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Ultrastructural studies on the interaction of *Plasmodium falciparum* ookinetes with the midgut epithelium of *Anopheles stephensi* mosquitoes

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Abstract. The interaction of *Plasmodium falciparum* ookinetes with the midgut epithelium of *Anopheles stephensi* is described by electron microscopy. *Plasmodium falciparum* ookinetes have been traced during the early stages of invasion and localization beneath the basal lamina of the midgut. It is generally assumed that ookinetes break through two membranes to reach the basal lamina of the midgut epithelium by an intracellular route. In the present study evidence is presented that the ookinete takes an intercellular route in traversing the epithelial layer. Compared to the mode of penetration and intracellular development of sporozoites and merozoites, an intercellular route for penetration of the ookinete is not inconsistent with its further extracellular development.

The mosquito infection with malaria parasites starts when infected blood containing gametocytes is ingested from a vertebrate host. In the mosquito midgut, gametocytes become gametes and fertilization into zygotes takes place (Sinden 1984). Transformation of the zygote to ookinete has been described extensively for *Plasmodium gallinaceum* (Mehlhorn et al. 1980; Aikawa et al. 1984). Ookinete penetration of the mosquito midgut was only studied to a limited extend.

Garnham et al. (1962) reported on the fine structure of ookinetes of *Plasmodium gallinaceum* and *P. cynomolgi* and made some initial observations on the penetration of the midgut epithelium by these parasites. They speculated that the ookinete dissolved the host cell membrane while entering, since the site of interaction of the ookinete with the epithelial cells was obscured. This view, although sometimes questioned (Davies 1974), has remained the accepted one until recently (Sinden 1984; Sinden 1985). In the present study we have followed by transmission electron microscopy the route taken by *P. falciparum* ookinetes during the early stages of invasion and localization beneath the basal lamina of the mosquito midgut.

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Materials and methods

Culturing of mature gametocytes

Plasmodium falciparum (isolate NF54) was selected for routine work based on the number of gametocytes produced, the duration of gametocytogenesis and the ability of the gametocytes to reach maturity (Ponnudurai et al. 1982a). Parasites were maintained in a semi-automated minitipper apparatus and the gametocytes produced in a normal tipper system (Ponnudurai et al. 1982b). Mature gametocytes were produced 14 days after the cultures were initiated.

Infections of mosquitoes

Parasite suspension harvested from the tipper after washing and the addition of fresh red cells in human serum were fed to *Anopheles stephensi* (Kasur strain) mosquitoes through a membrane feeder. To monitor infection, control mosquitoes were examined for midgut oocysts at day 7 after feeding.

Electron microscopy

Fed mosquitoes were randomly selected for fixation at 24, 28, 32, 36 and 48 h. The midguts of the mosquitoes were dissected into 2% glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.3) at room temperature. The midguts were punctured with a fine needle to assure good fixation. The guts were placed in eppendorf cups in fresh fixative for 24 h, washed 3 times in 0.1 *M* phosphate buffer and immersed for 1 h in 1% osmium tetroxide. After another wash in distilled water, they were stained en bloc for 1 h in 1% aquaeous uranyl acetate, dehydrated and embedded in Epon 812. Transverse and longitudinal sections of midguts were made, double contrasted with uranyl acetate and lead citrate and examined in a Philips EM 300 electron microscope.

Immunofluorescence

Live parasites in wet preparations of infected mosquito midguts were visualized using an anti-25 kDalton monoclonal antibody (32F71) conjugated to fluorescein isothiocyanate which outlines the outer membrane of the zygote and ookinete (Vermeulen et al. 1985).

Results

By the earliest fixation at 24 h, the peritrophic membrane (PM) has begun to form on the mosquito midgut wall (Fig. 1). At this time ookinetes were only found in the midgut lumen. Mature ookinetes did not seem to be hindered initially by the secreted PM. At 32 h an ookinete was seen in the process of entering between midgut cells (Figs. 2–4). The microvillous border of the midgut epithelium was pushed aside and a track of the ookinete was visible in the formed PM (Figs. 2, 4). In fresh preparations, the trail left behind by the movement of the ookinete could be observed by fluorescence microscope with a specific anti-ookinete monoclonal antibody (Fig. 4 inset). Several ookinetes were found at 32 and 36 h during their passage through the midgut epithelium (Figs. 5, 7). The convergence of the lateral midgut epithelial cell membranes towards the surface of the parasite strongly suggested an intercellular position. Both the epithelial cell and parasite membranes are, due to tangential sectioning, difficult to see. A limiting epithelial cell membrane is visible at high magnification (Fig. 6). The apical region of the ookinete was not always found pointed towards the basal lamina, indicating that the parasite was probably moving in a rotational fashion at the moment of fixation (Figs. 5, 6). During its passage through the midgut the parasite seemed to have already secreted the first cyst material. This process continued when the ookinete arrived beneath the basal lamina (Fig. 8) at the exterior of the midgut wall.

From 32 to 48 h many ookinetes (young oocysts?) were observed on the external surface of the mosquito midgut epithelium separated from the haemocoel by a basal lamina and two muscle layers (Figs. 8, 9). The cyst wall was not well formed, although it was more apparent in a tangential section of the parasite (Fig. 8). The ookinete became more rounded, the 55–60 subpellicular microtubules (Fig. 6) remaining clearly visible until about 48 h.

An aggregate of virus-like membrane-bound particles, each measuring approximately 42 nm, was sometimes present in the ookinete (Figs. 3, 4, 9). This aggregate, referred to as crystalloid, was also seen in parasites that had reached the basal lamina (Fig. 9). The crystalloid was closely associated with a system of tubular vesicles and randomly ordered in structure. Some individual particles could be seen in the oocyst/ookinete cytoplasm (Fig. 9). Dedifferentiation was not only marked by the rounding up of the parasite but also by partial disruption of the inner pellicular membranes (Fig. 9). At 28, 32 and 36 h several ookinetes were found "trapped" in the PM (Figs. 10, 11).

Discussion

Late sporogonic development of malaria parasites has been extensively studied using rodent parasites (Garnham 1965; Vanderberg et al. 1967), avian parasites (Terzakis et al. 1966) or primate parasites (Terzakis 1971). There is only one electron microscopic study on the late (6 day and older) sporogonic development of *P. falciparum* from mosquitoes that had fed on naturally infected volunteers (Sinden and Strong 1978). There are few studies of ookinetes inside the mosquito wall and these refer only to rodent and avian parasites. In earlier work on the ookinetes of rodent malaria parasites, it was not possible to conclude whether an inter- or intracellular route was taken during penetration of the midgut wall (Garnham et al. 1969; Canning and Sinden 1973; Davies 1974). Garnham and colleagues (1962) described the ookinete as traversing the midgut epithelium intracellularly, but Canning and Sinden (1973) suggested that an intercellular route should not be discounted. We have now presented evidence that *P. falciparum* ookinetes traverse the midgut epithelium by an intercellular route. To disrupt desmosomal contacts between the cells, the parasite might secrete chelating agents. Dedifferentiation of the ookinete into the oocyst is predominantly extracellular although occasionally, as has been reported for *P. berghei,* may also occur intracellularly (Vanderberg et al. 1967; Beaudoin et al. 1974). Therefore some ookinetes might actually penetrate the midgut epithelial cells, although we have not found this in the present study. Ectopic oocyst development has never been found in mosquitoes infected with *P. falciparum* (Sinden and Strong 1978). Little morphological change occurs in the ookinete of *P. falciparum* up to 48 h. Cristate mitochondria, similar to those found in gametocytes, and typical fenestrated buttons remain visible (Ponnudurai et al. 1986). By contrast, mitochondria in ookinetes of the rodent parasite, *P. berghei,* undergo a change in morphology from acristate to cristate in early oocysts (Sinden 1978).

The function and origin of the crystalloid in

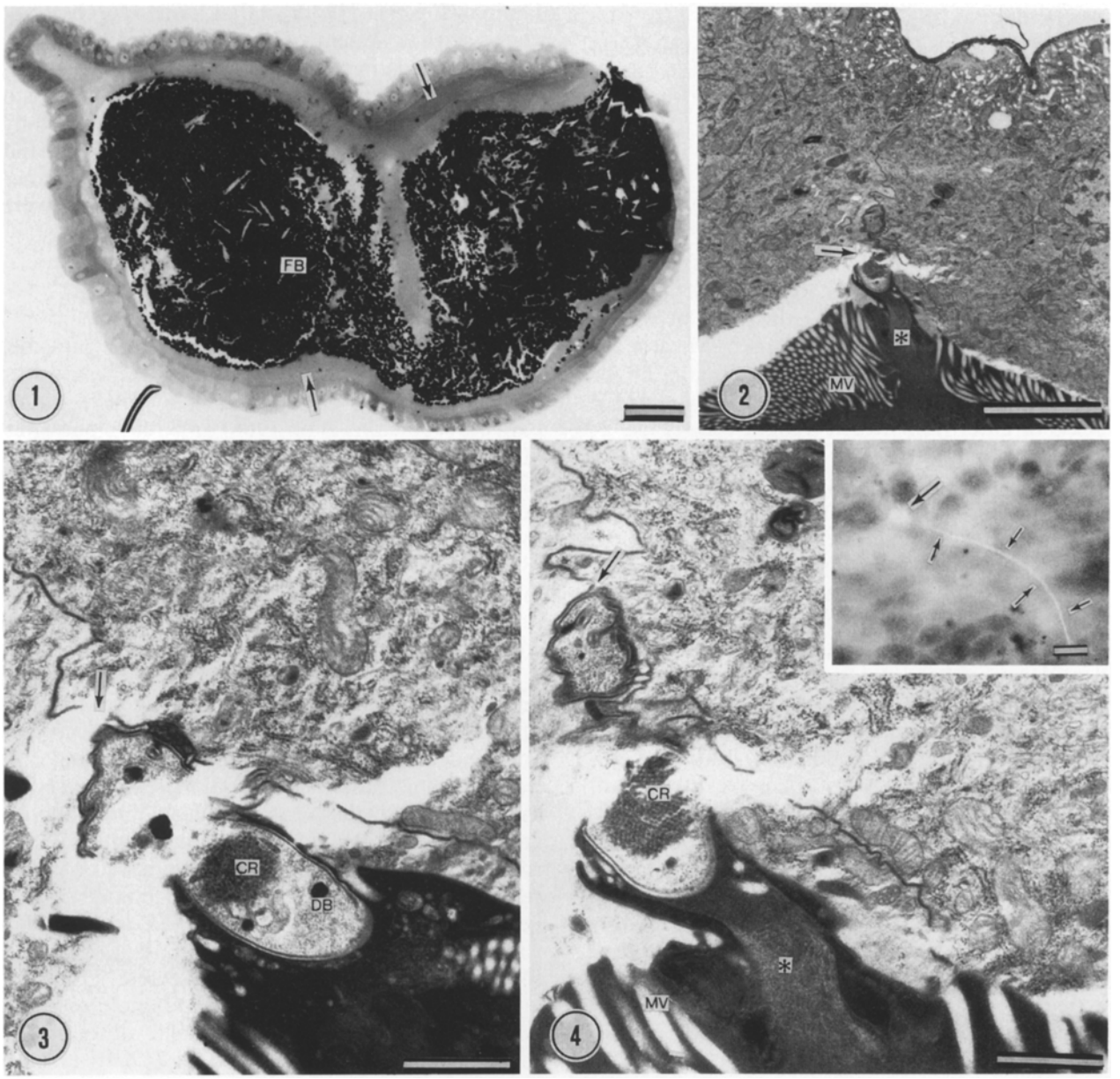

Fig. 1. Semi-thin epoxy section through the midgut of *Anopheles stephensi* 24 h after the blood feed. Around the foodbolus (*FB*) a perithrophic membrane (*PM*) (*arrows*) is formed and the epithelial cells have become hypertrophic. It is difficult to discern ookinetes in the epithelium. *Bar,* 50 μm

Fig. 2. Low power electron micrograph showing a *P. falciparum* ookinete (*arrow*) which had just migrated through the microvillous (*MV*) layer of the epithelial cells leaving a track in the PM (*asterisk*). *Bar,* 5 μm

Figs. 3, 4. Two serial sections of a *P. falciparum* ookinete at 32 h. The ookinete is between two epithelial cells (*arrow*) of the midgut epithelium. A track of the ookinete in the perithrophic membrane is still clearly visible (*asterisk*). Crystalloid (*cr*) and dense bodies (*db*) are present. *Bar,* 1 μm. *Inset:* immunofluorescence of a *P. falciparum* ookinete (*arrow*) from a wet midgut preparation showing a trail (*thin arrows*) left behind during movement (shedding phenomenon?). *Bar,* 10 μm

malaria parasites, first described by Garnham et al. (1962) in ookinetes of *P. gallinaceum* and *P. cynomolgi bastianellii,* is still unclear. Subsequently this structure was also described in ookinetes of *P. berghei* (Garnham et al. 1969; Terzakis et al. 1976) and in oocysts of *P. berghei* (Davies et al. 1971; Canning and Sinden 1973; Terzakis et al. 1976), *P. cynomolgi bastianellii* (Terzakis et al. 1976), *P. gallinaceum* (Terzakis et al. 1976) and *P. falciparum* (Sinden and Strong 1978). In the present

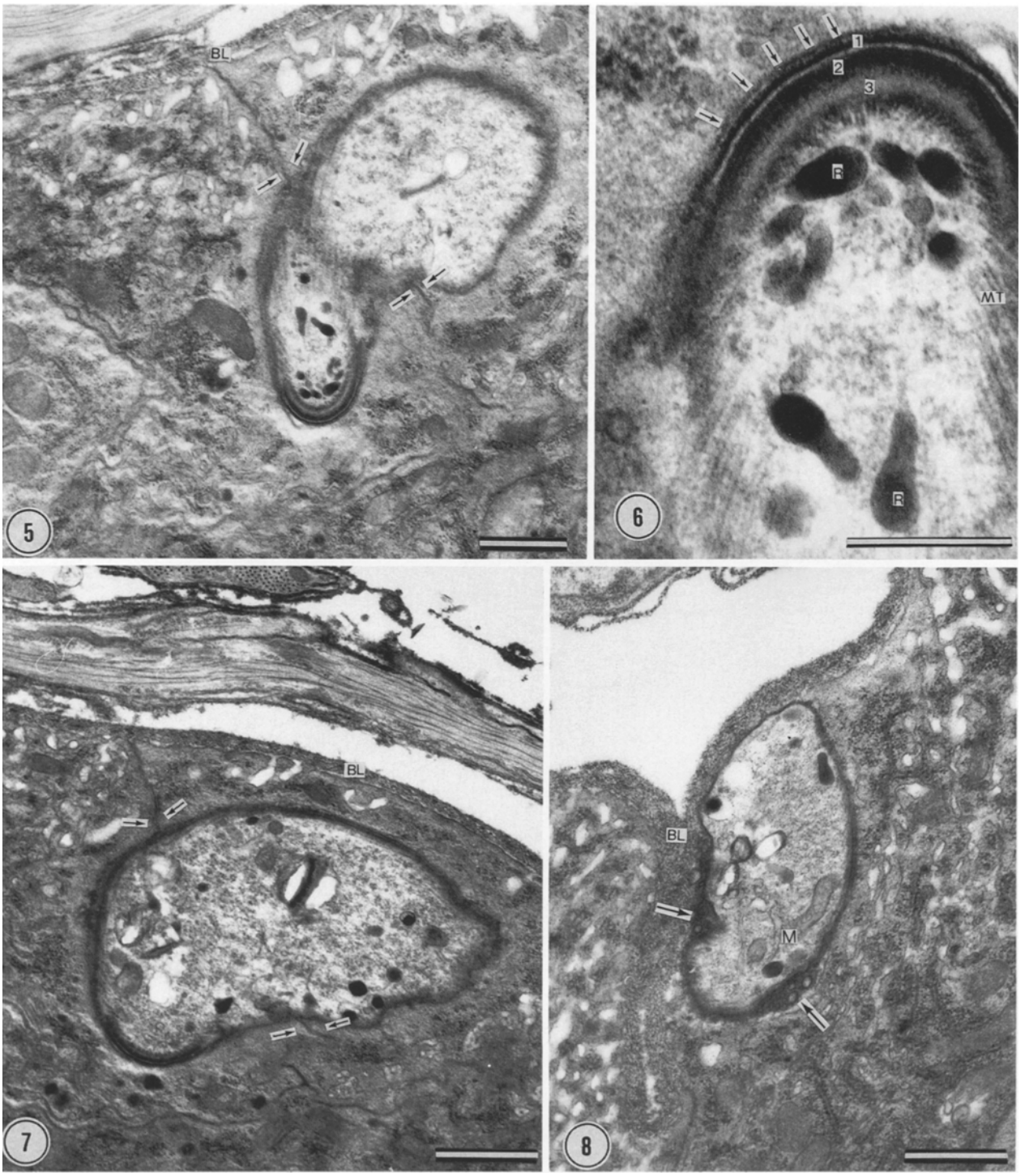

Fig. 5. Ookinete fixed in passage through the midgut epithelium at 36 h. The convergence of the lateral midgut cell membranes towards the surface of the parasite (*arrows*) strongly suggests an intercellular position. The apical end of the ookinete is not pointed towards the basal lamina (*BL*). *Bar*, 1 μm

Fig. 6. Detail of Fig. 5 showing the apical part of the ookinete with 56–60 subpellicular microtubules. Due to tangential sectioning, a membrane surrounding the parasite is not clearly visible. Only at the apical part this membrane is apparent (*arrows*). Note parasite plasmalemma (*1*), the inner pellicular membranes (*2*) and a section through the apical ring (*3*). *Bar*, 0.5 μm

Fig. 7. Extracellular ookinete between midgut epithelial cells. Note the convergence of the epithelial cell membranes to the parasite (*arrows*). Around the ookinete these membranes, as well as the parasite's plasmalemma, can hardly be discerned due to tangential sectioning. *Bar*, 1 μm

Fig. 8. Ookinete has reached an extracellular position beneath the basal lamina (*BL*) at 36 h. In a tangential section the cyst wall is visible (*arrows*). *M*, mitochondrion. *Bar*, 1 μm

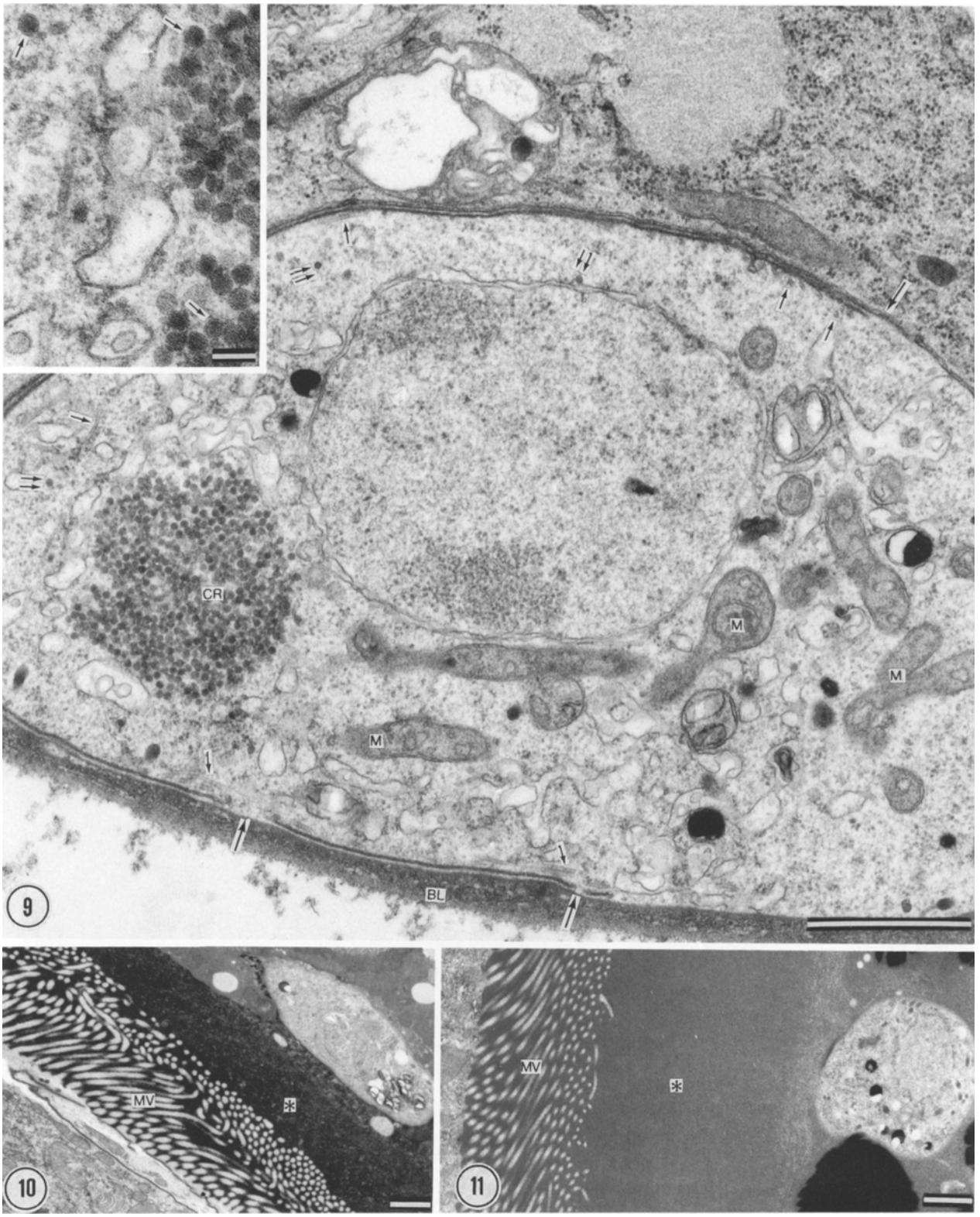

Fig. 9. Young ookinete/oocyst beneath the basal lamina (*BL*) at 36 h. The inner pellicular membranes have started to disrupt at some places (*large arrows*). A crystalloid with a diameter of 1–1.5 μm consists of individual particles and is associated with the ER. Individual particles are distributed in the cytoplasm (*double arrows*). Microtubules can be seen in the cytoplasm and in a subpellicular position (*thin arrows*). *Bar*, 1 μm. *Inset:* higher magnification of the crystalloid, showing membrane-bound 42 nm viruslike particles (*arrows*). *Bar*, 100 nm

Fig. 10. Ookinete attached to the PM (*asterisk*) at 32 h. *Bar*, 1 μm

Fig. 11. Ookinete attached to the PM (*asterisk*) at 36 h. *Bar*, 1 μm

study this crystalloid was also found in ookinetes and partly transformed ookinetes of *P. falciparum*.

Trefiak and Desser (1973) suggested that the crystalloid in several haemosporina might act as store of energy for the developing ookinete. However, considering the typical morphology (aggregations of membrane-bound 42 nm particles) and the limited presence of the crystalloid, we agree with other authors on its virus-like nature (Davies et al. 1971; Davies and Howells 1973; Terzakis 1969; Terzakis et al. 1976).

The influence of the PM on ookinete invasion is unclear. Recent light microscopical work on *P. berghei* ookinetes and *A. atroparvus* (Sluiters et al. 1986) indicated that the PM would not function as a physical barrier against migrating ookinetes. However, in our electron microscopic observations on *P. falciparum* ookinetes, we frequently found parasites "stuck" on to the PM. If the PM is dissected out 36 h after the mosquitoes had fed, one can find many ookinetes attached to its external surface (Ponnudurai et al., unpublished data). These ookinetes, although morphologically still intact, were probably non-viable. Another possibility is that the ookinete was capable of penetrating the newly formed, but not the thickened and hardened PM 36 h after the blood meal. Failure to cross this barrier might result in the tryptic digestion of the ookinete. As the PM develops progressively, it may be responsible for a substantial loss of ookinetes and hence oocysts on the midgut wall. Similarly, retarded penetration also increases the length of exposure of ookinetes to mosquito trypsin which might influence survival of the ookinetes (Gass 1978). In double feeding experiments young ookinetes of *P. gallinaceum* were found by Gass (1977) to be sensitive to digestive enzymes of mosquitoes and he suggested that parasites in the center of the blood meal are able to develop relatively unaffected by trypsin-like enzymes.

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