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DEMONSTRATION OF A PERSISTING EXO-ERYTHROCYTIC CYCLE IN PLASMODIUM CYNOMOLGI 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 such 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 parthenogenetic 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 Coatney (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. cynomolgi* 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. cynomolgi*. The mosquitoes were then ground up, suspended in serum-saline, and inoculated into the same monkey 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.

Feb. 26 : A third similar operation. March 1 : Blood contained rings and maturing schizonts. March 10 : Numerous mature gametocytes present. April 1 : Blood negative. April 7 : Scanty infection of blood. April 24 : Condition of blood similar. May 5 : Blood negative. May 22 : Blood negative. May 26 : Blood negative. June 1 : Blood negative. Piece of liver removed by open operation. June 2 : Considerable numbers of very early ring forms (23 in 100 fields of 1/12 oil-immersion lens). June 4 : Scanty mature schizonts.

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*.

Description

The description given applies to the two exo-erythrocytic parasites encountered. The first was situated in a parenchyma cell of the liver and extended over four serial sections cut at 4 μ in thickness. This would give the parasite a diameter of 16 μ, which was confirmed by actual measurement of the longest diameter. The stain in use was the modified Giemsa previously employed by us for pre-erythrocytic stages of *P. cynomolgi* in sections of liver.

The parasite was an almost spherical schizont lying in a parenchyma cell, the cytoplasm of which was separated from the schizont by a clear area either due to shrinkage or to some cytolytic effect. In one part of the periphery of the infected liver cell was the nucleus, itself somewhat flattened but not obviously degenerate.

The parasite had a pastel-blue-staining cytoplasm, markedly and coarsely granular and with a single sharply differentiated spherical blue staining mass, the origin and explanation of which is obscure. In this cytoplasm were distributed irregularly shaped masses of chromatin staining a magenta colour and numbering about 130 when counted over the four sections in which the parasite appeared. This schizont was similar in appearance to those described by us as occurring in the pre-erythrocytic development of *P. cynomolgi*, and in size and general morphology was comparable to a parasite between the fifth and sixth days of pre-erythrocytic development. The parasite appeared to be bounded by a wavy line which might indicate a containing membrane.

It does not, of course, follow that the size of this exo-erythrocytic form would necessarily imply that it was of the same developmental age as a pre-erythrocytic parasite of the same size, but this is probably a justifiable conclusion in the absence of any contrary evidence.

The second parasite encountered, almost spherical in shape, was in an advanced stage of development, comparable to the eighth-day stage of the pre-erythrocytic cycle, and measured 30 μ in the longest diameter. The blue cytoplasm showed commencing segregation to form merozoites, the chromatin particles of which numbered over 500. The cytoplasm exhibited two small vacuoles and several very densely stained blue masses which we interpret as residual bodies. The outline of the parasite was wavy as in the smaller form.

After the exo-erythrocytic cycle has been in progress for some time synchronism among the parasites is lost and exo-erythrocytic forms in various stages of development may be found, presumably from their first entry into the liver cells up to mature schizonts. The relative rarity of these bodies is indicated by the fact that 412 sections were examined in finding two schizonts.

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."

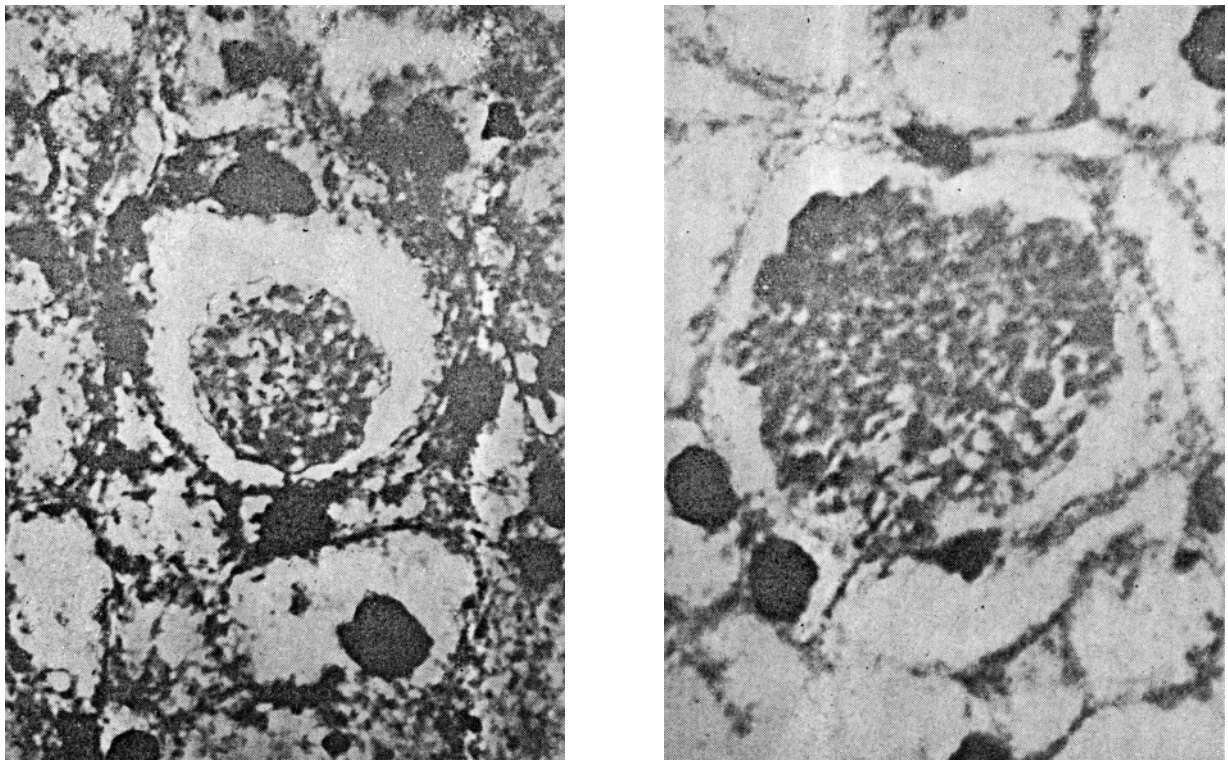
Huff (1947) states: "There is a suggestion, though no clear proof, in these statements that any hypothetical phanerozoic stages in human malaria may arise principally, if not wholly, from the, as yet, hypothetical pre-erythrocytic stages."

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 regain 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.



Two exo-erythrocytic schizonts of *Plasmodium cynomolgi*, in sections of liver, the probable cause of relapses in simian malaria, photographed on the same scale, ×1700.

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.

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