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Plasmodium falciparum: Studies on Mature Exoerythrocytic Forms in the Liver of the Chimpanzee, *Pan troglodytes*

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MEIS, J. F. G. M., PONNUDURAI, T., MONS, B., VAN BELKUM, A., VAN EERD, P. M. C. A., DRUILHE, P., AND SCHELLEKENS, H. 1990. *Plasmodium falciparum:* Studies on mature exoerythrocytic forms in the liver of the chimpanzee, *Pan troglodytes*. *Experimental Parasitology* 70, 1–11. Mature exoerythrocytic forms (EEF) of *Plasmodium falciparum* from the chimpanzee were examined by light- and transmission electron microscopy from a liver biopsy taken on Day 6 after sporozoite inoculation. Infectivity of the sporozoites obtained from whole mosquitoes which were membrane fed on cultured gametocytes was about 4–6%. In comparison, salivary gland sporozoites added to human hepatocytes *in vitro* had only a developmental percentage of 0.02 to 0.05% at Day 5. The EEF found in the liver biopsy were not all at the same stage of development. Immature compact parasites were seen simultaneously with stages with fully formed merozoites, indicating a rapid final maturation or asynchrony. At Day 7.5, large numbers of rings were already seen in the peripheral blood, indicating a duration of the liver development of *P. falciparum* in the chimpanzee of about 5.5–6 days. The process of merogony at the fine structural level was comparable to that described for rodent and other primate parasites *in vivo*. Compared to the fine structure of EEF *in vitro* in cultured human hepatocytes, the parasites described here were much more advanced in development. There appeared to be some cell infiltration with collagen deposition around the intracellular parasite; however, no marked degeneration of EEF was observed.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Plasmodium falciparum;* Protozoa; Malaria; Exoerythrocytic forms (EEF); Sporozoites; Merogony; Parasitophorous vacuole (PV); Liver, hepatocytes; Chimpanzee, *Pan troglodytes;* Ultrastructure; Transmission electron microscopy.

INTRODUCTION

Exoerythrocytic forms (EEF) of *Plasmodium falciparum* were first described by Shortt *et al.* (1951) in a liver biopsy taken from a human volunteer 5¾ days after infection by the bites of very large numbers of mosquitoes. Jeffery *et al.* (1952) subsequently published a study on *P. falciparum* EEF involving 14 patients selected for malaria therapy. Infection was by bite of heavily infected mosquitoes, intravenous inoculation of dissected salivary glands, or both. Only in the liver biopsy of the patient receiving the highest sporozoite inoculum could EEF be detected by microscopy. All other patients became patent from Days 7 to 13. Since it is not feasible to routinely study the liver EEF of *P. falciparum* in humans, alternative hosts have been sought. Bray (1958, 1960) and Bray and Gunders (1962, 1963) succeeded in infecting the chimpanzee with *P. falciparum* sporozoites and studying the development of EEF in detail. Patent parasitemia was obtained in the intact nonsplenectomized animal. After splenectomy, however, parasitemias became much higher. The susceptibility of

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other nonhuman primates for *P. falciparum* sporozoites has been studied subsequently by several investigators (for review see Coatney *et al.* 1971). Sodeman *et al.* (1969) reported EEF in biopsies taken at Days 6 and 7 from the owl monkey, *Aotus trivirgatus,* which appeared retarded as compared to EEF in the chimpanzee and in man. The owl monkey did not develop patent blood infection, which was thought to indicate abnormal development of the EEF. Druilhe *et al.* (1982, 1984) described *P. falciparum* EEF in the capuchin monkey, *Cebus apella,* and in the squirrel monkey, *Saimiri sciureus.* In these monkeys, 30- to 40-μm 5-day-old forms and 60- to 100-μm 7-day-old forms were found, the latter appearing as fully mature schizonts. However, a blood infection was not seen. These results suggested that the host specificity for liver forms may be less restricted than to blood forms. Therefore, to date, only man and chimpanzees are regarded as susceptible to all stages of *P. falciparum* infections. The ultrastructure of *P. falciparum* EEF in primate or human hosts is not well known. Boulard *et al.* (1982) described the fine structure of a 5-day EEF from the liver of the capuchin monkey, which does not produce a blood infection. Recently, the ultrastructure of 5- and 7-day *P. falciparum* EEF was published after cultivation in human hepatocytes *in vitro* (Meis *et al.* 1986a).

As part of a collaborative program to study the effect of immunizing chimpanzees with a *P. falciparum* liver stage antigen (Guerin-Marchand *et al.* 1987), a control animal was used to check the infectivity of sporozoites obtained from cultured gametocytes and to study the developing liver stages by light- and electron microscopy. Here we present the first fine structural study of EEF of *P. falciparum* in the liver of a host susceptible both to liver and to blood infection.

Materials and Methods

Sporozoites. P. falciparum sporozoites were obtained from *Anopheles stephensi* (Sind-Kasur strain). Mosquitoes (3–5 days old) were infected by feeding on mature gametocytes (isolate NF 54) obtained from the tipper automated culture system through a membrane (Ponnudurai *et al.* 1989). Unfed and partially fed mosquitoes were removed. The infected mosquitoes were kept at 26°C and 80% relative humidity. At Day 7 after the infectious bloodmeal, midguts were examined to determine the percentage of infected mosquitoes and oocyst load. Infection was 95% with a geometric mean of 38 oocysts per gut. At Day 19 after infection, sporozoites were harvested by crushing 712 mosquitoes in a tissue grinder with Medium 199 and 10% homologous chimpanzee serum and centrifuging at 70*g* for 5 min. The supernatant was collected and the debris resuspended and centrifuged again.

Inoculation. Sporozoites were counted and a total of 7.8×10^6 in 17 ml were slowly injected intravenously into an anesthesized chimpanzee, *Pan troglodytes.* To screen for the presence of mosquito bacterial flora, samples were plated on blood–agar. Antibiotic sensitivity tests were performed on the cultured bacteria, in case the animal would need antimicrobial therapy.

The female chimpanzee (identified as Sabine) was captive bred, born August 14, 1981 and had a weight of 35 kg.

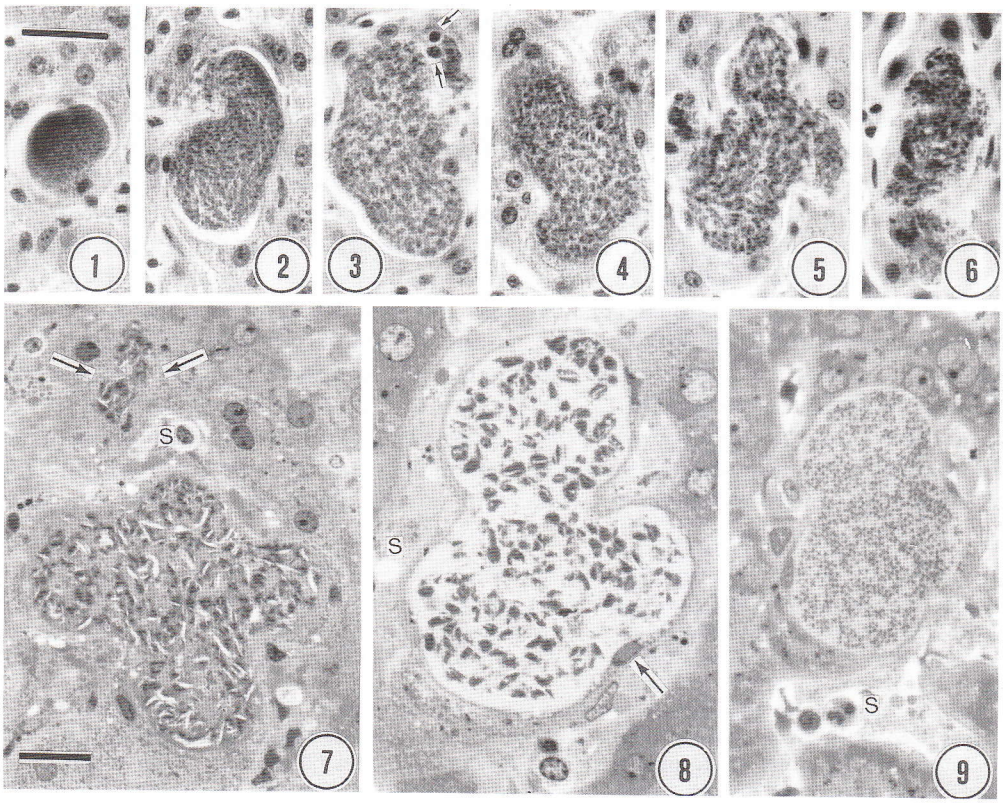
Liver stages. At Day 6 after sporozoite inoculation, a liver wedge biopsy (12–15 cm^3) from the left lobe was obtained by laparotomy. The liver tissue was cut into small pieces with a razor blade and immersion fixed for light- and electron microscopy. Other liver pieces were immediately frozen in liquid nitrogen. The tissues studied were immersed in 1 or 2% glutaraldehyde in 0.1 *M* phosphate buffer or directly in 2% (w/v) OsO_4 in distilled water. The former tissues were postfixed in 1% OsO_4 for 2 hr. The latter remained in the fixative for 48 hr at 37°C (Meis *et al.* 1986b). The samples were dehydrated and embedded in Epon 812 or alternatively in LR-White resin (Polysciences, U.K.). Semithin sections were cut with a histo diamond knife (Diatome) and stained with 1% (w/v) toluidine blue in 1% (w/v) sodium borate. Thin sections were double contrasted in uranyl acetate and lead citrate and examined in a Philips EM 301. For light microscopy, tissues were fixed in Carnoy or buffered formaldehyde, embedded in wax, sectioned at 5 μm, and stained with Giemsa or Hematoxylin–Eosin (H&E).

Results

Parasitemia and animal health. The chimpanzee tolerated the injected sporozoites and unavoidable bacterial contamination remarkably well. The gram negative *Serratia marcescens* was cultured from the inoculum, which was fully susceptible to most antibiotics. It was, however, not necessary to treat the animal. The blood smears examined on Day 7.5 revealed a

parasitemia of 0.01% by FACS analysis and microscopy. At Day 9 the animal received chloroquine base (200 mg im daily for 3 days).

Light microscopy. The density of EEF in the biopsy was 2-3/cm^2 tissue section and 400-600/cm^3 liver. Therefore, the infection rate of the inoculated sporozoites as seen by the development of mature EEF was 4–6%. In contrast, the infection rate of salivary gland sporozoites for human hepatocytes *in vitro* was only 0.02–0.05%. At 6 days, the EEF measured roughly 60–110 μm in their longest dimensions (Figs. 1–9). The parasites often exhibited an irregular contour with lobose arms, extending into neighboring hepatocytes (Fig. 7). The larger parasites seemed to dislocate adjacent hepatocytes and compress sinusoids (Figs. 7 and 8). There were prominent clefts and spaces in their cytoplasm. The study of several different EEF demonstrated that



FIGS. 1–6. Light micrographs of *P. falciparum* EEF from parafin sections and stained with H&E. All material was prepared using Carnoy fixation, except in Fig. 6 which represents formaldehyde fixation (bar = 40 μm).

FIG. 1. Compact, fine granular, still immature EEF.

FIGS. 2–6. Different parasites demonstrating coarse granular cytoplasm and lobose extensions. Two mononuclear cells (arrows) infiltrate the parasite (Fig. 3).

FIGS. 7–9. Light micrographs of *P. falciparum* EEF from 1-μm-thick LR-White sections stained with toluidine blue (bar = 20 μm).

FIG. 7. Lobose parasite giving the impression of penetrating hepatocyte at distance (arrows) with separated cytomeres. The prominent clefts are probably fixation related.

FIG. 8. Large parasite with clearly visible host hepatocyte rim impressing the sinusoids (S). The host nucleus is flattened and pushed to the periphery (arrow).

FIG. 9. EEF with thousands of merozoites still enclosed by the host hepatocyte.

they were not all at the same stage of development. Immature parasites with compact, solid cytoplasm without any visible differentiation (Fig. 1) as well as parasites with clefts and spaces (Figs. 2–8) and those completely differentiated with merozoites were observed (Fig. 9). EEF were preferentially located around periportal areas. Mononuclear cells had accumulated around some parasites, although no degeneration of parasites could be observed (Fig. 3). Vacuoles inside the parasites were not seen. The clear vacuole observed around some parasites in this (Figs. 1–3) and previous studies should be regarded as a shrinkage artifact.

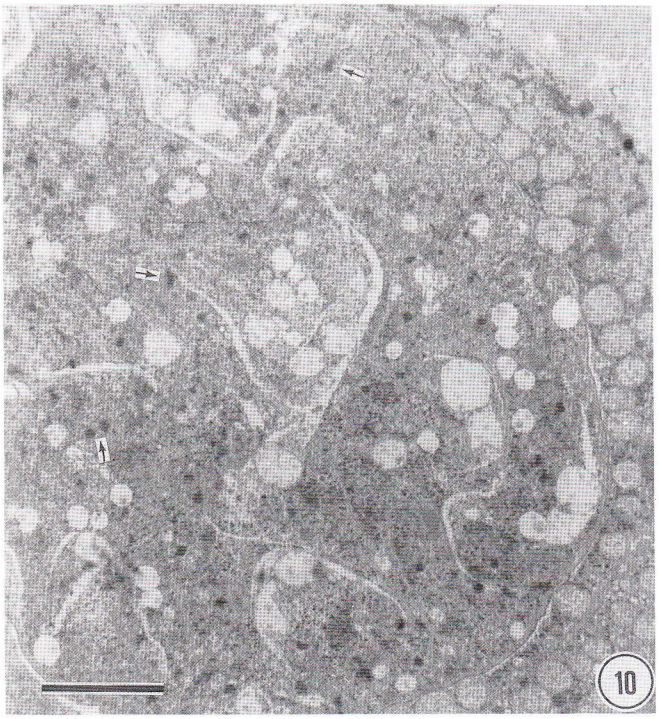
Electron microscopy. As with light microscopy, different developmental stages were found. The solid parasites with little differentiation (Fig. 10) were enclosed by a parasitophorous vacuole membrane (PVM). The parasitophorous vacuole (PV) between PVM and parasite cytoplasm had a floccular appearance and was 150 nm wide. The PVM had a continuous electron-dense layer on its parasitic surface (Fig. 11). In more advanced stages of development, the interaction of the parasite with the host cell involved a very convoluted contact, with extensions of the parasite penetrating into the host cell cytoplasm (Fig. 12). The solid appearance of the parasite has changed and now shows an enlarged PV filled with amorphous flocculent material and deep invaginations of the parasite plasmalemma forming the so-called pseudocytomeres (Fig. 12). These islands of parasite cytoplasm are generally composed of nuclei, RER, immature rhoptries, and lipid droplets (Fig. 12). Nuclei were about 1 μm long, with distended nuclear envelopes and spindle figures. Vacuoles with the flocculent material were not visible in mature parasites with completely formed cytomeres, only lipid droplets remained. In mature parasites, no fenestrated buttons or remnants of the sporozoite pellicle were visible. The irregular, not membrane-bound clefts in the parasite cytomeres (Figs. 16–18) are probably fixation-related artifacts. The parasitized hepatocyte contained numerous 200–250 nm vesicles (Figs. 13 and 14), which were even more clearly demonstrated in a tangential section through the host/parasite interface (Fig. 14). The host hepatocyte became extremely attenuated by the expanding parasite and in some areas, only a very thin rim remained (Figs. 15–17). We have

FIG. 10. Low power transmission electron micrograph of a *P. falciparum* EEF with solid cytoplasm. Several vacuoles of different diameter and density can be seen. Nuclear fragments can be recognized by the dense nuclear spindles (arrows). The PV is still inconspicuous. The "cracks" are probably fixation related (bar = 5 μm).

FIG. 11. Detail of the interface between parasite and hepatocyte host. The PV has widened to 150 nm, is filled with a flocculent material, and is bordered by a thin parasite plasmalemma and a thick PV membrane. Invaginations of the parasite plasmalemma produce clefts filled with a similar floccular material (arrows). In the host hepatocyte, numerous 150- to 200-nm vesicles are visible. Two types of vacuoles are present: lipid droplets (L) and vacuoles (*) with a flocculent content. The retraction of the hepatocyte cytoplasm from the PV membrane is an artifact (bar = 1 μm).

FIG. 12. An EEF in a more advanced stage of development. The solid cytoplasm is subdivided by invaginaton of the parasite plasmalemma. The vacuoles with the flocculent material have now disappeared. The highly irregular contact of the thick PV membrane with the very thin rim of hepatocyte host cytoplasm (H) is apparent. Collagen fibers (C) are observed closely associated with the hepatocyte host. The pseudocytomeres embedded in floccular material are connected with eachother in a reticulum. They contain nuclear fragments (N), RER, and lipid droplets (L). Mitochrondria are inconspicuous. First indications of rhoptry formation are visible (arrows) (bar = 1 μm).

FIG. 13. Detail of host/parasite interface demonstrating that the hepatocyte host (H) contains numerous 200- to 250-nm vesicles (bar = 1 μm).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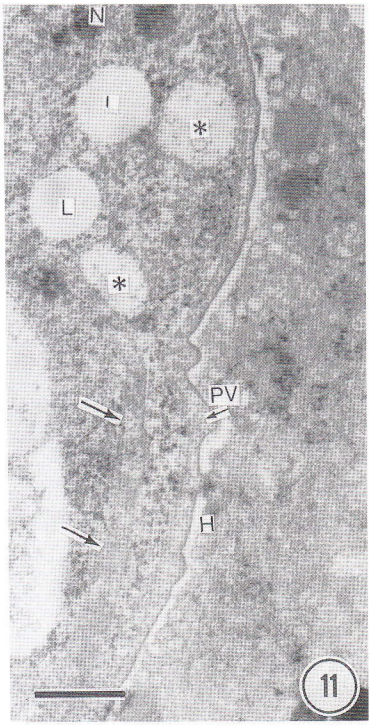
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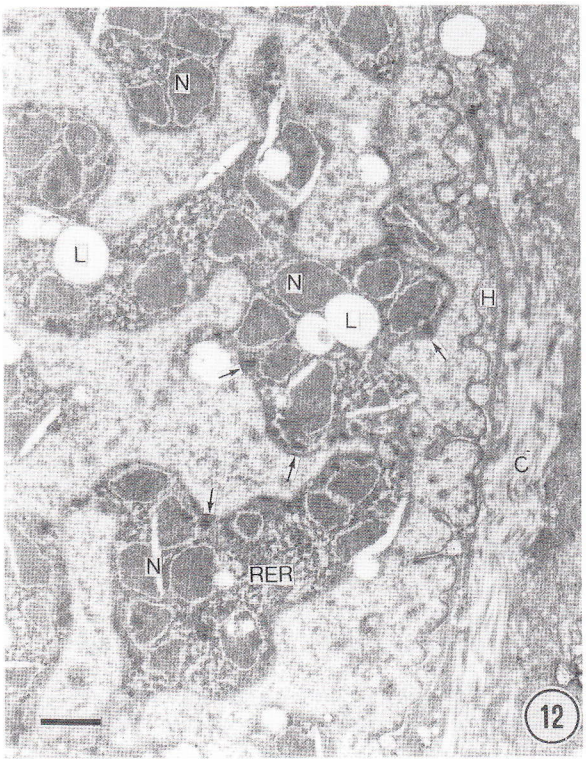
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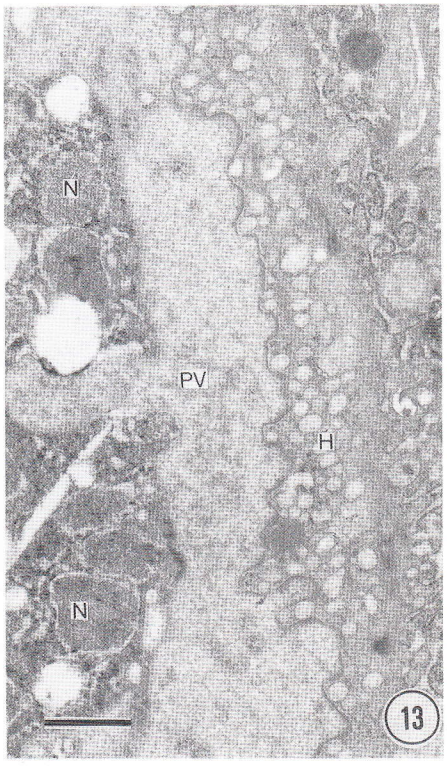
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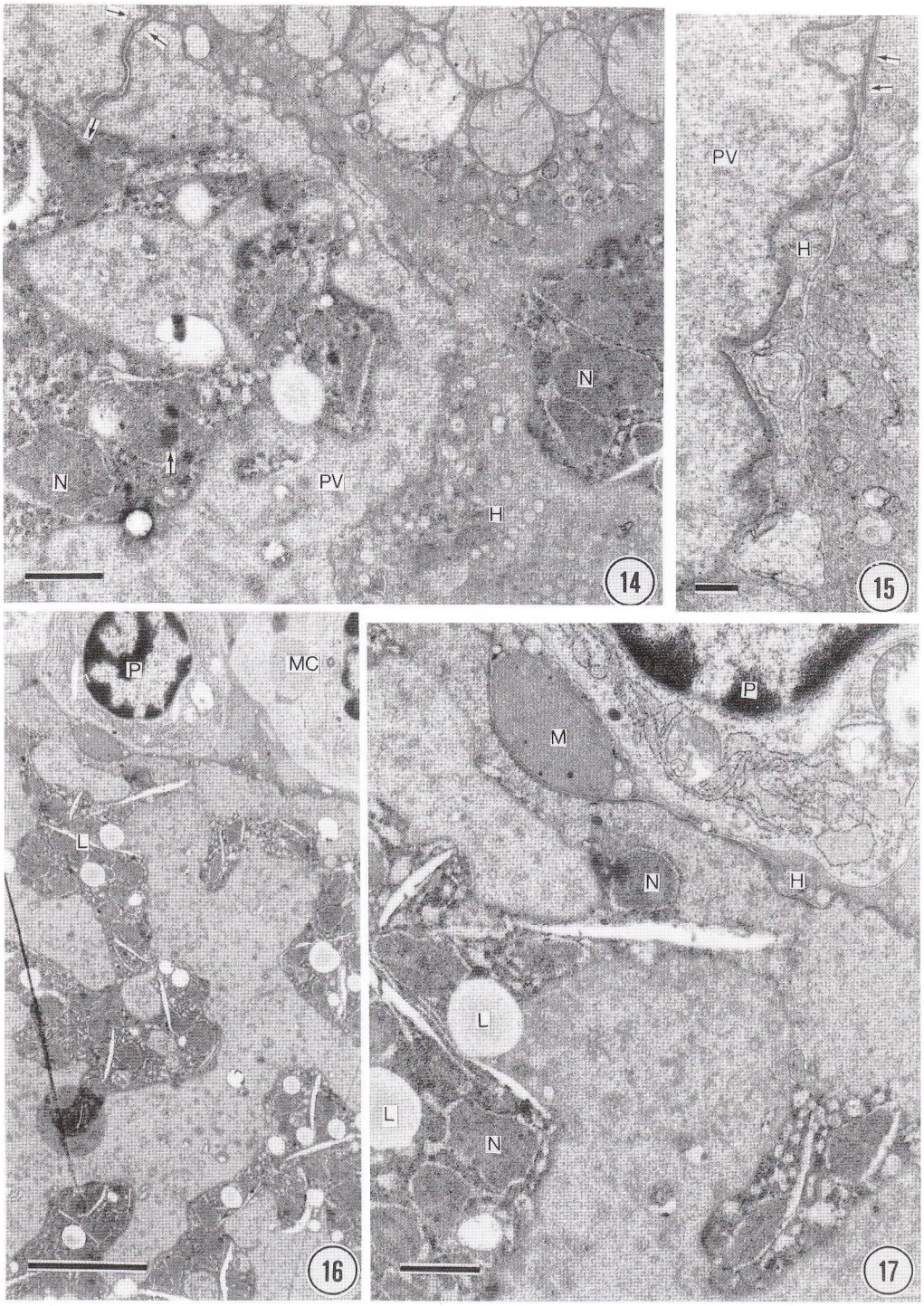

FIG. 14. A tangential section through the host/parasite interface shows the vesicles in the host hepatocyte (H), a portion of which is separating the parasite due to the irregular interface. Nuclear spindles (arrows) in the nuclei (N) are obvious. A deep invagination of the PV membrane is marked by arrows (bar = 1 μm).

FIG. 15. The hepatocyte containing a mature parasite is greatly attenuated with only a small rim of cytoplasm left around the EEF. Note the cell junction of the host with the neighbour hepatocyte (arrows) (bar = 250 nm).

FIG. 16. A plasma cell (P) and a monocytic cell (MC) can be observed in very close contact with the outer host hepatocyte membrane. The parasite does not seem to be affected by their presence (bar = 5 μm).

FIG. 17. Detail of Fig. 16 showing part of the EEF and the plasma cell. The attenuated host hepatocyte (H) with a mitochondrion (M) lays between parasite and infiltrating cell (bar = 1 μm).

not observed a continuous contact of the parasite with the exterior environment, but monocytic and plasma cells could be seen in very close contact with host hepatocytes (Figs. 16, 17, and 19). Collagen deposition was seen associated with the host hepatocyte (Figs. 12, 18–20). Because collagen deposits were also seen around noninfected hepatocytes, it is probably not a parasite-related observation. In the routinely fixed parasites, many poorly defined vacuoles were visible. However, using osmium fixation only, it could be demonstrated that most of these vacuoles were lipid droplets (Figs. 21 and 22). The cytomeres become progressively smaller until merozoites are formed. Between the parasite plasmalemma and nucleus, rhoptry precursors are formed together with the deposition of an inner membrane (Fig. 22). Merozoites can subsequently be seen embedded in the floccular material and still enclosed by the host hepatocyte (Fig. 23).

Discussion

The unsplenectomized chimpanzee supported the growth of *P. falciparum* EEF after intravenous inoculation of sporozoites of the NF 54 strain. The duration of the EEF development was probably about 5.5–6 days, since at Day 7.5 a large number of ring stages could already be detected. This is in agreement with the duration of development of EEF in the human volunteer experiments (Shortt *et al.* 1951; Jeffery *et al.* 1952) and is shorter than that described by Bray (1958, 1960) in chimpanzees. Garnham (1966) states that the earliest prepatent period for sporozoite-induced falciparum malaria in man is 5.5 days.

Only a percentage (4–6%) of the inoculated *P. falciparum* sporozoites reached maturity. Bray (personal communication) found with the highly infectious parasite *P. cynomolgi* an infectivity percentage of up to 60% when only salivary gland sporozoites were used. This relatively low infectivity rate could be due to the isolation procedure, allowing that noninfectious sporozoites from the whole mosquito and damaged ones were also injected. Furthermore, it is well known that not all sporozoites start development or reach maturity after hepatocyte penetration (Meis *et al.* 1986a) and many would be destroyed by macrophages. In contrast with *in vivo* findings, infectivity of the same *P. falciparum* sporozoites obtained from cultured gametocytes *in vitro* for hepatocytes was much lower than *in vivo,* ranging from 0.02% (Meis *et al.* 1985; present study) to a maximum of 0.5% (Mazier 1986). Whatever the reason for the more efficient sporozoite transformation *in vivo,* this experiment indicates that extrapolation of *in vitro* experiments to the *in vivo* situation should be done with utmost care.

The previously described mature, about 6-day old, EEF found in human liver measured from 55 to 60 μm in length (Shortt *et al.* 1951; Jeffery *et al.* 1952). Bray (1958) considered 8-day *P. falciparum* EEF in the chimpanzee to be nearly mature and described them as resembling the 6-day EEF from humans. Garnham (1966) described the *P. falciparum* EEF in the chimpanzee at Day 6 as measuring about 40 μm in greatest dimensions. In the present study, EEF measured from 60 to 110 μm support recent results also obtained from the chimpanzee (Szarfman *et al.* 1988a, b) and former ones obtained in the squirrel monkey (P. Druilhe *et al.,* unpublished observations). *P. falciparum* EEF cultured *in vitro* were at Day 7 up to 30 μm (Mazier 1986; Meis *et al.* 1986a), and therefore much smaller and more immature than those obtained *in vivo* from chimpanzee, squirrel monkey, and man.

The first report on the ultrastructure of EEF of primate malaria parasites in the rhesus monkey liver was by Sodeman *et al.* (1970) on 7-day parasites of *P. cynomolgi.* The latter monkey parasite (Atkinson *et al.* 1989) and *P. vivax* (Uni *et al.* 1985) were

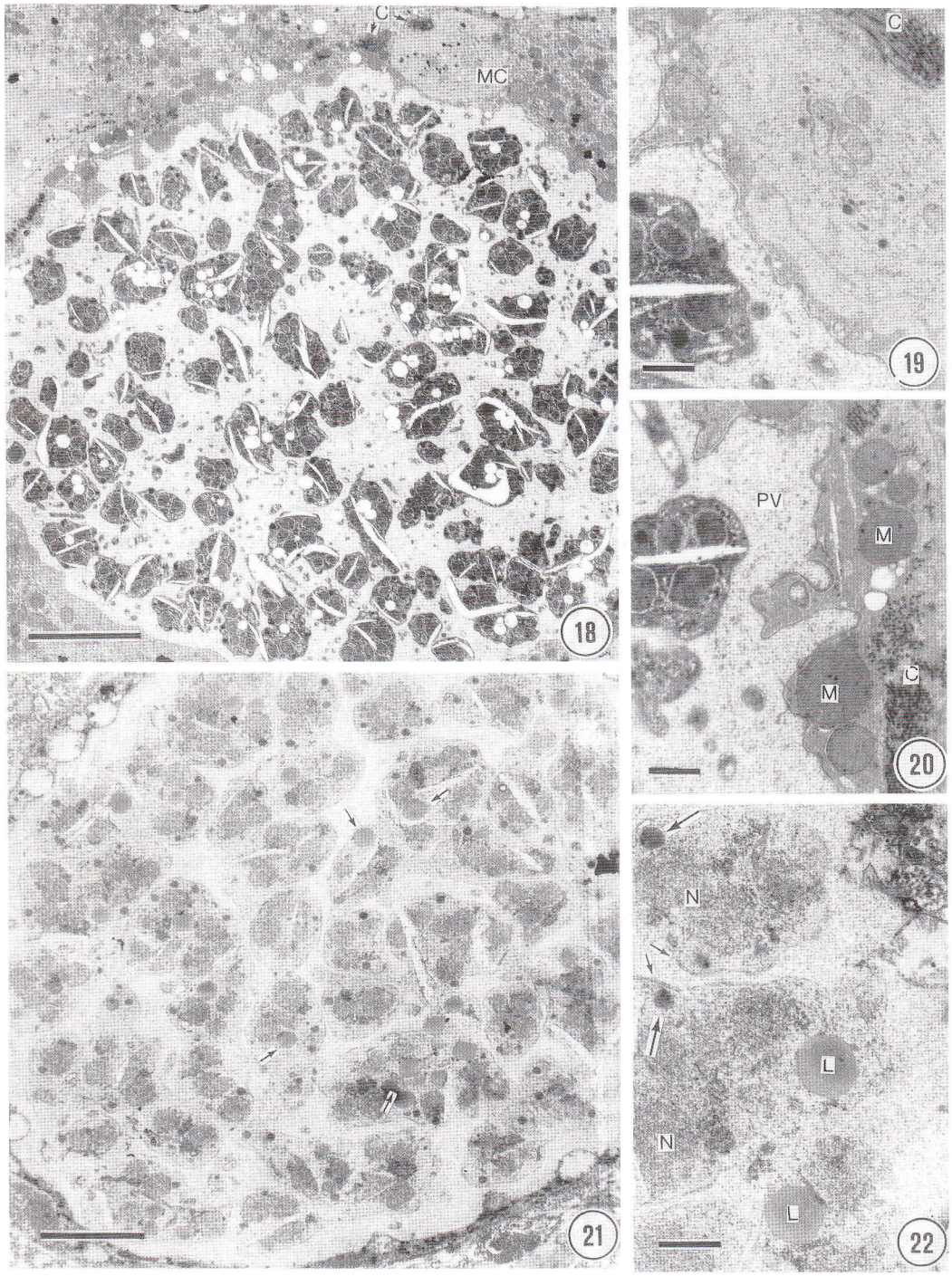

FIG. 18. Low power micrograph of a still more advanced stage of merogony. Many separate cytomeres, each with five to six nuclear fragments, are visible in a flocculent matrix. The "empty" vacuoles are lipid droplets. Note the monocytic cell (MC) and collagen (C) deposits (bar = 10 μm).

FIG. 19. Detail of Fig. 18 showing the monocytic cell with its typical light staining cytoplasm in close contact with the parasite (bar = 1 μm).

FIG. 20. Detail demonstrating the highly irregular host/parasite interface and the collagen (C) deposits. Host hepatocyte mitochondria (M) (bar = 1 μm).

FIG. 21. Low power micrograph of an EEF fixed solely in OsO_4. The lipid droplets are now well preserved in this fixative in contrast to routine fixation (for example, Fig. 18) (bar = 5 μm).

FIG. 22. Detail demonstrating the cytomeres with lipid (L), nuclear fragments (N), and rhoptries (arrows). Between the latter and the parasite plasmalemma, an inner membrane complex starts to appear (small arrows), signifying the beginning of merozoite formation (bar = 1 μm).

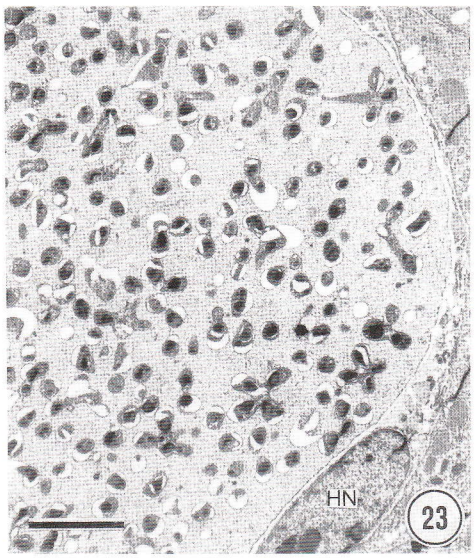

FIG. 23. Low power micrograph showing numerous merozoites, embedded in a floccular matrix and still enclosed by the PV membrane and the hepatocyte. Note the hepatocyte nucleus (HN) (bar = 5 μm).

subsequently studied at the fine structural level after culture conditions for liver stages became available. In these studies, different vacuoles were described in the parasite. The two types of vacuoles designated type I and II were probably similar to the lipid droplets and vacuoles with fluffy content described in the present study. Similar observations were recently made for *P. knowlesi* and confirmed for *P. cynomolgi* in the macaque monkey (Sinden *et al.* 1989). However, the latter authors described a third type which was commonly found and had an electron-dense appearance. Additionally, we find electron-dense vesicles in an advanced stage of merogony, which are the rhoptry precursors of the merozoites. Boulard *et al.* (1982) were the first to describe the fine structure of a 5-day EEF of *P. falciparum* from the liver of a capuchin monkey. In these immature 40-μm forms, numerous vacuoles containing fluffy or granular material were found, with most of them being close to the PV and some fusing with PVM depositing this granular material in the PV space. At a later stage in mature EEF, these vacuoles have disappeared and the fluffy material is only present in the PV. In still further-developed EEF, this fluffy material is distributed between the cytomeres and finally the merozoites. All primate parasites appear to have a vacuolar system, which produces cytomere formation as is also seen with rodent parasites (Meis and Verhave 1988). However, in contrast to the latter, *P. falciparum* seem to produce gradually smaller cytomeres from the multinucleate mass until in a last division, merozoites are separated. Thus we confirm the later part of the aposchizogony described by light microscopy (Bray 1960) in *P. falciparum* EEF. The early part of schizogony involving a large internal vacuole developing about the end of the fifth day (Bray 1960) could not be confirmed. The marked destruction of host hepatocyte cytoplasm described for *P. cynomolgi* and *P. knowlesi* (Sinden *et al.* 1989) in the rhesus monkey and *P. falciparum* in the cebus monkey (Boulard *et al.* 1982) was not seen in our study. Although host hepatocytes were extremely attenuated, no destruction or leaching of host cytoplasm was observed. Most of the infected hepatocytes contained numerous small vesicles. This was also described previously *in vitro* with a rodent parasite (Meis *et al.* 1984) and was thought to have some role in parasite nutrition. Of interest in the fine structure is the presence of osmiophilic vesicles or "fenestrated" buttons in the EEF from the *Cebus* (Boulard *et al.* 1982) as well as in EEF cultured *in vitro* in human hepatocytes (Meis *et al.* 1986a). These vesicles can be found in different avian parasites and in other developmental stages of *P. falciparum,* e.g., gametocytes (Ponnudurai *et al.* 1986). In the present study these vesicles are absent even from the morphologically youngest parasite, suggesting either that these could be differences due to

the type of host cells used or that the forms grown *in vitro* and in the blood stage-resistant host are very immature or degenerate compared to the parasites grown in the chimpanzee. In previous studies on *P. berghei* EEF in rat liver, we noted leukocytic infiltration around the intracellular parasite (Meis and Verhave 1988). Here we also describe monocytic and plasma cell infiltration around some morphologically healthy parasites. Earlier studies on *P. falciparum* EEF (Jeffery *et al.* 1952) specifically mentioned no leukocytic infiltration into areas around immature parasites. Only when merozoites were released was cell infiltration noted (Garnham 1966). The above finding is important in view of possible cell-mediated defense mechanisms against EEF and would be important to study in animals previously primed by sporozoites and/or liver stage antigens during a subsequent infection.

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