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Scanning Electron Microscope Observations of *Plasmodium berghei* Ookinetes in Primary Mosquito Cell Cultures[1]

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Plasmodium berghei-infected blood from mice was inoculated into primary cell cultures (PCC) obtained from the mosquito *Anopheles stephensi*. Immature and mature ookinetes of *Plasmodium berghei*, which developed in these cultures were studied with the scanning electron microscope. Immature ookinetes had a bulbous-like structure at the posterior end and a slightly wrinkled surface. Mature ookinetes were smoother in appearance and somewhat longer than immature forms. Shallow spiraling waves were observed on the surface of some ookinetes, especially in the anterior half of the body. Such waves may be involved in ookinete locomotion. Penetration of cultured cells by ookinetes was not observed. Infected red cells, which were present in the inoculum, had small depressions on the red cell surface, whereas some uninfected red cells had accentuated concavities. Mouse blood cells adhered closely to PCC cells; some attached red cells were irregular in shape.

INTRODUCTION

Although many fine structural studies of the various stages of malaria parasites (Aikawa, 1971) have been made, few of the stages have been examined with the scanning electron microscope. We report herein some observations on the development of *Plasmodium berghei* ookinetes in primary cell cultures from the mosquito, *Anopheles stephensi*.

MATERIALS AND METHODS

Ookinetes of *Plasmodium berghei* in primary cell cultures (PCC) (Rosales-Ronquillo and Silverman, 1974), obtained from fragments of newly hatched larvae of *Anopheles stephensi* (Rosales-Ronquillo et al., 1972), were studied with the scanning electron microscope (SEM). Ten- to 28-day-old PCC cells, grown on 10 × 50 mm coverslips in Leighton tubes at 21°C, were inoculated with *P. berghei*-infected blood obtained from mice. At 6-hr intervals between 12 and 48 hr after inoculation of infected blood, coverslips were removed from Leighton tubes and fixed for 2 to 12 hr in Karnovsky's fixative (Karnovsky, 1965) in 0.2 *M* cacodylate buffer, *p*H 7.4. They were then rinsed in 0.2 *M* cacodylate buffer for 2–12 hr, post-fixed for 15–30 min in osmium tetroxide, rinsed in two 15-min changes of buffer, dehydrated in a series of ethanol, placed in isoamyl acetate and critical point freeze-dried. Coverslips were then fractured and small pieces mounted on specimen pegs with conductive silver paint. Specimens were placed in a vacuum evaporator, coated with about 10–15 nm of gold while revolving 360 degrees through an arc of 85 degrees, and then examined in a MINI-SEM, Model-MSM-3 (International Scientific Instruments, Inc.). Micrographs were recorded on 4" × 5" Polaroid type 55 P/N and Dupont COS-7 negative film.

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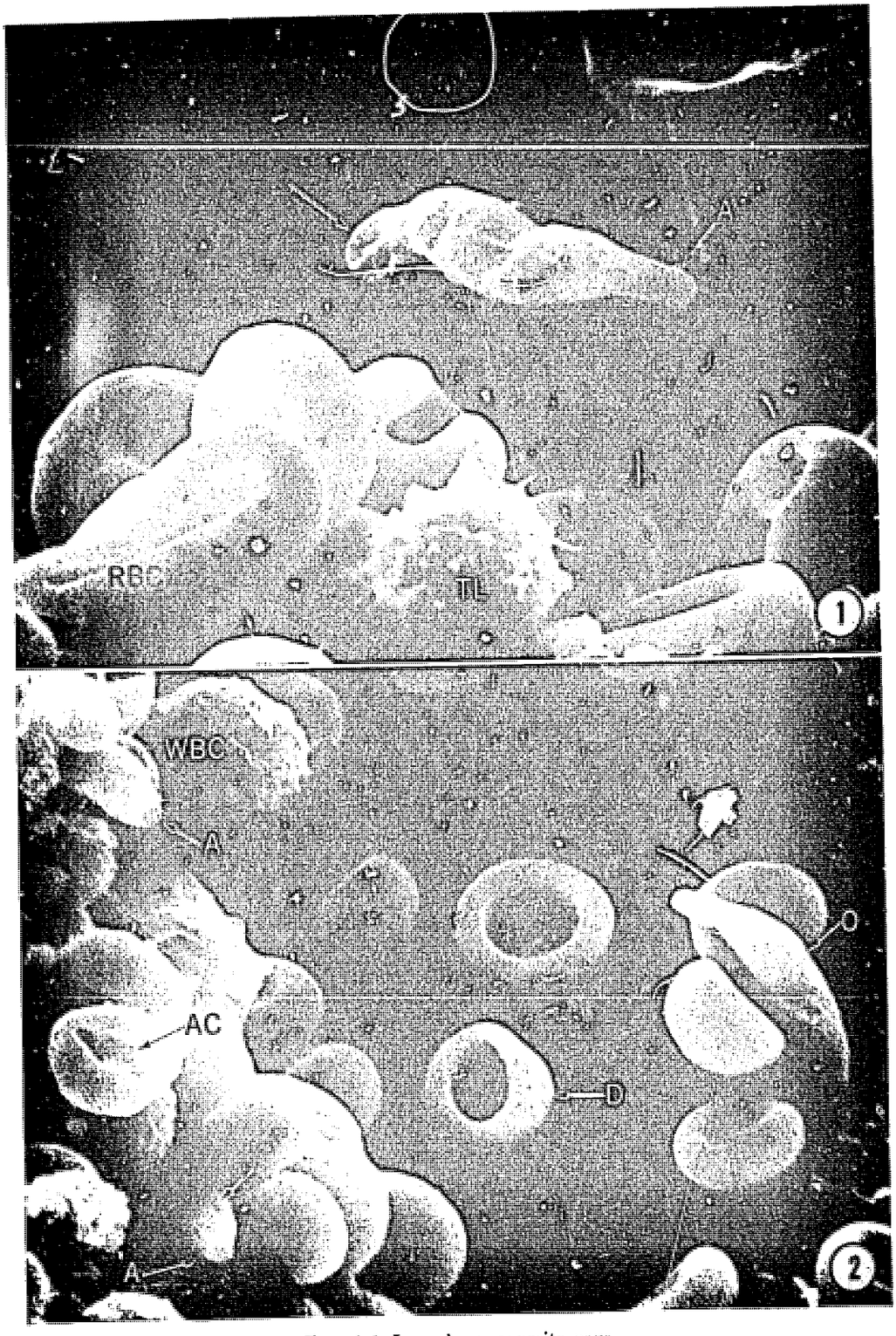

FIGS. 1–3. Legends on opposite page.

FIGS. 1–3. Scanning electron micrographs of *Plasmodium berghei* ookinetes in primary mosquito cell cultures. Age in days of primary cell culture, interval in hours between inoculations of *P. berghei*-infected blood into culture and fixation, and magnification are listed in parentheses. A, anterior end of ookinete; AC, accentuated concavity of uninfected red cell; BL, B-lymphocyte; D, depression on surface of infected red cell; O, ookinete; PC, primary mosquito cell; RBC, red blood cell; TL, T-lymphocyte; WBC, white blood cell.

FIG. 1. Immature ookinete; note small bulbous-like structure (arrow) at posterior end and wrinkling of ookinete surface, and T-lymphocyte with surface projections (21; 18; ×6700).

FIG. 2. Three ookinetes, two of which are located within clumps of mouse blood cells, the "free" ookinete is a mature form; note spiral wave (arrow) in one ookinete (21; 24; ×4500).

FIG. 3. Ookinete with short anterior end located in aggregate of cells; note apparent twisting of ookinete body, depressions (single arrows) on surface of one RBC, fibers (double arrow) arising from PC cell, and B-lymphocyte with relatively smooth surface (12; 40; ×8600).

RESULTS AND DISCUSSION

Exflagellation of plasmodial microgametocytes and fertilization of macrogametes, which then develop into ookinetes, normally occur in the midgut of various mosquitoes. Recently, Rosales-Ronquillo and Silverman (1974) reported successful development of *P. berghei* ookinetes in primary cell cultures as well as an established cell-line culture, both obtained from immature stages of *Anopheles stephensi*. After inoculation of *P. berghei*-infected hamster blood, exflagellation was observed within 7–10 min, fertilization apparently occurred soon thereafter and zygotes developed to mature ookinetes within 24 hr. We used the same primary mosquito cell cultures in the present investigation to obtain development of *P. berghei* ookinetes for observation with the SEM.

Ookinetes (Figs. 1–3) were somewhat vermiform, tapering slightly more at the

anterior end than at the posterior, and had a relatively smooth surface. Immature ookinetes (Fig. 1) were slightly shorter (10.9 × 2.4 μm) in length than mature forms (11.8 × 2.4 μm) (Fig. 2), had a bulbous-like expansion of variable size at the posterior end and a somewhat wrinkled surface, especially in the posterior one half of the ookinete. Mature ookinetes have a well-developed pellicle consisting of two unit membranes and subpellicular microtubules which extend along the whole length of the parasite (Garnham et al., 1969). This pellicular complex apparently makes these forms more rigid and wrinkle-free during fixation and processing for SEM than immature forms, which have an incomplete pellicle in which the double-membrane complex and subpellicular microtubules are not fully formed. Some ookinetes had a relatively short anterior end, averaging 0.6 μm in length (Figs. 2, 3), whereas others had a longer anterior end, 2.3 μm in length (Figs. 1, 2). This difference in length is probably associated with extending and retracting the anterior end. In all specimens, the anterior end was approximately 1 μm in width.

Most ookinetes appeared twisted on their longitudinal axis. In some of these specimens, shallow and slightly spiraling longitudinal waves were observed in the anterior half of the ookinete. Similar wavelike elevations, which were believed to be involved in locomotion, have been seen in electron micrographs of the pellicle of *P. berghei yoelii* ookinetes (Garnham et al., 1969) as well as in sporozoites of the coccidian, *Eimeria larimerensis* (Roberts et al., 1971). By using time-lapse cinemicrography, Freyvogel (1966) studied the motility of the ookinetes of four *Plasmodium* species. In ookinetes of *P. gallinaceum*, he found prominent annular waves of contraction, which formed at the anterior end and then moved varying distances into the posterior half of the ookinete. In the present study ookinetes which had spiral waves were perhaps in the process of moving at the instant of fixation. These waves may result secondarily from twisting of the ookinete body or may actually be involved in locomotion of the ookinete.

Numerous ultrastructural investigations of red cells infected with malaria parasites have been made. In a recent SEM study, Bodammer and Bahr (1973) found two types of alterations on the surface of red cells from *P. berghei*-infected animals. Some uninfected red cells had accentuated concavities, whereas infected red cells had randomly located depressions. We found similar accentuated concavities (Fig. 2) in uninfected red cells and depressions (Figs. 2, 3) in infected ones.

Usually, mouse blood cells adhered closely to PCC cells. Many of the red cells which appeared attached to PCC cells by a small portion of the red cell surface had transformed from typical biconcave discs into distended irregular- or pear-shaped forms (Fig. 3). Collagenlike fibers (Fig. 3) arising from the surface of PCC cells occasionally encircled and entwined clumps of red cells. Ookinetes were sometimes seen within aggregates of these "entrapped" red cells. A few ookinetes with the anterior end located close to red cells or PCC cells were seen; however, ookinetes in the process of penetrating cells were not observed. Garnham et al. (1962) reported that ookinetes of *P.* (*Haemamoeba*) *gallinaceum* and *P. cynomolgi bastianelli* penetrated the gut of the mosquito and apparently migrated intracellularly to reach a location at the external border of the gut, where development into the oocyst stage occurred. Freyvogel (1966) is the only investigator to report the passage of living *Plasmodium* ookinetes, *P.* (*H.*) *gallinaceum*, through the peritrophic membrane and into the epithelial layer of the mosquito gut. He studied the penetration process in peritrophic membranes which had been laid flat and cleaned of blood remnants or in intact membranes enveloping the entire blood coagulum. Owing to the comparatively poor depth definition inherent in the light microscope

lens, no details of ookinete shape or movement could be seen. Although the entrance of ookinetes into cells was not observed in the present study, the use of this in vitro system as well as scanning electron microscopy should enable such observations to be made.

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