Epidemiology and Infectivity of *Plasmodium falciparum* and *Plasmodium vivax* Gametocytes in Relation to Malaria Control and Elimination

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INTRODUCTION

Malaria remains one of the most important causes of morbidity and mortality in the tropical regions of the world. Current estimates suggest that approximately 2.4 billion people are at risk of stable or unstable *Plasmodium falciparum* transmission (178), with 350 to 500 million clinical episodes and 1 million deaths annually (191). For the first time, similar global estimates are also available for *P. vivax*, and while there is considerably less mortality attributed to this species, its geographical reach is far greater. An estimated 2.9 billion people are at risk for vivax malaria (179), with an estimated 80 million to 300 million clinical cases annually (296). These global estimates are a direct result of an increasing ability to collate and assimilate large data sets that also allow the monitoring of trends in malaria incidence and parasite prevalence. These broad-scale estimates from countries where malaria is endemic (178), together with specific country examples (23, 24, 35, 87, 174, 227, 328, 329), have highlighted the recent trend of decreasing malaria transmission intensity in many areas of endemicity, and these observations, at least in part, have stimulated (or restimulated) the malaria elimination agenda (8b, 276). While there is considerable debate in the malaria community about the rationale and likely success of elimination programs, the ongoing discussions have led to a reevaluation of current strategies to reduce or abrogate the transmission of malaria parasites. For *P. falciparum*, the theoretical solution is that if one were able to clear infections in all humans and render *P. falciparum* noninfectious to mosquitoes for a period longer than the mosquito life span (8 weeks, for example), then transmission would stop. This panglossian ideal is of course unlikely. Nevertheless, the addition of interventions specifically aimed at reducing transmission from humans to mosquitoes would greatly enhance our ability to control and potentially eliminate malaria. Elimination of *P. vivax* is further complicated by the presence of hypnozoites, dormant liver-stage schizonts that can form a reservoir for transmissible parasites for many months after the initial infection.

Transmission from an infected human host to a susceptible mosquito is mediated through highly specialized sexual-stage parasites, i.e., gametocytes (Fig. 1). The gametocytes of *P. falciparum* hold a prominent place in the history of malaria, in that it was the exflagellating male gametocyte that first led Laveran to describe malaria in the late 19th century (8a). Exflagellation is a highly active process whereby motile male microgametes free themselves from the red blood cell in order to locate and fertilize a female macrogamete. Also, on routine microscope blood films, the gametocytes of *P. falciparum*, with their unique crescent shape, are prominent.

Transitioning from the relatively protected and stable environment within the red blood cell of the human host to being an exposed parasite in the lumen of a mosquito midgut obviously requires considerably different characteristics. Not surprisingly, therefore, molecular sequencing and proteomics have identified 250 to 300 sexual stage-specific

genes, 75% of which were hypothetical at the time of identification (149, 155, 239, 247). These proteins will almost certainly be involved in some of the fundamental steps of gametocyte development. What stimulates gametocyte production? Where and how do gametocytes sequester? Do they preferentially localize in the dermis? What signals allow gametes to interact at low densities? These basic biological questions are central to our understanding of how the parasite transmits itself at the population level. This issue has extra complexity related to differences in malaria exposure, transmission intensity, and age of the individual infected as well as to how treatment and control measures such as drugs might affect transmission. Ultimately, these factors combine to define the infectious reservoir of malaria within an area, i.e., the people capable of transmitting malaria to mosquitoes. This human infectious reservoir forms the part of the population that would need to be targeted with transmission-reducing interventions in order to reduce malaria or abrogate malaria transmission.

In the subsequent sections, we discuss these issues to provide a current situational assessment of our knowledge of *P. falciparum* gametocytes and the related gaps in our understanding. Whenever gametocytes are mentioned without specifying the malaria species, we refer to *P. falciparum* gametocytes. Toward the end of this review, we discuss factors associated with gametocytemia and transmission of *P. vivax*, whose transmission stages have been studied less well than those of *P. falciparum*. At present, control tools that significantly target gametocytes are limited, and those that are available have not been developed suitably for wide-scale use. Research into these gametocyte-specific knowledge gaps may yet yield a tool to augment our malaria control capabilities and go some way toward achieving malaria elimination.

GAMETOCYTE BIOLOGY AND MORPHOLOGY

P. falciparum gametocytes are markedly different from their asexual precursors. Transcriptome analyses identified 250 to 300 genes that are specifically upregulated at the mRNA level during gametocyte development (417, 528). The *P. falciparum* proteome revealed more than 900 proteins in gametocytes, 315 of which were found exclusively in gametocytes (239). Furthermore, a sex-specific proteome study using the rodent malaria model *P. berghei* indicated that a large proportion of these proteins are expressed exclusively in either male or female gametocytes (220). These findings reflect the highly specialized nature of gametocytes and the individual roles of both sexes. Some of the key antigens are summarized in Table 1 and discussed below. Gametocyte development can be divided into five morphologically recognizable stages (Fig. 1) (190), during which the gametocyte grows and elongates to gradually occupy the majority of the erythrocyte (21). The most striking morphological features of gametocytes are the presence of the subpellicular microtubule-based cytoskeleton and the surrounding double membrane, which create the characteristic crescent shape (280, 464). The differences between male and
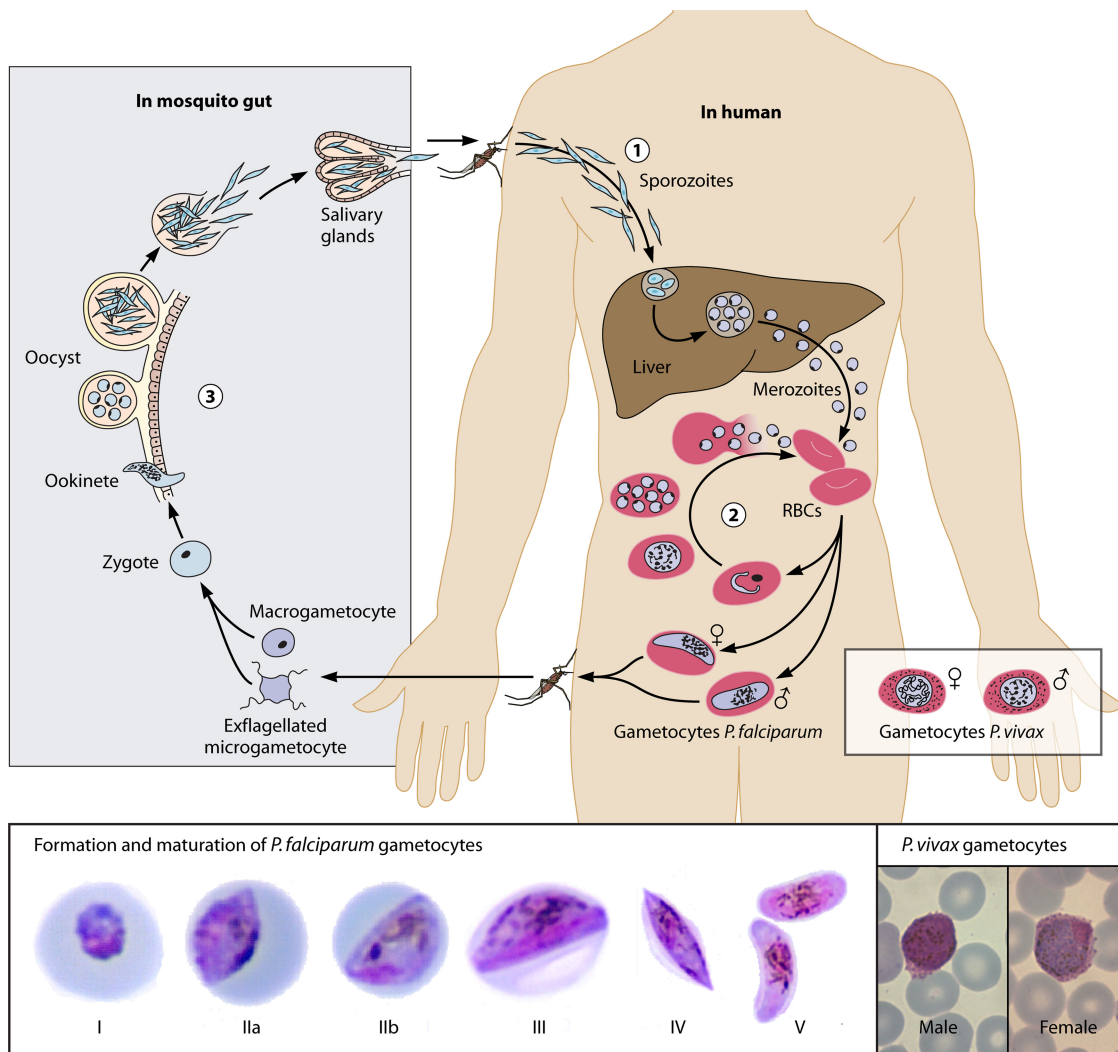
FIG. 1. (Top) Life cycle of *Plasmodium falciparum* and gametocyte development. Malaria parasites enter the human bloodstream in the form of sporozoites that are injected by infected female *Anopheles* mosquitoes taking a blood meal. The majority of sporozoites migrate to the liver, where they invade hepatocytes and multiply. Merozoites are formed that are released into the bloodstream, where they invade red blood cells, initiating the asexual multiplication cycle. A fraction of merozoites that are released from infected red blood cells form gametocytes, the transmissible parasite form. The formation and maturation of gametocytes take place in five morphologically recognizable stages. Early-stage gametocytes are sequestered, and only mature stage V gametocytes circulate in the peripheral blood, where they can be taken up by mosquitoes. Once ingested by mosquitoes, each individual gametocyte forms 1 female macrogamete or up to 8 male microgametes. In the mosquito midgut, the fusion of gametes results in the formation of a zygote that develops into a motile ookinete that can penetrate the midgut wall to form oocysts. The oocysts enlarge over time and burst to release sporozoites that migrate to the mosquito salivary gland, rendering the mosquito infectious to human beings. (Bottom) The five developmental stages of *P. falciparum* gametocytes and mature *P. vivax* gametocytes. (The *P. falciparum* gametocyte photographs are reprinted from reference 410 with permission; the *P. vivax* gametocyte photographs are courtesy of Debbie Nolder, Malaria Reference Laboratory, London School of Hygiene and Tropical Medicine, United Kingdom, reproduced with permission.)

female gametocytes become morphologically most apparent from stage IV onwards, when gametocytes are characterized by an elongated shape with pointed ends. Female gametocytes are characterized by a relatively small nucleus with a nucleolus and concentrated pigment. The nucleus is larger and the pigment is more diffuse in male gametocytes, which appear to lack a nucleolus and appear in Giemsa-stained blood films as pink cells, as opposed to violet females. Sexually committed ring-stage parasites and the crescent-shaped mature gametocytes (stage V) can be found in the human peripheral blood (404). Developmental-stage parasites are thought to be sequestered

in the bone marrow, and possibly the spleen (427, 475), but can circulate in splenectomized hosts (17).

Sequestration of Gametocytes

Immature *P. falciparum* gametocytes are sequestered away from the circulation, presumably to avoid immune clearance in the spleen. Once gametocytes are mature (stage V), they are released into the circulation and are thereby accessible to mosquitoes taking a blood meal. However, it may take 2 to 3 days before circulating and morphologically mature gametocytes

TABLE 1. Key antigens in *P. falciparum* gametocyte development and transmission-blocking vaccines

Antigen(s)	Localization[a]	Function	Reference(s)
PfEMP-1	RBC membrane	Sequestration	105, 193
RIFIN	Unknown	Sequestration?	351, 505
STEVOR	RBC membrane	Sequestration?	273
Pfs16	PVM	Unknown	21, 22
Pfpeg3 and -4	PVM	Early gametocyte development	417
PF14 744 and PF14 748	PVM	Gametocyte development	138
Pfg377	OB	Gametocyte maturation, osmiophilic body formation, and egression of macrogametes	6, 108
Pfg27	GCT cytoplasm	Gametocyte formation/integrity; evidence for RNA binding	21, 78, 253, 408
Pfs48/45[b]	GM	Fertilization: male fertility factor attachment of male microgametes to fertile female macrogametes	493, 494
Pfs47	GM	Fertilization: female fertility factor (not essential)	498
Pfs230[b]	GM	Fertilization: adherence of male gametes to red blood cells; may protect parasite from contents of blood meal; complement required for antibody function	137, 369, 514
HAP-2	GM (male)	Fertilization: fusion of gamete surface membranes	40, 251
Pfs25 and Pfs28[b]	OM	Midgut penetration, ookinete survival, oocyst formation	28, 130, 477

[a] RBC, red blood cell; PVM, parasitophorous vacuole membrane; OB, osmiophilic bodies; GCT, gametocyte; GM, gametocyte/gamete membrane; OM, zygote and ookinete membrane.
[b] Malaria transmission-blocking vaccine candidate in the most advanced stage of development.

become infectious to mosquitoes (243). Early-stage gametocytes (stages I and IIa) seem to depend on CD36 as the principal host ligand for gametocyte adherence (106, 193, 436); ICAM-1, CD49c, CD166, and CD164 receptors that are present in human bone marrow epithelial and stromal cell lines have been suggested as host ligands for later gametocyte stages (stages III and IV) (113, 390). Parasite ligands that mediate adherence are less well characterized but may include the parasite surface proteins PfEMP-1 (*P. falciparum* erythrocyte membrane protein 1) (105, 193, 411), RIFIN (repetitive interspersed family) (17, 505), and STEVOR (subtelomeric variable open reading frame) (273, 458). The *var* genes encode PfEMP-1 molecules (432), which are among the most extensively described parasite surface proteins. PfEMP-1 is the primary mechanism for cytoadherence and sequestration of asexual parasites (94, 322, 409). PfEMP-1 transcripts are also found throughout gametocyte development, with transcription profiles that appear independent of the phenotype of their asexual progenitors (411). PfEMP-1 is expressed in knob structures on erythrocytes infected with early-stage gametocytes (stages I and IIa) (105, 193), making a role in gametocyte sequestration particularly plausible in the first days of gametocyte development (193). Erythrocytes infected with late-stage gametocytes no longer have knob structures, and PfEMP-1 expression appears confined to the cytoplasm of the parasite (193). It is unclear why gametocytes switch to a different adhesion mechanism during maturation or which parasite ligands play a role in sequestration of the later stages. STEVOR transcripts are most abundant in early-stage gametocytes (273, 411), while the encoded protein is also localized at the infected erythrocyte membrane in late-stage gametocytes (273). This is consistent with a role in gametocyte sequestration, although the presence of STEVOR proteins on the surfaces of erythrocytes infected with nonsequestering stage V gametocytes makes a role in cytoadhesion speculative (351). There is recent evidence that RIFINs are transcribed in gametocytes (351, 505), but their role in gametocyte adhesion remains to be confirmed.

Preparing for Environmental Change

After approximately 6 days of gametocyte development, protein synthesis and hemoglobin digestion cease (21, 79, 192). Gametocytes appear developmentally arrested at the G_0 phase of the cell cycle (423). There is no genome replication in gametocytes (370), and nucleic acid synthesis in gametocytes is restricted to RNA synthesis. Rapid genome replication and nuclear division occur only once gametocytes are activated in the mosquito midgut (21). In preparation for this activation, mRNAs encoding proteins that are required after ingestion by mosquitoes, such as the P25 and P28 proteins, are synthesized in gametocytes but translationally repressed (182, 234, 342). This translational repression depends on RNA helicases (259, 260), and the mRNAs for several sexual stage-specific proteins can be detected in gametocytes (14, 277, 403, 404, 421). Among them is *pfs16* mRNA, the earliest marker of sexual stage development, present in all gametocyte stages and in sexual stage-committed asexual parasites 24 h after merozoite invasion (69, 404). Pfs16 protein is located on the parasitophorous vacuole membrane until it is lost during gametocyte activation (21, 22). Other early gametocyte proteins include Pfpeg3 and Pfpeg4 (stage II gametocytes) (417) as well as PF14 744 and PF14 748 (138). Pfg27 is transcribed at ~30 h postinvasion (83, 408) and is hypothesized to play a role in the extended period of gametocyte development (21) that is so specific to *P. falciparum*. *pfs25* mRNA transcription starts in mature (stage V) gametocytes (14, 15, 404). Once mature gametocytes are ingested by *Anopheles* mosquitoes that are taking a blood meal, they become activated and prepare for fertilization in a process called gametogenesis. Within 20 min after being taken up by a mosquito, gametocytes have differentiated into gametes in response to a variety of stimuli, including a drop in temperature of ~5°C, a rise in pH from 7.4 to 8 to 8.2, and the presence of the mosquito-derived gametocyte-activating factor xanthurenic acid (36, 37, 200, 423). During gametogenesis, gametocytes are activated to differentiate into spherical cells and shed their erythrocyte membrane. Male gametes then undergo three

rounds of DNA replication, and subsequently, up to eight motile microgametes are released during exflagellation (200, 343, 400, 420, 422, 423).

GAMETOCYTOGENESIS

Mature gametocytes are detectable in the bloodstream at days 7 to 15 after the initial wave of asexual parasites from which they are derived (105, 136). Gametocytogenesis is the process whereby male and female gametocytes develop from these asexual parasites. Our current understanding of molecular mechanisms involved in gametocytogenesis was recently reviewed by Baker, by Alano, and by Dixon et al. (5, 21, 113). Here we describe some of the key elements in gametocytogenesis and epidemiological factors that are associated with gametocyte production.

What Triggers the Sexual Pathway?

Commitment to the sexual pathway occurs in the cycle prior to the actual development of gametocytes, with all merozoites from a single schizont committed to either the sexual or the asexual pathway (68, 464). Recent data indicate that asexual parasites committed to the sexual pathway can be present as early as the first round of asexual replication after *P. falciparum* infection (404), and work with *P. berghei* indicated that some of the merozoites produced by exoerythrocytic schizonts may grow directly into gametocytes without an intervening erythrocytic schizogony (222). These initial gametocyte densities are likely to be very low, but they suggest a level of developmental flexibility important for onward transmission. Once committed to the sexual pathway, merozoites become either all male gametocytes or all female gametocytes (416, 435). Gametocyte production differs between parasite isolates (1, 133, 173), and continuous parasite culture may lead to a loss or reduction of gametocyte production related to the loss of genetic information in the right arm of chromosome 9 (89, 107). These findings indicate that there are intrinsic parasite factors related to gametocyte production and a role for a gene on chromosome 9 in gametocytogenesis. Subsequent work demonstrated that parasite strains with a disrupted *Pfgig* gene within this region of chromosome 9 had 5-fold less gametocyte production (154).

Limited experimentation has shown that gametocytogenesis can be induced *in vitro*, in parasite lines where gametocytes had not been observed during several years of continuous culture (330, 332), by addition of ammonium compounds (330) or after a short exposure to Berenil, an inhibitor of nucleic acid synthesis (332). Other studies found that gametocyte production *in vitro* can be induced by red blood cell lysate (86) or by the addition of mammalian hormones to culture (250, 265). Although these strategies are valuable for obtaining gametocytes from culture, they are unlikely to be the natural trigger for gametocytogenesis in natural infections. Signaling pathways may be involved in the molecular mechanism that triggers gametocytogenesis, potentially including the phorbol ester-inducing pathway (481) and the cyclic AMP (cAMP) signaling pathway (66, 377, 481). Additional findings on increased gametocytogenesis *in vitro* after addition of cholera toxin (132) led to the conclusion that a G-protein-dependent signaling system may mediate gametocytogenesis in response to environmental factors. These data suggest that multiple factors may contribute to the decision to form gametocytes such that the parasite is able to respond to a variety of environmental stimuli.

Gametocytogenesis is sometimes described as a stress response of the parasite that allows it to escape an increasingly unfavorable environment (21, 133). Although "stress" is unlikely to be essential for gametocyte production, as indicated by the commitment to sexual development early in infections (404) and in asymptomatic untreated infections (52, 131), gametocyte production is clearly affected by several factors during the course of an infection. In culture, gametocyte production may increase at higher parasite densities (68), in the presence of soluble factors from parasite-conditioned medium (513), and upon addition of human serum and lymphocytes (428), reticulocytes (480), or antimalarial drugs (72, 77, 202). All of these factors can be linked to increased gametocyte production in natural infections.

Host immunity, asexual parasite density, and gametocytogenesis. Naturally acquired immunity of the human host affects asexual parasite densities and therefore the production of gametocytes from their asexual precursors. Immune responses may also influence gametocytogenesis more directly. Waves of gametocytemia typically follow, but do not necessarily coincide with, clinical episodes of uncomplicated malaria (131, 448). This suggests a potential role in gametocytogenesis for nonspecific immune effectors associated with symptomatic malaria, such as increases in tumor necrosis factor alpha (TNF-α) (236, 237), which has antiparasitic properties (236). However, this association between gametocytogenesis and nonspecific immune responses could also explain the delayed appearance of *P. falciparum* gametocytes as a strategy to evade potentially toxic levels of human cytokines during febrile paroxysms (218; see the section on *P. vivax*).

Several lines of evidence also indicate a relationship between malaria-specific immune responses and gametocytogenesis. *P. falciparum* gametocyte production in culture was increased after addition of lymphocytes and sera from naturally infected Gambian children but not those from malaria-naïve Europeans (428). Increased *in vitro* gametocyte production was also observed after addition of anti-*P. falciparum* antibodies produced by hybridoma cell lines (331). In animal models, gametocytogenesis in *P. yoelii* was induced after immunization of the rodent host with a recombinant heat shock-like protein that is expressed throughout the parasite's life cycle (295). Similarly, increased gametocyte production was observed for *P. chabaudi* in the early parts of infection after partial immunization of mice with infected erythrocytes that were subsequently cleared by antimalarial treatment (73). These observations are clearly indicative of some relationship between immune responses and gametocytogenesis. In naturally occurring malaria infections, this relationship is difficult to disentangle from the impact of naturally acquired immunity on asexual parasite densities. The lower gametocyte prevalence in adults than that in children (51, 120, 131) (Fig. 2) is explained at least partly by acquired immunity that allows the semi-immune adult host to control asexual parasite densities better (115, 457), resulting in lower gametocyte densities. In contrast to this overall trend, commitment to the sexual pathway may actually
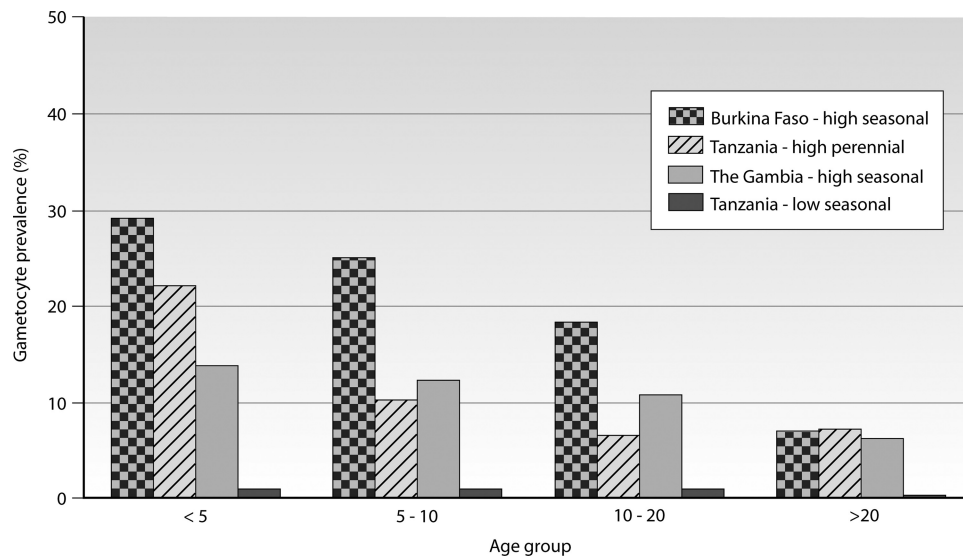
FIG. 2. Prevalence of gametocytes in different transmission settings and age groups. Gametocytes were detected by microscopy (thick blood films; screening of 100 fields at a magnification of ×1,000, using oil immersion) in cross-sectional surveys in areas of high and seasonal transmission intensity in Burkina Faso (336) and the Gambia (121), high perennial transmission in Tanzania (121), and low seasonal transmission intensity in Tanzania (413). Gametocytes are detected in a larger proportion of the population in settings of high endemicity, where gametocyte prevalence depends on age; in settings of low endemicity, gametocyte carriage is rare and more evenly distributed across age groups.

increase with acquired immunity. Both microscopic and sub-microscopic densities of gametocytes relative to asexual parasites increase with age in cross-sectional surveys (49, 120, 334). While the proportion of gametocyte carriers among parasite carriers is highest for children, the proportion of parasites that are gametocytes may therefore be highest for those with acquired immunity (120, 334). These observations require confirmation in longitudinal studies but suggest further adaptability of gametocytogenesis in response to acquired immunity. An analogous phenomenon has been seen with *P. chabaudi*, where the net gametocyte production may decrease as a result of acquired immunity against asexual parasites, but with an incomplete compensatory mechanism that increases gametocytogenesis by those asexual parasites that survive (73).

Host anemia and gametocytogenesis. Studies from Thailand and the Gambia observed that hemoglobin concentrations were lower in gametocyte carriers (310, 360, 455, 502) and negatively correlated with peak gametocyte counts and the duration of gametocyte carriage (310). The proportion of gametocyte carriers in the population is also associated with seasonal fluctuations in the prevalence of anemia (306). These epidemiological associations do not provide evidence for a causal role of anemia in triggering gametocytogenesis. A larger proportion of gametocyte carriers among anemic individuals could merely indicate a longer duration of infection and hence a longer time to develop gametocytes (360, 383, 440, 450). However, a more direct role is suggested by *in vitro* and animal studies.

There are strong indications that the triggering of the gametocyte pathway is affected by the presence of reticulocytes or very young erythrocytes (479, 482), which are characterized by a relatively high RNA content, hemoglobin synthesis, and the fact that they are less dense than mature erythrocytes (479). Reticulocytes are the preferred cells for gametocyte development (157), and their formation is increased in erythropoiesis, induced by the production of erythropoietin (EPO) by anemic hosts. EPO is implicated as a stimulant for gametocyte formation (379) and also influences gametocyte sex allocation (343, 346) (see "Gametocyte Sex Ratios"). Reticulocytosis also results in increased *in vitro* gametocyte production in different *P. falciparum* strains (479) and in rodent *P. berghei* (291) and *P. chabaudi* (157). It is unclear which signal associated with reticulocytes triggers gametocytogenesis; one suggestion is that one of the proteins that are shed from maturing reticulocytes may activate the promoter of the *pfs16* gene (479). Some observations from malaria transmission experiments suggest that anemia may have a role in other elements of transmission as well. A positive correlation between host hematocrit and the infectiousness of gametocytes (129) has been observed, though this was significant only at higher gametocyte densities. Conversely, a slight reduction in the host's hematocrit increases the feeding speed and quantity of blood uptake (470), thereby possibly enhancing the chance of successful mosquito feeding. The optimal hematocrit values for transmission success may therefore need to balance these associations (305).

Other factors associated with gametocytogenesis. Several other conditions that are unfavorable for asexual growth or parasite survival have been associated with increased gametocytogenesis. As an evolutionary strategy, it would be advantageous for parasites to alter their investment in transmission stages in response to the absence or presence of mosquito vectors. This phenomenon has been demonstrated for other vector-borne parasites, such as the increased presence of microfilaria in the peripheral blood during the night, when vectors are present (148, 272). An increase in gametocyte production in infections early in the transmission season was described in some epidemiological studies (336, 492) and was

hypothesized to be a result of the induction of gametocyte production by an increase in bites from uninfected mosquitoes (345). However, no direct evidence for this association exists from naturally acquired infections, and the rodent malaria parasites *P. chabaudi* and *P. vinckei* also do not increase their rates of gametocytogenesis in response to mosquito probing (415). There is also little evidence that gametocyte production is regulated in a way that ensures peak densities during peak mosquito biting hours (see "Sampling Issues: When and Where Do Mosquitoes Bite?").

Gametocyte Sex Ratios

Similar to the molecular mechanism that triggers gametocytogenesis, factors that determine the sex of gametocytes are poorly understood. *Plasmodium* parasites have no sex chromosomes (82); a single parasite clone can produce both male and female gametocytes (422) and can self-fertilize. The sex of gametocytes is determined early in sexual stage commitment, and all merozoites released from one sexually committed schizont become either all male or all female gametocytes (416, 435). The resulting gametocyte sex ratios are typically female biased, which is intuitively correct, since male gametocytes can produce up to eight microgametes and therefore fertilize several female gametes, each derived from a single female gametocyte (343). Considerable levels of inbreeding in malaria parasite populations (347) could suggest that the optimal transmission strategy is to ensure a balance between male microgametes and female macrogametes in the mosquito midgut. However, there is more to gametocyte sex ratios than simply counterbalancing the differences in gamete production by gametocytes. The ratio of female to male gametocytes has an immediate impact on transmission success (284), and the optimal ratio differs in different circumstances. Ratios of 3 or 4 females to 1 male gametocyte are commonly observed in natural *P. falciparum* infections (215, 385, 386, 445, 464), but there are variations in the sex ratio between clones (76, 373, 378), during the course of infections (207, 343, 344, 465), between regions (215, 385, 386, 442, 445), and between years in the same region (442).

A longer duration of infection reduces hemoglobin levels as a result of asexual parasite proliferation. The related induction of erythropoiesis is linked not only by a general increase in gametocyte production (see "Host anemia and gametocytogenesis") but also specifically, by an increase in male gametocytes (344, 386). Changes in gametocyte sex ratio may reflect a response of the parasite to overcome (sex-specific) immune responses that are mounted during the course of an infection and may influence the survival or infectivity of gametocytes, as discussed in more detail in the section on immune responses against gametocytes and transmission-blocking immunity. Gametocyte density may also define the optimal ratio for transmission success, where a more male-biased ratio becomes important at lower densities (344, 378). The presence of different parasite clones will favor shifting the female-biased sex ratio to a more even ratio to enhance the chances of transmission of an individual parasite clone (343, 378).

Reports on the impact of antimalarial treatment on gametocyte sex ratios *in vivo* are inconclusive. Antimalarial treatment may be followed by an increase (442–445, 447, 465) or decrease (445, 452) in the proportion of male gametocytes that may (444, 445, 452) or may not (465) be influenced by the type of treatment. The uncertainties are underlined by the fact that changes in sex allocation were sometimes reported within several days after the initiation of treatment (443, 445, 447, 452), while the environmental cues for sex determination must occur 7 to 10 days before mature gametocytes are observed (343). These findings may indicate sex-specific effects of drugs on male or female gametocytes or simply reflect the difficulty in studying parasite transmission strategies *in vivo* in humans. Studies using microscopy to quantify and sex gametocytes typically observe fewer than 10 gametocytes in a blood film, not all of which can be sexed reliably. These small numbers limit the precision of sex ratio estimates. The importance of this complication was shown by Robert and colleagues, who observed that as sex ratios became less female biased, the proportion of infected mosquitoes rose, as did the oocyst density in infected mosquitoes. However, this relationship was observed only for individuals for whom the sex ratio was based on sexing of ≥15 gametocytes, not for individuals for whom fewer observations were available (385).

Recent studies have begun to evaluate the use of molecular quantification of sex ratios using the sex-specific *P. falciparum* dyneins Pfs377 and Pf alpha-tubulin, though they have yet to provide sufficiently discriminatory data (407). The development of molecular tools that can sensitively quantify male and female gametocytes in epidemiological settings could assist in shedding light on the relevance of clonal diversity and sex ratios in malaria transmission (221, 317, 319, 385).

GAMETOCYTE LONGEVITY

For *P. falciparum*, the mean circulation time per gametocyte in the peripheral blood has been estimated by microscopy as 3.4 to 6.4 days (136, 429), and these estimates were recently confirmed using molecular tools to detect gametocytes (57). However, the true duration of gametocyte carriage depends greatly on the *de novo* production of gametocytes by asexual parasites. An untreated infection may result in asexual parasite carriage for many months (143, 314). Findings of continuous production of gametocytes for several months in natural infections (1, 7, 283) are likely to reflect this ongoing production of gametocytes by (low-density) asexual infections. After cessation of gametocyte production, i.e., after drug or immune clearance of asexual parasites, the duration of gametocyte carriage is determined by the maximum duration of gametocyte sequestration and the maximum circulation time following gametocyte release into the bloodstream. The circulation time of gametocytes in the bloodstream is then affected by the natural decay of gametocytes, antigametocyte immunity, and gametocytocidal drugs. The maximum gametocyte sequestration time was previously estimated to be 12 days (mean, 7.4 days); the maximum circulation time was estimated to be 22.2 days (mean, 6.4 days) (136). As a consequence, gametocyte carriage in individual patients may be as long as 3 to 6 weeks after asexual parasites are cleared (52, 136, 211). A recent modeling exercise based on findings with molecular gametocyte detection tools concluded that gametocytes may persist for well beyond 1 month after clearance of asexual parasites and that drugs clearing immature and mature gametocytes can lead to

substantial reductions of the duration of gametocyte carriage (57). There is currently no evidence for sex-specific differences in longevity or mortality of *P. falciparum* gametocytes (429), although experiments with *P. chabaudi* indicate that female gametocytes may be lost from the circulation at a higher rate than male gametocytes (380).

EPIDEMIOLOGY OF GAMETOCYTES

Patterns in gametocyte carriage in naturally infected individuals define the contributions of different populations to the human infectious reservoir for malaria (i.e., those individuals who are infectious to mosquitoes). By microscopy, gametocytes are invariably detected in fewer than 50% of clinical (25, 55, 147, 279, 412, 455, 532) and asymptomatic (52, 121, 337) *P. falciparum* infections. The question "why so few gametocytes?" has been postulated in reviews by Taylor and Read (469) and by Talman et al. (464). Since these reviews, an increasing body of evidence has indicated that a large proportion of gametocyte carriers remain undetected by microscopy (reviewed in reference 16; see below), and gametocytes may actually be present in the majority of infections, albeit at low densities. However, while prevalence may be increased, the density of gametocytes relative to asexual parasites remains low. Observations from neurosyphilitic patients undergoing malaria therapy suggested that, on average, 1 gametocyte is produced per 156 asexual parasites (136); in most endemic settings, fewer than 5% of the detectable parasites are gametocytes (469). These low levels of sexual-stage parasites are hypothesized as a strategy to evade the induction and/or effects of immune responses against gametocytes or gametes (354, 392, 469) and/or to prevent damage of the mosquito midgut by developing gametes that would negatively influence transmission success (223, 258, 469). In recent years, some new evidence has emerged to suggest that human immune responses can affect the longevity of gametocytes (392), thus supporting the notion that limiting gametocyte production will reduce a stimulus for antigametocyte immune responses.

In the following sections, we discuss the variation in gametocyte carriage between different age groups, transmission settings, and seasons and the possible role for human genetic factors in determining gametocyte carriage.

Age-Dependent Carriage of Gametocytes

The dynamics of *P. falciparum* gametocytes have been described most extensively for symptomatic malaria cases, where waves of asexual parasitemia and accompanying fever are followed by increases in gametocyte prevalence and density (131, 268). Gametocytes can be present in up to 40% of children upon presentation at a health center with symptomatic malaria, according to microscopy results (25, 55, 147, 279, 412, 455, 532). Gametocytes can also be found in asymptomatic individuals, although at a lower rate (52, 121, 337). Broadly, the prevalence of gametocytes in asymptomatic individuals closely follows that of asexual parasites, both by microscopy (52, 121) and by molecular detection tools (337, 413). In areas of high malaria transmission intensity, gametocyte carriage is most prevalent in younger age groups (Fig. 2), who also have the highest prevalences and densities of asexual malaria para-

sites (44, 121, 160, 255, 334, 478). In areas of low transmission intensity, the association between gametocyte prevalence and age is weaker, and smaller proportions of all age groups carry gametocytes (1, 51, 171, 413). While gametocyte prevalence appears to follow a predictable pattern, gametocyte densities do not appear to do so. In gametocyte-positive individuals, the density of gametocytes relative to asexual parasites increases with age. A recent study reported that gametocytes make up <2% of the total parasite population in the youngest children, but this proportion gradually increases to >15% in adults (Fig. 3) (334). This finding was observed with both microscopy and quantitative molecular methods (334) and is in line with previously published patterns (120).

While in many endemic settings children have the majority of high-density parasite infections and clinical attacks, both of which are frequently accompanied by gametocytes, this does not necessarily translate into a majority contribution to the human infectious reservoir. Because of their larger representation in the population, higher likelihood of subpatent untreated infections (337) that are frequently also accompanied by gametocytes (337, 413), and potentially higher level of commitment to the sexual pathway of low-density asexual infections (120, 334), adults contribute considerably to the infectious reservoir. A study that determined the contributions of different age groups to the human infectious reservoir concluded that adults are probably responsible for 28 to 38% of mosquito infections (121), a proportion that could be greater if the larger body size and higher exposure of adults to mosquitoes (357) are taken into account.

Gametocyte Carriage in Relation to Season and Transmission Intensity

Malaria infections and symptomatic malaria episodes are most frequent in the transmission season, usually when mosquitoes are most abundant (following seasonal rains) (321, 336). Two longitudinal studies in areas of markedly different transmission characteristics in Burkina Faso indicated that patterns of gametocyte carriage followed that of asexual parasites, increasing during the peak transmission season (44). However, in both settings, there were some inconsistencies, with relatively high rates of gametocyte carriage during times when asexual parasite carriage was low or declined and stable rates of gametocyte production during the dry season (44). The absolute number of gametocyte carriers is evidently lowest in the dry season, but a larger proportion of the relatively scarce asexual infections in the dry season may produce gametocytes, and the density of gametocytes in these infections relative to that of asexual parasites may be higher in the dry season (46, 120, 264). Although these associations have been described across a range of endemic settings, there is no definitive evidence, as the data are from cross-sectional rather than more informative longitudinal studies. A study where parasite isolates were collected at different time points in the season and submitted to short-term culture to determine their gametocyte-producing capacity did not show a higher production level of gametocytes in the dry season. On the contrary, isolates collected during the months of peak transmission were more likely to produce gametocytes and produced gametocytes at higher densities (39).
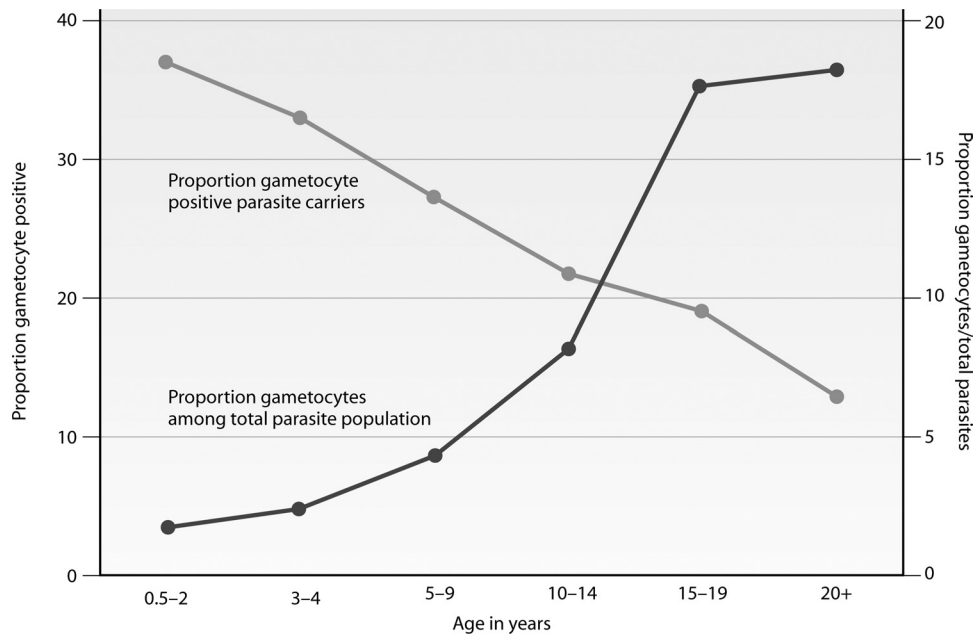
FIG. 3. Age-dependent carriage of gametocytes in an area of high and seasonal malaria transmission in Burkina Faso. Gametocytes were detected by microscopy (thick blood films; screening of 100 fields at a magnification of ×1,000, using oil immersion) in cross-sectional surveys. The proportion of infections with concurrent gametocytes is highest in children and decreases with age. Among infections with gametocytes, the density of gametocytes relative to the total parasite density increases with age (334).

Similarly, there is limited evidence for differences in gametocytogenesis between areas of different transmission intensity or after reductions in transmission intensity. Although gametocyte carriage is generally more common in areas of higher transmission intensity (120, 255, 302) (Fig. 2), the investment in transmission stages may be higher in settings of low endemicity. In Kenya, 18% of the parasite carriers living in an area exposed to 10 infectious bites per person per year (ibpy) harbored gametocytes, compared to 11% of the parasite carriers from an area exposed to 20 to 50 ibpy (120, 303). In Tanzania, gametocyte prevalence was 17% among parasite carriers exposed to ~100 ibpy, compared to 24% among carriers exposed to ~1 ibpy (120, 123).

In the landmark Garki project, gametocyte prevalence increased in some age groups after transmission intensity was successfully reduced through a control program (290). Similarly, interventions with insecticide spraying and mass drug administration (MDA) that reduced asexual parasite rates in Netherlands New Guinea in the 1950s were followed by increased gametocyte production in surviving infections (281). This increase in relative gametocyte production was mentioned as an explanation for the rapid recovery of *P. falciparum* parasite rates after an initial successful reduction (281).

Other Factors Associated with Gametocyte Carriage

Several studies have determined risk factors for gametocyte carriage. These studies were done almost exclusively with individuals visiting a health center for symptomatic malaria, and it is difficult to translate risk factors in this highly selective population to biological triggers for gametocytogenesis. In these symptomatic patients, gametocytes were observed more commonly in individuals with longer durations of infection

(360, 383, 440, 450), those who presented without fever (455, 502), and those with recrudescent infections (360). The association between mixed parasite infections and *P. falciparum* gametocytemia remains unclear. Gametocytemia was reported to be less common in mixed infections with *P. vivax* (360) but more common in infections with *P. malariae* (50, 269).

Host genetic factors may also affect gametocyte production. Lower gametocyte densities were observed in Fulani than in Mossi tribes in Burkina Faso, an observation that may not be explained completely by differences in asexual stage immunity (340). Gametocyte carriage and malaria transmission potential were also related to human genetic variation at the β-globin locus, with a higher gametocyte density and/or higher infection rate for individuals with the HbAS, HbAC, or HbCC genotype than for those with the HbAA β-globin genotype (164, 387). Further evidence of a genetic component of gametocyte carriage was provided by Lawaly et al. (240). They demonstrated a significant heritable contribution to gametocyte prevalence in individuals from Senegal and Thailand. This effect was apparent only in asymptomatic *P. falciparum* infections, not in symptomatic *P. falciparum* cases or *P. vivax* infections.

Coinfections with other pathogens may also influence the epidemiology of gametocytes. Infection with HIV has been associated with an increased susceptibility to malaria infection (150, 212, 512) and with higher malaria parasite densities (496). Antimalarial treatment efficacy may also be reduced in HIV-infected individuals or HIV-infected individuals with lower CD4 counts (38, 212, 497). Although there is currently no evidence for enhanced gametocyte production in HIV-infected individuals, these associations indicate the occurrence of more-frequent, longer, and higher-density asexual parasite carriage in HIV-infected individuals. It is therefore plausible

that, at population levels, HIV infection leads to increased malaria transmission (2, 495).

THE TIP OF THE ICEBERG: SUBMICROSCOPIC GAMETOCYTE DENSITIES

Gametocytes typically circulate at low densities, and microscopy is not a very sensitive tool for detecting all relevant densities of gametocytes. As early as the 1930s, it was realized that mosquitoes can become infected with *P. falciparum* (210, 297, 299) or *P. vivax* (61, 208) after taking a blood meal in which no gametocytes were observed by microscopy. In the last decade, evidence has accumulated that microscopy detects only a fraction of all gametocyte carriers.

Detecting Gametocytes by Microscopy

Longitudinal studies in regions where malaria is endemic frequently observe that microscopically detectable gametocytes are present at one time point and disappear at a follow-up visit, only to reappear later (52, 103, 126). This indicates that gametocytes often circulate at densities close to the threshold of microscopic detection. It has been estimated that routine examination of thick films (typically 100 fields at a magnification of $\times 100$, using oil immersion) results in screening of 0.16 to 1 μl of blood (31, 176, 217), although for reliable detection of a parasite density of 1/μl, a volume of at least 3 μl has to be examined (217). Routine microscopy examining 100 microscopic fields may therefore miss gametocyte densities as high as 20 to 50 gametocytes/μl (119, 217, 292). Gametocytes of nonfalciparum malaria species lack the distinctive crescent shape and resemble other stages of the life cycle; they may therefore go undetected at even higher densities. In line with this low sensitivity of routine microscopy, gametocyte prevalence is commonly increased in studies where the efficiency of examination by microscopy is increased by either increasing the number of examined microscope fields or concentrating parasites prior to screening. Estimates of gametocyte prevalence were increased by 60% when the number of examined fields of a thick film was increased from 20 to 100 (4), by 29% when this number was doubled from 200 to 400 (289), and 3-fold when 1,000 instead of 100 high-power thick blood film fields were examined (98). Use of the quantitative buffy coat technique, in which a 55-μl blood volume is examined by use of acridine orange-coated capillary tubes (453), increased the prevalence of gametocytes from 4.6% to 19.1% (301). Magnetic deposition microscopy (MDM) is used to concentrate erythrocytic-stage malaria parasites that contain hemozoin by exploiting their paramagnetic characteristics using strong magnetic fields (529). MDM makes it possible to screen a larger blood volume, thereby improving the detection sensitivity compared to that of routine microscopy. The use of MDM increased *P. falciparum* gametocyte prevalence in 200 examined thick blood smear fields from 7.3% ($\sim$0.4 μl blood examined quantitatively and up to 4 μl examined qualitatively) to 45% by MDM ($\sim$40 μl blood examined) for symptomatic malaria cases in Papua New Guinea (216). The increasing sensitivities of these methodologies confirm that a large pool of gametocyte carriers are undetected by routine microscopy.

Molecular Gametocyte Detection Tools

Our understanding of the prevalence and relevance of low-density gametocyte carriage has been facilitated greatly by the use of molecular detection tools that can detect, quantify, and characterize gametocytes genetically. Molecular gametocyte detection tools are based on the amplification of RNA that is expressed exclusively in gametocytes (14, 15, 277, 403, 404, 421). RNA is required specifically for gametocytes, as asexual parasites also carry the DNA encoding gametocyte-specific RNA transcripts. *pfs25* mRNA has formed the basis of gametocyte detection and quantification by reverse transcriptase PCR (RT-PCR) (1, 15, 16, 286), quantitative nucleic acid sequence-based amplification (QT-NASBA) (15, 16, 404), and RT loop-mediated isothermal amplification (RT-LAMP) (70). The gametocyte-specific protein gene *pfg377* is also transcribed exclusively in gametocytes (6, 239) and has a similar sensitivity for gametocyte detection to that for *pfs25* (277), but it is more polymorphic than *pfs25* (15). Single-nucleotide polymorphisms and size differences in the *pfg377* transcript allow identification of at least 21 distinct *pfg377* alleles (319) and, thereby, identification of distinct gametocyte-producing parasite clones within single infections (1, 277, 319). These molecular detection tools have sensitivities in the range of 0.02 to 0.1 gametocyte/μl (403), or perhaps as low as 0.002/μl or 2 gametocytes/ml (70). In reality, the sensitivity is limited by the volume of blood that can be collected and processed ethically and practically. Clinical and epidemiological studies that have determined gametocyte carriage by microscopy and RT-PCR or QT-NASBA are summarized in Fig. 4. In five clinical trials that used microscopy and molecular gametocyte detection tools, gametocytes were detected at enrollment in 4 to 26% of the patients by microscopy, but 3- to 10-fold more often by QT-NASBA or RT-PCR (39 to 90%) (7, 55, 279, 403, 412). Similar proportional increases in gametocyte prevalence were observed in the general asymptomatic population living in areas where malaria is endemic. In areas of very low endemicity in Tanzania and Sudan, gametocytes were detected in 0 to 6% of the population by microscopy but in 12 to 52% of the population by molecular tools (1, 314, 413). In areas of more intense seasonal transmission in the Gambia and Burkina Faso, gametocyte prevalences ranged from 3 to 30% by microscopy and from 49 to 70% by use of molecular tools (319, 337, 340). These findings indicate that gametocyte carriage is consistently underestimated by microscopy. A recent meta-analysis of microscopy and molecular methods for asexual infection showed a 2-fold higher prevalence by PCR (325), but for gametocytes, this figure may be as high as 10-fold.

The upshot of this is that all malaria infections should be considered plausible gametocyte producers. Studies comparing Pfg377 alleles in RNA and genomic DNA extracted from the same infection tend to support this and indicate that although there is variation in gametocyte production between strains (1), most infections produce gametocytes at some time point (314, 319). In this way, chronic asymptomatic infections may also sustain gametocyte production in the absence of mosquitoes during the dry season (1, 13, 314).

The number of gametocyte-positive days and the area under the gametocyte density-time curve (455) are more informative representations incorporating the temporal element in describ-
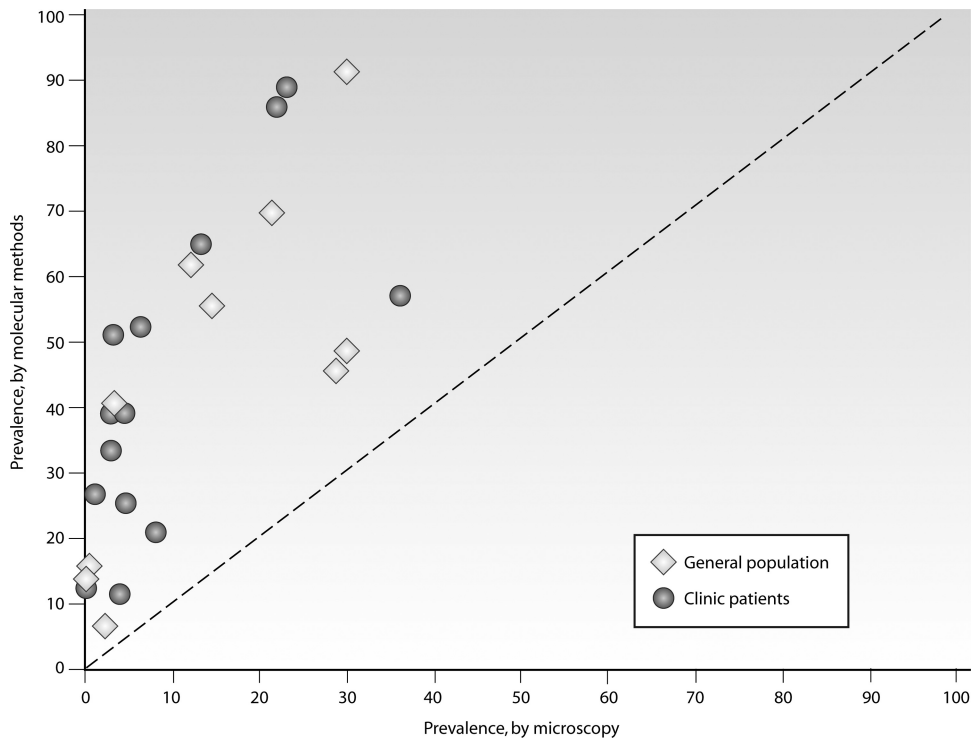
FIG. 4. Gametocyte carriage by microscopy and molecular detection tools. Gametocytes were detected by microscopy, typically screening 100 microscopic fields, and by *pfs25*- or *pfg377*-specific RT-PCR, LAMP, or QT-NASBA. Samples were derived from the general population in Burkina Faso, Tanzania, the Gambia, and Thailand (open diamonds) (70, 263, 319, 335, 337, 340, 413) and from people attending clinics in Kenya, Tanzania, Sudan, and Vietnam (closed circles) (1, 55, 140, 256, 279, 314, 412), mostly children participating in clinical trials. (Reproduced from reference 334 with permission.)

ing the reservoir of (submicroscopic) gametocyte carriage. These findings are of considerable epidemiological relevance, as gametocytes at submicroscopic densities can infect mosquitoes (335, 402) and contribute to malaria transmission, as we discuss below.

GAMETOCYTES AND MALARIA TRANSMISSION

Gametocytes are obviously essential for malaria transmission, but their presence in the peripheral blood does not necessarily equate to infectivity. The first step in the process is that a mosquito feeds on a gametocytemic host. This is determined by global and local factors influencing mosquito abundance (433), household and behavioral characteristics that influence mosquito exposure, and the attractiveness of humans to mosquitoes. The attractiveness of humans to mosquitoes is influenced by numerous factors, including body size (357) and human odor components (368). An increased attractiveness of gametocyte carriers to mosquitoes was suggested by Koella and colleagues (238); this finding requires confirmation in other settings but, if proven, would have implications for malaria control. After a mosquito has ingested gametocytes from a human host, the chance of infection depends on a combination of gametocyte maturity, concentrations of male and female forms, and human and mosquito immune responses. The infectiousness of gametocytes can be confirmed by offering gametocytes to *Anopheles* mosquitoes, followed by the detection of oocysts (300, 324, 394) or sporozoites (226, 394) in mosqui-

toes. Transmission experiments can involve direct feeding of mosquitoes on the skin of infected individuals (393) or offering a gametocytemic blood meal to mosquitoes through a membrane (300, 490) (see "Evidence for Naturally Acquired Transmission-Blocking Activity"). The former is closer to the natural situation, may be more efficient (111), and avoids some gametocyte sampling issues that are discussed below. However, the ethical preference in most settings appears to be membrane feeding experiments.

Gametocyte Density and Mosquito Infection Rates

Despite their close representation of a real-life scenario, membrane feeding experiments have almost unequivocally shown discrepancies between the presence of gametocytes and their infectiousness (47, 101, 170, 335, 402, 423). Thus, while there is an overall positive association between gametocyte density and the mosquito infection rate (59, 335, 402), the association is not very strong and is most variable at low gametocyte concentrations (490). High densities of gametocytes do not necessarily result in mosquito infection (151, 171, 402), and conversely, low densities do not exclude infectiousness (101, 297, 335, 402). The failure of high gametocyte densities to result in mosquito infection is partly the result of immaturity of gametocytes (183, 243). As stated above, after their release into the circulation, *P. falciparum* gametocytes require another 2 to 3 days to become infectious to mosquitoes (243, 429). However, more puzzling is low-density infectivity and how
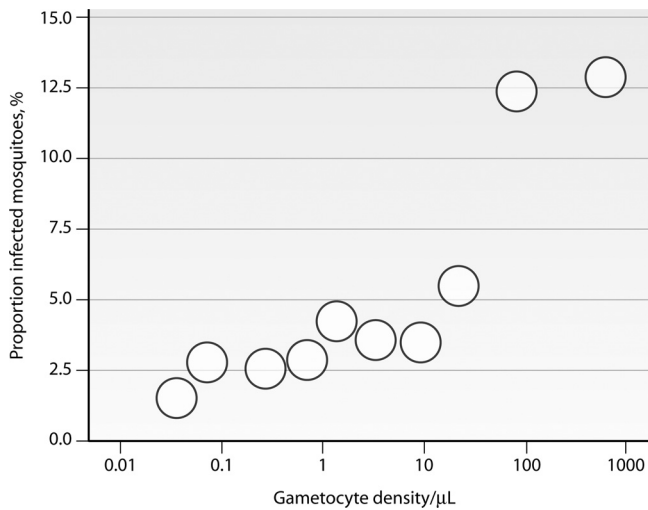
FIG. 5. Gametocyte densities and mosquito infection rates. Data from two transmission studies of asymptomatic children in Burkina Faso (335) and symptomatic children 2 weeks after antimalarial treatment in Kenya (55, 402) were combined. Both studies determined gametocyte carriage by Pfs25-specific QT-NASBA and offered venous blood to mosquitoes in a membrane feeding assay (see "Evidence for Naturally Acquired Transmission-Blocking Activity") to determine the proportion of infected mosquitoes. Circles indicate aggregated data, grouped in 10 categories of similar gametocyte densities.

some infections declared *P. falciparum* gametocyte free by routine microscopy were found to be infectious to mosquitoes in studies in the 1950s (210, 297, 299). Indeed, infectious reservoir determination by xenodiagnosis, where the infectiousness of humans is determined by mosquito experiments, was consequently considered to be more reliable than microscopic observations of gametocytes (297, 298). Despite these early observations, very few studies have since been conducted to determine the infectiousness of individuals to mosquitoes, independent of whether or not gametocytes were observed microscopically. A study in Burkina Faso indicated that the infectiousness of microscopically confirmed gametocyte carriers may be similar to that of individuals who are apparently gametocyte free by routine microscopy (45), while a study in Papua New Guinea did not find an important contribution of submicroscopic gametocytes to malaria transmission (171). Two studies that related gametocyte densities to mosquito infection rates by membrane feeding experiments using molecular detection tools indicated that mosquito infection is common below the microscopic threshold for gametocyte detection (Fig. 5). These field observations were confirmed by serial dilutions of cultured gametocytes that were offered to mosquitoes under laboratory conditions (402). In field and laboratory experiments, mosquitoes became infected after feeding on a meal containing gametocytes at a concentration below 1 gametocyte/μl. This finding highlights an important gap in our current understanding of malaria transmission. A mosquito blood meal is 2 to 3 μl, on average, and should contain at least one male and one female gametocyte to result in infection. Although the proportion of males may increase at low densities (344, 378), sex ratios are typically strongly female biased, with 3 or 4 female gametocytes for every male gametocyte (215, 385,

386, 445, 464). This makes the observed efficiency of transmission at low gametocyte concentrations even more intriguing. Unknown mechanisms may facilitate an aggregation of gametocytes that favors the encounter of males and females (352). One of these mechanisms may be a transmission-enhancing effect of antigametocyte antibodies (152, 195, 349), possibly directed against currently unknown antigens (459, 491). Other possibilities include preferential localization of gametocytes in the dermis prior to ingestion or enhanced chemotraction once in the midgut. Recently, the formation of membranous cell-to-cell connections was described for activated *P. falciparum* gametocytes. Up to 11 filaments were found on the surfaces of male and female gametes within minutes of gametocyte activation; no filaments were detected on non-activated gametocytes. It was hypothesized that these "nanotubes" have adhesive properties and play a role in locating, connecting, and pooling gametocytes and gametes within a blood meal, favoring successful fertilization (391). This is a field for further research to explore factors related to tropism and gametocyte sex ratios at low gametocyte concentrations.

Sampling Issues: When and Where Do Mosquitoes Bite?

Despite the value of membrane feeding assays, their current use has failed to answer some long-standing questions. Membrane feeding experiments are typically performed in a cross-sectional approach, sampling each individual once, either at enrollment or at a fixed time point after treatment. This approach is justifiable because of the ethical issues surrounding repeated venous bleeds on (young) gametocyte carriers. It is difficult, however, to appreciate the impact of interventions that reduce the duration of gametocyte carriage if observations are restricted to a single time point, given the potential for temporal fluctuations in infectiousness (183, 208, 270). A temporal association where peaks in infectiousness coincide with peaks in vector biting would be attractive from an evolutionary point of view, but this association remains to be proven. In transmission experiments conducted at 12- or 15-hour intervals, no significant fluctuations in the infectivity of *P. falciparum* gametocytes were observed (64, 161), although 48-hour fluctuations in exflagellation rates of gametocytes were described (190). A study of five Gambian infants suggested that gametocytes time their maturation in the peripheral blood according to regular daily temperature fluctuations in the host to coincide with peak vector densities (190), a hypothesis that was not supported by a study of eight Tanzanian children where gametocytes showed elevated peripheral densities between 1530 and 1930 h, well before the peak biting time of 0030 to 0330 h described for the local mosquito vector (257). Although it was suggested that due to the unknown temporal association between peaks in gametocyte densities in finger-prick samples and subdermal capillaries, gametocyte densities at mosquito sampling sites may still peak at the correct time for maximal transmission (459), the periodicity of malaria gametocytemia remains unresolved (156).

These examinations of the periodicity of gametocytemia and infectivity have highlighted another crucial hole in our knowledge, namely, the association between gametocyte densities in venous blood samples (typically used in transmission experi-

ments), finger-prick blood samples (typically used for microscopic detection), and subdermal capillaries. The latter, where mosquitoes take their blood meal, is the real site of interest. It is conceivable that infective gametocytes actively seek subdermal sites to enhance their transmission potential. Subdermal clustering has been hypothesized to be related to the crescent shape of *P. falciparum* gametocyte-infected erythrocytes (304), and clustering of gametocytes in mosquito blood meals suggests some additional mechanism for aggregation (352). A unique study from 1952 is often cited in this respect and provides direct, albeit incomplete, evidence for preferential sequestration in the subdermal capillaries. In this study, 200 individuals were examined for different stages of malaria parasites by skin scarification (91), a method with a sensitivity similar to that of skin snips for detecting *Onchocerca volvulus* infections (315). The prevalence of gametocytes in skin samples was 3-fold higher than that in thick smears from finger-prick samples from the same area (91). Although this finding was confirmed by the same research group in a different setting (488) and corroborates findings for rodent malaria showing that the blood meal the mosquito engorges directly from the rodent contains more gametocytes than a blood sample from the animal's tail (157), evidence is incomplete.

ANTIMALARIAL DRUGS AND GAMETOCYTEMIA

All antimalarial drugs that successfully clear asexual parasitemia will limit the duration of gametocyte carriage by preventing or interrupting the production of gametocytes from asexual parasites; the speed at which asexual parasites are cleared will determine how quickly gametocyte production is disrupted after the initiation of treatment. The impact of antimalarial drugs on *P. falciparum* gametocytes depends on the type of antimalarial drug and the level of drug resistance.

Effects of Drugs on Gametocytemia

Early-stage *P. falciparum* gametocytes are susceptible to most commonly used antimalarial drugs (77, 426, 510). Nevertheless, antimalarial treatment is not automatically followed by a decrease in gametocyte carriage. *P. chabaudi* and *P. falciparum* have been documented to increase their investment in gametocytes in response to subcurative drug therapy (71, 72), and after therapeutic doses of antimalarials, an initial increase in gametocyte carriage may also be observed. The use of sulfadoxine-pyrimethamine (SP) is associated with an increase in gametocyte carriage after treatment (55, 166, 167, 383, 440, 448, 466, 502). However, studies using molecular gametocyte detection tools suggest that this increase in microscopic gametocyte prevalence after treatment may simply reflect an increase in the density of gametocytes circulating pretreatment to a level beyond the microscopic threshold for detection (7, 403). The increase in gametocytemia after treatment is a likely result of the efflux of sequestered gametocytes rather than *de novo* gametocytogenesis (77). An efflux of not fully mature gametocytes may explain the finding of relatively low infectivity of gametocytes 1 week after SP treatment, despite high densities (30, 466), although this could also be related to sporonto-

cidal effects of persisting drug concentrations (229). In general, gametocytemia after treatment is a composite of (i) the ongoing production of gametocytes after initiation of treatment, possibly enhanced by drug-induced stress by slow-acting antimalarials; (ii) the release of sequestrated gametocytes; (iii) the activity of drugs against immature gametocytes, preventing their release into the circulation; and (iv) the activity of drugs against mature gametocytes, clearing the circulating gametocyte population. It is impossible to disentangle these effects in data from field trials. A high rate of gametocyte development after initiation of treatment (i.e., samples are gametocyte negative at enrollment but positive after treatment) can be interpreted as a limited activity of a drug against immature gametocytes; a long persistence of gametocytes that were detected at enrollment can be interpreted as a limited activity against mature gametocytes. The effects of several antimalarial drugs against gametocytes are summarized in Table 2. Non-artemisinin combination therapy (ACT) drugs, especially SP and chloroquine (CQ), have partial activity against immature gametocytes. This is reflected by a large proportion of treated individuals developing mature gametocytes in the week following treatment. SP, CQ, amodiaquine (AQ), and quinine have no or very limited activity against mature gametocytes (55, 77, 225, 429, 448), resulting in a large proportion of individuals who remain gametocyte positive after treatment (Table 2). In general, mature gametocytes may persist for several weeks after successful clearance of asexual parasites (55, 136, 211, 324, 429, 446); molecular gametocyte detection tools indicate that gametocyte carriage is likely to persist for more than 1 month after successful clearance of asexual parasites (57). Drugs that actively clear gametocytes may therefore increase the impact of drug treatment on malaria transmission.

The 8-aminoquinolines (pamaquine, primaquine [PQ], quinocide, and tafenoquine) occupy a unique position in their effects against mature gametocytes (510). PQ is the only drug of this group that has been used extensively in patients and is currently available for wide-scale use. The exact mechanism of PQ or its effector metabolites is unknown (401, 487, 510), but PQ treatment severely disrupts the metabolic processes of plasmodial mitochondria (197). PQ clears mature gametocytes of *P. falciparum* (75, 211, 363, 412, 471, 510), resulting in a shorter duration of posttreatment (submicroscopic) gametocyte carriage (57, 363, 412, 438). The clinical use of PQ has been limited by its hematological toxicity, particularly in individuals who have glucose-6-phosphate dehydrogenase (G6PD) deficiency (65, 381, 412, 471, 510, 521). The consequences for public health interventions are discussed in more detail in the section on gametocytes and malaria elimination strategies. The safety and gametocytocidal activity of alternative drugs from the same family as PQ (the 8-aminoquinolines), such as bulaquine (CDRI 80/53) (162, 163) and tafenoquine (WR-238605) (99, 100, 371, 487), require further investigation. Methylene blue, which together with quinine served as a structural starting point for the development of 8-aminoquinolines (153, 406), was recently suggested as an alternative safe gametocytocidal component of drug combinations (103, 531) and may be a promising gametocytocidal component of combination therapy.

TABLE 2. Impact of antimalarial drugs on development and persistence of *P. falciparum* gametocytes

Drug(s)[a]	Median gametocyte prevalence (% [IQR]) on day 7 for those who were gametocyte free on day 0	Median gametocyte prevalence (% [IQR]) for those who were gametocyte carriers on day 0		References
		Day 7	Day 14	
Non-ACT drugs				
CQ	35 (26–44)	76 (67–88)	60 (43–83)	4, 7, 127, 183, 333, 367, 383, 384, 440, 466
SP	55 (47–62)	87.1 (78.3–97.5)	71.4 (54.0–83.0)	7, 55, 93, 183, 287, 333, 367, 383, 440, 449, 466, 502
AQ	15 (8–23)	69 (58–74)	37 (22–46)	93, 440, 442, 456
SP + CQ	39 (38–44)	89 (58–100)	60 (33–74)	93, 183, 375, 461, 489
SP + AQ	10 (7–19)	56 (33–78)	33 (24–43)	55, 117, 144, 146, 287, 444, 454, 526, 530
ACT regimens				
SP + AS	8 (5–18)	38 (25–60)	18 (17–75)	55, 185, 287, 412, 466, 504
AQ + AS	5 (0–13)	35 (30–40)	13 (0–41)	74, 103, 117, 146, 147, 185, 189, 339, 454, 526
MQ + AS	1 (1–3)	13 (3–30)	1 (0–9)	11, 12, 146, 169, 205, 242, 266, 437, 451, 489, 532
AL	2 (1–4)	20 (18–41)	16 (4–19)	55, 74, 117, 144, 146, 147, 205, 213, 242, 266, 279, 339, 353, 374, 444, 461, 489, 527, 530, 532
DHA-PPQ	4 (3–5)	33 (15–49)	23 (17–29)	11, 12, 169, 189, 213, 279, 374, 437, 527, 530, 532
ACT-PQ regimen				
SP + AS + PQ	0 (0–0)	20 (0–40)	0 (0–0)	412, 506

[a] ACT, artemisinin combination therapy; CQ, chloroquine; SP, sulfadoxine-pyrimethamine; AQ, amodiaquine; AS, artesunate; MQ, mefloquine; AL, artemether-lumefantrine; DHA-PPQ, dihydroartemisinin-piperaquine; PQ, primaquine. Only drug combinations for which we were able to obtain raw data for two or more trials were included.

Gametocytes as an Early Marker for Spread of Drug Resistance

Antimalarial treatment failure has been associated with increased gametocyte carriage after treatment (360). CQ treatment failure is followed by an increased gametocyte prevalence and density (4, 188, 383, 440, 448, 476) and, as a consequence, increased posttreatment malaria transmission (188, 383). Similarly, posttreatment gametocytemia is also increased after treatment failure of SP alone (52, 53, 440, 476) or in combination with CQ (476). The rapid spread of CQ and SP resistance may be due to some degree to high gametocyte carriage after treatment and treatment failure (26).These associations are explained only partly by ongoing gametocyte production by asexual parasites that survive the failing antimalarial treatment. Not all genetically resistant parasites will result in treatment failure, which depends on parasite sensitivity, drug concentration, and host immunity (116, 142, 175, 474). Even if resistant parasites are cleared successfully, they appear to have a transmission advantage over fully sensitive (wild-type) parasite strains. Mutations in the *Pfcrt* and *Pfmdr1* genes, encoding CQ resistance, were associated with a higher gametocyte density and subsequent malaria transmission after CQ monotherapy (184, 460) or SP-CQ combination treatment (183) (Fig. 6). Similarly, mutations in the *dhfr* and *dhps* genes, encoding SP resistance, were related to increased gametocyte carriage following (30, 275, 288) or even prior to (288) treatment and to a longer duration of gametocyte carriage than that for wild-type parasites (25). A study by Méndez and colleagues showed that malaria transmission was enhanced 10-fold for parasites with low levels of resistance compared to fully sensitive parasites, despite high SP cure rates in Colombia (274). This may be explained partly by a longer parasite clearance time for *dhfr/dhps* mutant parasites allowing asexual parasites to differentiate into gametocytes (275), although other factors may also be important (25, 275). In addition to this transmission advantage of mutant parasites in the human host, through a higher gametocyte prevalence or density (184, 275), a selective advantage in the mosquito midgut has been suggested for *Pfcrt* mutants (184). In an area of low endemicity in South Africa, gametocyte carriage was highest for isolates with quintuple mutations in *dhfr* and *dhps* genes, intermediate for those with fewer mutations, and lowest for wild-type infections (25). In contrast, a study in an area of intense transmission in Kenya with high levels of SP resistance indicated that there may be no increase in gametocytemia for parasite strains with ≥3 mutations in the *dhfr/dhps* genes compared to those with a double mutation (320).

Given this relationship with resistance-associated mutations, gametocytes and posttreatment malaria transmission may serve as effective early parasitological indicators of reduced drug sensitivity (25, 183, 274). Two examples of the use of gametocyte carriage as a warning system for an increase in the prevalence of parasite resistance have been reported. In South Africa, the prevalence, density, and duration of gametocyte carriage after treatment increased between 1998 and 2002; in this period, the prevalence of *dhfr/dhps* mutant strains increased, although the cure rate of SP remained consistently high, with >90% successful parasite clearance (25). In Sri Lanka, the prevalence of gametocyte carriage in parasitemic individuals increased considerably after CQ resistance emerged. After control measures were implemented to contain CQ resistance, this gametocyte prevalence decreased again (188).

Combination Therapy and Gametocytes

ACT has been advocated widely as first-line therapy for treating uncomplicated malaria episodes since the early 2000s (327). Treatment with artemisinin derivatives results in rapid reductions in parasitemia (3, 363, 509), and treatment with
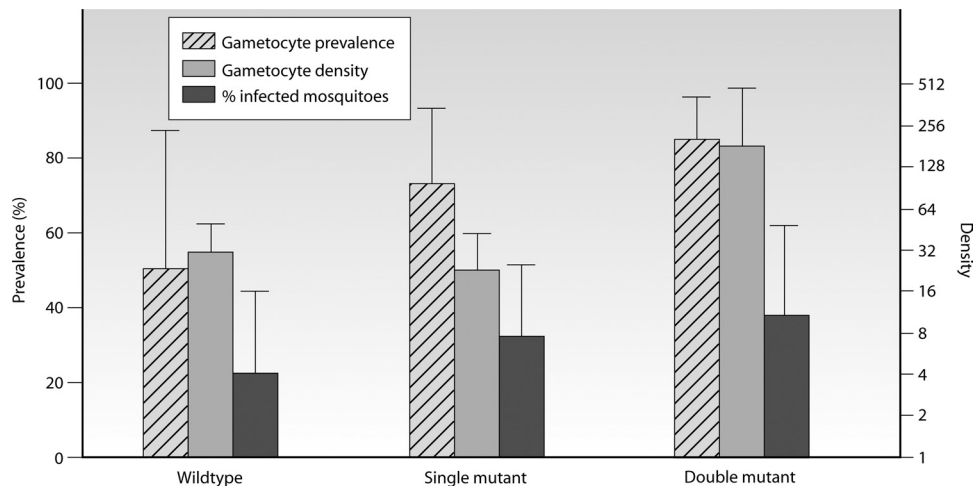
FIG. 6. Gametocytes as an early indicator of parasite resistance. The data presented are from a study in the Gambia where children were treated with chloroquine (184). The figure presents gametocyte prevalence by microscopy (thick blood films; screening of 100 high-power fields at a magnification of ×1,000, using oil immersion) on day 7 after treatment (left *y* axis; error bars indicate the upper limit of the 95% confidence interval), median gametocyte density by microscopy in gametocyte carriers on day 7 after treatment (right *y* axis; error bars indicate the upper limit of the interquartile range), and the mean proportion of infected mosquitoes determined by membrane feeding assays (right *y* axis; error bars indicate the upper limit of the 95% confidence interval). Wild type, no mutations detected at enrollment in two genes related to chloroquine resistance, *P. falciparum* multidrug resistance gene 1 (*Pfmdr1* 86Y) and *P. falciparum* chloroquine resistance transporter (*Pfcrt* 76T); single mutant, mutation detected in either *Pfmdr1* or *Pfcrt*; double mutant, mutation detected in both *Pfmdr1* and *Pfcrt*.

ACT is associated with lower rates of gametocyte carriage (55, 127, 184, 324, 361, 403, 419, 461, 466) and posttreatment malaria transmission (55, 127, 184, 324, 461, 466). Artemisinins are highly active against immature gametocytes (96, 235, 363, 466, 510), destroying a substantial proportion of those sequestered in the microvasculature, resulting in a significant reduction in the release of mature gametocytes into the peripheral blood (324). As shown in Table 2, this is reflected by a low gametocyte prevalence on day 7 for individuals who were gametocyte free at enrollment. It is assumed that artemisinins have little or no activity against mature gametocytes (96, 235, 348). This resulted in persisting gametocytes on days 7 and 14 in individuals who already harbored gametocytes at enrollment (Table 2). The persistence of mature gametocytes after ACT translates to posttreatment malaria transmission (55, 461, 466).

The gametocytocidal drug PQ lacks useful activity against *P. falciparum* asexual parasites and, presumably, early gametocytes (363) and should therefore be combined with an effective schizonticidal drug, preferably in a drug combination that also clears early gametocytes, such as an efficacious ACT regimen (363, 412, 438, 510) (Table 2).

IMMUNE RESPONSES TO GAMETOCYTES AND TRANSMISSION-BLOCKING IMMUNITY

Immune responses against gametocytes can act in three ways. First, immune responses may interfere with gametocyte sequestration and thereby enhance gametocyte clearance before they become mature and infectious. Second, circulating gametocytes may actively be cleared, also resulting in a lower density of gametocytes in the human bloodstream. Third, responses against parasite antigens that are expressed in the gamete or postgamete stages may reduce the transmission of gametocytes. Evidence for the first two mechanisms is cur-

rently inconclusive but is discussed in the following section; the third mechanism receives attention in the two subsequent sections.

Evidence for Immune Responses Influencing Gametocyte Concentrations

Gametocyte-specific and nonspecific parasite adhesion molecules may elicit immune responses that interfere with gametocyte maturation. Since the adhesion phenotype of young gametocytes appears indistinguishable from that of trophozoites (106), it is plausible that cross-reactive asexual- and sexual-stage immunity affects early-stage gametocytes. For PfEMP-1, it has been suggested that immunity may regulate the densities of both gametocytes and trophozoites by antibody-dependent cell-mediated cytotoxicity reactions (354) and prevention of sequestration (90). The scavenger receptor CD36 was found to mediate the uptake of erythrocytes infected with early-stage gametocytes (I and IIa) by nonopsonic phagocytosis by monocytes and macrophages (436), as was previously shown in the phagocytosis of trophozoites and schizonts (267). In addition to proteins involved in erythrocyte adhesion, molecules on the surfaces of erythrocytes infected with mature gametocytes may also elicit immune responses and may be involved in the clearance of circulating gametocytes. These molecules may be involved in spatial and temporal tropism, although their existence so far is speculative (459). There is some evidence for antibodies that recognize erythrocytes infected with mature (stage V) gametocytes, and the presence of these antibodies is associated with lower gametocyte densities in naturally infected individuals (392). This is supported by earlier epidemiological observations of protection against gametocytemia among individuals whose sera showed reactivity to gametocytes in immunofluorescence assays (19). In addi-

tion, the age-dependent recognition of gametocyte-infected erythrocytes, which closely resembled that of antibodies to the surfaces of trophozoites (354), could explain the negative association between the duration of gametocyte carriage and age (52, 131). There is, however, no conclusive evidence of the immune clearance of circulating gametocytes. Most *in vitro* studies on clearance of parasite-infected erythrocytes by phagocytosis (48, 88, 267, 483) or by destruction via free radicals or cytokines (159, 523) have focused on asexual parasites, and several studies suggest that intraerythrocytic gametocytes may not be susceptible to phagocytosis (194, 425). If, on the other hand, gametocyte surface antigens can be identified and their role in gametocyte clearance can be established, these would have potential for vaccine development (459).

Immune Responses Influencing Gametocyte Infectivity

Independent of immunity affecting gametocyte densities, gametocyte infectiousness can also be influenced by immune responses. There is some evidence for nonspecific immune effects on infectivity; for example, cytokines such as TNF-α can inactivate gametocytes in the presence of white blood cells (218, 312) and may be related to the lower infectiousness of gametocytes during paroxysm (165). Early evidence for specific transmission-blocking immune responses came from experiments with avian malaria *P. gallinaceum*, where transmission was reduced 95 to 99.9% after vaccination with formalin-treated or irradiated gametes (81, 181).

Sexual-stage parasites are most vulnerable when they leave the erythrocyte after their ingestion by mosquitoes. Inside the mosquito midgut, extracellular gametes are exposed to mosquito immune responses (reviewed in references 43, 282, and 511) and to human leukocytes, antibodies, and complement. Both human antibody-mediated and cell-mediated effector mechanisms may play a role in transmission-blocking immunity (467). Human leukocytes remain active and are capable of surviving for several hours in the mosquito midgut. There, human leukocytes can phagocytize extracellular activated and exflagellating gametocytes (425), although conditions in the mosquito midgut may reduce the activity of leukocytes to a level that is unlikely to hinder malaria transmission (425). Opsonization of gametes by antibodies against sexual-stage proteins is needed for successful phagocytosis inside the mosquito midgut (246). Additionally, the presence of human leukocytes in a blood meal may increase the transmission-reducing activity (TRA) of antibodies (244, 246).

Antibodies can be directed against a large number of sexual-stage antigens and appear to play a larger role in transmission-blocking immune responses than cell-mediated responses do. Proteomic information gathered after the availability of the *P. falciparum* genome (155) and two rodent malaria genomes (80, 182) led to the identification in sexual-stage parasites of a large number of predicted proteins with cell-adhesive motifs (149, 239, 417) that are accessible to human antibodies. The expression, function, and transmission-blocking potential of sexual-stage proteins were recently reviewed (358). Here we concentrate on proteins that are known to be recognized by naturally infected individuals and/or are major candidates for malaria transmission-blocking vaccines (MTBV). Antigens involved in transmission-blocking immune responses can be categorized as those that act before zygote formation (prefertilization antigens) and those that affect the subsequent development of mosquito stages (postfertilization antigens) (Table 1).

Prefertilization antigens. Two gamete-specific surface proteins have been shown to be involved in gamete-gamete interaction and male fertility: HAP2 (GCS1) (199, 251) and P48/45 (493). The HAP2 protein is present in many plant, protist, and animal genomes (294, 518) and has been shown to be essential for fertilization. Studies of rodent malaria *P. berghei* have indicated that HAP2 is localized on male gametocytes and microgametes (251) and that HAP2 is not necessary for adhesion of gametes but is essential for the fusion of gamete surface membranes (251). P48/45 belongs to a family of proteins that are characterized by six positionally conserved cysteines (6-Cys proteins) (494). All 6-Cys proteins contain a signal peptide, and most contain a putative glycosylphosphatidylinositol (GPI) anchor sequence, making it plausible that they are located on the surfaces of parasites or invasion-associated organelles (473, 498). P48/45 protein is detectable on the parasite membrane surface beginning in gametocyte stages and continuing up to the erythrocyte-emerged gamete and zygote stages within the mosquito midgut (233, 382, 499, 500). P48/45 is essential for the attachment of male microgametes to fertile female macrogametes in *P. berghei* (493). In addition to the Pfs48/45 gene, two more 6-Cys protein genes are expressed exclusively on *P. falciparum* gametes (Pfs47 and Pfs230 genes), suggesting a role in gamete recognition and fertilization (228, 494, 498, 499, 515). Pfs47 is a female-specific paralogue of Pfs48/45 located on the surfaces of female gametes following their emergence from erythrocytes, but it does not appear to be crucial for fertilization (498). Pfs230 is synthesized early in gametocyte development, similar to Pfs48/45 (233, 499), and is derived from proteolytic cleavage of a 360-kDa precursor molecule into 300- and 35-kDa or 307- and 47-kDa forms (67, 369, 500). The 300- and 307-kDa membrane-associated Pfs230 fragments that do not contain a GPI anchor remain on the gamete surface (500, 516) as a complex with Pfs48/45 (139, 232, 233). Pfs230 may be involved in protection of the parasite from the contents of the blood meal, in fertilization (514), or in the formation of exflagellation centers (137). Antibodies against Pfs230 can reduce oocyst development (369, 514) by lysis of gametes in a complement-dependent manner (196, 369, 376, 388). The presence of complement is essential for the transmission-blocking activities of Pfs230. Mutant parasites lacking Pfs48/45 or Pfs230 have reduced but not completely abrogated transmission potential (139, 493, 514), suggesting a possible redundancy of proteins and the presence of paralogous proteins that may compensate for their loss (498), as shown for P25 and P28 (477).

Postfertilization antigens. P25 and P28 are expressed on the surfaces of gametes after their emergence from erythrocytes, and this expression continues until ookinete formation (358, 418, 499). There is no expression of these proteins during the parasite's life cycle in humans. This has the advantage for the parasite that the protein is not subject to immune selection pressure in the human host (84, 484) and the disadvantage for vaccine development that boosting of immune responses by natural exposure to antigen is unlikely. P25 and P28 are structurally similar proteins that have been described for *P. falciparum* (130, 468), *P. vivax* (484), and several nonhuman malaria

species. Work with *P. berghei* has shown that the antigens play partially redundant roles in ookinete survival in the mosquito midgut, mediation of ookinete entry into midgut epithelial cells, and oocyst formation (28, 477). Double-knockout P25/P28 *P. berghei* parasites showed a greater reduction in oocyst formation than single-knockout parasites (477), suggesting that both target antigens may have to be included in transmission-blocking vaccines. Fusion proteins of Pfs25 and Pfs28 can induce potent transmission-blocking antibodies in mice (168). A strong association between anti-Pfs25 antibody concentration and a reduction in oocyst numbers was observed for mice, rabbits, monkeys, and humans (95). Encouragingly, antibodies against Pfs25 elicited in mice resulted in complete interference with oocyst development in four different field isolates of *P. falciparum* (10), and immunization of mice with protein conjugates of Pfs25 demonstrated that it is possible to elicit long-lasting transmission-blocking activity (231).

Evidence for Naturally Acquired Transmission-Blocking Activity

Naturally acquired transmission-reducing immune responses can be assessed in experiments where mosquitoes are offered gametocytes from culture or from naturally infected individuals in the presence or absence of a test serum (125, 300). In the standard membrane feeding assay (SMFA) (Fig. 7), *in vitro* cultured gametocytes are mixed with red blood cells and human serum and fed through membrane feeders to laboratory-reared uninfected *Anopheles* mosquitoes (355). The level of TRA is determined at approximately 1 week postfeeding, when the ratio of the number of oocysts developed in mosquito midguts feeding on test sera is compared to that in mosquitoes feeding on control sera (245, 490). With strict protocols to minimize the influence of day-to-day variations in transmission efficiency (490), the SMFA forms the gold standard for assessing TRA. The direct membrane feeding assay (DMFA) (Fig. 7) is an alternative for the SMFA that can be conducted in the field. In the DMFA, blood samples from naturally infected gametocyte carriers are fed to mosquitoes in the presence of autologous plasma or control serum, after a washing step (125, 300, 472). Advantages of the DMFA include the use of (i) a wide variety of parasite strains that are naturally circulating in areas of endemicity, not relying on a limited number of laboratory-adapted gametocyte-producing parasite strains; (ii) gametocyte densities found in natural infections; and (iii) locally caught and reared mosquitoes. The DMFA may more realistically represent natural infectivity, though a considerable disadvantage is that it is much less standardized than the SMFA. There is a positive but imperfect correlation between outcomes when samples are tested in both assays (125, 300). Both DMFA and SMFA are hampered by the labor-intensiveness of the assays (389), typically requiring the dissection of more than 20 mosquitoes per experiment to be statistically robust.

Setting these difficulties aside, DMFA has shown the presence of TRA in endemic populations through a combined analysis of experiments from the Gambia, Kenya, and Cameroon. In all studies, the proportion of infected mosquitoes and total oocyst burdens were higher when mosquitoes were fed in the presence of control serum than with autologous semi-

immune plasma (59). Naturally acquired TRA has been associated with immune responses to both Pf230 and Pfs48/45. A strong association between TRA and anti-Pfs230 antibodies was observed in Gambian (122, 125, 195), Tanzanian (49, 58), and Papuan/Papua New Guinean (54, 172) sera, but no statistically significant association was found for sera from Cameroon (300, 491) or Sri Lanka (359). Similarly, antibodies against Pfs48/45 were positively associated with TRA in most (49, 54, 58, 122, 125, 300, 389, 491), but not all (172, 359), studies. A number of individuals show TRA but have no demonstrable antibodies to either Pfs48/45 or Pfs230 (49, 58, 122), suggesting that other antigenic targets of TRA exist. As an adjunct to this, further questions remain as to the strain or isolate specificity of TRA. One study testing a panel of sera from Cameroon and the Gambia found sera to have variable TRA against different gametocyte isolates from naturally infected individuals (128). It is unclear if this was due to antigenic variation in parasites, though sequence variation in both Pfs48/45 and Pfs230 appears limited (102, 124). Antibody concentration is clearly important. While high concentrations of sexual-stage antibodies can reduce transmission, low concentrations have been associated with transmission enhancement in *P. vivax* (152, 349) and *P. cynomolgi* (311), but not *P. falciparum* (195, 356, 491).

TRA is observed in young children (122, 196), in individuals exposed to low-level transmission (58, 359), and in individuals shortly after (but not before) migrating from an area where malaria is not endemic to an area where it is endemic (54). These findings suggest that TRA may be relatively short-lived and may depend on recent exposure to gametocyte antigens, a hypothesis that is further supported by observations of higher prevalences of TRA toward the end of the peak transmission season and in children with a longer recent exposure to gametocytes (59). Similarly, the observation that TRA may decrease rather than increase with increasing age of Gambian children (122) could be explained by a longer recent exposure to gametocytes in the youngest children (Fig. 2) (51).

Current Status of Transmission-Blocking Vaccines

As a consequence of the renewed interest in malaria elimination, MTBV have moved to the center stage of malaria vaccine research. MTBV candidates that are currently in the most advanced stage of development for *P. falciparum* are based on the prefertilization proteins Pfs48/45 and Pfs230 and the postfertilization proteins Pfs25 and Pfs28. The cysteine-rich nature of the Pfs48/45 protein and its conformational structure made the production of a correctly folded recombinant molecule a major challenge that was only recently overcome (97, 338). It was demonstrated that the correctly folded C-terminal fragment of the Pfs48/45 protein induced high antibody titers in mice and transmission-blocking immunity in 90% of immunized mice (338). Experiments with the properly folded full-length soluble recombinant Pfs48/45 protein gave equally promising results. Sera from vaccinated mice and nonhuman primates displayed >90% reductions in oocyst numbers after a single immunization, reaching nearly complete blocking after a booster dose of the vaccine (97). Antibodies induced after vaccination with the *Escherichia coli*-expressed Pfs230 C region decreased the number of oocysts by 70 to 90%

Skin feeding

Direct Membrane Feeding Assay (DMFA)

Own serum   Control serum

Standard Membrane Feeding Assay (SMFA)

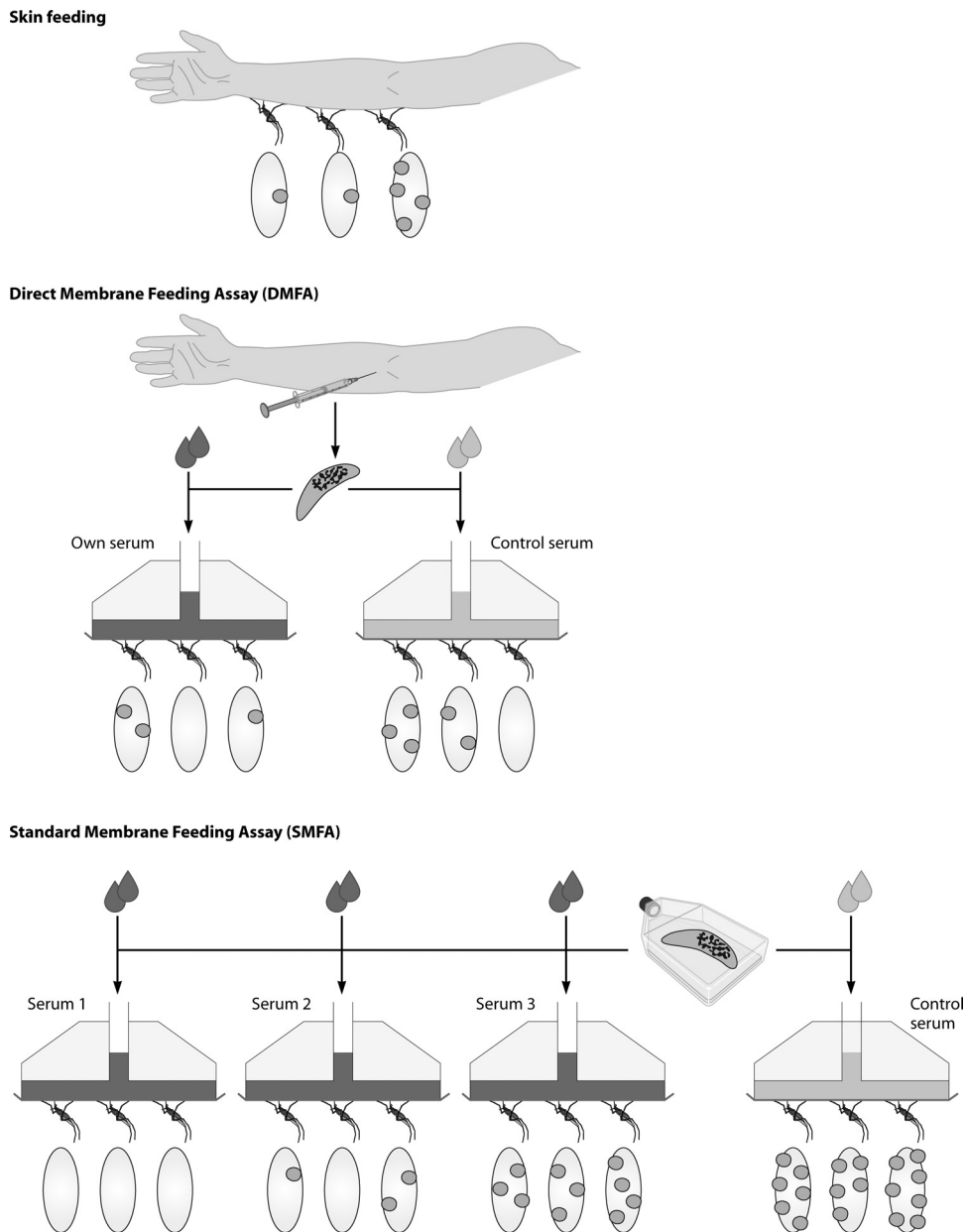Serum 1   Serum 2   Serum 3   Control serum

FIG. 7. Three different experimental setups for assessing malaria transmission and TRA. In direct skin feeding assays, reared (malaria-free) mosquitoes are allowed to feed directly on blood through the skin of a naturally infected individual. Oocysts can be detected on the mosquito midgut after dissection of the mosquito. This is mostly done 7 to 9 days after feeding. The presence of oocysts confirms infection; TRA cannot be determined by direct skin feeding. In the direct membrane feeding assay (DMFA), a venous blood sample is taken from a naturally infected individual. This blood sample forms the source of gametocytes and test plasma. Samples are centrifuged, and the red blood cells that contain gametocytes are subsequently mixed with autologous plasma or malaria-naïve control serum. This mixture is kept at 37°C and offered to reared mosquitoes through a membrane, commonly Parafilm. Seven days after feeding, mosquitoes are dissected. The prevalence or density of infection in mosquitoes can be compared in pairwise comparisons between the batch of mosquitoes that fed on gametocytes in combination with control serum and the batch of mosquitoes that fed on gametocytes with autologous plasma. In the standard membrane feeding assay (SMFA), cultured gametocytes are offered to mosquitoes in the presence of test or control sera. The prevalence or density of infection in batches of mosquitoes feeding on gametocytes with control serum is compared to that in batches with test serum. In the SMFA, a large number of test sera can be tested simultaneously.

(517). DNA vaccination with the immunogenic region C of Pfs230 with an additional GPI anchor also induced high IgG titers in mice (145). Because of its size and the difficulty in obtaining correctly folded protein, an alternative approach producing parts of the protein in tobacco plants is currently used (501). A similar approach is being pursued for Pfs48/45, Pfs28, and Pfs25.

Recombinant Pfs25, Pfs28, Pvs25, and Pvs28 all induce antibodies in mice that significantly reduce oocyst production (27, 41, 95, 130, 219, 230). Initial vaccine studies with Pfs25 resulted

in a measurable but weak immunogenicity in healthy volunteers (501). Considerable progress has since been made to enhance the immunogenicity of Pfs25 (231, 525), and several vaccine formulations based on Pfs25 and Pvs25 have been tested in phase I clinical trials in humans (262, 524). A trial of Pfs25 in combination with the Montanide ISA 51 mineral oil-based adjuvant had to be stopped after serious side effects in two volunteers that were attributed to the adjuvant. The trial showed >90% reductions in oocyst numbers in one of four individuals who completed all vaccine doses and 18 to 47% reductions in the other three (524). Other MTBV candidates include the parasite chitinase PfCHT (249) and HAP2. Promising work with rodent models demonstrated the potential of HAP2 for transmission-blocking vaccine development: anti-*P. berghei* HAP2 serum had no effect on gametocyte exflagellation but significantly inhibited ookinete and oocyst formation (40). In addition to these MTBV candidates that target gametocyte and gamete antigens, there are initiatives to target mosquito proteins that are important for parasite invasion. Antibodies raised in rabbits against the mosquito antigen anopheline midgut alanyl aminopeptidase (AnAPN1) blocked development of *P. vivax* by 98% in mosquitoes in Thailand and completely blocked *P. falciparum* development in mosquitoes in Cameroon (112, 501).

P. VIVAX GAMETOCYTES

P. vivax Gametocyte Biology and Gametocytogenesis

P. vivax gametocytes of both sexes are large and round or oval, filling nearly the whole enlarged and stippled host red blood cell (424). Studies on the biology of *P. vivax* gametocytes at the cellular and molecular levels have been complicated by the fact that the routine culture of *P. vivax* beyond a few weeks has yet to be established (486). This is in part because *P. vivax* merozoites preferentially invade reticulocytes, which are difficult to obtain routinely. The use of laboratory-produced erythroblasts may improve the possibility of culturing *P. vivax* in the future (341, 486), but our current understanding of *P. vivax* gametocytes is based largely on natural and experimental infections of humans.

Gametocyte production may start with the first generation of *P. vivax* merozoites, and gametocytes can be detected within 3 days after the first asexual parasites are observed (61, 62, 268, 424). Studies of individuals undergoing malaria therapy for neurosyphilis showed that the initial appearance of *P. vivax* gametocytes may be followed by nearly continuous gametocyte production for the first months of infection (270). There is no relevant cytoadherence of erythrocytes infected with *P. vivax* asexual parasites or gametocytes, and all developmental stages can be detected in the circulation. Although erythrocytes infected with *P. vivax* parasites increase in size, they remain flexible (463). This increased flexibility may reduce the chances of being trapped and cleared in the sinusoids of the spleen. Once gametocytes are ingested by mosquitoes, gametes emerge from the erythrocyte and undergo activation comparable to that of *P. falciparum* (206), with dramatic changes in protein transcript levels occurring during the process (507). Sporogony takes 8 to 10 days at 28°C and 16 days at 20°C for both parasite species, but *P. vivax* has a lower limit of development of 14.5°C, compared with 16°C for *P. falciparum* (180, 424). This is part of the explanation for the broader geographical distribution of *P. vivax* than that of *P. falciparum*.

Epidemiology of *P. vivax* Gametocytes

The epidemiology and control of *P. vivax* are greatly influenced by the presence of hypnozoites that can become activated weeks, months, or years after infection. Relapses occur in a seasonal manner (204, 296), resulting in seasonal patterns in asexual parasitemia and gametocytemia. Gametocytes are observed more commonly in *P. vivax* infections than in *P. falciparum* infections (271) and are detected by microscopy in the majority of or sometimes all infections (151, 189, 271, 350, 365, 394, 462), at densities of <10% that of asexual parasites (203, 309, 365). However, the duration of *P. vivax* gametocyte carriage is markedly shorter than that for *P. falciparum*. In the absence of treatment, *P. vivax* gametocytes circulate for a maximum of 3 days (82). Because of the high rate of gametocyte production by asexual parasites and their short longevity, the epidemiology of *P. vivax* gametocytes closely follows that of asexual parasites. In clinical malaria episodes, *P. vivax* gametocytemia has been associated with higher parasite densities (271, 365), younger age (151, 271, 309), lower hemoglobin levels (309), lower platelet counts (271, 309), and absence of fever (309). Similar to the case for *P. falciparum*, clinical episodes are most common in or shortly after the rainy season, but gametocyte carriage may be most common in the dry season (307). The mechanism behind this is poorly understood, but it was hypothesized that the investment in transmission stages is increased when fewer vectors are available, under the influence of seasonal patterns in UV radiation or of hematological or immunological characteristics (307). One study of hospitalized patients found no evidence for an association between hemoglobin E (a hemoglobinopathy with mild hemolytic anemia and mild splenomegaly in homozygotes and no symptoms in heterozygotes) and *P. vivax* gametocyte prevalence or the duration of gametocyte carriage (309).

P. vivax Gametocytes and Mosquito Infection Rates

Similar to the case for *P. falciparum*, the association between *P. vivax* gametocyte density and mosquito infection rates is often described as weak (34, 151, 171) or even absent (101). The limited sensitivity of microscopy for detecting all relevant gametocyte densities was demonstrated by Jeffery, who described the parasitic status and infectiousness of neurosyphilitic patients undergoing malaria therapy. These patients were infectious to mosquitoes shortly after asexual parasites were detected in the blood film but well before the appearance of microscopically detectable gametocytes (208). In all those individuals who infected mosquitoes, gametocytes were detected in the circulation in the days following the successful feeding (208). This infectivity of low or undetectable gametocyte densities has been confirmed by numerous studies (34, 61, 101, 151, 171, 208, 270, 350, 393, 394). The transmission success may depend on the duration of infection, i.e., the level of gametocyte maturation, and the proportion of male gametocytes (270). Two direct comparisons of *P. vivax* and *P. falciparum* transmission suggested that *P. vivax* malaria transmission

may be more efficient than that of *P. falciparum*, with lower gametocyte concentrations resulting in mosquito infection (61, 365). Molecular gametocyte detection tools offer possibilities for a more detailed characterization of the human infectious reservoir for *P. vivax*. A study that used QT-NASBA to detect gametocytes based on *pvs*25 mRNA reported an 8-fold increased gametocyte detection rate compared to that for microscopy (33). The association between gametocyte density and the intensity of infection in mosquitoes was also improved when a molecular tool for *P. vivax* gametocyte detection was used instead of microscopy (34).

Antimalarial Drugs and *P. vivax* Gametocytemia

Gametocyte longevity is reduced with all commonly used antimalarial drugs, considered to be effective against both immature and mature sexual stages of *P. vivax* (18, 104, 309). The median gametocyte clearance time is 24 h (309), and gametocytes are cleared from the vast majority of patients within 4 days after successful antimalarial treatment (365). Some studies that determined the infectiousness of individuals to mosquitoes following drug treatment described that the infectiousness to mosquitoes was reduced within hours after successful treatment with CQ alone (224, 225) or in combination with PQ (224). Mosquito infection was completely prevented within 2 days after initiation of treatment (209, 224, 225). ACTs are increasingly being used for *P. vivax* infections in areas where both *P. falciparum* and *P. vivax* are endemic (118). Similar to the case for *P. falciparum*, ACTs reduce the duration of gametocyte carriage compared to that with CQ (29, 308), with differences between different ACT regimens (374). Although PQ may not have a preferential effect on *P. vivax* gametocytes, it plays a key role in vivax malaria control because of its value for radical clearance of liver hypnozoites (510). In addition, it may reduce the gametocyte clearance time by a synergistic effect with chloroquine against asexual parasites (365, 366). Drugs that result in a slow and incomplete clearance of asexual parasitemia result in a longer duration of gametocyte carriage. For *P. vivax*, treatment with SP, against which high-level *P. vivax* resistance is reported (362), and antibiotics results in longer gametocyte carriage than that with more efficacious antimalarial drugs (248, 362, 364, 365). Similar to the case for *P. falciparum*, *P. vivax* gametocytemia after treatment may serve as a predictor for the later reappearance of asexual parasites (189, 365).

P. vivax Transmission-Blocking Immunity and Transmission-Blocking Vaccines

The infectivity of *P. vivax* gametocytes is affected during paroxysms by nonspecific immune mediators such as TNF-α (218), possibly through an interaction with white blood cells (313). In addition, specific immune responses can influence the transmission of *P. vivax* to mosquitoes. Both transmission-enhancing and transmission-reducing immune responses have been described (152, 349, 393), as they have for *P. falciparum* (491). Human monoclonal and polyclonal antibodies against sexual-stage *P. vivax* can have this dual action, enhancing transmission at low concentrations while reducing transmission at higher concentrations (349). Transmission-blocking immune responses seem most prevalent in younger children (152), who have the lowest transmission efficiencies per gametocyte (151). Transmission-blocking immune responses appear short-lived, as individuals who have not been exposed to malaria for several months may have similar sexual-stage immune responses to those with a primary infection (152, 372), suggesting that functional transmission-blocking immunity depends on recent exposure to antigen. The functionality of transmission-blocking immune responses is associated with antibody titer, measured by indirect immunofluorescence assay (152, 349, 372), but may also depend on the intrinsic infectivity of gametocytes (152).

Molecular targets of naturally acquired *P. vivax* transmission-blocking immunity have not been characterized. Despite this, there has been considerable progress in developing an MTBV for *P. vivax*. The principal candidates are the ookinete surface antigens Pvs25 and Pvs28. Both were reported to be highly conserved in parasite isolates from different continents (484), which could reflect the lack of selective pressure exerted by human immune responses to antigens expressed only in mosquito stages. However, there may be higher degrees of polymorphism in Pvs25 and Pvs28 than in their *P. falciparum* homologs, especially for Pvs28 (485). Recent findings suggest a rapid progress of genetic changes in Pvs25 and Pvs28 among Korean isolates (186). Recombinant proteins for both antigens have high levels of immunogenicity, and vaccination resulted in the induction of functional transmission-blocking antibodies in rodent and primate models (10, 41, 201, 285). Antibodies induced by vaccinating mice and rabbits with recombinant Pvs25 or Pvs28 significantly reduced oocyst development of several natural Thai *P. vivax* isolates (285, 395). Pvs25 is the most advanced MTBV candidate for *P. vivax* and has undergone extensive preclinical testing and testing in phase I clinical trials. A variety of vectors, as well as several adjuvants, have been used to express Pvs25 protein (252, 262, 524). The recombinant protein requires both conformation-dependent epitopes and a strong adjuvant to induce transmission-blocking antibodies (41). Phase I trials of humans with an alum adjuvant showed that the vaccine was well tolerated and induced antibodies associated with TRA. The functional activity of antibodies strongly depended on their concentration, which was generally low in vaccinated volunteers. Five sera with the highest antibody concentrations prevented infection in 20 to 30% of mosquitoes (262). A more potent adjuvant was used in a follow-up study. Pvs25 with the Montanide ISA 51 mineral oil-based adjuvant resulted in a large proportion of severe adverse reactions that were likely the result of the adjuvant (524). An alternative approach for Pvs25 or Pvs28 vaccine development has been the use of DNA vaccines, which resulted in promising antibody titers and TRA in mice (230).

GAMETOCYTES AND MALARIA ELIMINATION STRATEGIES

Several currently used malaria control tools reduce the transmission of malaria from humans to mosquitoes. Vector control efforts such as insecticide-treated nets (ITNs) not only have an impact on malaria morbidity and parasite prevalence, because of the protection they provide against bites from sporozoite-infected mosquitoes, but also prevent mosquitoes

from feeding on a gametocytemic human. This dual mode of action makes vector control measures powerful tools in malaria control and elimination strategies, although the impact of ITNs may be larger for *P. falciparum* than for *P. vivax* (42, 254, 296), possibly due to differences in biting behaviors of mosquitoes (42).

In addition to vector control measures, there are several tools to specifically prevent the transmission of gametocytes to mosquito vectors. MTBV reduce the infectiousness of gametocytes after their uptake by mosquitoes taking a blood meal from a vaccinated and gametocytemic donor. MTBV have objectives that are different from those of anti-infection or anti-disease vaccines and are not designed to protect vaccinated individuals directly but to form part of an integrated malaria control program in order to reduce malaria transmission. MTBV can play a role in (i) reducing mortality and morbidity in areas of medium to high transmission intensity; (ii) eliminating malaria in areas of low endemicity; (iii) protecting against epidemics or reentry of malaria; and (iv) protecting other vaccines or drugs against the spread of resistant parasites, thereby prolonging their effective life (396–399, 434, 522). In some settings, one could consider MTBV as a standalone vaccine, for instance, for preventing reintroduction of malaria in malaria-free areas or preventing the transmission of (drug-)resistant parasite strains from an area of low transmission intensity. In most situations, MTBV are more likely to be used in combination with vaccines against other parasite stages. Depending on the achieved antibody titer and the potential for natural boosting, one would expect an MTBV to reduce malaria transmission for a certain period after completion of the vaccination schedule. This is a major advantage over interventions with antimalarial drugs that may have a similar effect in reducing the transmission from parasitemic individuals but may need repeated administration in most endemic settings. An efficacious MTBV may therefore be "an ideal public good" (522) and have an impact against both *P. vivax* and *P. falciparum*. Although several candidates are currently in clinical development, MTBV are unavailable at present and therefore are unlikely to form an important element in control or elimination programs in the coming decade.

Community interventions with antimalarial drugs and ITNs are commonly targeted to specific risk groups for (severe) disease, such as children and pregnant women (9, 198, 278), to have a maximum impact on morbidity and mortality. Control interventions that specifically aim to reduce transmission would, on the other hand, target all individuals who contribute to malaria transmission, including individuals who are apparently healthy. These types of interventions are considered by some to be altruistic in the sense that they do not protect individuals themselves but the community as a whole, and therefore they require additional ethical considerations (20) and a new degree of community awareness and engagement (109). For *P. vivax* elimination strategies, a major challenge in elimination efforts is the clearance of hypnozoites that form reservoirs of infection that persist in the livers of infected people. There is an urgent need for safe and efficacious drugs to clear liver schizonts and hypnozoites. Effective clearance of liver-stage *P. vivax* currently depends on taking a 2-week radical curative regimen of PQ (i.e., 14 days of 0.25 to 0.5 mg base/kg of body weight/day) (510); this has several logistical and clinical consequences, some of which are discussed in the

TABLE 3. Gaps in the current knowledge of *P. falciparum* gametocytemia

Category	Unanswered question or area for research
Biology	What triggers gametocytogenesis in *P. falciparum*?
	What parasite adhesins are involved in gametocyte sequestration?
	Are there tropins that mediate the fertilization of male and female gametocytes at low gametocyte densities?
	Do gametocytes preferentially localize in the small capillaries?
	Are there circadian patterns in gametocyte concentration or infectivity?
Control	What is the contribution of chronic asymptomatic parasite carriers to malaria transmission?
Tools	Sensitive tool for the detection of asymptomatic parasite/gametocyte carriers for public health purposes
	Sensitive tool for sexing gametocytes at low gametocyte densities
	Safe and efficacious gametocytocidal component for combination therapy
	Sensitive parasite detection methods for field evaluation of transmission-reducing interventions in settings of low endemicity

section on drugs for mass drug administration. For *P. falciparum*, clearance of liver-stage parasites is less important in elimination strategies, while the long-lived gametocytes require specific attention. In the previous sections, we identified a number of areas related to *P. falciparum* gametocyte carriage, infectivity, and drug treatment in relation to transmission control. However, even with this knowledge, we are still short of knowing how best to use transmission-reducing interventions. Some of the key gaps in our current understanding are summarized in Table 3.

Who Should Be Targeted with Drug-Based Interventions?

Effective antimalarial treatment reduces transmission (510), but the impact of treatment on the level of malaria transmission experienced on a community level depends on the proportion of parasite carriers reached with antimalarial drugs, drug characteristics, and malaria epidemiology. Although the switch from ineffective malaria monotherapy to efficacious ACTs may result in or contribute to a reduction in transmission intensity (23, 24, 318), there are limitations to reducing transmission by changing the first-line antimalarial drug. The asymptomatic reservoir of parasite and gametocyte carriers plays a major role in this respect. In many regions where malaria is endemic, the majority of infections do not result in clinical symptoms (8, 32, 63, 261, 337), and even in children, long-term asymptomatic parasite carriage may occur (261). Mathematical models suggest that gametocytocidal properties of antimalarials used as first-line treatment have the most impact in low-transmission settings, while prophylactic effects have more impact in areas with high transmission levels (323). Thus, the impact of ACT in high-transmission settings (e.g., above 20% baseline slide prevalence) is smallest due to a combination of a smaller proportion of infections being treated

and the different dynamics of infection prevalence (323). The prophylactic effect of a drug can also contribute considerably to the overall transmission-reducing effect, because preventing infection also reduces the potential for gametocyte development. Therefore, an ACT with the gametocytocidal effects of the artemisinin derivative and the prophylactic effect of a longer-acting partner drug is predicted to have the greatest impact on transmission across all areas (323).

MSAT of Asymptomatic Individuals

We previously argued that all parasitemic individuals can be considered potential current or future gametocyte producers. Interventions that aim to reduce malaria transmission should therefore ideally include all symptomatic and asymptomatic parasite carriers. Mathematical models unanimously indicate that this will increase the impact of interventions at all levels of transmission intensity (177, 430, 508). There are two approaches to do this by drug interventions: mass screening and treatment (MSAT) and mass drug administration (MDA). In MSAT, individuals are screened for the presence of malaria parasites, regardless of their symptoms, and are treated only if parasites are detected. The advantage of this approach is that it reduces exposure of nonparasitemic individuals to antimalarial drugs that may have disadvantageous effects in terms of side effects and an increased drug pressure in the community that may promote the spread of drug-resistant parasites (114). One of the disadvantages of this approach is that the success of identifying all parasite carriers in the population will depend on the sensitivity of the screening tool. Microscopy and rapid diagnostic tests will miss a substantial proportion of carriers. Especially in areas with low levels of endemicity, a relatively large proportion of parasite carriers will remain undetected by microscopy (325). The sensitivity of detection tools may be solved in the coming years by the development or improvement of molecular parasite detection techniques, such as PCR, LAMP, or QT-NASBA (187, 405, 439), which should become affordable and suitable for large-scale testing in the field. The use of molecular detection tools for MSAT was piloted in an elegant but laborious study in two Sudanese villages where parasite carriers were detected by PCR in the long dry season and subsequently cleared of parasites with ACTs, resulting in a significant reduction in parasite carriage in the wet season (141). An additional disadvantage of MSAT is that people who are exposed to malaria shortly after being found parasite negative during the MSAT campaign will not experience any beneficial prophylactic effect of the drug. This disadvantage can be overcome only by using an alternative (re)treatment strategy.

MDA

The logistical difficulties in detecting (submicroscopic) parasite densities prior to drug administration can be circumvented by treating individuals in MDA campaigns, regardless of parasitemia. Several MDA campaigns to reduce malaria transmission have been conducted since the 1930s (reviewed in reference 503). The success of these past MDA interventions is difficult to evaluate because of trial designs that we would currently consider inadequate and the concurrent employment of vector control measures (214). Most interventions resulted in only modest and short-term reductions in transmission intensity (503). In a review on historical MDA initiatives, it was noted that the failure to achieve long-term results could be explained by insufficient drug coverage, use of suboptimal drugs, too-high baseline levels of transmission intensity, and reintroduction of infections by movement of infected human and mosquito populations (503, 504). Studies in the Gambia using a combination of SP and a single dose of artesunate (AS) recorded only a moderate and transient difference in malaria incidence between control versus intervention villages (504). One explanation for this was the persistence of mature gametocytes unaffected by the single dose of AS. A more concerted drug regimen which included regular doses of CQ and SP, together with PQ as a gametocytocidal agent, had considerable success in a small island population in Vanuatu. From an initial prevalence of >20% in the late 1980s, *P. falciparum* was reported to be eradicated, and only sporadic cases of *P. vivax* were recorded by the year 2000 (214). Since SP and CQ are no longer viable drugs, current MDA initiatives need to use alternative drugs. In Cambodia, dramatic reductions in parasite rates, from >50% to <5%, were observed in a pilot MDA study of more than 3,000 individuals receiving two doses of artemisinin-piperaquine (AP) and adults receiving regular PQ. In villages where parasites remained, a second round of AP 1 year later reduced the parasite prevalence to 0% in children. The authors concluded that their approach was effective, safe, and affordable (441). Nevertheless, field trials that determine the impact of MDA alone or in combination with other interventions remain rare. This is perhaps not surprising given the complexity of conducting and evaluating these trials, and mathematical models could provide a convenient framework for comparing various combinations of drugs in areas with different levels of endemicity.

Predicted impact of MDA in areas with different levels of endemicity. Okell and colleagues showed that at a high transmission level (50% parasitemia), a single round of MDA had a short-term effect, with baseline parasitemia restored within 1 year. At a low transmission level (5% parasitemia), however, the effect lasted considerably longer (326). Logically, these types of interventions are targeted at the nadir in transmission, when the parasite reservoir is at its minimum and can be enhanced with repeated MDA. In Fig. 8, we present two different epidemiological scenarios for settings with seasonal transmission and contrasting parasite rates and anopheline densities. The top panel shows transmission characteristics in the absence of interventions, and the bottom panel shows the effects of one or two rounds of MDA with a drug that has the properties of artemether-lumefantrine. Not surprisingly, the effect of MDA on the parasite rate was most pronounced in the setting with a low level of endemicity, and there was an advantage in repeating the administration. In the simulated setting of high endemicity, two rounds of MDA were highly unlikely to lead to malaria elimination. For the setting of low endemicity, the model indicates a 10% chance of elimination after two rounds of MDA in a population of 1,000 individuals and a 0.8% chance of elimination in a population of 3,000 individuals. The high basic reproduction rate of malaria (R_0) (here we assume that one infected individual may potentially lead to more than 100 new infections in a susceptible population living in a setting where malaria vectors are highly prev-
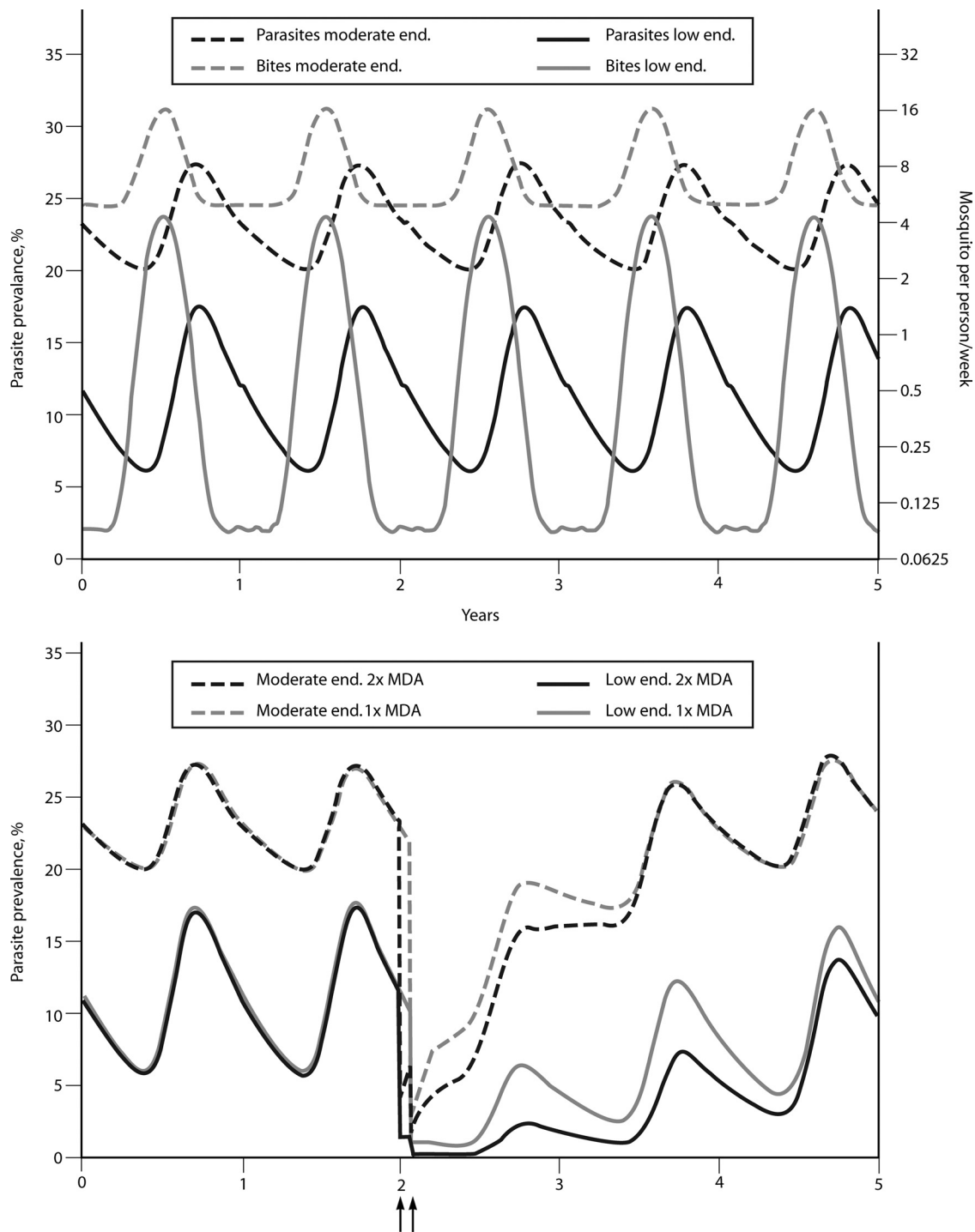
FIG. 8. Predicted impacts of mass drug administration in two settings of malaria endemicity, using two different transmission scenarios. (Top) Situation of seasonal transmission in an area of moderate endemicity and an area of low endemicity. In the area of moderate endemicity, parasite prevalence is ~20% in the general population in the dry season, when people are exposed to ~5 mosquito bites/person/week; in the wet season, parasite prevalence increases to ~28% and mosquito exposure to 16 bites/person/week. In the setting of low endemicity, parasite prevalence is ~5% in the dry season, when people are exposed to an average of one mosquito bite/2 months; in the wet season, parasite prevalence increases to ~18% and mosquito exposure to 4 bites/person/week. (Bottom) Simulated impact of one or two rounds of MDA toward the end of the dry season. The timing of the MDA is indicated with an arrow; in the scenario where MDA is repeated, the gap between the two rounds of MDA is 1 month, and the correlation between coverage in the first and second rounds of MDA is 0.5. The drug used for MDA has the characteristics of artemether-lumefantrine, with an efficacy against asexual parasites of 95% and a duration of (submicroscopic) gametocyte carriage of 13 days after initiation of treatment. The coverage with the intervention is 95%.

alent [431]) warns that eliminating local transmission may not lead to long-term results if malaria can be reintroduced by parasite carriers. Thus, MDA, like other transmission-blocking interventions, is unlikely to be used alone (434).

Choice of drug for MDA. A major reservation about MDA highlighted by several authors (114, 503, 510) is that individuals who are parasite negative receive a treatment that they do not need, and there are legitimate concerns that introducing large quantities of a drug into a population may have hastened the development of drug resistance. The choice of drug for MDA therefore requires careful consideration not just related to its likely efficacy; interventions are unlikely to be conducted with first-line treatment. At an individual level, concerns with drug use are particularly acute for PQ. As discussed above, this drug is unique in it that it has a strong gametocytocidal effect (363, 412, 438), but it also induces hemolysis that is related to G6PD deficiency (20, 92, 381, 414). Hemolysis may occur after a single dose in G6PD-deficient individuals (381, 412, 414) and may be particularly severe in individuals with the Mediterranean (B−) G6PD genotype (20, 158). Although the effect of PQ on hemoglobin levels is generally mild (412, 414) and self-limiting (110, 412), severe and life-threatening hemolysis may occur in some individuals (158, 381). Our current understanding of PQ sensitivity phenotypes is incomplete (20, 414), and Baird and Surjadjaja recently stressed that the reliable information is currently "inadequate by almost any contemporary standard of safety in chemotherapeutics, especially in the context of dosing demonstrably vulnerable populations" (20). Further safety data are particularly important for regions where malaria is endemic, where not only the prevalence of G6PD deficiency is high (316) but also mild and severe anemia can be common due to infections (e.g., malaria and helminth infections) and other genetic conditions (e.g., thalassemia and sickle-cell anemia). Further studies to determine the optimal dosage and timing (20, 241) of PQ to have a maximal impact on gametocyte persistence but minimal hemolysis and the development of practical means to screen against potentially dangerous G6PD variants in tropical settings (20) are therefore highly desirable.

Targeted Interventions

Tailored versions of both MDA and MSAT will become increasingly viable as transmission decreases and elimination becomes a possibility. At these low levels of transmission, a specific gametocytocidal therapy may be added to routine treatment, as already done in several countries, such as Sri Lanka and Mexico (20, 293).

In this later context, control will become more efficient for specifically targeting the foci of clinical and asymptomatic transmission (85, 520). These hot spots of transmission amplify transmission by transmitting malaria parasites to a large number of mosquitoes (431, 519). How one identifies these hot spots is an increasingly vibrant area of research (32, 56, 60). The recent suggestion that there are stable and less-stable hot spots of transmission (32) begs the question of how gametocyte carriage contributes. It may be that stable malaria hot spots are associated with more consistent low-level gametocyte carriage (e.g., chronically infected adults), whereas unstable hot spots are due in some part to short-term highly infectious individuals

(e.g., infected children with high parasite densities). These potential hot spots of gametocytemic individuals can be targeted specifically by focal MDA/MSAT. Practical issues for this type of administration will be the size of the hot spot and how widely drugs are administered. If, for example, an index case is identified, then the issue is whether to treat individuals—all of those testing positive—in the same house, in neighboring houses, or to an arbitrary distance of 500 m. Assessing the efficacy of transmission-reducing measures will be similar to that for other approaches to control. The issue of the sensitivity of detection methods, such as those for determining parasite end points or directly sampling transmission intensity (infected mosquito end points), is applicable in all settings but will become more acute at the lowest transmission levels (293).

CONCLUDING REMARKS

In this article, we have attempted to present a current assessment of our knowledge on malaria gametocytes, with a particular emphasis on epidemiology and factors affecting their distribution within endemic populations. It remains surprising that despite their pivotal role in transmission, some of the most fundamental questions about gametocyte biology remain unknown. For *P. falciparum*, the within-host distribution of both immature and mature forms remains almost a complete mystery. While genomics and proteomics have made the first initial steps in confirming the considerable change in emphasis in protein transcription and production in preparing the parasite for life in the mosquito midgut, few specific targets—ligands or receptors—have been identified. Molecular amplification techniques have shown us that conventional blood sampling measures vastly underestimate the number of individuals who are potentially infectious to mosquitoes. At the level of an individual with a very low gametocyte density, how are single male and female gametes able to fuse and mate in a mosquito blood meal with approximately 10^7 uninfected red blood cells? At the level of a population living in a region where malaria is endemic, a reassessment of our concept of the infectious reservoir is required. Fewer than 10 attempts to measure and define the infectious reservoir have been conducted in the last 50 years, yet more will be needed if we are to optimize any existing or develop new gametocytocidal control tools. These control approaches will need to be integrated with more detailed knowledge of the underlying epidemiology concerning the spatial and temporal patterns of gametocyte carriage and infectivity. Control attempts can be enhanced further if hot spots of infection are associated with hot spots of gametocyte carriage or groups of individuals who are "super spreaders" and potentially amenable to specific targeting. Ultimately, less predictable economic and political factors may define the success of malaria control and elimination; however, improving our understanding of gametocytes will be a huge step in the right direction.

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