

ERYTHROCYTE ENTRY BY MALARIAL PARASITES

A Moving Junction between Erythrocyte and Parasite

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ABSTRACT

Invasion of erythrocytes by merozoites of the monkey malaria, *Plasmodium knowlesi*, was investigated by electron microscopy. The apical end of the merozoite makes initial contact with the erythrocyte, creating a small depression in the erythrocyte membrane. The area of the erythrocyte membrane to which the merozoite is attached becomes thickened and forms a junction with the plasma membrane of the merozoite. As the merozoite enters the invagination in the erythrocyte surface, the junction, which is in the form of a circumferential zone of attachment between the erythrocyte and merozoite, moves along the confronted membranes to maintain its position at the orifice of the invagination. When entry is completed, the orifice closes behind the parasite in the fashion of an iris diaphragm, and the junction becomes a part of the parasitophorous vacuole. The movement of the junction during invasion is an important component of the mechanism by which the merozoite enters the erythrocyte.

The extracellular merozoite is covered with a prominent surface coat. During invasion, this coat appears to be absent from the portion of the merozoite within the erythrocyte invagination, but the density of the surface coat outside the invagination (beyond the junction) is unaltered.

KEY WORDS malarial parasites · *Plasmodium knowlesi* · erythrocyte · junction · invasion mechanism · electron microscopy · cell interaction

The asexual malaria parasite infects erythrocytes and there develops to a mature schizont that is made up of many individual merozoites. Upon rupture of the schizont-infected erythrocyte, the merozoites are released and are capable of infecting other erythrocytes. In 1969 Ladda et al. (15) reported on the invasion of erythrocytes by merozoites of *Plasmodium berghei* and *P. gallinaceum*,

and established that merozoites enter within an invagination of the erythrocyte membrane rather than by penetrating it. They found that merozoites approached erythrocytes with the apical end and formed a focal depression on the erythrocyte membrane. With deeper invagination of the erythrocyte membrane, the resulting cavity conformed to the shape of the merozoite. The orifice of the invaginated erythrocyte membrane fused upon completion of the entry. They also noted that granular material (a surface coat) covered the entire surface of the extracellular merozoite and was removed upon completion of the merozoite

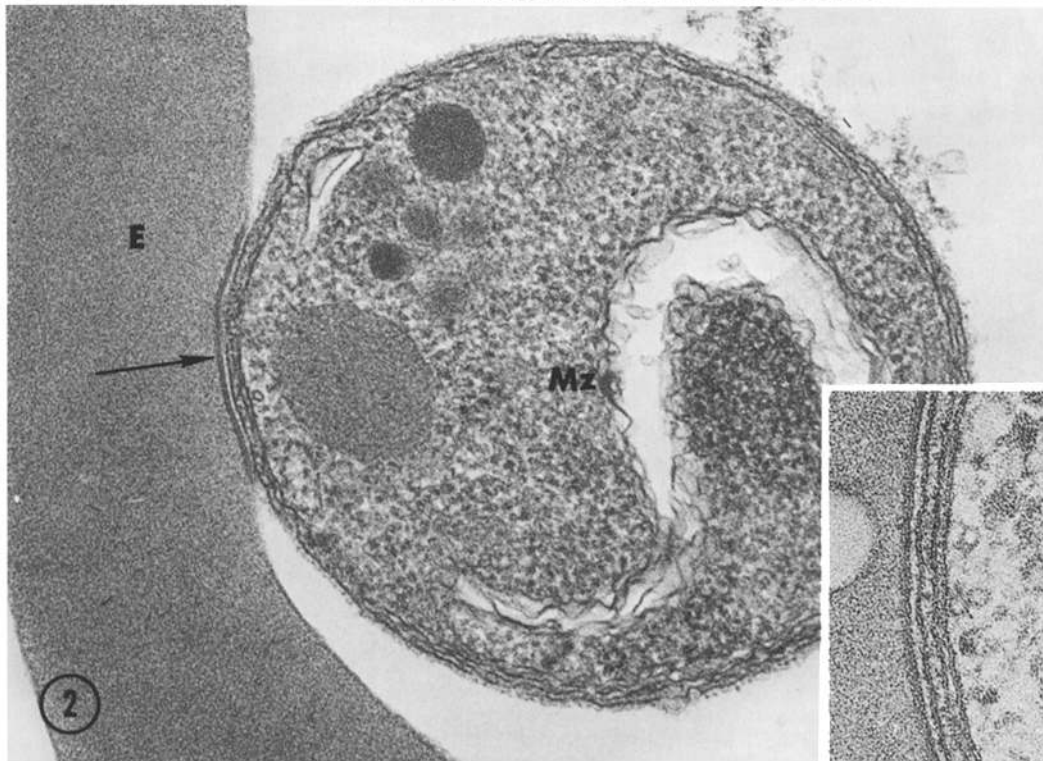
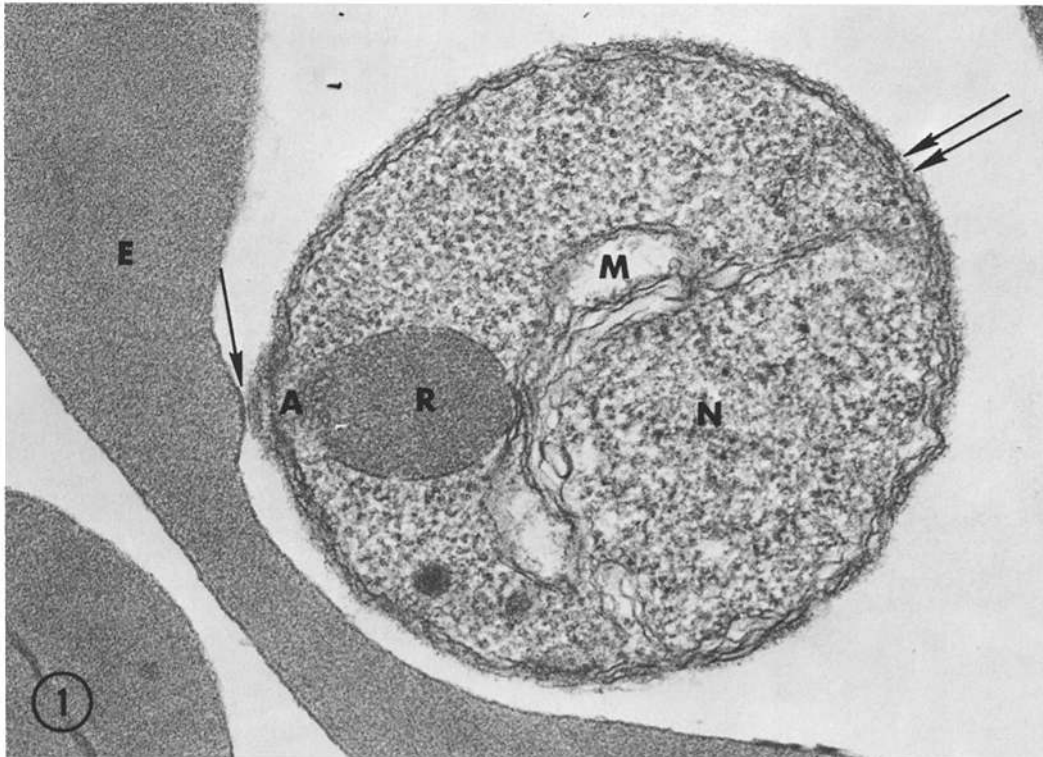


FIGURE 1 Electron micrograph of a merozoite of *P. knowlesi* at the initial contact between the merozoite's apical end (arrow) and an erythrocyte (*E*). The erythrocyte membrane is slightly raised at the point of the interaction. The merozoite shows an apical end (*A*), a rhoptry (*R*), a nucleus (*N*), and a mitochondrion (*M*). The surface is covered with a surface coat (double arrow). $\times 50,000$.

FIGURE 2 Electron micrograph of a merozoite (*Mz*) contacting an erythrocyte (*E*). The erythrocyte membrane is thickened (15 nm) at the attachment site (arrow). $\times 54,000$. Inset, higher magnification micrograph of the erythrocyte-merozoite attachment site showing the thickened erythrocyte membrane. $\times 112,000$.

entry. The surface coat was at least in part of parasite origin (16). Dvorak et al. (7) studied by light microscopy the invasion of erythrocytes by *P. knowlesi* and observed that the invasion consisted of attachment of the apical end of the parasite to the erythrocyte, deformation of the erythrocyte, and entry of the parasite by invagination of the erythrocyte membrane. The entire invasion sequence was complete in approximately 30 s.

Ultrastructural studies on invasion were hindered by sampling problems. Recently, Dennis et al. (6) reported a new method for the collection of large quantities of free, viable merozoites of *P. knowlesi* which could invade erythrocytes. By applying this method, Bannister et al. (4) confirmed the previous electron microscope observations on invasion. We have used an approach similar to that of Bannister et al. (4) and report several new findings on the invasion process. They include (a) a junction between erythrocytes and merozoites, (b) movement of the junction during invasion, and (c) the fate of the surface coat on merozoites. These findings will be presented and discussed in this paper.

MATERIALS AND METHODS

Parasitized erythrocytes were obtained from rhesus monkeys (*Macaca mulatta*) infected with the Malaysian strain of *Plasmodium knowlesi* (5) when the parasitemia was 10–30%. The majority of parasites were schizonts with 8–10 nuclei. The blood was mixed with heparin and adenosine diphosphate and passed over glass beads to remove platelets and some white cells. The cells were washed one time at room temperature in culture medium that consisted of medium 199 plus 10 mM glucose, 6.6 mM glycylglycine, 25 μ g/ml of gentamycin, and 10% (vol/vol) heat-inactivated fetal calf serum. Packed cells were pipetted into 2 mm \times 10 cm glass tubes that were sealed at one end. To separate the low-density schizonts from the other erythrocytes, the cells were spun at 1,200 g for 5 min. The schizonts formed a brown layer

at the top of the tube. The tube was broken at the interface between schizonts and other erythrocytes, and the schizonts were added to the culture medium. Approximately 3×10^8 schizonts were added to 35 ml of culture medium in the culture chamber and incubated at 37°C.

The culture chamber was built according to the specifications of Dennis et al. (6). The bottom of the culture chamber was covered with a Nuclepore filter (Nuclepore Corp., Pleasanton, Calif.) with 1.5- μ m holes. Fresh medium was continuously added through the wall in the side of the chamber and carried out through the filter in the bottom at a rate of approximately 1.5 ml/min. Merozoites freely passed through the holes in the filter; few schizonts could pass this filter. This permitted collection of viable merozoites soon after their release from schizont-infected erythrocytes. 1 ml of the merozoite suspension ($\sim 20,000$ merozoites/mm³) was mixed with 0.1 ml of washed rhesus erythrocytes (100,000/mm³) and incubated at 37°C with continuous agitation. After 1 and 5 min, the cell suspension was added to glutaraldehyde fixative (2% glutaraldehyde, 0.05 M phosphate buffer, pH 7.4, and 0.116 M sucrose). Transmission electron microscopy was performed as previously described (1). Thin sections were stained with uranyl acetate and lead nitrate and were examined with a Siemens Elmiskop 101 electron microscope.

To visualize the merozoite surface coat clearly, photographic enhancement of the surface coat was accomplished by masking the merozoite image. With the merozoite masked, only the surface coat was projected on the photographic paper. The mask was then removed and the whole image (merozoite and surface coat) was reprojected on the same sheet of paper. The net result was a 1 \times exposure for the merozoite and a 2 \times exposure for the surface coat.

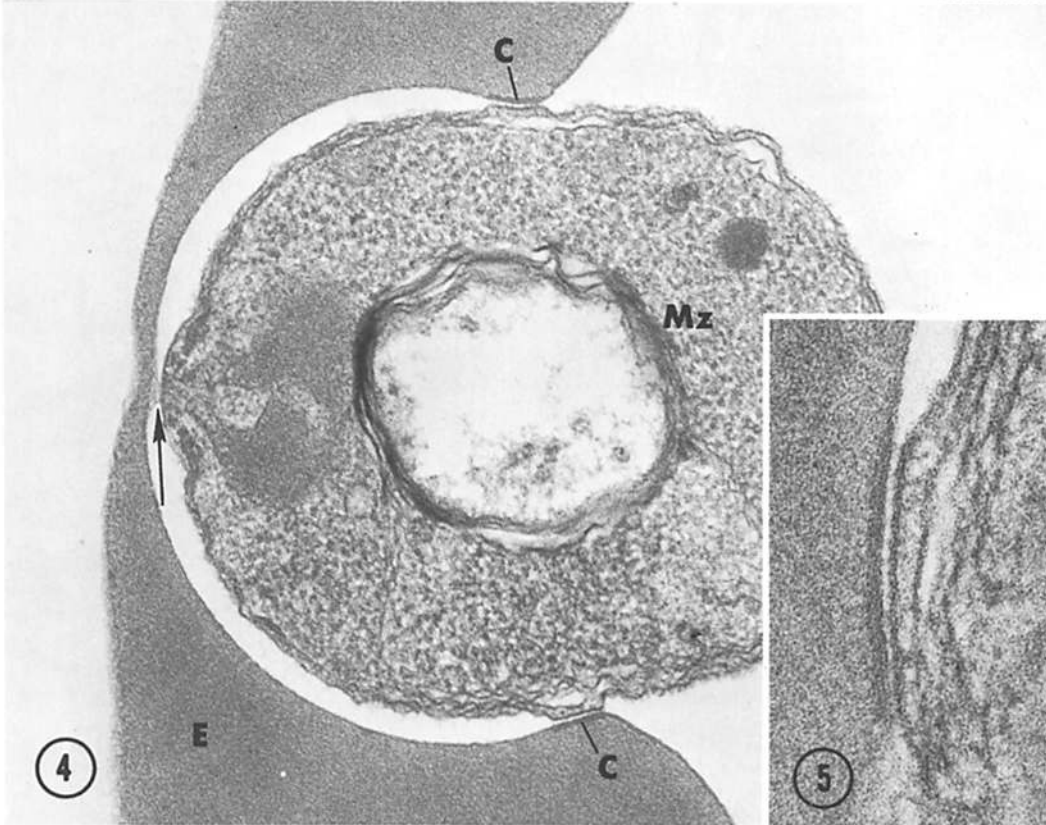
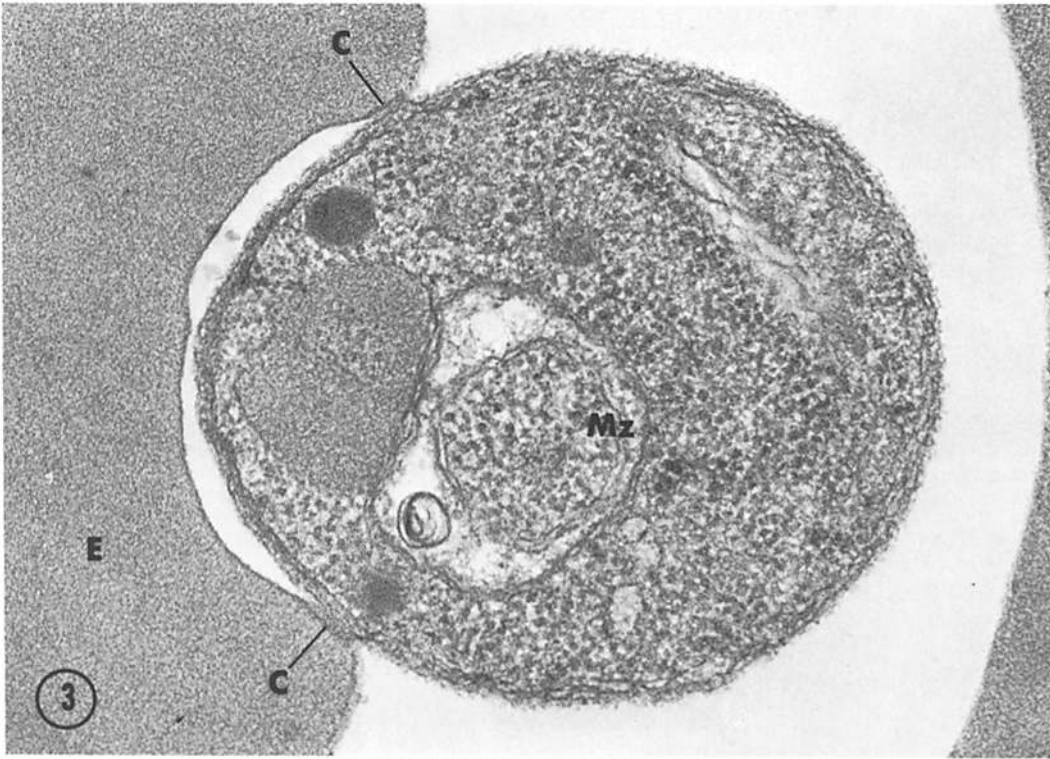
RESULTS

Inasmuch as the fine structure of merozoites of *P. knowlesi* has been reported by several investigators (1, 3, 4, 16), only a brief description of the parasite is presented here (Fig. 1). The entire surface of the merozoite is covered with an electron-dense surface coat measuring ~ 20 nm that

FIGURE 3 An advanced stage of erythrocyte (E) entry by a merozoite (Mz). The invagination of the erythrocyte is created by the merozoite. Note a junctional attachment (C) at each side of the entry orifice. $\times 54,000$.

FIGURE 4 A further advanced stage of erythrocyte (E) entry by a merozoite (Mz). The junction (C) is formed between the thickened membrane of the erythrocyte and merozoite plasmalemma. The invagination deepens and conforms to the curvature of the merozoite. An electron-opaque projection connects the apical end and the erythrocyte membrane (arrow). $\times 50,000$.

FIGURE 5 Higher magnification micrograph of the junction showing the thickened erythrocyte membrane and fine fibrils between the two parallel membranes. $\times 144,000$.



consists of fine fibrils. Two electron-dense rhoptries and a few electron-dense micronemes are located in the apical region. A mitochondrion and nucleus are located in the posterior portion.

The invasion of the erythrocytes by the merozoites of *P. knowlesi* is initiated by the merozoite contacting the erythrocyte with the apical end oriented against the erythrocyte membrane (Fig. 1). The erythrocyte membrane at the point of the interaction is slightly raised initially (Fig. 1), but eventually a depression is created in the erythrocyte membrane (Fig. 2). The erythrocyte membrane to which the merozoite is attached becomes thickened (Fig. 2, inset).

As invasion progresses, the depression in the erythrocyte deepens and conforms to the curvature of the merozoite (Figs. 3, 4, 6, and 7). At this time the thickened, electron-dense zone on the erythrocyte membrane is no longer observed at the point of the initial attachment but now appears at the orifice of the merozoite-induced invagination of the erythrocyte membrane (Figs. 3-7). This thickened area of the erythrocyte membrane measures ~15 nm in thickness and ~250 nm in length, and appears to be a thickening of the inner leaflet of the bilayer (Fig. 5). (The membrane of a normal erythrocyte is 7.5-10 nm in thickness.)

The thickened erythrocyte membrane forms a junction with the merozoite. The merozoite membrane often appears pulled toward the thickening in this area (Fig. 5). It is likely that there is a relatively greater shrinkage of merozoite than erythrocyte during specimen preparations. Because of this, the erythrocyte and merozoite remain in contact only at the junction and apical region. The gap between these two membranes is ~10 nm, and fine fibrils extend between these two parallel membranes. The junction forms a circumferential interaction at the orifice of the invaginated erythrocyte membrane, as it is always located at each side of the orifice as seen by transmission electron microscopy, regardless of the plane through which the section passes. When entry is completed (i.e. the merozoite is within the erythrocyte), the junction appears to fuse at the posterior end of the merozoite (Fig. 8), closing the orifice in the fashion of an iris diaphragm. The merozoite membrane still remains in close apposition to the thickened erythrocyte membrane (Fig. 8, inset) at the point of the final closure.

During the invasion process the distribution of the surface coat alters. Before invasion, mero-

zoites are covered with a uniform surface coat of ~20 nm thickness (Fig. 9). During invasion, no surface coat is visible on the portion of the merozoite within the erythrocyte invagination, whereas the surface coat on that portion of the merozoite remaining outside the erythrocyte appears to be similar to that seen on free merozoites (Fig. 10). No accumulation of the surface coat is seen at or beyond the junction. This becomes more apparent when the surface coat is photographically intensified by a double-exposure technique.

Throughout invasion, the apical end remains in contact with the erythrocyte. In some specimens, several small vacuoles are seen in the erythrocyte cytoplasm in this region as originally described by Bannister et al. (4). There is an electron-opaque band between the tip of the apical end and the erythrocyte (Figs. 4 and 11). This band appears to be continuous with the common duct of the rhoptries. The common duct is formed by the meeting of ductules which lead from each of the rhoptries (Fig. 11). The common duct is less electron dense than the rhoptry itself.

DISCUSSION

Host-parasite interaction is of major importance to parasitic protozoa inasmuch as their survival depends on host cells which supply environmental and nutritional requirements. They cannot live apart from their host cells or host-cell nutrients. In recent years, there has been a great interest in the mechanism by which protozoa attach to and enter their host cells. Malaria parasites and other related protozoa such as *Toxoplasma* (2, 13), *Babesia* (18), *Eimeria* (12), and *Lankesteria* (20) enter within an invagination of the host cell membrane, apical end first. In the case of malaria parasites, invasion is probably explained other than by the merozoite pushing its way into a cell or by the cell ingesting the merozoite. The merozoite has limited motility and the host cell, the mature erythrocyte, is nonphagocytic. The observations in the present study provide a possible mechanism for erythrocyte membrane invagination by the merozoite.

Invasion of erythrocytes by merozoites requires a number of distinct steps. They include (a) initial attachment of the merozoites to the erythrocyte membrane, (b) invagination of the erythrocyte membrane around the merozoite to form a parasitophorous vacuole, and (c) sealing of the erythrocyte after completion of merozoite invasion. The initial attachment involves an interaction be-

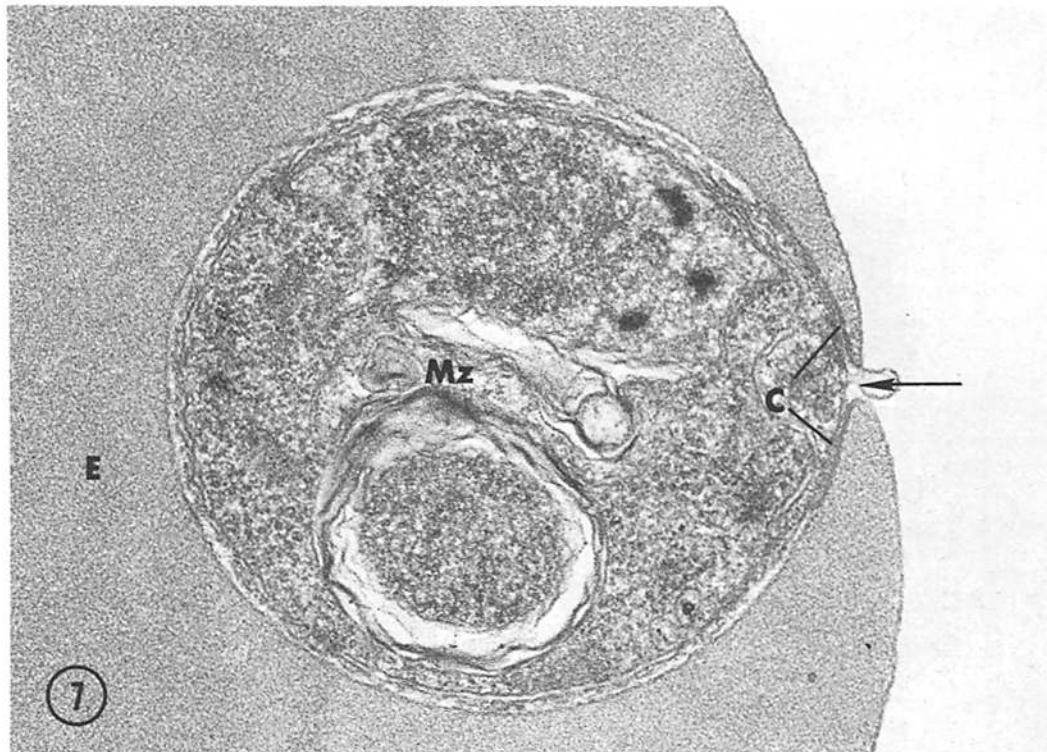
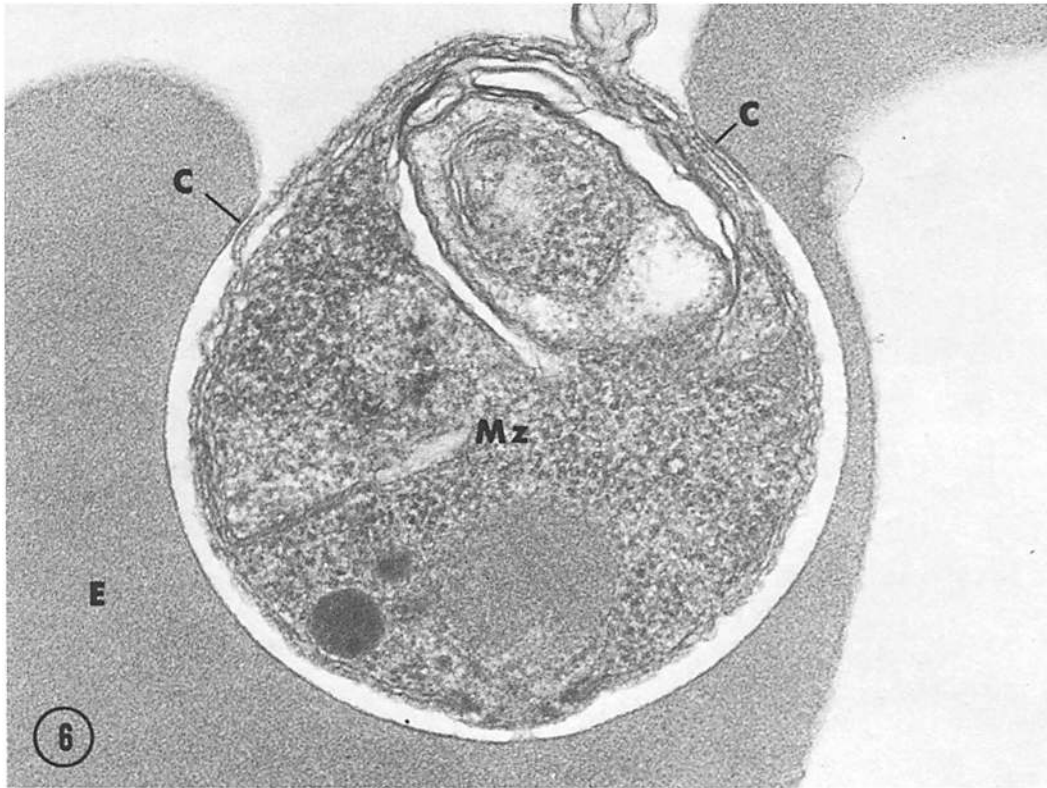


FIGURE 6 A further advanced stage of erythrocyte (*E*) entry by a merozoite (*Mz*). The junction (*C*) is always located at the orifice of erythrocyte entry and is now located at the more posterior portion of the merozoite than in Figs. 3 or 4. $\times 48,000$.

FIGURE 7 Erythrocyte (*E*) entry by a merozoite (*Mz*) is almost completed and a small orifice (arrow) is seen at the posterior end of the merozoite. The junction (*C*) is now moved to the posterior end of the merozoite. $\times 54,000$.

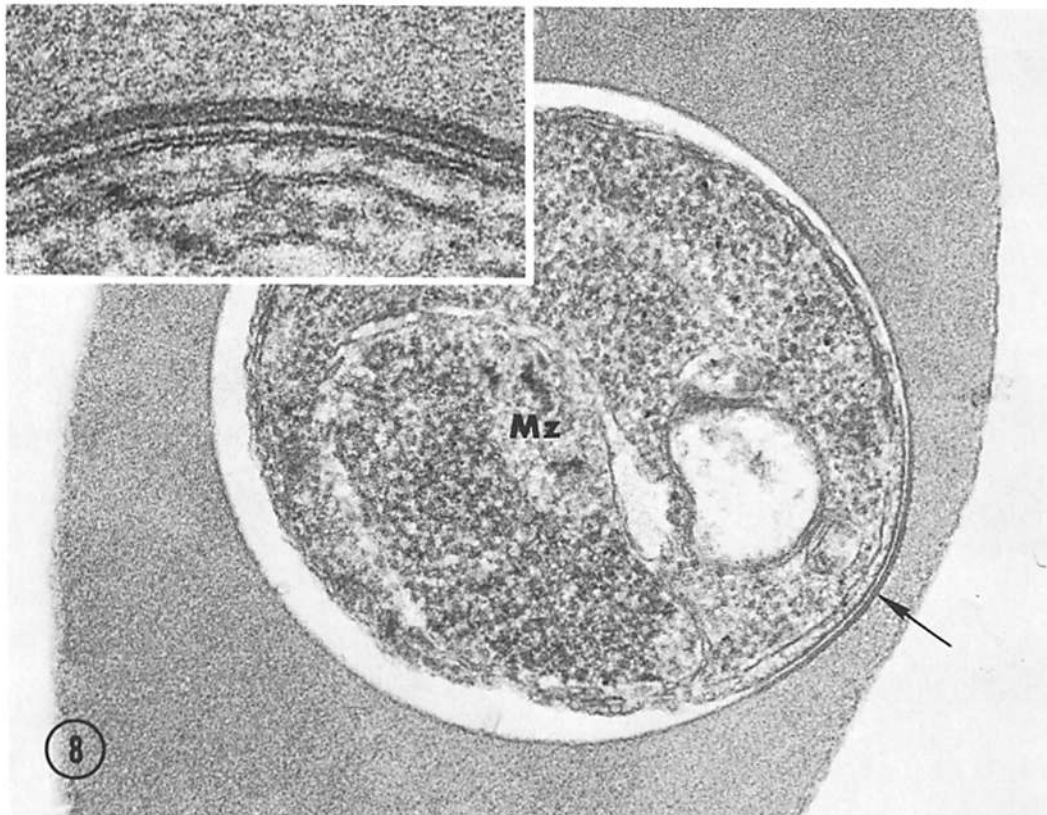


FIGURE 8 A merozoite (*Mz*) is now inside of an erythrocyte. However, the posterior end of the merozoite is still attached (arrow) to the thickened erythrocyte membrane, and the other portion is separated from the surrounding membrane by a clear space. $\times 50,000$. Inset, higher magnification micrograph of the thickened erythrocyte membrane which is attached to the merozoite. $\times 300,000$.

tween the erythrocyte membrane and the apical end of the merozoite. Miller et al. (17) reported that initial recognition and attachment between *P. knowlesi* merozoites and erythrocytes probably involve specific determinants, probably associated with Duffy blood group related antigens. After contact, a junction forms between the merozoite and host cell. The junction appears to be a circumferential attachment at the orifice of the parasitophorous vacuole as shown in Fig. 12. As the junction moves over the merozoite, the merozoite is brought within the invaginated erythrocyte membrane. The junction fuses at the posterior end of the merozoite, and the merozoite is then inside the erythrocyte within a vacuole originating from the inverted erythrocyte membrane.

Are these events that occur during invasion related to endocytotic processes by which phagocytic cells ingest particles, other cells, and microorganisms? Griffin et al. (8) proposed alternative

hypotheses for endocytosis of particles, namely, specific attachment triggering endocytosis and zippering. Triggering requires specific receptors for attachment, but the ingestion is independent of receptors outside of the attachment zone. Zippering is attachment to receptors around the circumference of the particles. Zippering may require a metabolically active cell, such as macrophage phagocytosis of cells via Fc or C3b receptors (8, 9), or may be a passive process, such as envelopment of Sendai virus by ganglioside-containing liposomes (10). Neither model, however, appears to explain the observation during invasion by malarial parasites.

Therefore, we would propose the following alternative models for invasion of erythrocytes by malarial merozoites, although we have no idea whether the parasite, the erythrocyte, or both supply the energy for the event. (a) Movement of the junction at the level of the membrane. This

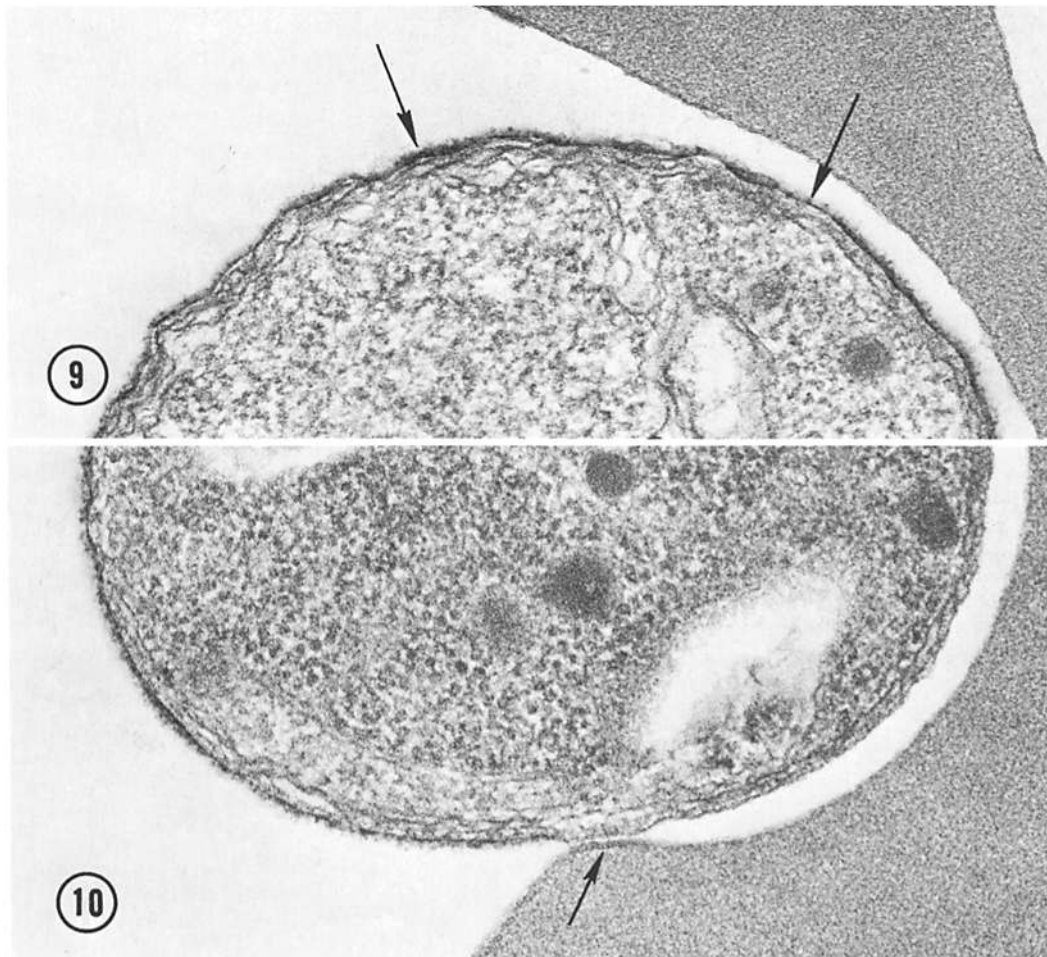


FIGURE 9 A free merozoite showing a surface coat (arrow) covering the entire surface. $\times 68,000$. (This surface coat was photographically intensified by a double-exposure technique.)

FIGURE 10 A merozoite entering an erythrocyte. No surface coat is visible on the portion of the merozoite surface which has invaginated the erythrocyte membrane, whereas the prominent surface coat is visible behind the attachment site (arrow). No accumulation of the surface coat is seen at the site of the junction. $\times 68,000$. (This surface coat was photographically intensified by a double-exposure technique.)

movement may be related to the lateral displacement of the junction by the agency of membrane flow, which is now a well-established phenomenon (21). However, it should be pointed out that this would necessitate simultaneous parallel flow of membrane components in both cells. (b) Attachment-detachment (modified zipper) model. The junction itself may be capable of migrating on the surface of a relatively stable plasma membrane. This would require that the leading edge of the moving junction becomes attached and the following edge becomes detached, perhaps by enzymatic cleavage.

During invasion, alterations occur in the merozoite within the vacuole. The merozoite coat that is evenly distributed over the merozoite surface before invasion (16) is now missing (4, 15, and present study). Although Bannister et al. (4) suggested that the surface coat accumulates at the orifice of the entry site of the erythrocyte by the merozoite, there is no evidence to suggest such an occurrence in our present study. The surface coat beyond the junction appears to be unaltered in density. Therefore, simple capping does not seem to fit our observations.

In the early 1960's, investigators reported on

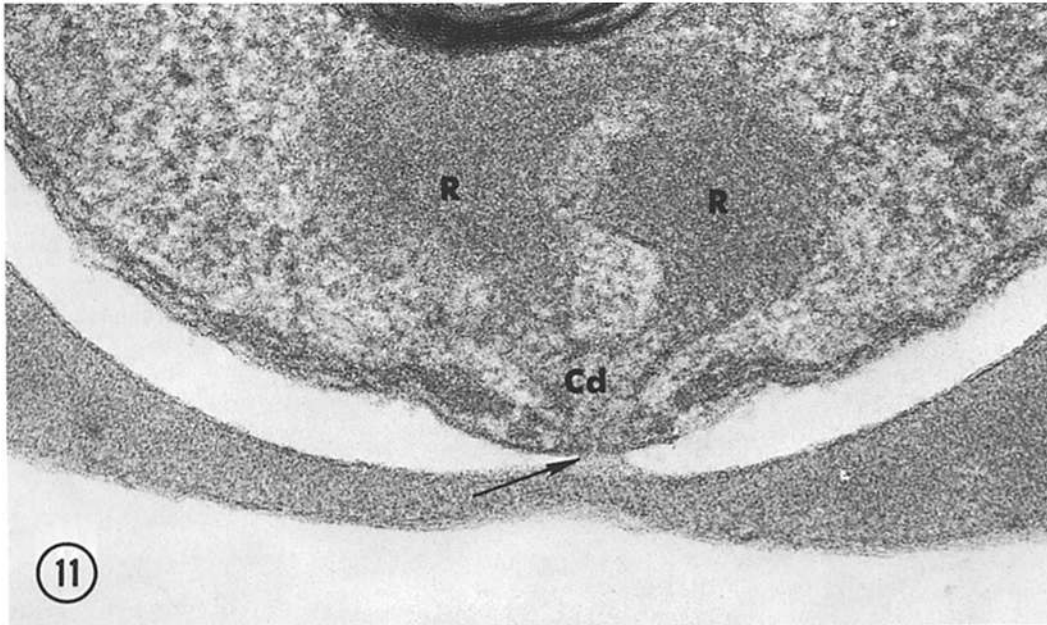


FIGURE 11 Higher magnification electron micrograph showing two rhoptries (*R*) at the apical end. The common duct (*Cd*) is formed by the meeting of ductules which lead from each rhoptry. An electron-opaque projection connects the apical end and the erythrocyte membrane (arrow). $\times 120,000$.

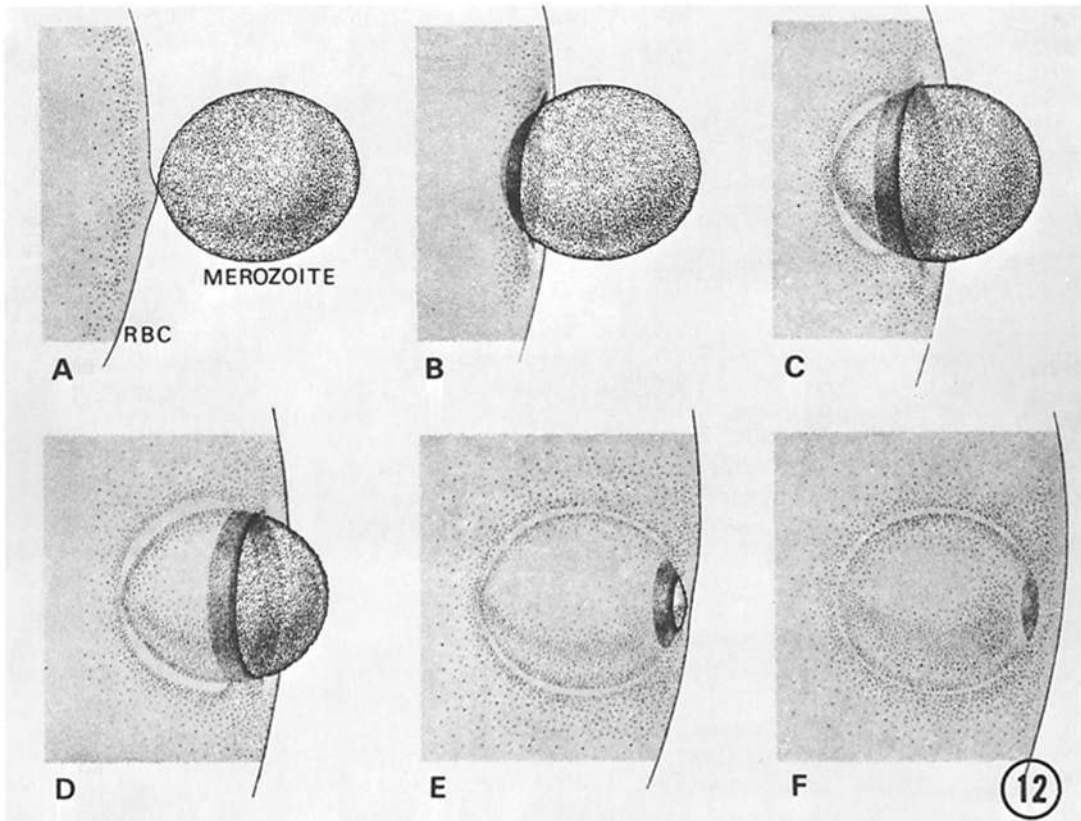


FIGURE 12 A diagram of a moving circumferential junction between merozoite and erythrocyte. The moving junction brings the merozoite within an invagination of the erythrocyte membrane.

electron microscope studies indicating that the motile forms of these intracellular protozoa possess anterior organelles, rhoptries, and micronemes, which were thought to be associated with host cell entry (1). In *Plasmodium*, *Babesia* (18), *Eimeria* (11), and *Besnoitia* (19), a ductule runs from the rhoptries to the apical end which is the point of initial contact between the protozoa and host cell. During invasion, the lower electron density in the duct suggests a release of rhoptry contents. Kilejian (14) suggested that the rhoptries and micronemes of an avian malarial parasite, *P. lophurae*, contain a histidine-rich protein which invaginates the erythrocyte membrane. Our observation on the connection between the rhoptries and the erythrocyte membrane presented here supports the supposition that the rhoptries play a role in merozoite entry into the erythrocyte. However, the identification of the function of rhoptries and micronemes must await the isolation of the contents of these organelles for the analysis of their chemical and physical properties.

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