillance using less intensive surveillance methods than those employed earlier in the process of development.

Samples collected during phase-I and phase-II trials can provide a great deal of detailed, sequential information on the humoral and cellular immune responses to the antigen(s) in the vaccine because it is possible to collect relatively large amounts of blood on repeated occasions from healthy volunteers. Studies of humoral immunity may include measurement of overall concentrations of antibodies to the vaccine antigen(s), their sub-class and their affinity.

Cellular studies might include the measurement of lymphoproliferative responses to the vaccine antigen(s) and measurement of cytokine production either in conventional culture or using the ELISPOT technique [36]. The latter technique probably reflects T-cell cytotoxic activity and is easier to perform than direct measurement of cytotoxic T-cell activity which is technically difficult [37]. If experimental challenge is done, detailed comparisons can be made between the immune responses of volunteers who were protected and of those who were not. When this has been done for vaccines based on the CSP of *P. falciparum*, some correlation has been found between protection and high antibody concentrations to this antigen although this has been only partial [8, 10, 12]. In general, it has been found that protected volunteers had a high antibody level although not all subjects with a high antibody level were protected.

Unfortunately, there are no generic immunological tests that can be used to indicate whether vaccination has induced protective immunity against either pre-erythrocytic or blood stages of the parasite; current functional assays are not sufficiently validated. However, it is a reasonable assumption that a vaccine that induces antibodies that provide strong transmission-blocking activity in mosquitoes, as demonstrated using the membrane-feeding technique, would induce transmission-blocking immunity when used in exposed populations. Thus, measurement of transmission-blocking antibody levels in samples obtained during phase-I trials of transmission-blocking vaccines may provide valuable information on factors such as dose, number of injections and optimum adjuvant directly relevant to the vaccine's use in the field.

Phase-II Trials

Phase-II trials, often conducted on larger numbers of subjects than phase-I trials, can provide important additional information on safety and immunogenicity but their main purpose is to provide preliminary evidence of efficacy in volunteers. A great deal of work has been done at the Walter Reed Army Institute for Medical Research and elsewhere on the development of a standardised and reproducible challenge model which, using well-characterised parasites and mosquitoes, ensures that control subjects consistently become infected without overwhelming the defences of volunteers with some protective immunity. The former is important because group sizes for challenge studies are usually small and efficacy is unlikely to be demonstrated at a statistically significant level, even if present, unless the attack rate in the control group is very high. Many trials of pre-erythrocytic vaccines have now been undertaken using this very useful model (table 1).

An alternative challenge model has been developed by Cheng et al. [38] in Australia which involves the induction of infection by inoculation of infected blood and the measurement of parasite multiplication in the recipient using a semi-quantitative polymerase chain reaction technique which is so sensitive that it can detect several cycles of parasite multiplication before the volunteer becomes ill. The model assumes that, in the presence of immunity to the blood stage of the parasite, the rate of multiplication will be delayed, even at very low parasite densities. This model has the advantage that any protection demonstrated in the vaccinees must have been achieved as a result of an immune response directed at the blood stage of the infection. In contrast, demonstration of a protective effect with a blood-stage vaccine using the mosquito-challenge model cannot exclude the possibility that protection was achieved through some unanticipated cross-reactivity with the pre-erythrocytic stages of the parasite. The model has the disadvantage that it would not detect the beneficial effect of a vaccine that induced a protective effect on parasite growth that was density dependent. In addition, there are concerns that regardless of the rigorous nature of the investigations undertaken to ensure the safety of the blood used to initiate the infection, the possibility of the transmission to the volunteer of some unidentified pathogen cannot be totally excluded and this challenge model has, so far, not been adopted widely.

Challenge studies in non-immune volunteers are especially useful in the evaluation of pre-erythrocytic vaccines because the first appearance of parasitaemia can be used as the trial end-point, allowing prompt treatment of the volunteers before they become too unwell, although most develop some symptoms before parasites are detected. Both the prevalence of infection and the time to infection are useful end-points. A delay of a few days in the mean time to the detection of parasitaemia in the vaccinated subjects indicates a substantial degree of pre-erythrocytic immunity, even if all the vaccinees eventually become infected. This was the case in the trial of NYVAC-Pf7; only 1 volunteer was completely protected but the pre-patent period of the remaining 33 challenged volunteers was extended by a mean of about 4 days (fig. 2) [16]. The mosquito-challenge method is less well suited to the evaluation of blood-stage vaccines in non-immune volunteers because of the need to treat infected subjects as soon as parasitaemia is detected. Detection of a delay in the mean time to patent parasitaemia in vaccinated subjects could indicate the presence of an immune response that delayed parasite multiplication but this model would not detect the protective effect of a blood-stage vaccine that reduced parasite density and the incidence of severe disease, but which did not reduce the incidence of infection.

Semi-immune adults are less suitable for phase-II challenge studies than non-immune volunteers because some of the subjects allocated to the control

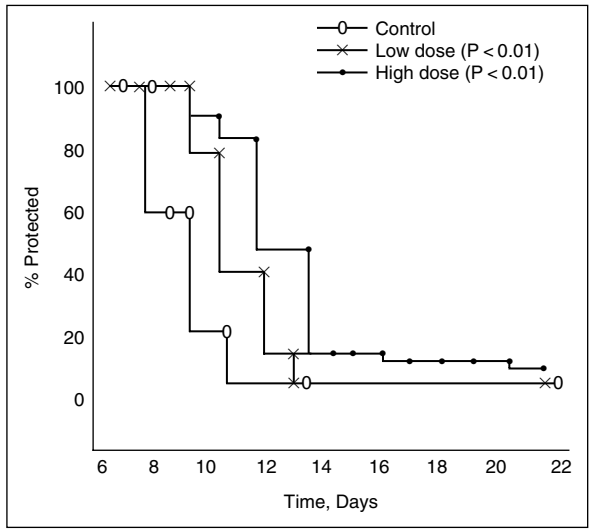

Fig. 2. Production of a delay in the pre-patent period of volunteers immunised with the multivalent vaccine NYVAC-Pf7 [16].

group are likely to resist infection as a result of prior acquisition of natural immunity. Semi-immune volunteers could be used in challenge studies to look at the protective effects of a blood-stage vaccine on parasite density and clinical disease under controlled conditions. However, as some control subjects would probably not develop a patent infection, the sample size for a trial of this kind would have to be large and would probably be similar to that required for a phase-III study. Thus, progression from a successful phase-II challenge study in non-immunes to a phase-III trial in semi-immunes may be the logical way forward, the route followed for the development of RTS,S.

Phase-III Trials

Phase-III trials are designed to evaluate the efficacy of a vaccine under conditions of natural challenge. Even in areas where levels of transmission are high, phase-III trials are likely to require several hundred subjects and to be demanding and expensive. Thus, a decision to progress to a phase-III trial is a major one. Encouraging results from a phase-II challenge study may provide strong grounds for moving on to a phase-III study, as was the case for RTS,S but, in some cases, a difficult decision may be required as to whether or not to undertake a phase-III trial when only immunogenicity and safety data are available.

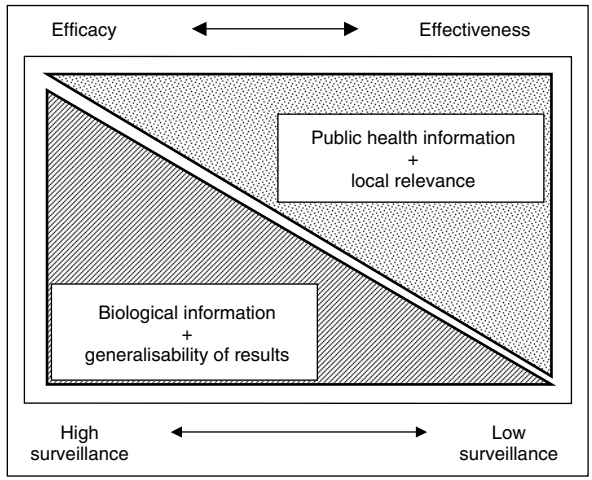

Fig. 3. Contrasting characteristics of efficacy and effectiveness trials.

The factors that need to be considered in the planning and execution of phase-III malaria vaccine trials have been reviewed extensively by WHO committees and other groups during the past few years and their conclusions have been published in a number of WHO reports [39] and elsewhere [40, 41]. In this chapter, we concentrate on some of the issues that have emerged when the approaches advocated in these guidelines have been put into practice.

Trial Objectives and End-Points

The first factor to be considered when a phase-III trial is being planned is the objective of the trial. Is the aim to show protection against infection, mild clinical malaria, severe malaria or death? The answer to this question will depend upon the type of vaccine under consideration, its stage of clinical development and the degree of transition between testing for efficacy or effectiveness (fig. 3). It is likely that initial trials will take malaria infection or mild clinical malaria as their main end-point rather than severe disease or death because adoption of the latter end-points will require a large sample size and there are likely to be concerns about the safety of embarking on a very large trial when only limited safety data are available. However, if a moderate degree of efficacy is detected against clinical infection in the first trial then, for ethical reasons, it may be difficult to progress to a larger randomised, placebo-controlled trial with severe disease or death as its main end-point. One possible approach to this dilemma, adopted for the second Tanzanian SPf66 trial [31], is to plan for a large trial with a severe disease end-point but to include an interim analysis

that will allow the trial to be stopped early if any safety problems arise or if no efficacy against milder end-points is detectable at a time when only a limited number of subjects have been recruited.

Study Design

An individually randomised, placebo-controlled trial is likely to be the first choice for evaluation of a pre-erythrocytic or blood-stage vaccine as this reduces the chance that confounding from one of the many genetic and environmental factors known to influence susceptibility to malaria, such as the presence of sickle cell trait or the use of insecticide-treated bednets, will influence the outcome of the trial. Ideally, controls should receive the adjuvant used in the malaria vaccine as it is possible that the adjuvant itself could have some protective effect. However, this may not be ethically acceptable, especially if a new adjuvant is being used which puts controls at risk with little prospect of any benefit. Thus, an alternative vaccine which would not normally be given may be needed for the control group. The nature of the vaccine chosen to fill this role will be determined by the age of the vaccinees and the immunisation policy in the study area (table 2). Possibilities include hepatitis B and inactivated polio vaccine for children and rabies or meningococcal vaccines for adults. Ideally, the control vaccine should employ the same adjuvant as the malaria vaccine.

Phase-III trials of transmission-blocking vaccines raise special design issues as the level of protection to an individual will be influenced by the number of subjects in the community who are immunised. Thus, community randomised trials will be required, ideally with buffer zones between vaccinated and control villages so as to reduce the impact of vector migration between communities. Isolation of study communities, thus minimising the effects of human migration, is also desirable. Valuable experience on how such trials might be conducted has come from community randomised trials of insecticide-treated bednets [42] and trials of mass drug administration of combination therapy to reduce malaria transmission [von Seidelin et al., in preparation].

Investigators planning phase-III trials in developing countries are often faced with the problem of deciding whether they are trying to prove the biological efficacy of the vaccine when given under optimum conditions (an efficacy study) or to determine what impact the vaccine has when used under the conditions prevailing in the community (an effectiveness study; fig. 3). The advantage of the first approach is that the results of the trial are likely to be transferable to other communities with a similar pattern of malaria, whilst the latter provides important information about the likely public health impact of the vaccine in a particular situation. For example a vaccine with a modest degree of efficacy would have little use if its immunisation schedule were so complex

that, when administered under routine conditions, few subjects were fully vaccinated. A case can be made for conducting both efficacy and effectiveness studies with the same vaccine. However, because these trials are demanding and expensive, initial trials are often a compromise between the two approaches, for example administration of the vaccine through the routine health services but with the provision of some additional support in terms of cold chain supplies and/or transport to the immunisation programme.

Study Site

The ideal malaria vaccine should provide protection against infection under all circumstances but a partially effective pre-erythrocytic or transmission-blocking vaccine might provide protection only under conditions of low challenge. Thus, a case can be made for first testing such vaccines under conditions of low challenge or, simultaneously; under conditions of high and low challenge, so as to ensure that a vaccine which could have a useful role in some geographical areas but not in others is not discarded prematurely. However, selection of a low-transmission area will mean that a large trial may be needed to give an adequate sample size. Because of the possible effect of the level of transmission on vaccine efficacy, some measure of the transmission rate in the population should be made whenever a vaccine trial is undertaken. This is especially important when trials are done at several sites as otherwise it may not be possible to explain discordant findings.

The operational issues that will influence the trial site [39–41] include political considerations, accessibility, level of health care, state of the immunisation services and, most importantly, the degree of commitment of the local population.

Study Population

The age group chosen for a trial will be influenced by the level of endemicity in the study area. In communities where the level of transmission is low and all are at risk, it may be appropriate to conduct phase-III trials in subjects of all ages. However, in areas of high transmission, where the main burden of clinical malaria is in the young, phase-III trials are likely to be undertaken mainly in children. The special problems of trials in infants are discussed above.

In communities where malaria is concentrated in specific sections of the community, for example in migrants or refugees, it may be appropriate to choose these groups for initial phase-III trials as was the case for the SPf66 trial undertaken in Thailand [29]. Pregnant women constitute another special at risk group who may be targeted for vaccination. The design of trials of malaria vaccines in pregnancy has received little attention so far, but such trials are likely to incorporate unique end-points such as the prevalence of placental malaria and

birth weight. Studies of intermittent treatment or chemoprophylaxis in pregnancy provide a model for the kind of trial that would be needed.

Because the incidence of malaria in individual members of the study population may be influenced by many different genetic and environmental factors, such as their sickle cell status, exposure to mosquitoes, use of bednets and access to treatment as well as by their vaccination status, it is important that as many as possible of these variables should be measured in the trial subjects. Randomisation should take account of most inter-individual variability but some adjustment for imbalances in the prevalence of risk factors at the start of the trial may be needed (see below).

Study Size

The size of a phase-III vaccine trial should be determined by the nature of the study end-point(s) selected, their prevalence or incidence in the community and the size of the effect required using standard sample size calculations [43]. Because of the very high prevalence of malaria in endemic areas, a vaccine that provided a level of efficacy (for example 30%) that was much less than that usually demanded for vaccines intended for widespread use, could still be a useful public health tool. Thus, most phase-III trials conducted to date have used a sample size sufficient to detect a vaccine effect of around this level.

Evaluation of potential combination vaccines constructed using the 'bottom-up' approach described above poses special problems as the potential number of study groups may be large. Recently, it has been suggested that one way to approach this problem is to use a factorial design, a technique used widely in agricultural research, and a simple end-point such as parasite density which necessitates only a small sample size for each cell [Smith and Genton, personal commun.]. Studies of this kind could provide a useful screen for deciding which combinations should be taken into larger trials.

End-Points

A number of different end-points can be used in the evaluation of malaria vaccines, each of which presents its own particular problems (table 6). For example, if the incidence of infection is chosen as the primary end-point, as might be the case in the evaluation of a pre-erythrocytic vaccine, then it has to be decided whether or not to clear any infection present at the beginning of the surveillance period by chemotherapy. The advantage of doing so is that any subsequent episodes of parasitaemia can be assumed to be new infections. However, treatment with some long-acting anti-malarials, such as pyrimethamine/sulphadoxine, will give a period of chemoprophylaxis and may interfere with immunity dependent upon the persistence of low-level parasitaemia. Surprisingly, different results were obtained in a trial of a MSP1/MSP2/RESA vaccine

Table 6. Possible end-points for phase-III malaria vaccine trials

End-point	Advantages	Disadvantges
Death	The most important public health end-point	Measurement requires a large trial
Malaria mortality		Difficult to measure
Severe clinical malaria	An important public health end-point	Requires a high hospital admission rate
Mild clinical malaria	Requires a relatively small trial	May miss an effect on severe disease
Parasitaemia	Easy to measure	May miss an important clinical effect

in children treated with sulphadoxine/pyrimethamine and in those who were not [32].

If clinical malaria is to be used as the primary end-point for a trial, it is necessary to decide how a clinical episode of malaria will be defined. It is now generally agreed that there is no absolute standard for the diagnosis of clinical malaria – the use of definitions that include the presence of fever or a history of recent fever, no other obvious cause for the fever and the presence of parasitaemia is probably the best that can be done. In areas of high malaria endemicity, nearly all children will be parasitaemic so many febrile illnesses due to other causes will be accompanied by parasitaemia. In these circumstances, measurement of parasite density can help to improve the specificity of the diagnosis of clinical malaria and methods have been developed for determining the parasite density which have maximum sensitivity and specificity for the diagnosis of malaria in a particular community [44]. Unfortunately, the optimum parasite density, usually in the range of 1,000–20,000 parasites/μl, varies with age and area. This approach to the definition of clinical malaria has been used in several phase-III malaria vaccine trials but, as the relationship between symptoms and the level of parasitaemia may differ in vaccinated and control subjects, there is a danger that one definition of clinical malaria may not be applicable to both groups.

Cases of clinical malaria may be detected either by active surveillance, which involves regular home visits, or by detection of cases who present to a clinic. These 2 groups of patients may differ from each other, even when the same definition of clinical malaria is used, as clinic cases are likely to have had a longer history of illness and to be sicker than cases detected at home. Thus, it is important that the method of case detection in any particular trial is defined carefully as it is theoretically possible that a blood-stage vaccine could show

efficacy against cases who present to a clinic but not against cases detected at home.

As far as we are aware, no malaria vaccine trial has yet used the incidence of hospital admissions with severe malaria as its main end-point but this end-point was used successfully in a trial of insecticide-treated bednets in Kenya [45] and could be used for vaccine trials conducted in communities where a high proportion of seriously ill subjects are admitted to hospitals.

Measurement of overall mortality in vaccine recipients should not present any major problems as methods for demographic surveillance which are suitable for use in malaria-endemic areas are now well developed. However, measuring malaria-specific mortality is much more difficult for many deaths from malaria occur at home and the post-mortem questionnaire technique, the only means of establishing the cause of death in such circumstances, has a poor specificity for the diagnosis of deaths from malaria [46, 47].

Parasitological end-points for a malaria vaccine trial are likely to include the incidence or prevalence of asexual infections with the parasite species against which the vaccine is directed, the density of any infection that occurs and the prevalence of gametocytaemia. Because of evidence for interactions between *P. falciparum* and *Plasmodium vivax*, such as an increase in the incidence of *P. vivax* infections after successful treatment of a *P. falciparum* infection [48], the effect of a vaccine on the incidence of infection with the heterologous parasite should be studied when a single species vaccine is used in communities where mixed infections occur frequently.

New molecular techniques have made it possible to study changes in parasite populations resulting from vaccination in more detail than has been possible using microscopy. Thus, it has been shown in both Tanzania and in The Gambia that the blood-stage vaccine SPf66 reduces the number of parasite genotypes present in vaccinated subjects even though it has little effect on the overall prevalence of parasitaemia [49, 50]. These findings suggest the occurrence of some form of density-dependent regulation in parasitaemia with SPf66 either reducing the incidence of new infections or accelerating the rate of parasite clearance. Molecular techniques can be used also to look for any strain-specific effect of a vaccine that contains antigens with polymorphic epitopes. This approach has been used in the evaluation of both SPf66 and RTS,S vaccines. Samples collected from subjects who had been vaccinated with SPf66 in The Gambia or Kenya were studied for polymorphisms in the region of the MSP1 antigen present in SPf66. No evidence for selection against the variant present in the vaccine was found [50, 51]. The way in which RTS,S protects against malaria is not known. There is evidence that antibody is important but protection could be mediated through T-cell responses directed against the Th2R and Th3R epitopes which are highly polymorphic. Thus, the distribution of Th2R and Th3R variants

present in parasites obtained from vaccinated and control subjects was studied during the Gambian RTS,S trial. The prevalence of infections with strains whose genotype corresponded to that of the parasite used to produce the vaccine was similar in vaccinated and control subjects, suggesting that the vaccine had not induced a strain-specific effect [Alloueche et al., in preparation].

Economic Evaluation

An important factor that will influence whether a partially effective malaria vaccine is introduced into routine use will be its cost efficacy in relation to other malaria-control measures [52]. Thus, it is important that an economic evaluation of the costs of vaccination incurred during a phase-III trial, for example the cost of an additional vaccine contact outside the routine EPI programme, are measured. Information may already be available for the cost of a case of malaria prevented for the study community, or a similar one, thus allowing a cost-efficacy estimate to be made [53].

Analysis

Analysis of the phase-III trials done so far has highlighted a number of issues that are especially relevant to malaria vaccine trials. The issue of the many potential confounders of vaccine efficacy has been discussed above and most studies have taken these into account to produce an adjusted as well as a crude measure of vaccine efficacy. If this is to be done, it is essential that the rules for doing so should be addressed clearly in the analytical plan and not after the vaccine code has been broken. Most phase-III studies done so far have used the first or only malaria episode as the primary end-point for the trial (fig. 4). However, from a public health point of view, the total number of cases saved by the vaccine during the observation period may be a more important measure. If subjects who develop malaria during the course of the trial are not censored at the time of their first attack but treated and allowed to remain in the trial, problems arise because of the potential chemoprophylactic effect of the drug used for treatment. One way round this problem is to exclude a subject from the denominator for a 4-week period after treatment with an antimalarial drug (including co-trimoxazole) has been given. If subjects who develop malaria are censored when they develop an infection, this may cause problems in the determination of vaccine efficacy for a vaccine that gives only a short period of protection. In such a situation, the proportion of subjects susceptible to malaria for reasons other than a lack of vaccine-induced immunity will be higher in the vaccine than in the control group during the latter part of the trial, giving an apparent increase in susceptibility to malaria in the vaccinated group in the latter part of the study. Special methods of analysis are needed to take account of this effect [Milligan et al., in preparation].

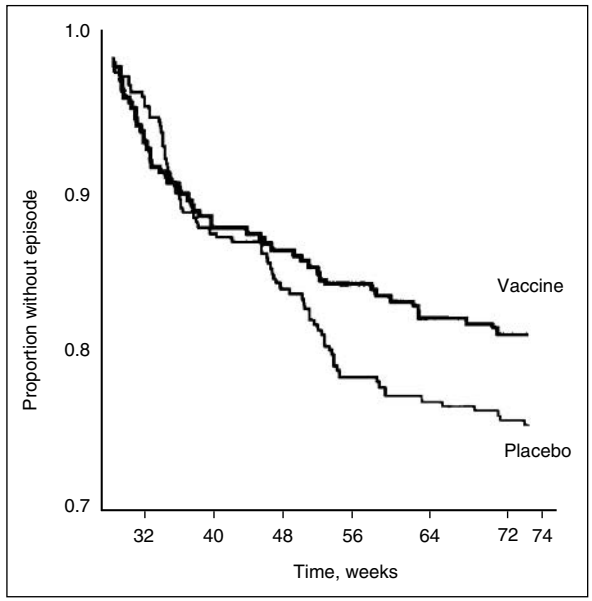

Fig. 4. Kaplan-Meier analysis of the time to first infection observed during a trial of SPf66 vaccine in Tanzanian children [24].

After the Trial Is Over

The responsibilities of the investigators to the study population are not finished once the results of the trial are obtained. Whatever its outcome, it is vital that the results of the trial are discussed in detail with the population; failure to do so is likely to prejudice their willingness to participate in further studies. Conduct of a malaria vaccine trial will often require strengthening of the local resources for vaccination and health care. Thus, there is a danger that on completion of the trial, standards of clinical care will decline in the study area. Everything possible must be done to prevent this, for example by supporting a limited number of extra staff during a transition period.

The extent of the responsibilities of the investigators and their sponsors to the study population will be influenced by the results of the trial. If the vaccine shows no protection and there is no reason to suspect the occurrence of late side effects, then long-term follow-up is unlikely to be needed. If vaccine efficacy is shown over a defined period, for example one malaria transmission season, then there will be considerable interest in learning how long efficacy, perhaps boosted by natural infection, is sustained. However, the ability to do this accurately may be compromised by loss of the control group. If substantial vaccine efficacy is established, for example 50% protection against clinical malaria, there will be strong

pressures on the investigators to vaccinate the controls and possibly even the whole population of the area in which the trial has been done. If this is done, it will be difficult to determine accurately the duration of protection provided by the vaccine. In the first trial of SPf66 undertaken in Tanzania, which showed a protective efficacy of 30%, control children were vaccinated [24, 25] but this was not done in the Gambian SPf66 trial as protection was not shown. Both vaccinated and control children were followed for 3 years after vaccination in the Gambian trial as a safety measure [27]. Deciding whether or not to vaccinate the controls is difficult. Whatever is decided, some rules should be set before the results of the trial are known and the study population should be told at the beginning of the trial what they can expect, as was the case in the Tanzanian trials.

A special problem posed in the evaluation of malaria vaccines in young children in highly endemic areas is the fact that age influences the clinical presentation of malaria. Cerebral malaria is seen more frequently in older than in younger children whilst severe anaemia shows the opposite pattern. An effective malaria vaccine given to young infants in a highly endemic area could shift the peak of infection from very young to older children. The implications of such a change on mortality are not known. Thus, when a vaccine given to young children is shown to be effective, long-term follow-up of the vaccine cohort is essential.

Phase IV

Once a malaria vaccine has been licensed, individual countries will have to decide whether or not to deploy it. Whether this takes place is likely to depend upon the level of efficacy obtained, its cost, the availability of alternative methods of malaria control and the finances available to support its introduction. Studies have shown that a vaccine that gave 50% efficacy over 3 years and which cost USD 10/course would be a good investment.

If a national decision to use a particular malaria vaccine is made, it is likely that the vaccine will be introduced in a phased manner. If this is the case, it provides an opportunity to learn more about the safety and efficacy of the vaccine. Establishment of low-level surveillance in the areas where the vaccine is first introduced, and in control areas where it will be introduced subsequently, may allow the detection of rare side effects that would not have been picked up during phase-III trials. If registration was based only on the efficacy of the vaccine in preventing clinical malaria, then a carefully designed phased programme of introduction, using some kind of step-wedge design [54], may provide additional important information on the efficacy of the vaccine in preventing less common outcomes such as admission to hospital with severe malaria or death. Information on efficacy can also be obtained during phase-IV studies using case-control techniques.

Ethics

Malaria-vaccine trials must meet internationally recognised ethical standards for clinical trials in areas such as the solicitation of informed consent and provision of good standards of clinical care for all study participants. Because most phase-III trials will be carried out in developing countries with the participation of scientists and vaccine producers in industrialised countries, the ethical aspects of these studies are likely to come under close scrutiny [55, 56]. Ethical approval will be required both in the country where the vaccine is developed and in the country where it is tested. Establishment of an effective data and safety management board is essential. Specific ethical issues that have arisen during the course of malaria vaccine trials conducted so far include vaccination of the controls, the duration of follow-up and the inadvertent determination of efficacy in a trial not powered to measure this end-point when information on the clinical incidence of malaria was collected and analysed as a safety measure [57, 58]. The ethical aspects of malaria and related vaccine trials have been debated at a number of workshops organised by the African Malaria Vaccine Testing Network.

Financing Vaccine Trials

Phase-III malaria vaccine trials, likely to involve several hundred or even thousands of individuals, are expensive and time-consuming. A budget of USD 1 million is not unreasonable for a phase-III trial. Thus, imaginative new approaches, such as the factorial design, are needed that could provide some form of screening to indicate which vaccines or vaccine combinations warrant larger studies. The development of surrogate markers of protective immunity is needed urgently. As more trials with a successful outcome are completed, it may become possible to identify immunological markers that correlate with protection, at least for a particular group of antigens.

Conclusions

A fear of many scientists involved in malaria vaccine development is that even if the enormously difficult task of developing an effective malaria vaccine is accomplished in the near future, malaria vaccines will be too expensive to be used in the areas where they are needed most, such as rural, tropical Africa. Experience with hepatitis B and *Haemophilus influenzae* type b vaccines has not been encouraging. However, increasing international support for vaccination

of the world's poorest populations through new initiatives such as the Children's Vaccine Programme and the Global Alliance for Vaccination and Immunisation provides hope that once an effective malaria vaccine has been developed, it will be used.

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