The fate of sporozoites
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In their tribute to Colonel Shortt on his 80th birthday, the renowned malariologists P.G. Shute and Sir Gordon Covell eloquently placed the discovery of the exoerythrocytic (EE) hepatic phase of mammalian malaria parasites in the following historical context (1). “Just as the name of Ross will forever be associated with the discovery that mosquitoes transmit malaria, so too, will the names of Shortt and Garnham will be remembered in connection with the primary tissue phase of the parasite.” It has been 52 years since Shortt & Garnham published their milestone finding (2) of the cyst-like body, filled with thousands of merozoites, in the liver of a rhesus monkey that had been inoculated 102 days before with Plasmodium cynomolgi sporozoites from 500 mosquitoes. With their bold experiment they solved a centuries-old mystery — the source of malaria’s parasitaemic relapses.

An outstanding parasitological goof had been the claim by Fritz Schaudinn (best remembered as the co-discoverer of the Treponema causation of syphilis) that the Plasmodium vivax sporozoite penetrated the erythrocyte (3). Schaudinn’s accompanying drawings showed “the theatrical picture of the entry of a malaria sporozoite into a red blood cell”, as Knowles commented (4). We may now wonder how such an erroneous observation could have been made by so distinguished and expert a protozoologist. We may also wonder at the pervasiveness of Schaudinn’s authority, so powerful that it overrode all the failures to substantiate his findings.

According to Shute & Covell (1) the first doubts of Schaudinn’s theory came from the malarialtherapy centres treating paretics. In practices that today would bring down the wrath of hospital patient oversight committees (and a phalanx of lawyers bearing malpractice briefs), malaria, mostly P. vivax, was induced either by direct inoculation of infected blood (continental European style) or by inoculating sporozoites by mosquito bites or in isolated salivary glands and ground-up thoraces (British style). The blood-inoculated patients were readily, radically, cured with quinine but the sporozoite-induced infections relapsed after the same therapy. The proof, albeit still circumstantial, that Schaudinn was totally wrong, that there was a missing link in the life cycle of human malaria, came from the remarkable experiment of Sir Neil Hamilton Fairley in Australia (5). Fairley showed that the blood of volunteers injected with large numbers of P. vivax sporozoites was infectious to other volunteers for only 30 minutes. The blood then became “sterile” until 7 days later when it once again became infectious to volunteers.

Although P. knowlesi had been known as a primate malaria since 1932 (6), during the first half of the 20th century — until the discovery of P. berghei (7) in a wild tree rat of the Congo — the avian malarias served as the main experimental models. Distinguished researchers of that period were bird malaria experts, e.g., Huff in the United States, Brumpt in France, Raffaele in Italy, and James in Britain. Bird malarias also relapsed after quinine treatment. Tissue and organ smears from infected birds revealed exoerythrocytic schizonts in reticulo-endothelial and hemoblastic cells (8–10). Prediction held that the exoerythrocytic stage of human and primate malarias would also be located in these tissues and the malaria birdmen were chagrined when the site turned out to be the hepatocyte.

Shortt was a traditionalist who held to the importance of lineage in science. He would peer over his half-glasses to issue a stern rebuke to a former student (who might by then be a full professor) for a serious “transgression”, such as straying into helminthology: “Remember who your teachers are!” he would say. That line of teachers, in his view, went from the student to Shortt to Rickard Christophers to Ross. In a sense, all present malaria researchers are
students in the lineage of Shortt (and, of course, Garnham) and it is fitting in this new millennium to be mindful of the far-reaching impact of the discovery of the EE bodies.

Consideration of virtually any aspect of malaria must now make some link with the search for an effective vaccine. Almost sixty years ago the pioneering experiments of Russell, Mulligan & Mohan (11) established the principle that each stage of the malaria parasite’s life cycle has its own unique antigenic signature. In now devising a stage-specific vaccine, the pre-erythrocytic schizonts have become an important target. An effective vaccine would prevent sporozoites from invading the hepatocyte and/or prevent maturation of exoerythrocytic schizonts to merozoites. Sporozoite ligands binding to hepatocyte receptors and liver stage-specific antigens have now been isolated. As adjuvanted vaccines they induce a cytotoxic T lymphocyte (CD8+)-mediated immunity which will kill the infected hepatocyte. It is also now proposed to use them as DNA vaccines either alone or in a multi-stage formulation (12–16).

We do not conventionally associate the awakening knowledge of the EE cycle with the massive, WHO-orchestrated Global Eradication of Malaria campaign (1955–72), but I believe that there was a crucial connection. The programme was proposed as a relatively short campaign after which there would be no continuous “maintenance”: with the cessation of transmission and the absence of reinfection, the malaria in a population (with the probable exception of P. malariae) would go to self-cure even without chemotherapeutic intervention. Since the discovery of the EE phase and its patterns in the human malaria, the source of the relapses was now known and the strategy, especially in respect of P. falciparum, could be applied with confidence. Thus Shortt & Garnham laid the logical foundation for the campaign.

The arguments are now fading with the years, as the graduate students and associates of these two remarkable men also now fade into history. A contention between them has been, “Did Garnham’s earlier finding of the liver stage of Hepatocystis kochi (17) point Shortt in the right direction?” My belief, undoubtedly influenced by being his last graduate student, is that Shortt, who directed the experiment, had no preconceived idea where the hidden parasites might be. The monkey was, literally, taken apart, tissue samples sectioned with the Department’s Spencer microtome, stained by the gorgeous Giemsa-colophonium method and scanned under Shortt’s new, prized Leitz binocular microscope which eventually revealed the “Eureka!” liver specimen.

My final words are of praise for these two famous men, each a physician-naturalist, each a devoted protozoologist. Shortt, a product of the Indian Medical Service, the relentless pursuer of all quarry — single-celled parasites, tigers, trout and houseflies — was a retiring man and yet generous and caring to his associates and students. Garnham, who came from the East African Medical Service, was a very different man: an aesthete who, as I recall, skied and played the cello. He was as comfortable in discussing the theatre, opera and literature as he was in eruditely explaining the lives and times of the Haemoporida. And although we here pay tribute to their discovery of the EE cycle we should also be mindful of their many other seminal contributions to medical protozoology.

References

DEMONSTRATION OF A PERSISTING EXO-ERYTHROCYTIC CYCLE IN PLASMODIUM CYNOMOLOGI AND ITS BEARING ON THE PRODUCTION OF RELAPSES

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From the earliest days of the modern study of malaria the phenomenon of relapses in this disease has been invested with a mystery which has been responsible for various hypothetical solutions made in an attempt to explain the known facts.

The first of these facts is that periods of patent infection accompanied by the demonstrable presence of parasites in the peripheral blood are often succeeded by more or less prolonged periods when no such parasitaemia can be demonstrated. This condition is described as latent malaria, and it may supervene in the natural course of the disease or may be produced artificially by the use of antimalarial therapy which destroys the erythrocytic infection. Another fact is that relapses are often precipitated by any happening which causes a temporary lowering of the host's resistance, and the problem here is the source of the parasites producing the relapse. A third fact is that the various species of malaria parasites vary in their tendency to relapse and in the period of years during which relapses may recur. A final fact relates to artificially produced infections. Those which are produced as the result of inoculation of blood show no tendency to relapse after antimalarial treatment, while those induced by sporozoite inoculations, either artificially or by mosquito bite, tend to produce relapses after the cessation of treatment.

The extent to which these facts are explained by the finding that is the subject of this communication is dealt with in the discussion. Up to the present time three commonly adduced explanations of relapses have held the field, but, in the absence of accurate knowledge of the life-cycle of malaria parasites, none could claim greater weight than a mere guess. These theories are: (a) The continued existence of a low-grade erythrocytic infection kept in abeyance by the host's immune mechanism but flaring up on any impairment of the latter; (b) the theory of the pathogenicetic development of the female gametocyte (Grassi, 1900; Schaudinn, 1902); (c) the existence of a cryptic stage in the internal organs capable of producing an erythrocytic invasion on any lowering of the host's resistance. The third of these theories gained added weight with the discovery of the exo-erythrocytic cycle in bird malaria, where these forms play an important, if not the essential, part in the causation of relapses.

The possibility that the hypothetical exo-erythrocytic cycle in mammalian malaria played a similar part in the production of relapses has been put forward at various times by many workers, and the names given in brackets are only a representative selection from workers who have made such a suggestion, after the first establishment of a definite exo-erythrocytic cycle in bird malaria by James and Tate in 1937. (James and Tate, 1937; Shortt, Menon, and Iyer, 1940; Fairley, 1945; Huff, 1947; Cooper, Ruhe, and Coatey (personal communication to Huff), 1947; Shortt and Garnham, 1948).

The discovery recorded in this paper of the continued existence in monkey malaria of the exo-erythrocytic cycle after establishment of the blood infection would appear to constitute the strongest proof that this form of the parasite is the aetiological agent concerned in the production of relapses. When we (Shortt and Garnham, 1948) published a detailed description of the pre-erythrocytic cycle in P. cynomoligi and P. vivax we suggested that if this cycle was found to persist after establishment of the blood infection it might play an essential part in the maintenance of the infection over long periods and in the production of relapses, so that the present work is the logical sequence to the experiment establishing the pre-erythrocytic cycle in mammalian malaria.

To investigate the theory of the persistence of the exo-erythrocytic cycle and its relation to relapses, examination of infected monkeys a considerable time after the establishment of sporozoite-induced infections seemed to offer the most direct approach to the problem. For this purpose we selected a monkey (Macaca mulatta) originally infected by sporozoites, in which the infection had reached a latent stage. This stage, with apparent absence of parasites in the peripheral blood, had lasted for over a month, and from our experience it was considered probable that a relapse would soon occur.

If we were correct in this assumption and if the hypothetical exo-erythrocytic forms, probably in the liver, were to be the source of parasites for the relapse, it would follow that examination of the liver would reveal these forms if in sufficient numbers to make this a practical proposition. This aggregation of "ifs" did not allow us to anticipate an easy or rapid conclusion to the investigation, but, as events turned out, the monkey chosen proved to be in the earliest stage of an imminent relapse and we were able to demonstrate the presence of an exo-erythrocytic stage. The finding is considered important enough to justify setting forth in detail the history of the monkey concerned.

This is given below.

Monkey 37 (Macaca mulatta). Weight 3 lb. Feb. 18, 1948: Fed on by 680 Anopheles maculipennis infected with P. cynomoligi. The mosquitoes were then ground up, suspended in serum-saline, and inoculated intraperitoneally and intramuscularly. Sporozoites were microscopically demonstrable in the 10-ml. volume of the suspension. Seven mosquitoes of this batch were fed, as a control, on Monkey 38, which developed malaria on March 2. On dissection of six of these mosquitoes five contained sporozoites in the salivary glands, showing that a very high proportion of the batch must have been infective. Feb. 21: A piece of liver removed by open operation. Feb. 23: A second similar operation.

The piece of liver removed on June 1 was placed in Carnoy's fixative and sections were prepared.  These showed the presence of exo-erythrocytic forms of *P. cynomolgi*.

**Discussion**

The finding of exo-erythrocytic schizonts of *P. cynomolgi* in the liver of a monkey nearly three and a half months after the production of a sporozoite-induced infection is unequivocal evidence of the persistence of the exo-erythrocytic cycle after establishment of the blood infection.  That this finding is the fulfilment of intelligent anticipation by various workers will be evident from the following quotations, which are only three chosen from many others.

Thus Fairley (1945) writes:  "The reappearance of erythrocytic forms in *P. vivax* after the blood has been completely cleared of parasites, no less than the tendency of benign tertian infections to relapse repeatedly despite prolonged antimalarial treatment, suggests the persistence of a tissue stage (exo-erythrocytic form) which, from time to time, throws off asexual parasites into the circulation for invasion of the erythrocytes."

Huff (1947) states:  "There is a suggestion, though no clear proof, in these statements that any hypothetical phanerozoic stages in human malaria may arise principally, if not wholly, from the, as yet, hypothetical pre-erythrocytic stages."

In 1948 we propounded an almost identical view in saying:  "If certain of the merozoites resulting from exo-erythrocytic schizogony enter fresh liver cells to maintain the local liver cycle, the destruction of the blood infection, either by specific immune response or by chemotherapeutic agents, would possibly leave intact the exo-erythrocytic cycle, which, under a suitable stimulus, could renew the blood infection."

It is true that the continuance of an exo-erythrocytic cycle is not clear scientific proof that this is the source of clinical relapses, but there are various facts which seem to make that a reasonable conclusion.  Thus in the course of an infection there are long periods when the presence of parasites in the circulation cannot be demonstrated even by inoculation of blood into susceptible hosts.  Again, in the case of sporozoite-induced infections, antimalarial treatment can suppress the erythrocytic cycle before a clinical attack is manifested or can apparently effectively sterilize the blood of the erythrocytic cycle when this is present.  Yet after a longer or shorter interval relapses will occur, and these presumably are caused by merozoites originating in the exo-erythrocytic schizonts in the liver.

Lastly, work on bird malaria and recent work on the exo-erythrocytic cycle in man (Shortt and Garnham, 1948) have shown that immunity against the erythrocytic parasite is not active against the exo-erythrocytic parasite.  This is possibly because the latter in their intracellular habitat in the parenchyma cells of the liver are protected from the host's immune mechanism and become susceptible only when the merozoites are released.  In these circumstances those merozoites which remain other liver cells are similarly protected and the liver cycle can go on for an indefinite period independently of the blood infection.

In the case of the monkey which was the subject of this experiment the fact that the blood was negative on the day of the operation and became positive on the day after would indicate that we were correct in expecting an early relapse and, in fact, that the relapse was imminent on the day of the operation.  The finding of a contemporaneous exo-erythrocytic cycle would supply a reasonable explanation of the source of the parasites producing the relapse if the interpretation of our findings given below is accepted.
Although it involves some repetition, we think it would be useful to give, in a few words, our interpretation of the findings obtained in our recent work on the exo-erythrocytic cycle in mammalian malaria.

The inoculation of sporozoites by the infected mosquito is followed by a pre-erythrocytic development in the parenchyma cells of the liver, with the ultimate production of merozoites. Many of these enter the erythrocytes to produce a parasitaemia and a clinical attack of malaria. Other merozoites enter normal liver cells and repeat the process of exo-erythrocytic schizogony. This latter process repeats itself indefinitely, irrespective of whether the erythrocytic cycle is present or is in abeyance as the result of antimalarial treatment or a naturally acquired active immunity. This active immunity is operative only against the erythrocytic parasites and destroys those merozoites liberated by the exo-erythrocytic schizonts which are destined to enter red cells. Those which enter liver cells to maintain the exo-erythrocytic cycle are protected from this immunity by their intracellular position outside the circulating blood.

If, for any reason, the active immunity of the host is impaired it no longer operates against the merozoites destined to start the erythrocytic cycle, and these enter the blood cells and initiate a clinical relapse.

The marked similarities both in erythrocytic and in pre-erythrocytic stages between *P. cynomolgi* and *P. vivax* make it reasonable to suppose that the course of events here described in the case of the former parasite will be applicable to the latter.

Until recently the fact that exo-erythrocytic development is the rule in the case of avian plasmodia, while it was not demonstrable in the case of mammalian plasmodia, tended to cause some misgiving in placing both groups in the same genus. The discovery, however, of the pre-erythrocytic cycle in simian and human plasmodia appeared to narrow the gap between the two groups, and now that the simultaneous existence of erythrocytic and exo-erythrocytic cycles has been demonstrated in a simian *Plasmodium* there seems still less justification for considering avian and mammalian plasmodia as other than very closely related.

Summary

The finding of exo-erythrocytic schizonts of *Plasmodium cynomolgi* in the liver of a monkey about three and a half months after a sporozoite-induced infection is evidence of the persistence of the exo-erythrocytic cycle after establishment of the blood infection.

Reasons are given for the assumption that this is the cycle responsible for the production of relapses.

As in earlier work in this investigation we wish to record the valuable technical assistance of our staff, Mr. W. Cooper and Mr. E. Blackie and Miss J. Stedman.

References

