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Studies on the life cycle of malaria parasites with special reference to recent research on relapses of « Plasmodium vivax » and allied species and the discovery of a new stage of the parasite (the hypnozoite) (**)

Before embarking on the main topic, I should like to draw attention to certain factors connected with the life cycle of malaria parasites of a more general character, which have not received much detailed study.

1. *The origin of malaria parasites.*

As parasites, they live in two hosts — vertebrate and invertebrate (Garnham, 1966). There is a tendency to assume that the vertebrate, the invertebrate and the parasite have roughly the same phylogeny; it seems likely that there is a range, applicable to all the hosts from the primitive to those exhibiting more advanced features, particularly in the exoerythrocytic [E.E.] stages.

Briefly, the sequence is as follows:

Reptiles (confined to tropics) malaria common in lizards; rare in snakes. Invertebrate hosts are varied — *Phlebotomus*, *Culicoides*, probably culicine mosquitoes.

Birds (cosmopolitan) wide range of vertebrate hosts; invertebrate hosts are specially *Aedes*, culicines, rarely *Anopheles*.

Mammals (confined to Old World with 2 minor exceptions — *P. brasilianum* and *P. odocoilei*) vertebrate hosts are specially primates, including Man; local species in other Orders, including African rodents. Invertebrate host is always

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Anopheles. Human species in the Americas probably represent old world importations.

In order to determine the evolutionary age of a parasite and its hosts, it is necessary to look at the sequences in the DNA structure, but this has rarely been attempted. Fossil evidence may give clues to the hosts, but not to the phylogeny of the parasite.

2. *Chronobiology*.

Each species has its characteristic time-table — e.g., tertian and quartan malaria which reflects the duration of schizogony in the blood. But chronobiology affects more than the blood stages, e.g. liver stages, sporogony, gametocytogenesis (Hawking phenomenon) etc. But the phenomena in any one species seem to possess a definite relationship. Golgi described the blood cycles (hence the name - the "Golgi cycle") but he did not explain why the growing schizonts fall into step, so that they mature at the same hour.

3. *Pathology*.

The primary feature in all species is the destruction of red blood cells, either following the rupture of the schizont, or from a non-specific and unknown cause. Immunity follows.

4. *Gametocytes*.

a) What is the stimulus for gametocyte production?

b) What is the factor which inhibits gametocytogenesis, e.g. after frequent blood passage of the parasite?

c) Modern culture methods (e.g. of *P. falciparum*) result in the production of viable crescents, but the precise cytological details are unknown.

5. *Rodent malaria*.

Puzzling questions remain in research on these parasites, including:

a) The very short exoerythrocytic (E.E.) stage - occupying only 2 or 3 days (in contrast to the 5-15 days in primate malaria). Cultural features might provide a clue.

b) Unique absence of pyrexia in rodent malaria.

c) Absence of the micropore in the sporozoites, though it is present in all other stages.

d) There is no stippling of the infected corpuscle in rodent malaria.

Three more practical, but major problems relate to the following:

(i) *Resistance of parasites to drugs* — new compounds are needed, and possibly the Chinese drug, Qinghasu, derived from the plant *Artemesia annua* may be useful, as it is of a different nature from the old drugs.

(ii) *Resistance of mosquitoes to insecticides.*

(iii) *The strong immunity* which occurs in malaria suggests that vaccines may be useful and this subject is being exhaustively studied today (see Working Group of Pontificia Academia Scientiarum 1982).

Recent Work on the Relapse Phenomenon

Relapses in malaria have been recognised since the time of Hippocrates in 400 B.C. Since that time, people have been fully aware of relapses, but practically no attempt was made to investigate their nature. Of course, the problem was insoluble until basic knowledge had been acquired of the cause of the disease, *viz.* the discovery of the parasite in the blood and its cycle in the erythrocyte, its transmission by mosquitoes and its development in the parenchyma cells of the liver.

I do not need to describe these 3 cycles (see fig. 1, Bray and Garnham, 1982) in detail, but might just point out that development in the mosquito requires the presence of viable male and female gametocytes in the imbibed blood, and that subsequent development in the vertebrate is entirely dependent upon the introduction of sporozoites. Grassi (1901) knew, of course, the details of these first two cycles and even had an inkling of the third. In his *schema*, he placed a question mark between the sporozoite and the blood cycle. Today, we can substitute the hypnozoite for the blank. The first clue to the origin of relapses was provided by the discovery of exoerythrocytic development in the liver.

First, let us clarify 2 points: 1) the species of malaria parasites in which relapses occur and 2) the terminology of "relapses". For some unknown reason, it is only those species which produce Schuffner's dots in the infected red blood cells that exhibit the relapse phenomenon *viz.* in man, *P. vivax* and *P. ovale*, and in monkeys, *P. cynomolgi*, *P. simiovale*, *P. fieldi*; also *P. schwetzi* of chimpanzees and gorillas and *P. silvaticum* of orang-utans.

The drafting committee of WHO limited the true "relapse" to renewed manifestations of parasitaemia which are the direct product of exoerythrocytic schizogony, and which can only occur after sporozoite inoculation. A "recrudescence" on the other hand is a renewed manifestation of parasitaemia due to the survival of *erythrocytic* forms of the parasite and may occur in all species of malaria parasite e.g. *P. falciparum* and *P. malariae* in man or *P. knowlesi* and *P. inui*, etc., in the monkey (Garnham, 1966).

Corradetti has often pointed out that it is impossible to distinguish, in practice, between a relapse and a recrudescence. He (1981) emphasized the

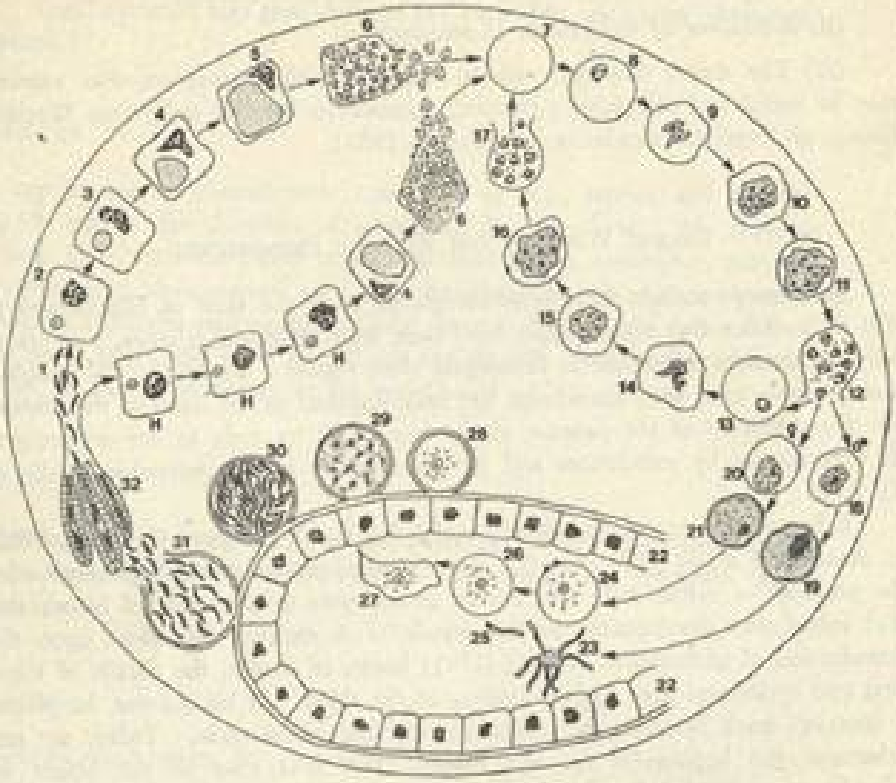

Fig. 1. - The life-cycle of a primate malaria parasite.

1: sporozoites injected into skin by the mosquito; 2: 2-day-old exoerythrocytic form in a hepatocyte; 3, 4, 5: growing exoerythrocytic schizonts; H: hypnozoites in hepatocytes; 6: mature exoerythrocytic schizonts bursting, releasing merozoites into the blood; 7: erythrocyte; 8, 9: growing trophozoites; 10, 11: growing schizonts; 12: mature schizont releasing merozoites; 13, 14, 15, 16, 17: erythrocytic cycle repeated; 18, 19: growth of the microgametocyte; 20, 21: growth of the macrogametocyte; 22: mosquito has taken gametocytes up into its mid-gut; 23: exflagellation of the microgametocyte; 24: macrogametocyte escapes from erythrocyte to become a macrogamete; 25: microgamete; 26: macrogamete about to be fertilized; 27: zygote or oökinete; 28, 29, 30: oöcyst growth on the mid-gut surface; 31: oöcyst bursting, releasing sporozoites; 32: sporozoites in the mosquito salivary glands.

gross inaccuracy of using the term "relapse" for those cases of *P. vivax* malaria in temperate or subtropical regions, in which delayed prepatency — up to 8 years or more — is a characteristic feature. He rightly calls them delayed primary attacks.

When Colonel Shortt and I (1948b) first drew attention to the presence of E.E. schizonts in the liver of monkeys three and a half months after we had infected them with the sporozoites of *P. cynomolgi*, we assumed that here were the bodies responsible for relapses and we quickly formulated the theory that repeated E.E. schizogony took place in the liver and that a few merozoites re-invaded hepatocytes instead of erythrocytes. Immunity gradually developed and cut short the blood infection destroying the stream of merozoites from the liver; immunity eventually declined, the animal became susceptible, the merozoites proceeded to develop in the erythrocytes, and initiated a relapse. But according to our original theory, showers of E.E. merozoites should continue to be produced and *immunity* would persist as the antigen would always be present.

Several people, including Sir Neil Hamilton Fairley (1949) stated that such a theory was unacceptable also because:

1. What happens during prolonged prepatency, *i.e. when* parasites do not appear in the blood until long after (months or even years) the normal incubation period? According to our original theory there should be a limited number of histotropic merozoites at the end of each schizogonic generation in the liver and either parasitaemia must ensue, or immunity may inhibit the multiplication of the parasites in the blood. But, no parasites are found in the blood until the end of the prolonged prepatent period. And, no immunity is present, because, if blood parasites are inoculated during this negative period, a normal parasitaemia will at once arise, showing that immunity must be absent.

2. A reduction in the number of sporozoites of *P. vivax* (*P. cynomolgi* and *P. simiovale*) inoculated, is followed by a similar reduction in the number of relapses (Contacos and Collins, 1973).

Latency of the parasite in some form or other seems to be the only explanation of these queries, and for a long time, the existence of a special body — the X body of Shute (1946) or the dormozoite of Markus (1976) (a name later changed to hypnozoite by Garnham, 1977) — was strongly suspected. But all attempts to *find* a parasite in a state of latency *failed* — until the recent work of Krotoski and his collaborators (1980), when hypnozoites were demonstrated.

Hypnozoites are only found in infections of species which exhibit relapses — such as the *vivax group*. On the contrary no hypnozoites could be found in species in which relapses were absent, such as *P. knowlesi* (Bray *et al.*, 1985), or *P. malariae*. The hypnozoite theory of relapses was confirmed by many later experiments.

Colonel Shortt disagrees with our new theory and has an important paper

expressing his views in the October number (1983) of the *Transactions of the Royal Society of Tropical Medicine and Hygiene*. The paper is followed by our refutation (Garnham *et al.*, 1983) of his argument, though I disliked to renegue from the Shortt-Garnham theory.

I now propose to describe shortly the events which started in 1978 at the 4th International Congress of Parasitology in Warsaw. Dr. Krotoski approached me with the suggestion that he and I should try to find what happened to the sporozoite after it entered the body of the primate — and thus complete the story of the life cycle (except for the interpretation of the relapse phenomenon). The date was exactly 30 years after the original discovery of Shortt and myself. In the interval, Krotoski had developed with much success the technique of immuno-fluorescence to demonstrate E.E. parasites in the liver; he had shown me his work with W.E. Collins at Atlanta in 1976 and I was happy to become associated with a new star in the firmament of malariologists.

We arranged that my colleagues and I in England should inoculate rhesus monkeys with millions of sporozoites of *P. cynomolgi* from infected salivary glands of mosquitoes, then to take biopsies of the liver at appropriate intervals in order to watch the development of the sporozoite in the organ. The Carnoy-fixed material was examined in England by the special Giemsa-Colophonium technique and a duplicate set was sent to Dr. Krotoski in New Orleans for the application of the immuno-fluorescence test (IFAT).

I shall just make a brief mention of the negative result of both methods (IFAT and Giemsa) to detect 24 hour forms in the liver and indeed fewer than expected E.E. schizonts in the 46 hour biopsies. I ascribe this failure to the possibility that, when the sporozoite penetrates the host cell, it loses its outer membrane; the latter contains the antigen which reacts in the IFAT. The antigen takes nearly 2 days to become reformed in the surface cost of the developing E.E. schizont. The normal duration of growth to maturity of *P. vivax* and allied species is 8 days.

It thus seemed that our first attempts to detect the earliest development of the sporozoite were a failure. We repeated the experiments several times, and extended the time of biopsies at both ends of the scale, *viz.*, as early as 5 minutes after inoculation of sporozoites and up to 229 days later. As before, nothing was visible in the liver during the first 36 hours, but the later biopsies showed, of course, the normal cycle of growth of the E.E. schizont up to 8 days (and persistence of hypnozoites up to 229 days).

Our first objective, *i.e.*, to observe the earliest development of sporozoites in the liver was thus a complete failure! But, instead, in the course of the same experiments we solved the mystery of relapses, and demonstrated the long-sought latent stage of the malaria parasite — the hypnozoite — in the parenchyma cells of the liver.

In the 7 day material, Krotoski's eye was suddenly struck by the appearance of tiny, strongly fluorescent, bodies in the sections exposed to the IFAT. He immediately phoned us in England and we replied, "Mark the exact location

of the fluorescent bodies and restain the sections by the Giemsa-Colophonium technique*. He did so and the bodies were now seen to be 5-6 µm in diameter, with a heavily stained single nucleus and lying in a parenchyma cell of the liver. We then re-examined our Giemsa-stained material and with great difficulty were able to detect the hypnozoite. The examination of the tissue has to be done under 1/12 objective, and every single parenchyma cell must be scrutinised, so that, even in heavily infected material, it takes many hours to find a hypnozoite. Of course, with IFAT, the fluorescent object is much more easily observed — and under a lower magnification. I need to stress the importance of using very large numbers of viable sporozoites (up to 80 million), and relatively small monkeys with correspondingly small livers. The biopsy must be taken after laparotomy, in order to get a good piece of tissue. A biopsy needle is quite useless.

Hypnozoites of *P. c. bastianellii* have now been found at the following times: 3 days, 5 days, 7 days, 12 days, 14 days, then at weekly intervals until 56 days; then at 77 days, 91 days, 105 days in the first experiment. In the second experiment, the timing of the biopsies was different, because they were taken at 2 day intervals between the 49th and 62nd days (7 biopsies) in order to see if a synchronous growth of reactivated schizonts could be detected. But unfortunately at this stage of the infection, *i.e.* starting 7 weeks after the inoculation of sporozoites, reactived schizonts were too rare to get a clear picture. In this experiment, the numbers of hypnozoites were much higher, but they began to decline after about 13 weeks — corresponding to the expected time of the first relapse in *P. cynomolgi* infections. Our theory is that some hypnozoites become reactivated about this time and in this strain, but many remain latent — as was seen in another experiment when biopsies were continued until Day 229, when hypnozoites were found also at 140, 168, 196 and 229 days.

There are 2 points which I wish to emphasize:

1. The size and morphology of the hypnozoite remain practically the same from the 7th day to Day 229. On Days 3 and 5, the size is slightly smaller — *viz.* 3.8 µm on Day 3; on Day 7, the average dimension is 4.7 µm, and this size persists until the last biopsies, with a slight increase to 5.7 µm.

2. The hypnozoite throughout the periods remains uninucleate. The nucleus is a remarkably distinct object after Giemsa staining; it is usually angular or irregular in outline and is surrounded by a pale pink halo. The cytoplasm is granular and light blue in colour. A thin but distinct membrane encloses the parasite. A target-like appearance is most characteristic and after practice one is able to identify the hypnozoite with increasing facility.

Our hypnozoite theory was confirmed (1982) by experiments that we carried out, using 2 strains of *P. vivax*, in splenectomised chimpanzees. As in the simian species, we found identical uninucleate bodies, 4-5 µm in diameter, 7 and

10 days after inoculation of 2 million sporozoites of the Chesson strain and 10 million of the North Korean strain. We had previously characterised these two strains in observations on human patients suffering from cerebral syphilis who were being treated by "malaria therapy". This was in collaboration (1976) with our Rumanian colleagues, Lupascu, Branzei and Ungureanu. I shall not go into detail but would point out that the Chesson strain has a normal incubation period of (minimum) 8 days and late relapses while the North Korean very frequently exhibits prepatency for 250 days — or in 1 case 629 days. The latter strongly resembles the Moscow strain of *P. vivax* which Nicolaiev (1949) named *P. v. hibernans* because it invariably "hibernated" after sporozoite induction. Very recent work by Chinese parasitologists, Jing Bo Jiang, at the Sun Yat-sen University in Canton (Guangzhou), has revealed another form of vivax malaria with a prolonged incubation period — which was named *P. vivax multinucleatum*. Seven volunteers had incubation periods of 236-441 days.

It is clear that many strains or subspp of *P. vivax* exist and until they are characterised by their isoenzymes or by other biochemical properties, the taxonomy must remain provisional.

Hypnozoites have now been demonstrated in several subspecies of *P. cynomolgi* of monkeys and in 3 strains of *P. vivax*. True relapses have been found in *P. ovale* of man and in simian and ape species — *P. fieldi*, *P. simiovale* and *P. schwetzi*; hypnozoites have not yet been seen in the last three parasites, but they surely exist. All these species have another common character — the infected red blood cells are heavily stippled with Schüffner's dots.

We have further negative evidence to support the hypnozoite theory. It is only those species of malaria parasites with long term relapses which exhibit hypnozoites. The experiments, using very heavy doses of sporozoites of *P. knowlesi*, revealed no hypnozoites in biopsies done on the 5th and 42nd days after sporozoite infection. Similarly no hynozoites could be found by the IFAT on old liver blocks containing very numerous exoerythrocytic schizonts of *P. malariae* and *P. falciparum*, while the examination of old blocks containing *P. cynomolgi* and *P. vivax* revealed hypnozoites without difficulty.

Primaquin, in contrast to blood schizonticidal drugs, has a direct action on exoerythrocytic stages of the parasite. We therefore watched its action on a monkey which had been inoculated with 74 million sporozoites of *P. c. hastinellii*; 8 days later parasites appeared in the blood, and hypnozoites were found in its liver on the 5th, 21st and 28th days. On the 35th day it was given primaquin (0.5 mg/kg) daily for 14 days; two further biopsies were taken on the 49th and 91st days — but hypnozoites were absent. In the control monkey (inoculated with 46 million sporozoites) hypnozoites were found in 9 biopsies up to Day 105. Examinations were done under "blind" conditions. The parasiticidal effect of the drug indicates that the hypnozoites were viable.

Other confirmatory evidence regarding the nature of the hypnozoite was provided by substituting an immunoperoxidase technique in place of IFAT on

the liver sections (Cogswell *et al.*, 1983). Similar results were obtained, and the advantage of this method is that the sections can be preserved indefinitely.

We began this research with another objective, which was not achieved. However, in the meantime the fate of the sporozoite on entry into the liver was discovered by several groups of investigators, chiefly with *P. berghei* and working by 2 methods: 1) *in vitro* observations on tissue cultures of hepatocytes infected with sporozoites by Lambiotte and Landau *et al.*, (1981) in Paris, and Hollingdale and Nussenzweig (1984) in the US, who observed growth to maturity of the parasite and 2) *in vivo* infection of rats in which specimens of liver were taken 10, 15 minutes or later for electron microscopy, by Meis, Meuwissen *et al.*, (1984) in Nijmegen. These Dutch workers showed clearly that sporozoites entered the Kupffer cells and remained viable for a short time; if they survived, they passed through the Kupffer cell and into the hepatocyte; others however went directly into the liver cell. Mazier *et al.*, (1985) has had some success with cultures of hepatocytes, infected with sporozoites of Brazilian and African strains of *P. falciparum*. None of this work unfortunately has thrown any light on the ultimate fate of hypnozoites of the vivax group, which would entail maintaining the cultures alive for months.

I ended my refutation of Col. Shortt's criticism of our hypnozoite theory, by listing a few of the outstanding problems which still need research. I mention three here because it may be that the Accademia Nazionale delle Scienze will be able to stimulate work on some of them:

1. The nature of the trigger which reactivates the hypnozoites.

2. The nature of the difference between sporozoites of the Chesson (with no delayed prepatency) and the N. Korean (usually *with* delayed prepatency) possibly by the use of monoclonal antibodies, or by observing the ultrastructure.

3. The transformation of the hypnozoite into the active stage. The most feasible method would be prolonged culture of the 2 types of sporozoite.

4. Search for an easier simian model for further hypnozoite studies and relapses, e.g. substitute *Aotus* for chimpanzees for *P. vivax* work; initiate work on *P. simiovale* and *P. fieldi* using rhesus; similar experiments with *P. ovale*.

5. Characterise biochemically a) strains of *P. vivax* and b) strains of *P. malariae*, *P. brasilianum*, *P. inui* (as control).

Such work would need the following.

1. *Expertise*. Suitable candidates would need training in special methods, e.g. at the London School of Hygiene and Tropical Medicine (Draper; Peters); Imperial College of Science and Technology (Bray, Killick-Kendrick, Sinden); Musée National d'Histoire Naturelle, Paris, (Chabaud, Landau); Division of Parasitology, New York University Medical School (Vanderberg, Nussenzweig); National Institutes of Health, Bethesda (Gwadz, Miller); Hansen's Disease Cen-

tre, Carville, Louisiana (Krotoski); Centers for Disease Control, Atlanta, Georgia (Collins) and certain other Institutes such as Istituto Superiore di Sanità, Rome; University of Nijmegen, Medical School, Holland.

2. *Fully equipped parasitological laboratories*, including first class insectories and accommodation for primates.

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