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Electron microscopic studies on the interaction of rat Kupffer cells and *Plasmodium berghei* sporozoites

J.F.G.M. Meis[1], J.P. Verhave[1], A. Brouwer[2], and J.H.E.Th. Meuwissen[1]

[1] Department of Medical Parasitology, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

[2] Institute for Experimental Gerontology TNO, P.O. Box 5815, 2280 HV Rijswijk, The Netherlands

Abstract. The interactions between *Plasmodium berghei* sporozoites and Kupffer cells in rat liver were studied by transmission electron microscopy. Between 10 and 45 min after inoculation, sporozoites were found in the process of entering Kupffer cells and inside phagolysosomes. The sporozoites entered the Kupffer cells by phagocytosis as determined by the presence of pseudopods and local accumulations of aggregated microfilaments and the resulting exclusion of other organelles in the phagocyte cytoplasm beneath the attached parasite. Sporozoites were taken up either with their anterior end first, or backwards. Scanning electron microscopy of in vitro sporozoite Kupffer cell interaction confirmed these observations. It was concluded that sporozoites are taken up in a normal phagocytic way by the Kupffer cells, regardless of their initial place of contact or position. Thirty min after inoculation sporozoites found in phagolysosomes were still morphologically intact but after 45 min we could encounter completely digested sporozoites.

Introduction

The fate of sporozoites of *Plasmodium berghei* when injected into the blood has been investigated in morphological (Meis et al. 1982) and kinetic studies (Sinden and Smith 1982; Danforth et al. 1982). These have shown that the sporozoites are cleared from the blood and rapidly end up in the phagocytic Kupffer cells of the liver. Some of them eventually escape from the Kupffer cell and subsequently penetrate the neighbour hepatocyte (Meis et al. 1983a). The invasion process of the parenchymal liver cell appears to be an active process of the sporozoite (Meis et al. 1983b).

Although sporozoites are often found inside Kupffer cells (Meis et al. 1983a), it is still not clear whether their interiorization is due to phagocytosis by the host cells or to active penetration by the parasite. Evidence for

Offprint requests to: J.F.G.M. Meis

interaction of *P. berghei* sporozoites with macrophages has only been provided by in vitro experiments (Danforth et al. 1980). The results of the latter work indicate that the sporozoites penetrate the mouse peritoneal macrophages actively, but it is difficult to extrapolate from the in vitro situation with peritoneal macrophages to the role of Kupffer cells in the infection process in vivo. Sinden and Smith (1982) suggest that retention of sporozoites in the liver is due to phagocytosis by the Kupffer cells. This phagocytosis has been shown to be a non-specific process, i.e., non-viable and heterologous "non-infective" sporozoites are retained with the same efficiency as the viable homologous sporozoites (Danforth et al. 1982). These results indicate that the process described in vitro (Danforth et al. 1980) for sporozoite interaction with peritoneal macrophages does not necessarily occur in vivo with Kupffer cells. In the present electron microscopic study on the uptake and fate of *P. berghei* sporozoites in rat Kupffer cells in vivo and in vitro, we demonstrate the involvement of pseudopodia and microfilaments in the process of sporozoite uptake, which indicates active phagocytosis.

Materials and methods

Parasites. Sporozoites of *Plasmodium berghei* ANKA were obtained and handled as described previously (Vermeulen et al. 1982).

Laboratory animals. Brown Norway rats (BN/BiRij) originating from TNO, Rijswijk, The Netherlands, were bred in the Central Animal Laboratory of the Nijmegen University.

In vivo sporozoite Kupffer cell interaction. Four-week-old female Brown Norway rats were injected directly into the portal vein with five million sporozoites. To facilitate the detection of the injected sporozoites the flow of the portal blood was restricted to the two right liver lobes by ligation of the blood vessels leading to the median, left lateral and caudal lobes (Meis et al. 1981). In view of the short period of time during which sporozoites can be demonstrated in the circulation, the rats were sacrificed 10, 30 or 45 min after inoculation. Their livers were perfused with 1.5% (v/v) glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.3; 350 mosmol) at 20° C for 5 min at a rate of 2 ml/min. The liver tissues were immersion-fixed for another 2 h at 4° C in the same fixative. The tissues were postfixed in 1% (w/v) osmium tetroxide, dehydrated and embedded in Epon. Semi-thin sections (0.5 μm) were cut with glass knives and examined after staining with toluidine blue. To increase the chances of finding sporozoites in ultra-thin sections the areas around afferent portal tracts were selected because of the high density and the higher phagocytic activity of Kupffer cells in those regions (Sleyster and Knook 1982).

Ultra-thin sections (60 nm) were cut with a diamond knife (Diatome) on a Reichert OM U3 ultramicrotome. These sections were contrasted with uranyl acetate and lead citrate, and examined in a Philips EM 300 electron microscope using an accelerating voltage of 60 kV.

In vitro sporozoite Kupffer cell interaction. Sinusoidal liver cells were isolated from 3-month-old female BN/BiRij rats by selective enzymatic destruction of parenchymal cells by pronase E (Merck, Darmstadt) as previously described (Knook and Sleyster 1976). Subsequently Kupffer cells were purified from the sinusoidal cell suspensions by using a JE-6 elutriator rotor (Beckman Instruments, Palo Alto, CA) in a J-21 type Beckman centrifuge at a speed of 2500 rpm (750 *g*). Sterile conditions were maintained during these isolation and purification procedures. Cell viability was estimated by trypan blue exclusion. The purity of the isolated Kupffer cell preparation was at least 85%. Purified Kupffer cells were suspended in Dulbecco's modifi-

cation of Eagle's medium and cultured on round glass coverslips in 24-well Costar plates at 37° C in a 5% CO_2 atmosphere according to Brouwer and Knook (1982) for 24 h. Approximately 10^4 Kupffer cells were spreaded per coverslip. Sporozoites were obtained by sterile dissection of mosquitoes in 10% normal rat serum and suspended at a final concentration of 10^4/0.5 ml. Sporozoites were incubated with the Kupffer cells at 37° C and allowed to sediment for 10 min. Then after incubation periods of 2, 10, 30 and 45 min, the coverslips were washed twice with medium and fixed for 1 h with 2% glutaraldehyde 0.1 *M* cacodylate buffer (pH 7.2). Following postfixation with 0.1 *M* cacodylate-buffered 1% OsO_4 and dehydration the glass coverslips were critical point dried, coated with palladium-gold, and examined in a Philips SEM 500 scanning electron microscope.

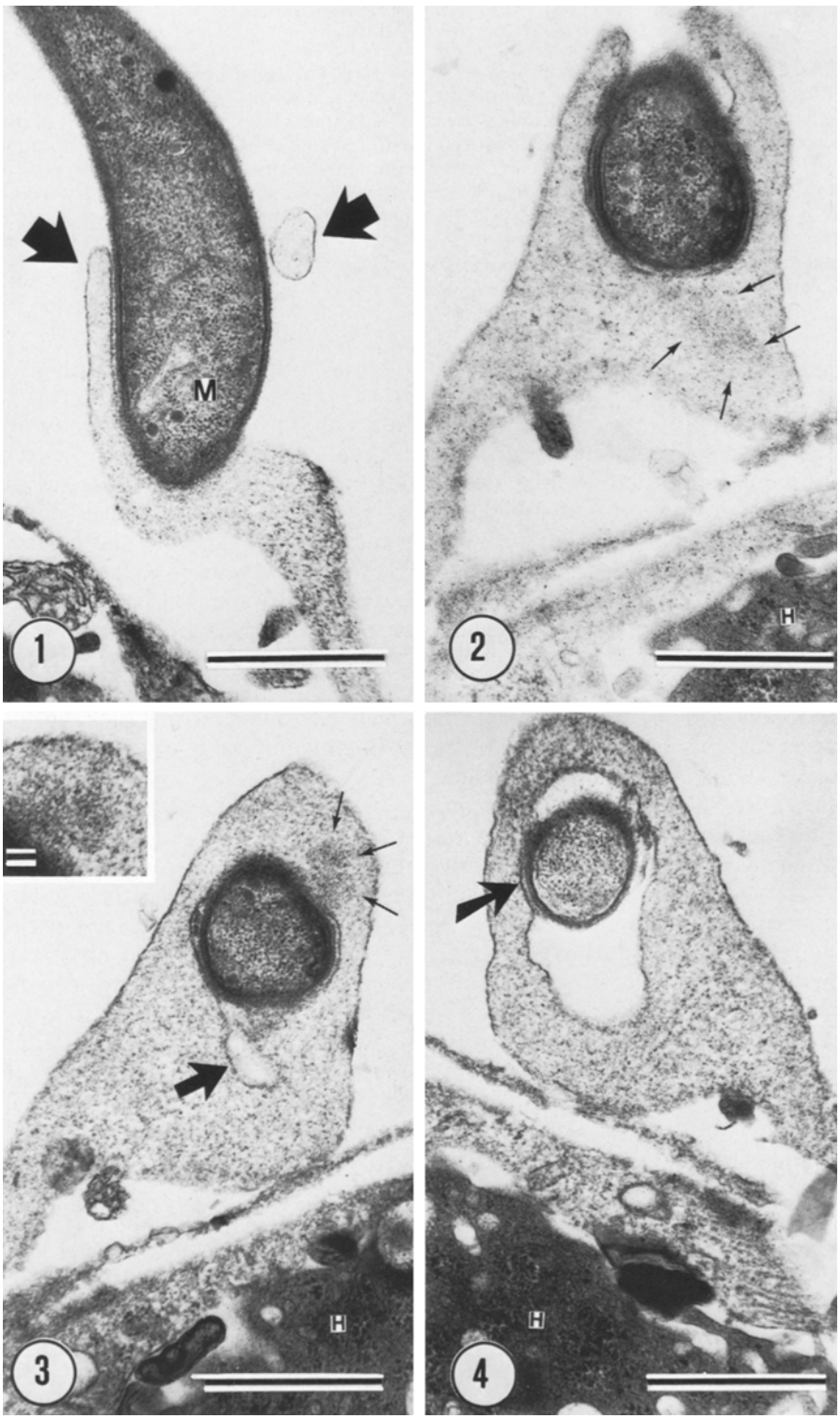
Results

Among the many sporozoites lying inside Kupffer cells, few were found in the process of entering these sinusoid-lining cells. Serial sections of liver from a rat inoculated 10 min before fixation revealed different phases of pseudopod formation and attachment of parasite and macrophage (Figs. 1–5). One part of the sporozoite was in intimate contact with the macrophage. A sparce surface coat on the sporozoite appeared to bridge the gap between the parasite and the phagocytic membrane. Local accumulations of cytoskeletal elements beneath the enveloping phagocyte membrane were clearly visible. The microfilaments were organized as large aggregates and no other structures were seen in the hyaloplasm of the Kupffer cell pseudopods. The part of the sporozoite which was already inside the Kupffer cell had lost the close contact with the limiting membrane of the endocytotic vacuole that was formed meanwhile.

We have never observed disruption of the macrophage plasmalemma during invasion of the parasite. Thus, when inside a vacuole the parasites were enclosed by a macrophage-derived membrane.

After 2- and 10-min incubation sporozoites were observed in various interactions with Kupffer cells in vitro. These cells stretched out their pseudopods to make contact with sporozoites which could be taken up at the tail end (Fig. 6). After 30 and 45 min no sporozoites were found with scanning electron microscopy in vitro. With transmission electron microscopy sporozoites were found in the process of entering Kupffer cells 30 min after inoculation, also with their posterior end first (Figs. 7–8). The anterior end that can be recognized by the presence of micronemes, was still lying free in the sinusoidal lumen. A nearby granulocyte did not show any sign of phagocytozing the sporozoite. In fact, we have never found granulocytes ingesting sporozoites whilst they did ingest bacteria.

At 30-min post-inoculation other sporozoites could be found in phagolysosomes of Kupffer cells. The parasite (Fig. 9) was morphologically still fairly intact and clearly recognizable, although the formation of vacuoles around osmiophilic bodies may be a first sign of degradation. A mitochondrion was simultaneously visible in the phagolysosome containing the sporozoite. Bacteria could be found in the same Kupffer cell (see also Fig. 7). This cell showed a very active cytoplasm with several large lysosomal structures.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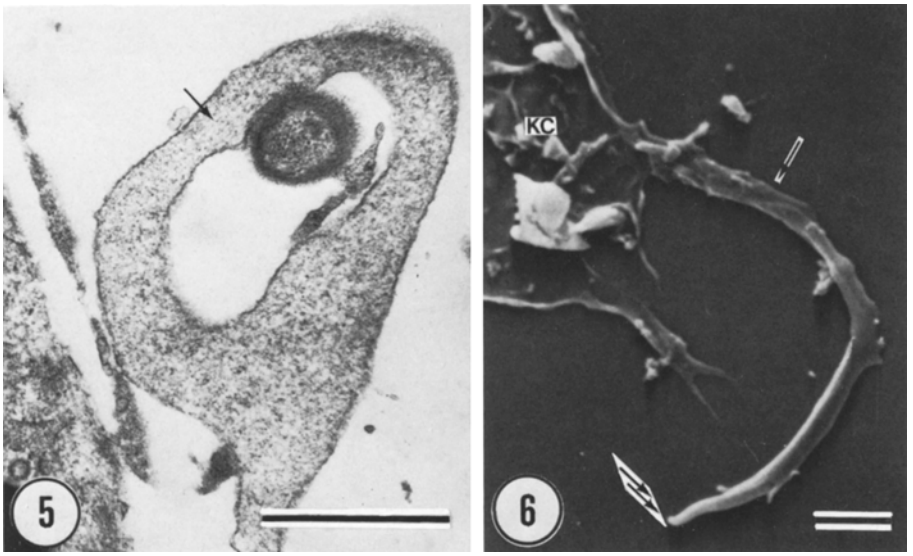


Fig. 5. Transmission electron micrograph of section through the presumed end of the sporozoite taken up by a Kupffer cell 10 min after inoculation. The vacuole in the Kupffer cell is greatly enlarged. Due to the oblique section, the parasite membranes are not clearly outlined. Microfilaments of the macrophage are located under the endocytotic vacuole limiting membrane (*arrow*). Bar = 1 μm

Fig. 6. Scanning electron micrograph of sporozoite/Kupffer cell (*KC*) interaction in vitro after 10-min incubation. The posterior portion (*arrow*) is already engulfed by pseudopods, whilst the anterior portion, recognized by its tapered end, lies free (*double arrows*). Bar = 2 μm

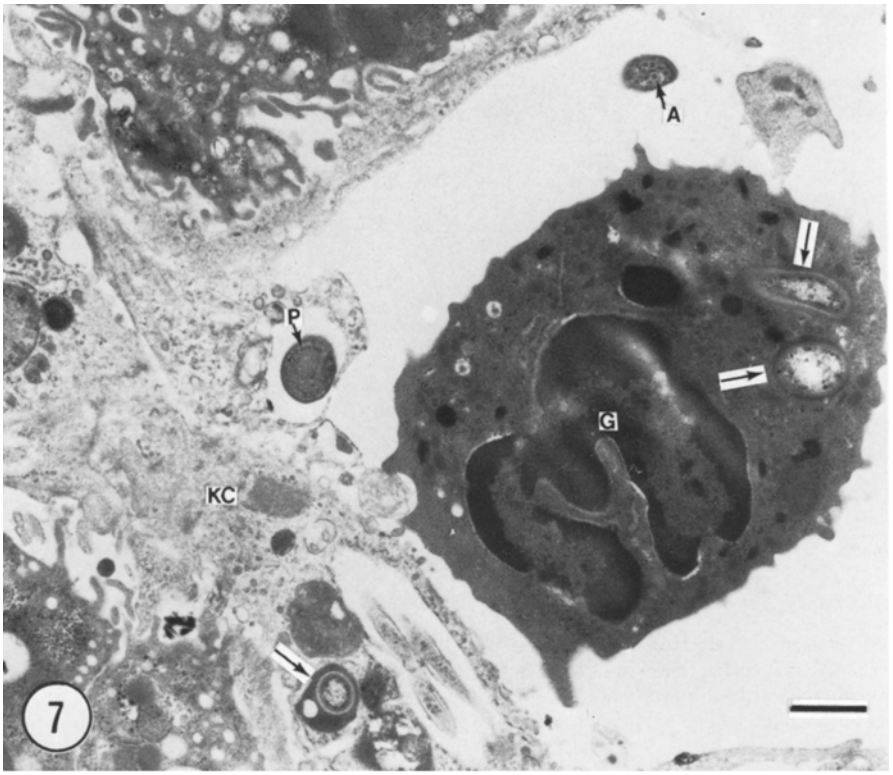
Figs. 1–4. Transmission electron micrographs of serial sections showing attachment and uptake of a sporozoite of *Plasmodium berghei* by a Kupffer cell 10 min after inoculation. Bar = 1 μm

Fig. 1. The sporozoite is adhering to the Kupffer cell pseudopods (*arrows*). The surface coat material seems to bridge the gap between the parasite and Kupffer cell membrane. *M* = sporozoite mitochondrion

Fig. 2. The Kupffer cell seems to engulf the sporozoite. Microfilaments (*arrows*) are visible in the Kupffer cell cytoplasm. Hepatocyte (*H*) is visible in the right lower corner

Fig. 3. Interiorized part of the sporozoite. The appearance of an endocytotic vacuole is visible (*thick arrow*). Note that there is extensive aggregation of microfilaments (*arrows*) in the Kupffer cell cytoplasm. (Inset; Bar = 0.1 μm)

Fig. 4. An advanced section shows that the sporozoite is surrounded by a wide space which makes only at one side close contact with the limiting membrane of the endocytotic vacuole (*arrow*)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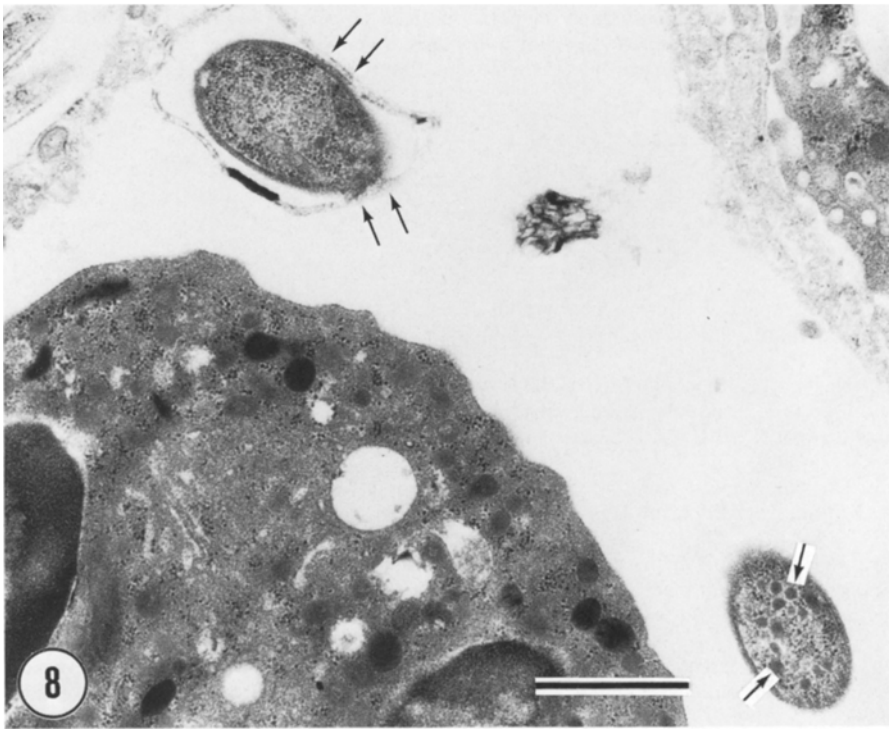
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After 45 min and later, sporozoites were seen degraded inside phagolysosomes of Kupffer cells and lying in a coiled shape. Their cytoplasm had become very granular, but the apical structures and subpellicular microtubules were still visible (Fig. 10).

Discussion

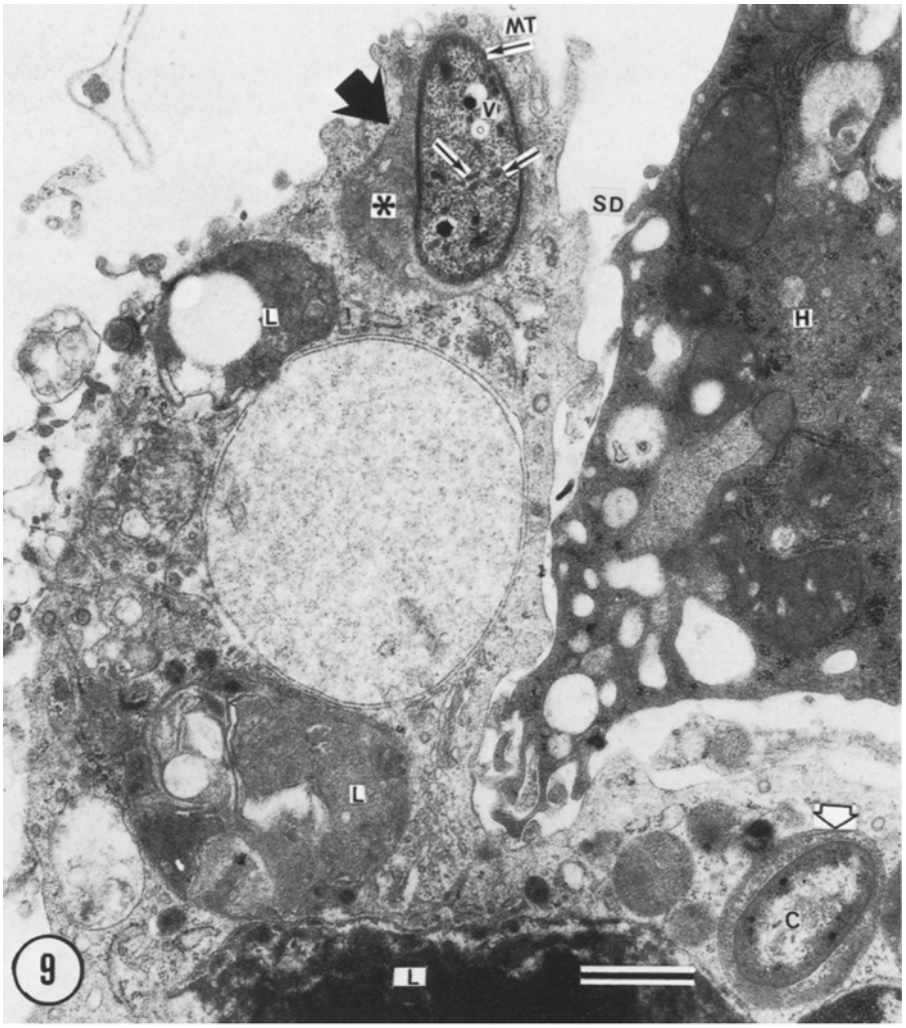
In previous studies we showed morphologically intact sporozoites in Kupffer cell endocytotic vacuoles 10 and 15 min after inoculation (Meis et al. 1983a). Even after 1 h intact sporozoites could be found, but these were thought to be not successful in leaving the phagocytes and penetrating the hepatocytes (Meis et al. 1983b). Danforth et al. (1980) have shown in vitro that only sporozoites incubated with immune serum are degraded by macrophages while sporozoites incubated in normal serum are not degraded within about 60 min. Thus the presence or absence of antibodies on the sporozoite induces different types of interaction with and inside macrophages. Sporozoites which are immature or otherwise non-infective end in and are destroyed by macrophages regardless of the presence of immune serum (Sinden and Smith 1982). In this study we can confirm that part of the sporozoites are degraded in phagolysosomes of Kupffer cells. However, this process takes more than the 2 min that have been reported for Sprague Dawley rat Kupffer cells (Shin et al. 1982).

We have observed major differences in the susceptibility to sporozoites between Sprague-Dawley and Brown-Norway rats and also in the growth rate of exoerythrocytic forms. The former results point to a difference in the phagocytic and lytic action of the Kupffer cells in these rats (Verhave et al. 1985). If we assume that a proportion of the sporozoites can leave the Kupffer cells and infect hepatocytes, it is understandable that in resistant Sprague Dawley rats, sporozoites are readily degraded in their Kupffer cells while only the quickest ones manage to escape to the space of Disse and penetrate hepatocytes. Brown Norway rat Kupffer cells, not so fast in degrading sporozoites, would allow more parasites to leave these phagocytes (Meis et al. 1983a).

In the present study we have seen that the uptake of sporozoites from the circulation by Kupffer cells was accomplished by pseudopod formation and by a very close contact between the parasite and the Kupffer cell membrane. The narrow gap between the two was bridged by a thin surface coat on the sporozoite. We have never noticed a surface coat on the sporo-

Fig. 7. Electron micrograph showing a neutrophilic granulocyte (*G*) and a sinusoid lining Kupffer cell (*KC*). Both phagocytes have incorporated bacteria, in phagolysosomes (*arrows*). The Kupffer cell is at the same time in the process of engulfing a malarial sporozoite, posterior end (*P*) first. The anterior portion (*A*) lies free in the sinusoid. Bar = 1 μm

Fig. 8. Higher magnification of the same area as Fig. 7 in another section. The sporozoite has only at few sites a close connection with the Kupffer cell pseudopods (*double arrows*). The anterior portion of the sporozoite is recognizable by the micronemes (*arrows*). Bar = 1 μm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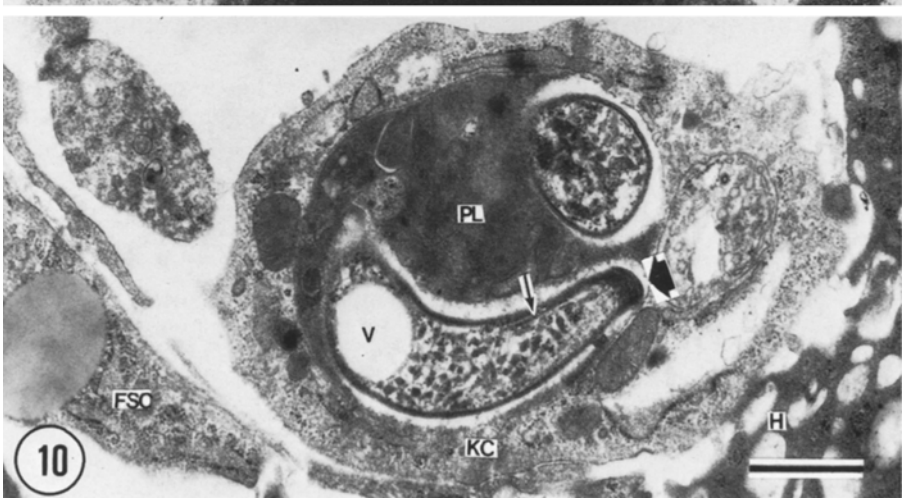
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zoite before, neither when they were interiorized nor on free ones in the sinusoid (Meis et al. 1983a), although the presence of a very thin coat has been reported earlier (Cochrane et al. 1976). We have tentatively suggested that this coat was lost during Kupffer cell invasion, similar to what was described by Langreth et al. (1978) for *P. falciparum* merozoite invasion into erythrocytes.

The local accumulation of cytoskeletal elements in the Kupffer cell and the appearance of engulfing pseudopods together with the observation that the sporozoites also entered with their posterior end first, indicate that they are taken up by the Kupffer cells rather than that the sporozoites actively enter these cells. The fact that the actual process of sporozoite uptake is found only in the period shortly after inoculation and that many more are seen completely intracellular (Meis et al. 1982) is another indication that the Kupffer cells act quickly, and effectively clear the sporozoites from the circulating blood.

On the basis of cytochalasin B experiments, Danforth et al. (1980) concluded that phagocytosis is not essential for sporozoites to become intracellular into peritoneal macrophages in vitro. However, it has been shown recently that phagocytosis by these cells diminished markedly after anchorage of the cells to a substrate (Goldmacher 1984). After detachment from the substrate, the cells regained their phagocytotic activity. This observation may explain the variability of data on phagocytosis in vitro.

When studying the fate of *P. berghei* sporozoites in vivo Danforth et al. (1982) found indications that immature sporozoites from oocysts, which are hardly infective, are retained in the liver as effectively as mature salivary gland sporozoites. The same was demonstrated for non-viable sporozoites by Sinden and Smith (1982), which further indicates that some other mechanism than active penetration by the sporozoite must be present.

Almost all studies on the interaction of intracellular parasites and vertebrate cells were done in vitro with mouse peritoneal exudate macrophages as host cells (for reviews see Blackwell and Alexander 1983; Thorne and Blackwell 1983). Kupffer cells are quite different. Especially the periportal

Fig. 9. Kupffer cell with lysosomal structures (*L*) found 30 min after inoculation of sporozoites. A sporozoite is incorporated in a phagolysosome (*thick arrow*). A structure resembling a cristate host mitochondrion is also visible (*asterisk*). The morphology of the sporozoite still showed subpellicular microtubules (*MT*) and micronemes (*arrows*). A bacterium incorporated in a phagolysosome of the same Kupffer cell is also still morphologically intact (*open arrow*). This picture clearly demonstrates the difference between the sporozoite and the bacterium. The former has a more differentiated cytoplasm, while the bacterium possesses an electron-lucent core with some fine fibrillar DNA strands (*C*). The Kupffer cell borders the space of Disse (*SD*) and the hepatocyte (*H*). Bar = 1 μm

Fig. 10. Degenerated sporozoites can be found 45 min after inoculation. The parasite has become rounded up and is localized in a phagolysosome (*PL*). The cytoplasm is granulated and large abnormal vacuoles (*V*) are visible. The only organelles which are still recognizable are the subpellicular microtubules (*arrow*) together with the apical complex (*thick arrow*). A hepatocyte (*H*) and a fat-storing cell (*FSC*) are clearly visible in this picture. Bar = 1 μm

Kupffer cells show a high phagocytic activity and are mainly involved in uptake of foreign particles from the blood stream (Sleyster and Knook 1982). Indeed most of our electron micrographs were taken in periportal areas. We have demonstrated previously that most exoerythrocytic forms develop in the periportal areas (Meis et al. 1983a).

Conclusion

We suggest that in vivo there is a non-specific initial interaction of sporozoites and Kupffer cells, followed by active phagocytosis of the parasites (Verhave et al. 1985; Danforth et al. 1982; Sinden and Smith 1982). The interaction of ligands on the sporozoites and receptors on Kupffer cells and hepatocytes in vivo has to be further explored. Once in contact with the hepatocyte surface, other events could take place resulting in active invasion of this ultimate host cell (Hollingdale and Schwartz 1983). This receptor-mediated endocytosis can be inhibited by monovalent fragments (Fab) of monoclonal antibodies against the surface antigen on sporozoites, which do not promote degradation in Kupffer cells (Potocnjak et al. 1980; Yoshida et al. 1980; Aikawa et al. 1981). Therefore, it is possible that this antigen plays an important role as a ligand in the process of recognition and in the subsequent penetration of the sporozoite into the hepatocyte, while it is irrelevant in the interaction with Kupffer cells.

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