CLINICAL MICROBIOLOGY REVIEWS, Jan. 1992, p. 26–35
0893-8512/92/010026-10$02.00/0

Vol. 5, No. 1

The Hypnozoite and Relapse in Primate Malaria

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INTRODUCTION

The recurrence of malaria is a phenomenon that was known to the ancients and first recorded by Horace in his third satire. A popular misconception even today is that malaria is not completely curable, although radically curative drugs have been available since 1952. It is only recently that the riddle of the malarial relapse, a phenomenon that has intrigued parasitologists for over a century, has begun to be understood.

MALARIA FUNDAMENTALS

Malaria is caused by a protozoan parasite, phylum Apicomplexa, genus *Plasmodium*. There are four species of *Plasmodium* responsible for human malaria: *Plasmodium falciparum* (malignant tertian or falciparum malaria), *P. vivax*, (benign tertian or vivax malaria), *P. malariae* (quartan malaria), and *P. ovale* (ovale malaria) (7).

The life cycles of all *Plasmodium* species causing human malaria are essentially the same, comprising a sexual phase followed by sporogony in an anopheline mosquito and an asexual phase including both erythrocytic and preerythrocytic schizogony in the human host.

The cycle of malaria in the human host is initiated by the female anopheline mosquito which, before taking a requisite blood meal, injects malarial sporozoites contained in an allergenic, anticoagulant saliva (Fig. 1). The sporozoites are thought to leave the bloodstream within minutes (20, 76), and the developing schizonts can be found in the liver 48 h later (49). Recent studies have shown that the sporozoites appear to invade hepatocytes directly and do not have an obligate phase in a Kupffer cell (76).

A portion of these parasites then undergoes early preerythrocytic schizogony (nuclear division and an increase in cytoplasmic volume) for 5 to 15 days depending on the species. The mature schizont (up to 70 μm in diameter) enlarges the hepatic parenchymal cell, actually pushing the nucleus to one side (Fig. 2). Upon reaching maturity, the schizont ruptures and releases merozoites into the bloodstream, where they invade erythrocytes and initiate a schizogonic cycle in the blood. Clinical manifestations (chills and fever) are associated with the release of succeeding generations of merozoites from erythrocytes prior to their invasion of new erythrocytes (27). Microgametocytes (male) and macrogametocytes (female) are produced after two or more cycles and circulate in the bloodstream to be taken up by the mosquito, in which they develop into gametes and in which fertilization occurs. The resulting zygotes develop into ookinetes which migrate through the stomach wall of the mosquito and form oocysts on the outside of the midgut. Each successful oocyst grows to maturity, the nuclei dividing repeatedly, until it bursts and releases thousands of motile sporozoites. These sporozoites make their way to the salivary glands to make ready for a sojourn in another human host.

HISTORICAL PERSPECTIVE

Although malaria has been known as a disease since the beginning of recorded history, its cause was not understood until 1880, when Laveran discovered the parasite in the blood of malaria patients in Algeria (54). His discovery was met with some skepticism, as medical pundits of that time thought that malaria was bacterial in origin. One of those who early became convinced of Laveran's theory was Golgi (whose primary interest was actually the nervous system). He observed that the parasites divided simultaneously and that this division coincided with the onset of fever. In 1893, presaging discovery of tissue stages of human malarial parasites by over 50 years, Golgi suggested that the parasites might have an undiscovered tissue phase in endothelial cells (31).

Arguably the first to publish a theory regarding the existence of a tissue stage of the parasite was Pel, whose ideas were published in 1886 (65). As translated by Meis and Verhave (60), Pel's explanation of long-term malarial latency was as follows.

"During the latent period the germ is fixed somewhere or not able to reproduce, until by some cause its conditions for life become more favorable. Then the germs can multiply or shift to another more active stage of development, reach the blood and cause particular disease symptoms."

In 1897, Ross first described the parasite in the insect vector of avian malaria (67). His findings were confirmed and expanded upon by the Italian school of malariologists, notably, Bignami and Bastianelli (2, 3) and Grassi and coworkers (32, 33). Manson documented anopheline trans-

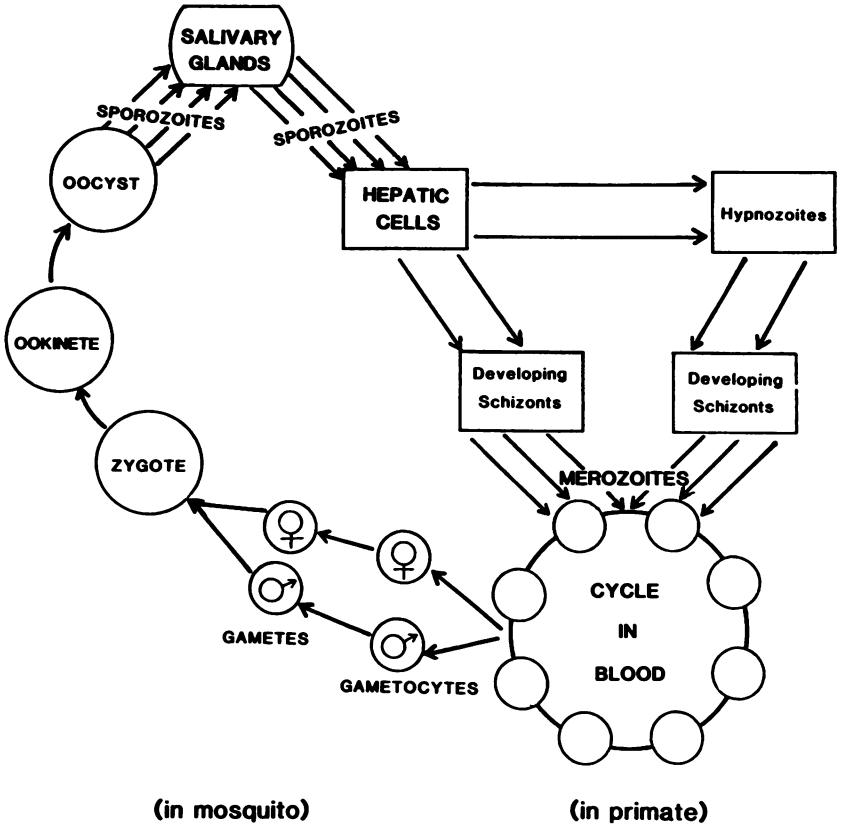

FIG. 1. Generalized life cycle of relapsing primate malaria parasites.

mission of human malaria by letting infected mosquitoes feed on his adult son (57). It was the younger Manson who described in detail his own relapse 9 months after a supposedly complete cure with quinine (58). Another pertinent description of relapse with vivax malaria was given in 1903 by Fearnside, who described his own relapse 8 months after treatment (21).

The earliest known record of true relapse is that of Thayer, published in 1897, in which he quotes a physician experienced in the clinical features of malaria who sustained a series of chills and fever almost 2 years after his initial attack (84). The physician had not been in a malarious area since his first episode. Thayer published a series of lectures in which he speculated that there must be a heretofore undescribed form of the parasite which remained "within the cell body of certain phagocytes" and would explain the latent period between the initial parasitemia and relapse (84).

Unfortunately, the eminent German protozoologist Schaudinn described in 1902 what he thought to be the direct invasion of erythrocytes by malaria sporozoites (70), thereby diverting attention from a theoretical latent stage. Schaudinn's scientific stature and the detail with which he described his observations were most convincing and served to send malaria research on a meandering detour for almost 30 years.

DEFINITION OF RELAPSE

Relapse is a term used widely in medicine to mean a return of the clinical symptoms of a disease after its apparent cessation. The term as applied to malaria, however, is

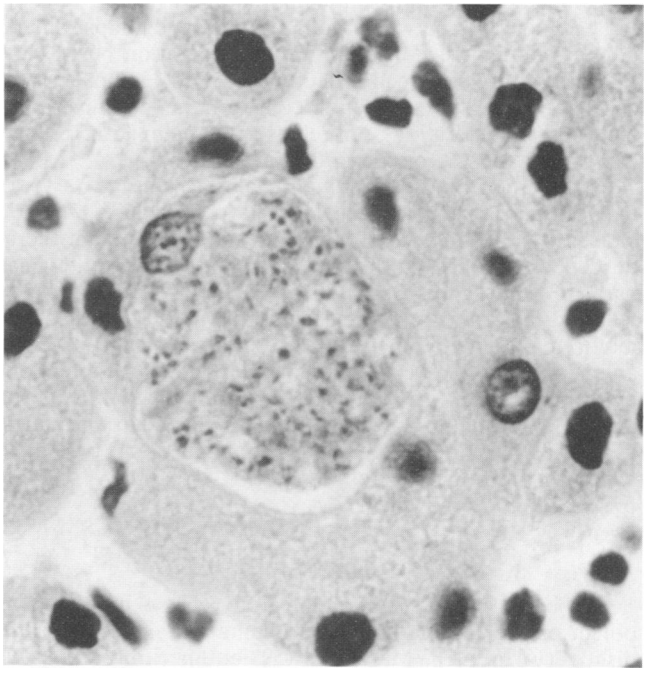

FIG. 2. Mature schizont of *P. vivax* Chesson 7 days after sporozoite inoculation: Giemsa-colophonium stain after IFA. Magnification, ×800. Reprinted from the *American Journal of Tropical Medicine and Hygiene* (51) with permission of the publisher.

TABLE 1. Primate malarias

Species	Natural host	Hypnozoites found[a]	Reference(s)
Known to relapse			
P. vivax	Human	+	47, 51
P. cynomolgi	*Macaca* sp.	+	45, 50, 53
P. ovale	Human	ND	7
P. simiovale	*Macaca* sp.	+	10, 12
P. fieldi	*Macaca* sp.	ND	7
May relapse			
P. silvaticum	Orangutan	ND	26
P. simium	Howling monkey	ND	26
P. schwetzi	Chimpanzee and gorilla	ND	7, 26
Do not relapse			
P. falciparum	Human	ND	7, 26
P. fragile	*Macaca radiata*	ND	7, 26
P. knowlesi	*Macaca* sp.	−	46
P. coatneyi	*Macaca* sp. and *Presbytis* sp.	ND	7,26
P. malariae	Human	ND	7,26
P. inui	*Macaca* sp.	ND	7,26
P. brasilianum	New World monkeys	ND	7,26

[a] ND, not done. Symbols: +, hypnozoites found; −, hypnozoites not found.

somewhat more specific and must be distinguished from relapse denoting simple recurrence.

The recurrence of malaria due to the incomplete elimination of the blood stage of the parasite, whether due to inadequate treatment with blood schizonticides, to waning immunity, or to evolution of new variants, is termed recrudescence. True malarial relapse is defined as the "re-appearance of parasitemia in a sporozoite-induced infection following adequate blood schizonticidal therapy" (6).

The distinction between recrudescence and relapse should not be minimized. An important consideration here is the fact that blood-induced infections cannot relapse. Only sporozoites can infect hepatic cells, and hence only sporozoite-induced infections can relapse. A true malarial relapse is always exoerythrocytic in origin.

Of the four kinds of human malaria, only two, vivax malaria and ovale malaria, show true relapse. Malaria caused by *P. falciparum* does not relapse, although recrudescences are common after inadequate treatment if the parasite is drug resistant (26) or a new variant appears. Quartan malaria is not thought to exhibit true relapses, but may remain asymptomatic for up to 53 years (34). Primate animal models of relapsing human malaria include infections by *P. cynomolgi* (an analog of *P. vivax*), *P. simiovale* (an analog of *P. ovale*), and *P. fieldi*, all from Asian macaques, as well as *P. schwetzi* from the chimpanzee and gorilla, *P. simium* from howler monkeys, and *P. silvaticum* from the orangutan (7, 26) (Table 1).

THEORIES OF RELAPSE

The exact mechanism of malarial relapse has been an object of speculation and experimentation since Laveran's discovery. In 1926, Marchoux (59) outlined three possible mechanisms to account for relapse: (i) parthenogenesis of macrogametocytes; (ii) persistence of schizonts in small numbers in the blood where their multiplication is inhibited by immunity and this immunity disappears; and (iii) reactivation of an encysted body in the blood. As discussed by Garnham (26), Marchoux developed these theories before the full life cycle of malaria parasites was disclosed. He was strongly in favor of parthenogenesis as the correct explanation for relapse, in part because of the description of parthenogenesis by Schaudinn (70), a description that was later found to be erroneous. In 1900, Bignami and Bastianelli found that they could not infect an individual with blood containing only gametocytes (3), and 30 years later, Garnham (23) also failed in this endeavor, finally squelching the theory of parthenogenesis of gametocytes.

The second theory, a persistent blood stage infection, had been proposed by Ross and Thompson in 1910 (68) and was still championed until recently by some malariologists, notably, Corradetti (17). This theory is, in fact, correct in the case of *P. malariae*, which can remain in the blood at undetectable levels for many years (55).

It was suggested by James in 1931 that, perhaps after being injected by the mosquito, the sporozoites are carried to internal organs, where they enter the reticuloendothelial cells and undergo a cycle of development (39). This conclusion was based on the observation that treatment with quinine had no effect when the drug was administered before clinical symptoms appeared. By 1935, Huff and Bloom had demonstrated the exoerythrocytic stages of avian malaria (38). Accumulating observations about the tissue stages of bird malaria (40) made it plausible that these stages occurred in primate *Plasmodium* species as well.

Fairley reported in 1945 that inoculation of blood from a patient with *P. vivax* may fail to induce malaria in a susceptible recipient, although the donor may subsequently develop overt malaria. He speculated that this constituted evidence of persistent tissue forms of *P. vivax* (19).

In 1946, Shute (82), who was infecting large numbers of mosquitoes with vivax malaria for malariotherapy of neurosyphilis, noticed that, even though heavily infected mosquitoes fed on a patient, an immediate malarial infection did not always result, although symptoms would be exhibited several months later. He speculated that this was due to a "resting parasite." Sapero proposed in 1947 that perhaps a link existed between a tissue stage not yet discovered in patients with malaria and the phenomenon of relapse (69). A year later such a stage had indeed been found.

The 1947 discovery by Garnham of exoerythrocytic schizogony by the related parasite *Hepatocystis kochi* (24) directly led to the 1948 discovery by Shortt and Garnham of the liver stages of *P. cynomolgi* in the monkey (79). A human volunteer then consented to receive a massive dose of infected sporozoites of *P. vivax* and undergo a liver biopsy, allowing Shortt et al. (81) to demonstrate the tissue stage of a human malarial parasite.

Because the developing schizonts were found 3.5 months after sporozoite inoculation and because of the analogy with avian malaria, the original theory of relapse involved a tissue "cycle" (80). It was thought that merozoites erupting from mature schizonts would reinvade hepatic parenchymal cells in a more or less continuous cycle until waning immunity allowed them to invade erythrocytes and initiate another blood cycle. This theory was attractive, but it did not account for several subsequent observations.

EXAMINATION OF THE CYCLIC THEORY OF RELAPSE

The demonstration of the tissue stages of *Plasmodium* in primate malaria has been clearly and repeatedly confirmed,

but the accompanying theory of a preerythrocytic cycle has not been as fortunate. The theory failed to account for evidence in four areas of experimentation.

First, in 1949, Cooper et al. (16) showed that blood transfusions performed during the long latency between the primary parasitemia and the first relapse of *P. vivax* infections failed to produce symptoms in susceptible individuals, although the donors eventually experienced a characteristic relapse. The absence of circulating parasites during the latent period argued against a continuous tissue cycle, although Corradetti charged that donors would have to be totally exsanguinated to be absolutely sure that no circulating parasites were sequestered in "the capillaries of internal organs" (17).

Second, Cooper et al. in 1947 showed that individuals were susceptible to a homologous blood-induced infection of *P. vivax* during the latent period (15). This would seem to be potent evidence against waning immunity as the trigger for reappearance of parasites in the blood.

A third and most telling argument against the cyclic theory of relapse concerns the regular relapse patterns found in patients infected with various strains of *P. vivax* (8, 9). Long latent periods are characteristic of *P. vivax hibernans* (64), *P. vivax multinucleatum* in China, and the North Korean strain of *P. vivax* which exhibits a marked latency of up to a year or more (29, 83). With other strains causing benign tertian malaria, e.g., the St. Elizabeth's strain, characteristic relapse patterns are seen in each infection irrespective of the host's immune response (14).

Results from a fourth area of inquiry also cast doubt on the cyclic theory of relapse. Contacos and Collins in 1973 had demonstrated that not all of the monkeys receiving calculated numbers of *P. cynomolgi* sporozoites showed the relapses expected if a tissue cycle existed, and they claimed that the hepatic cycle theory of relapse "does not obtain" (13). Warren et al. in 1974 (87) also showed that the number and frequency of relapses seemed to depend on the number of sporozoites injected. Ungureanu et al. in 1976 (85) demonstrated that the duration of prepatent periods with a tropical strain of *P. vivax* in human volunteers was not dependent on the number of sporozoites injected. These results contrasted with those of Shute et al., who in 1976 found a direct correlation between low sporozoite numbers injected and long prepatent periods when a temperate strain of *P. vivax* was used (83). Ungureanu et al. explained this discrepancy by advocating the theory of two populations of sporozoites, i.e., fast developing and slow developing. Unlike the temperate strain (North Korean), the tropical strain of *P. vivax* (Chesson) did not contain greatly unequal proportions of the two types of sporozoites, and long prepatent periods due to slow-developing sporozoites were not evident with the Chesson strain, even with an inoculum as low as 10 sporozoites. Ungureanu et al. surmised that both the long latent periods and the characteristic relapses in *P. vivax* infections were part of the same phenomenon.

In 1977, elaborating on the ideas of Moshkovsky (63), Lysenko et al. (56) suggested a series of postulates to explain the phenomena of long incubation periods and relapses. They theorized that the duration of the preerythrocytic development of *P. vivax* is a polymorphic characteristic controlled by several gene loci and that sporozoites are divided into two complex groups of phenotypes, i.e., the slow-developing and fast-developing types advocated by Ungureanu et al. (85). Lysenko et al. stressed that the strains studied in the laboratory do not fully represent natural populations of parasites, which have a much larger gene pool and consequently greater variation of genes.

In a studied review of the theories of relapse in primate malaria, Garnham in 1967 came to discount the cyclic theory of relapse and reintroduced the concept of a latent cycle to account for the shortcomings of the cycle theory (25).

Just 1 year before the centennial of Laveran's discovery, a latent tissue stage was found by Krotoski and coworkers in the liver of a monkey heavily infected with *P. cynomolgi* (53).

THE LATENT STAGE THEORY OF RELAPSE

The development of immunofluorescence techniques permitted the detection of even earlier forms of the parasite in the liver (49). In 1981, Krotoski et al. described the 48-h preerythrocytic form of *P. cynomolgi* by using the indirect fluorescent-antibody method (IFA) (48). Routine use of this technique to examine heavily infected monkey liver eventually led to the discovery of a uninucleate stage of the parasite seen initially at 7 days postinfection in animals infected with *P. cynomolgi* (50). These uninucleate forms, found by immunofluorescence and restained with Giemsa-colophonium stain, were thought to be the long-sought dormant stage of the parasite.

Experiments were undertaken to establish the true nature of this form, and it was subsequently found to be present from 3 to 229 days after sporozoite inoculation and to remain virtually unchanged during that period (5). This finding served to underscore the latent nature of this stage, named the hypnozoite (sleeping animalcule) stage by Garnham (26).

Developing theory of relapse must now include this newly discovered stage, and to be considered as the true relapse body, the hypnozoite must meet certain testable criteria (43): (i) hypnozoites must be present in all *Plasmodium* species that cause relapsing malaria; (ii) they must be absent in *Plasmodium* species that cause nonrelapsing malaria; and (iii) they must be shown to decline in absolute numbers after relapse(s).

In 1985 it was demonstrated that hypnozoites were in fact present in two strains of *P. vivax* (47, 51), the first such demonstration in a human malaria species (Fig. 3). The two malarial strains have disparate relapse patterns: malaria caused by the Chesson strain (New Guinea-South Pacific) shows frequent relapses, and that caused by the North Korean strain is characterized by long latent periods. The numbers of hypnozoites found in relation to the number of developing schizonts and to the relapse pattern were as expected according to the hypnozoite theory.

To determine whether hypnozoites were present in a nonrelapsing type of malaria, Krotoski and Collins examined liver biopsy samples from monkeys infected with *P. knowlesi* (46). When the same sensitive IFA was used, no hypnozoites were seen after examination of 3 cm^2 of liver tissue, which by analogy to *P. cynomolgi*-infected liver would have been expected to contain 35 to 50 such forms.

Hypnozoites had now been found in two species of *Plasmodium* causing relapsing malaria and had not been found in a *Plasmodium* species causing a nonrelapsing malaria. These stages had been shown to be dormant, present in 229 days after sporozoite inoculation and undoubtedly malarial in nature (Fig. 4). In keeping with the history of malaria research, however, the theory had its detractors.

In 1981, Shortt, one of the original discoverers of malaria tissue stages, took issue with the preliminary report of the

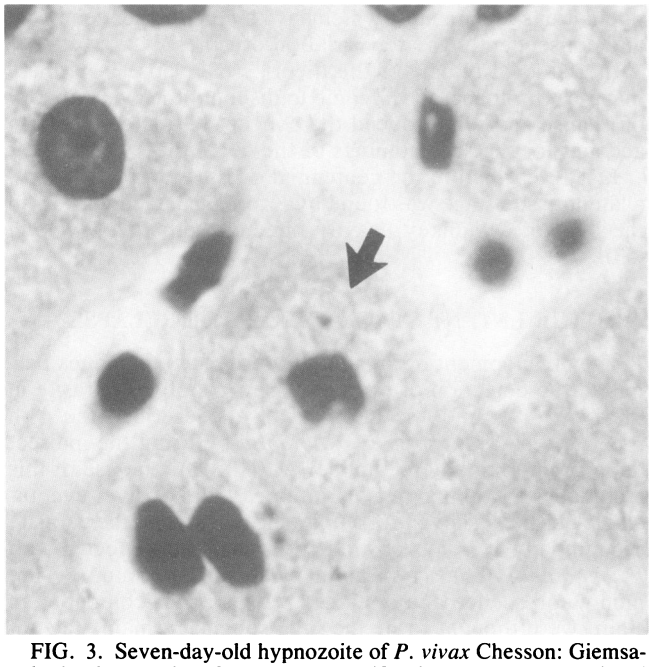

FIG. 3. Seven-day-old hypnozoite of *P. vivax* Chesson: Giemsa-colophonium stain after IFA. Magnification, ×1,260. Reprinted from the *American Journal of Tropical Medicine and Hygiene* (50) with permission of the publisher.

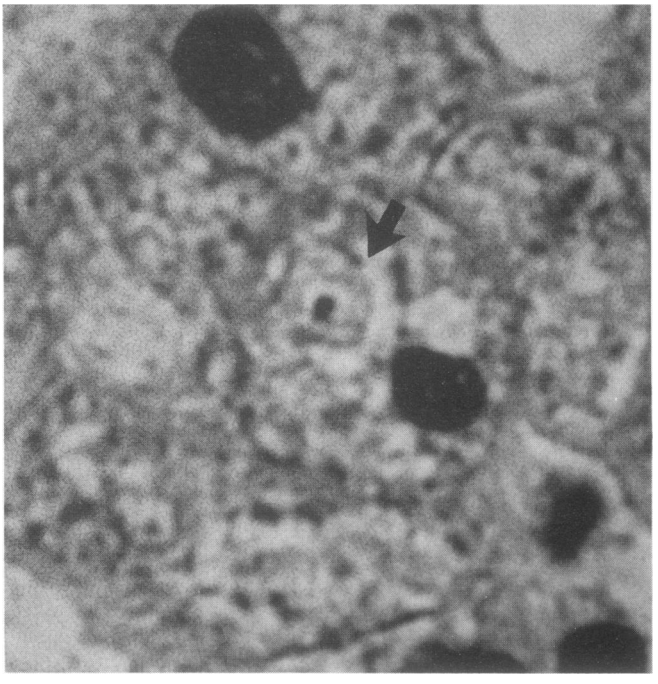

FIG. 5. Hypnozoite of *P. simiovale* 8 days after sporozoite inoculation: Giemsa-colophonium stain after IFA. Magnification, ×1,250. Reprinted from the *American Journal of Tropical Medicine and Hygiene* (10) with permission of the publisher.

discovery of hypnozoites (77, 78). He questioned their malarial nature, speculating that they might be contaminants from the mosquito (microsporidia, etc.) or even merozoites from early schizonts that had reinvaded liver cells. His objections were answered systematically by Garnham in a published reply and in subsequent reports of continuing work (28, 30).

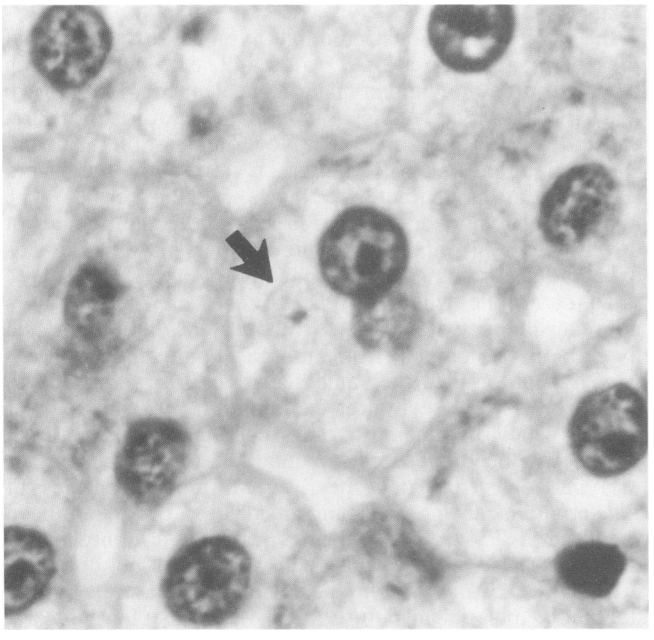

FIG. 4. Hypnozoite of *P. cynomolgi bastianelli* 229 days after sporozoite inoculation: Giemsa-colophonium stain after IFA. Magnification, ×1,260. Reprinted from *Progress in Clinical Parasitology* (44) with permission of the publisher.

The malarial nature of hypnozoites is underscored by their fluorescence with specific antisera. They are very different in size and morphology from merozoites and can be found at 3 days after sporozoite inoculation, well before preerythrocytic schizonts would be expected to mature.

Questions were also raised by Schmidt (71–73), who thought that the cyclic theory of relapse was more compatible with the number of relapses, the relapse interval, and the regularity of relapse he had documented in his extensive work with rhesus monkeys infected with *P. cynomolgi* (74). Close examination of Schmidt's voluminous data shows that the interval values were obtained by averaging observations from a large number of monkeys, and, in fact, the ranges of intervals for the duration of relapse were fairly long. Schmidt's concerns were addressed systematically by Knell (42), who pointed out that the regularity of relapses observed by Schmidt were somewhat artifactual, since they were the result of treatments at the first sign of parasitemia. Untreated infections show little such regularity. Schmidt did not comment on his own data, which showed that the size of the sporozoite inoculum is directly related to the frequency of relapses and that the frequency of relapse declined as the infection progressed. Both of these observations tend to support the latent stage theory of relapse.

Doubts were also expressed by Corradetti (18), who, while acknowledging the existence of hypnozoites, maintained that they were sporozoites that never developed. This conclusion is incorrect since the two stages are morphologically distinct.

Recently, hypnozoites have been found in monkeys infected with *P. simiovale* (10) (Fig. 5), an analog of the relapsing human malarial parasite *P. ovale* (12). The pres-

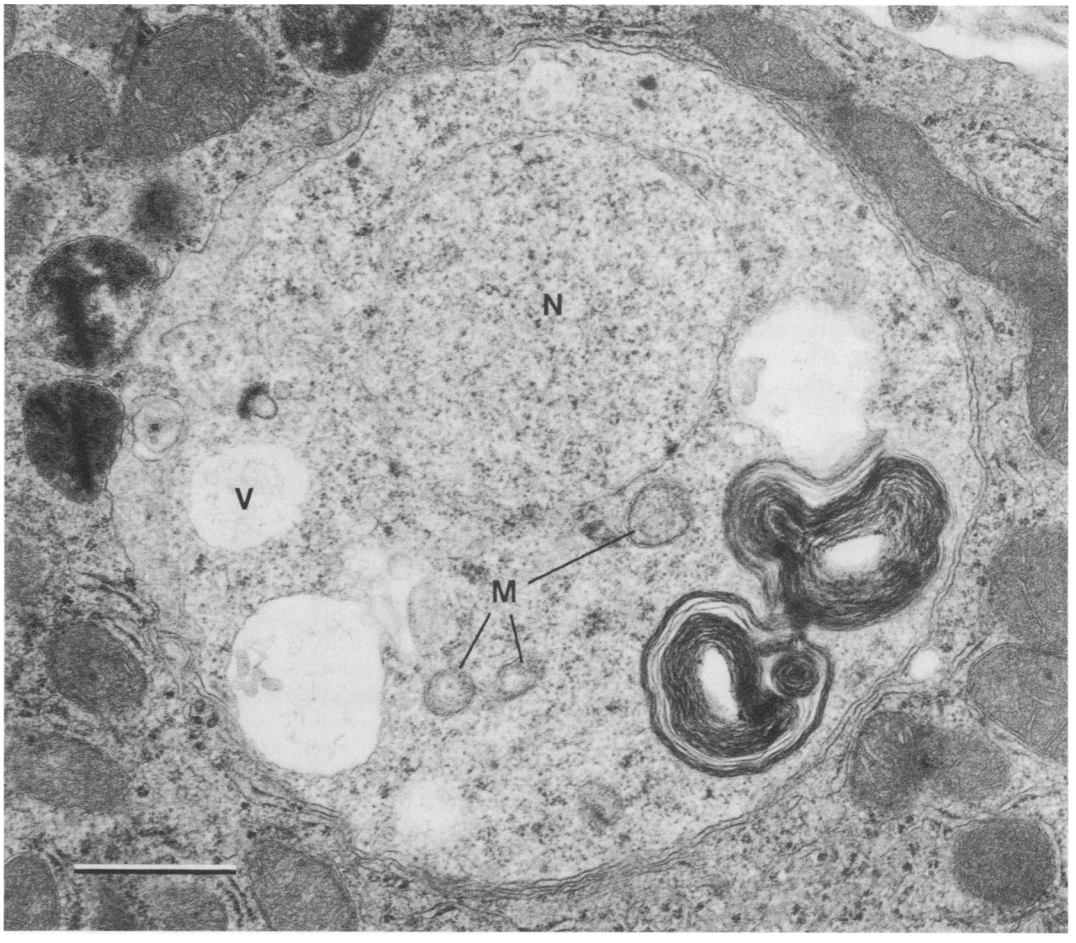

FIG. 6. Five-day-old uninucleate tissue form (presumably a hypnozoite) of *P. cynomolgi* from in vitro culture of rhesus hepatocytes. This parasite had remained uninucleate after other tissue forms had begun schizogony and is compatible in appearance with the hypnozoite. Bar, 1 μm. N, nucleus; M, mitochondria; V, vacuole. The sample was prepared as described by Atkinson et al. (1). (Photo courtesy of C. T. Atkinson and M. Aikawa.)

ence of this form in an ovale-type *Plasmodium* species once again supports the role of these forms of relapse bodies.

There is no experimental proof of a preerythrocytic cycle in malaria. A useful construct that has stimulated a valuable area of research and guided a generation of parasitologists, it fails to account for the long latent periods in some strains of *P. vivax*. The theory of latent tissue stages best accounts for relapse patterns, delayed prepatent periods, and sporozoite dilution experiments. The existence of the hypnozoite in three species of *Plasmodium* that cause relapsing malarias provides morphological confirmation of this theory.

THE HYPNOZOITE OF PRIMATE MALARIA

Hypnozoites are most easily located with an indirect immunofluorescence (52) or immunoperoxidase antibody (11, 37) technique in tissue preserved with Carnoy's fixative. They appear as round to nearly oval, well-defined, brightly fluorescing bodies approximately 5 μm in diameter within the cytoplasm of a hepatic parenchymal cell (45). Restained with Giemsa-colophonium, hypnozoites exhibit a light blue, slightly variegated cytoplasm with a distinct limiting membrane. The nucleus appears to be characteristic chromatin, with staining properties indistinguishable from those of the nuclei of schizonts. There is sometimes a partial "halo" of pinkish, clear cytoplasm around the nucleus, presenting a "target" appearance at low magnification. The immunofluorescence technique has a deleterious effect on subsequent staining of both schizonts and hypnozoites, and even with increased staining times (2 h), the process is less than satisfactory.

Only one instance of what appeared to be a dividing nucleus has been reported. In 1985, Bray et al. (5) recorded a hypnozoite of *P. cynomolgi* having two nuclei at 49 days after sporozoite inoculation. Growing schizonts were found on days 51, 55, and 56, and it is probable that this double nucleus was a dividing form.

In 1989, Atkinson et al. (1) published what may be the first electron micrograph of a hypnozoite (Fig. 6). The 5-day-old forms of *P. cynomolgi* were grown in primary cultures of hepatocytes and were labeled by monoclonal antibody to circumsporozoite antigen. Labeling was densest on the surface of the parasite, with some scattered labeling in the cytoplasm. A nucleus is visible, as are several nuclear pores. Atkinson et al. described both small uninucleate parasites (3.26 μm) and larger uninucleate forms (4.6 μm) at 5 days after sporozoite inoculation. The larger forms exhibited mitochondria in the cytoplasm and vacuolated areas. The

smaller forms contained a nucleus with numerous nuclear pores, mitochondria, scattered vacuolated areas, and randomly distributed electron-dense granules. The same tissue at 5 days contained multinucleated schizonts with numerous large vacuoles containing electron-dense flocculent material, suggesting that the small forms are dormant or delayed in developing. The suggestion of dormancy and the uninucleate appearance are consistent with the definition of hypnozoites.

The first to observe hypnozoites in culture were Hollingdale et al. in 1985 (36). They observed persistent nondividing *P. vivax* parasites in cultured hepatoma cells. The small forms (5 to 6 μm) were strongly reactive to 2F2 antisporozoite monoclonal antibodies and remained apparently unchanged in the culture at 15 days, which was 3 days after the last merozoites were produced. Both the penetration of sporozoites of *P. vivax* into cultured hepatocytes and their development into uninucleate forms seen by using an immunoperoxidase system (37) were documented.

In contrast, Krotoski et al. (50) had failed to find preerythrocytic forms in vivo earlier than 36 h when they used both anti-blood-stage and antisporozoite sera. Uninucleate forms were also reported in vitro by Millet et al. (62) 5 days after hepatocyte cultures were inoculated with *P. cynomolgi* sporozoites. It seems that in vitro culture will allow us to investigate early development of these preerythrocytic stages.

One of the tenets of the developing hypnozoite theory holds that more of the latent forms should be found in a strain that causes frequent relapses than in a strain with a long latent period. It is also true that ratio of hypnozoites to developing schizonts should be greater in a strain that causes frequent relapses than in a strain that causes few relapses (43, 44). This has been shown by Hollingdale et al. to be the case in the in vitro culture of two strains of *P. vivax* with disparate relapse patterns. Sporozoites of the North Korean strain of *P. vivax* invaded the hepatoma cells and differentiated predominantly into persisting, nondividing forms. In contrast, the Chesson and ONG strains of *P. vivax* differentiated into essentially equal proportions of schizonts and hypnozoites (35).

To date, hypnozoites of *P. cynomolgi* (three distinct strains) *P. vivax* (both North Korean and Chesson strains), and *P. simiovale* have been found in animal models. It is expected that these forms will be present in the life cycles of *P. ovale*, *P. fieldi*, and *P. schwetzi*. These stages were not found in monkeys infected with *P. knowlesi*, which causes a nonrelapsing malaria. It is expected that hypnozoites will not be a component of the life cycles of *P. falciparum*, *P. fragile*, *P. coatneyi*, *P. malariae*, or *P. inui*, each of which causes a nonrelapsing malaria.

TREATMENT OF LATENT STAGES

The most effective treatment of hypnozoites at present is primaquine, one of the 8-aminoquinolines thought to interrupt mitochondrial electron transport. Although drug levels sufficient to kill blood stage parasites are accompanied by unacceptable toxicity to the host, the drug is effective against all preerythrocytic forms. A likely explanation for this phenomenon is the ability of the blood stage parasites to synthesize further mitochondrial material. The dormant hypnozoites, not carrying out active synthesis, are then more susceptible to the drug, according to Warhurst (86).

Conversely, the diaminopyrimidines (e.g., pyrimethamine) and the biguanides (e.g., proguanil) are antimetabolites and are active against growing blood and tissue schizonts but ineffectual against mature gametocytes and hypnozoites, according to Jiang et al. (41) and Bray (4).

Ferreira et al. (22) and Schofield et al. (75) have recently shown that gamma interferon inhibits the development of preerythrocytic forms of malaria parasites. Quinoline esters were found by Puri and Dutta (66) to prevent relapses in monkeys infected with *P. cynomolgi*.

TOPICS FOR FURTHER RESEARCH

A number of questions about hypnozoites and their role in malarial relapse remain to be answered. To fulfill one of the basic tenets of the latent stage theory of relapse, hypnozoites should be found in all species of *Plasmodium* that cause relapsing malaria and should be absent from species that cause a nonrelapsing infection. To date, only three types of relapsing malaria have been examined and only one species of *Plasmodium* causing a nonrelapsing malaria has been investigated for hypnozoites. Other relapsing and nonrelapsing malarias must be searched for latent tissue stages to confirm the hypnozoite theory.

Another area of interest involves the relationship of the hypnozoite to its host cell. If the longevity of hepatic parenchymal cells is estimated to be less than 1 year, how are we to explain the occurrence of relapse after more than a year in some instances, e.g., in cases of infection with *P. vivax*? Although little is experimentally known about the life span of liver cells, the longevity of relapsing malaria cases suggests that the estimate of 1 year is less than precise. It is conceivable that the hypnozoite could survive in a daughter cell upon hepatocyte division. This topic remains one of some interest and considerable speculation and might be addressed experimentally by in vitro cultivation of hepatic stage parasites.

An additional area that merits attention concerns the early forms of preerythrocytic stage parasites. Why have forms less than 36 h old not been found in vivo even after diligent searching with both anti-erythrocyte-stage and antisporozoite antisera? These forms have been reported from culture (36) so their absence from biopsy samples remains an interesting enigma. Perhaps the use of more sensitive molecular biological techniques, e.g., polymerase chain reaction or in situ hybridization, would permit detection of these early stages in biopsy samples.

The search for evidence of the pleiotropic action of genes controlling the duration of tissue phases of the parasite, as proposed by Lysenko et al. (56), also remains a fertile area for experimentation. Studies at the genetic and molecular levels may serve to answer perhaps the most important question in regard to the dormant liver stages, i.e., what is the trigger for their activation?

Recent studies showing that preerythrocytic stages of *P. cynomolgi* induce a polyvalent immune response (61) point out the need for molecular characterization of both the schizont and the hypnozoite stages.

ACKNOWLEDGMENTS

This report could not have been produced without the invaluable guidance of W. A. Krotoski, mentor and friend, who provided editorial assistance, encouragement, and photographs but selflessly declined authorship. I also thank Sharon Nastasi for proofreading the manuscript and Murphy Dowouis for developing the photographs and drawing the life cycle. The editorial assistance of Norbert Lanners and Mario Philipp is gratefully acknowledged.

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