Freeze-Fracture Study on the Erythrocyte Membrane during Malarial Parasite Invasion

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ABSTRACT Invasion of erythrocytes by malarial merozoites requires the formation of a junction between the merozoite and the erythrocyte. Migration of the junction parallel to the long axis of the merozoite occurs during the entry of the merozoite into an invagination of the erythrocyte. Freeze-fracture shows a narrow circumferential band of rhomboidally arrayed particles on the P face of the erythrocyte membrane at the neck of the erythrocyte invagination and matching rhomboidally arrayed pits on the E face. The band corresponds to the junction between the erythrocyte and merozoite membranes observed in thin sections and may represent the anchorage sites of the contractile proteins within the erythrocyte. Intramembrane particles (IMP) on the P face of the erythrocyte membrane disappear beyond this junction. When the erythrocytes and cytochalasin B-treated merozoites are incubated together, the merozoite attaches to the erythrocyte membrane and a junction is formed between the two, but the invasion process does not advance further and no movement of the junction occurs. Although there is no entry of the parasite, the erythrocyte membrane still invaginates. Freeze-fracture shows that the P face of the invaginated erythrocyte membrane is almost devoid of the IMP that are found elsewhere on the membrane, suggesting that the attachment process in and of itself is sufficient to create a relatively IMP-free bilayer.

Invasion of the erythrocytes by malarial merozoites requires the formation of a junction between the merozoite and the erythrocyte (1). Migration of the junction parallel to the long axis of the merozoite occurs during the entry of the merozoite into an invagination of the erythrocyte. As observed by thin-section electron microscopy, this junction is a region of close apposition between the merozoite and the erythrocyte, where the inner leaflet of the erythrocyte membrane appears thickened (1). The invaginated erythrocyte membrane beyond the junction has been shown by freeze-fracture to be devoid of intramembrane particles (IMP) (11). Cytochalasin-treated merozoites that can attach to but not enter into the erythrocyte also form a junction between the apical end of the merozoite and the erythrocyte (12). Even though the cytochalasin-treated merozoite never enters the erythrocyte, membrane invaginations occur within the erythrocyte in the region of the attached parasite. To further characterize the junctions and the erythrocyte membrane invaginations, we have analyzed them by freeze-fracture during normal invasion and after attachment of cytochalasin-treated merozoites to the erythrocytes.

MATERIALS AND METHODS

Viable, invasive merozoites of a Malaysian strain of *Plasmodium knowlesi* were isolated according to the method of Dennis et al. (2) as modified by Johnson et al. (5). The merozoites were released into a protein-free medium containing medium RPMI 1640/15 mM HEPES/34.5 mM HCO_3 gassed with 5% CO_2 in air. 1 ml of the merozoite suspension contained ~5×10^7 merozoites. For invasion studies, 200 μl of heat-inactivated fetal calf serum and 100 μl of rhesus monkey erythrocytes (5×10^8/ml) in medium 199/10% fetal calf serum were added to 2 ml of the merozoite suspension. The cell suspension was mixed in a 37°C water bath for 2 min and then added to 13 ml of glutaraldehyde fixative (2% glutaraldehyde/116 mM sucrose/50 mM phosphate buffer, pH 7.4). For attachment studies, cytochalasin B in dimethyl sulfoxide (DMSO) was added to 1 ml of merozoite suspension so that the final concentration was 10 μg/ml of cytochalasin B in 0.1% DMSO. After a 1-min incubation at room temperature, 100 μl of rhesus erythrocytes in medium 199/10% fetal calf serum (10^8/ml) were added, and the suspension was mixed for 4 min at 37°C. The suspension was centrifuged for 2 min at 1,000 *g*, the supernate was removed, and the pellet was resuspended in the final drop of medium. The cells were then fixed in 2 ml of glutaraldehyde fixative, dehydrated in an ascending alcohol series, and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and examined with JEOL 100CX electron microscope.

For freeze-fracturing, the samples were fixed in glutaraldehyde fixative for 1 h, washed several times in 50 mM phosphate buffer, and soaked for 1 h in the

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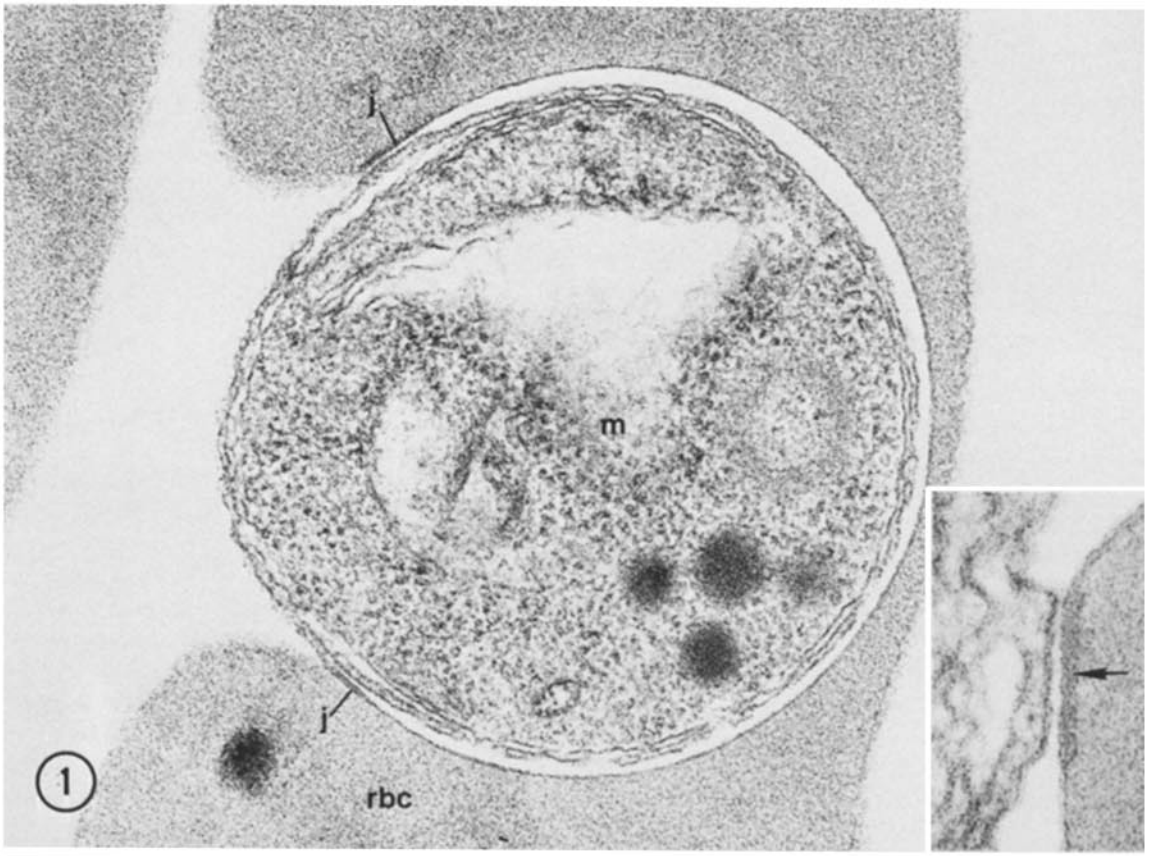
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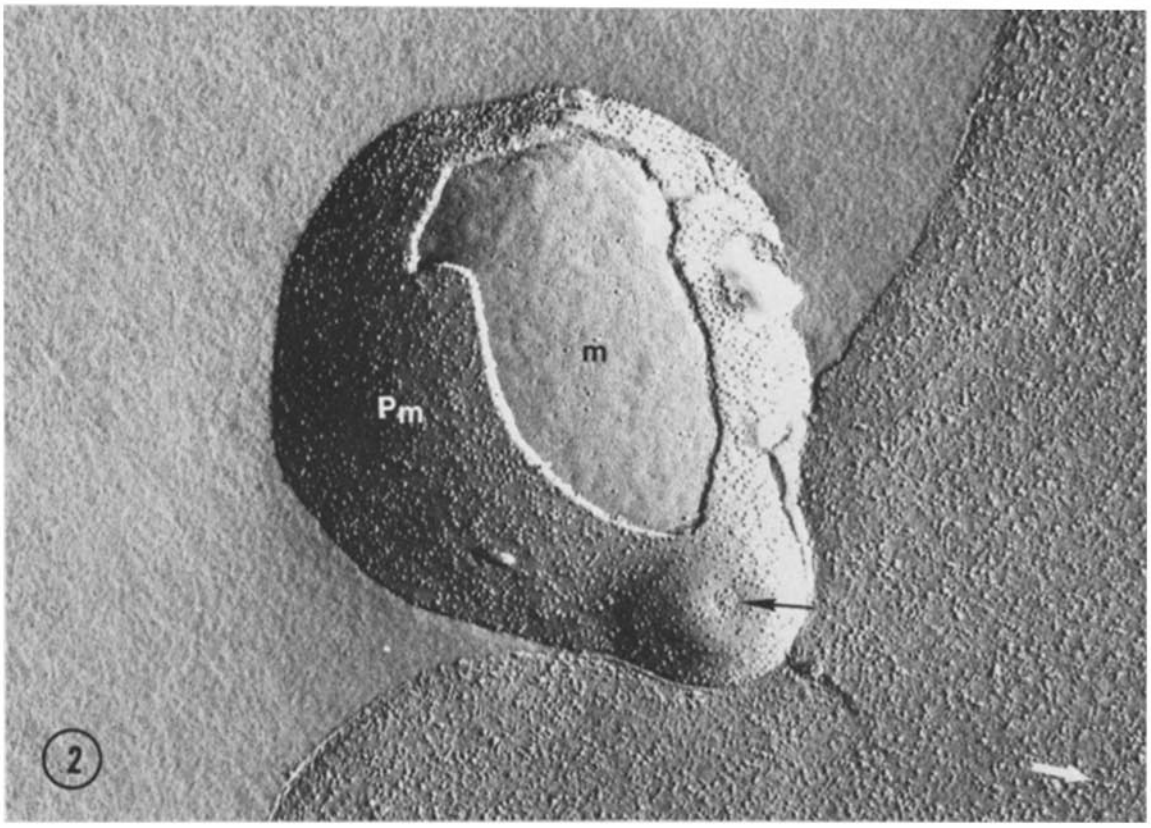
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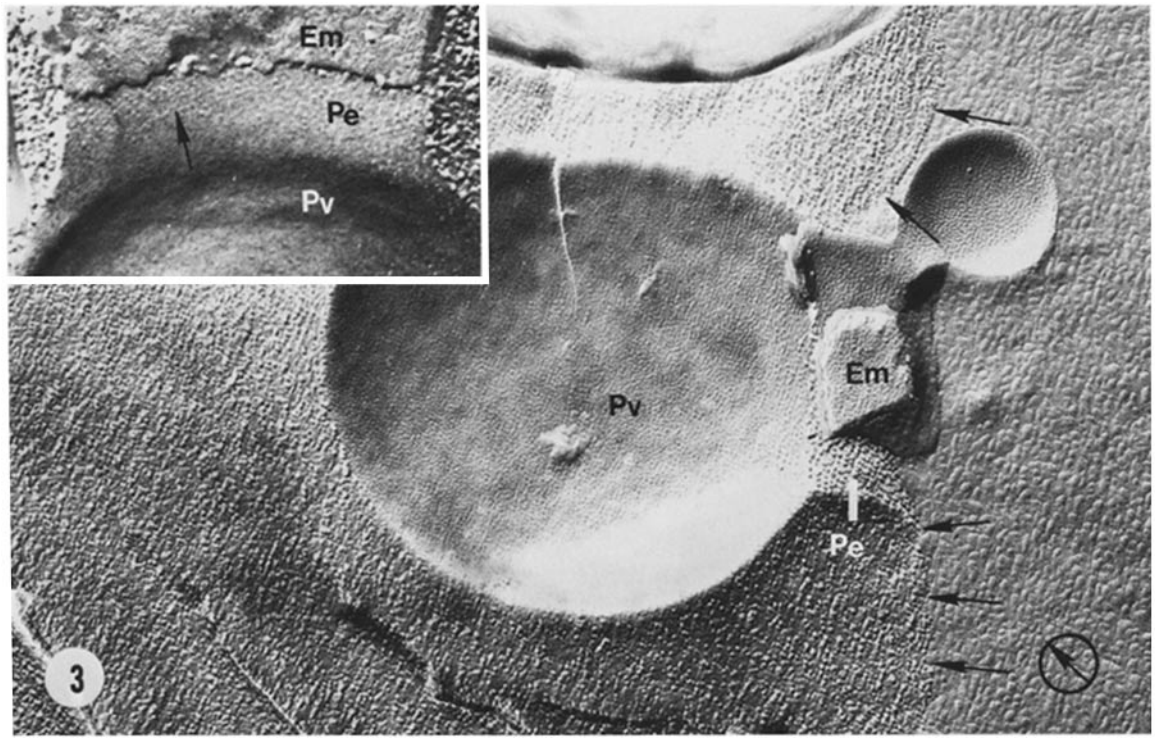

FIGURE 3 Freeze-fracture electron micrograph showing the P face (*Pv*) of a Florence flask-shaped parasitophorous vacuole that is created by the invagination of the erythrocyte membrane during a merozoite invasion. The P face (*Pe*) of the erythrocyte membrane at the neck of the invagination is covered with IMP but they disappear beyond the point where the neck of the invagination abruptly expands. Arrows outline the fracture edge of the erythrocyte membrane. × 52,000. *Inset:* high-magnification electron micrograph of the P face (*Pe*) of the erythrocyte membrane at the neck of the invagination. A band of rhomboidally arrayed particles (arrow) is seen at a point just before the neck of the erythrocyte membrane invagination abruptly expands into the parasitophorous vacuole. × 85,000.

same buffer containing 20% (vol/vol) glycerol as the cryoprotective agent. Freeze-fracturing was performed in a Balzers (Hudson, N. H.) freeze-fracture etching unit. Fracturing was carried out at −120°C under a vacuum of 2×10^{-7} torr. The surface obtained was replicated with platinum and carbon. Replicas were cleaned in 2% sodium hypochlorite to remove adherent organic material and then rinsed in distilled water. Particle counts were made by counting the number of IMP that fell in a 0.62 μm^2 scribed square placed over each membrane face (11). At least 20 such counts were made to give values of density/square micron. Differences between particle counts were evaluated by Student's *t* test.

RESULTS

Studies on Erythrocytes during Invasion by Merozoites

Because thin-section transmission electron microscopy on the invasion of the erythrocyte of *P. knowlesi* merozoites has been reported in detail (1), only a brief description of this process is presented here. Initially, the apical end of the merozoite makes contact with the erythrocyte and creates a depression in the erythrocyte membrane (Fig. 1). The zone of the erythrocyte membrane to which the merozoite is attached becomes thickened (to ~15 nm), probably by the accumulation of electron-dense material along its inner face, and forms a junction (intercellular space measures ~10 nm) with the plasma membrane of the merozoite (Fig. 1). Fine fibrils appear to span the intrajunctional space. As the merozoite enters the invagination of the erythrocyte surface, the junction moves along the confronted membranes to maintain its position at the neck of the invagination (Fig. 1). When entry is completed, the neck closes behind the merozoite in the fashion of an iris diaphragm and the junction becomes a part of the parasitophorous vacuole. The merozoite is now situated within a parasitophorous vacuole, the membrane of which originated from the erythrocyte plasmalemma.

FIGURE 1 In any of Figs. 1-8 in which it occurs, an arrow at the lower right-hand corner indicates the direction of shadowing. Abbreviations used in the figures are: *Pe*, P face of the erythrocyte membrane; *Pv*, P face of the vacuole membrane; *Pm*, P face of the merozoite membrane; *Ee*, E face of the erythrocyte membrane; *Ev*, E face of the vacuole membrane; *Em*, E face of the merozoite membrane.

Fig. 1 is a thin-section electron micrograph of erythrocyte (*rbc*) entry by a merozoite (*m*). The merozoite is located within an invagination of the erythrocyte membrane. A junction (*j*) formed between the merozoite and the erythrocyte membranes is always located at the orifice of the invagination. × 54,000. *Inset:* high magnification of the junction shows thickening of the erythrocyte membrane where the merozoite is attached (arrow) and fine fibrils that appear to span the intrajunctional space. × 160,000.

FIGURE 2 Freeze-fracture electron micrograph showing that the merozoite (*m*) is creating a slight invagination of the erythrocyte membrane during the initial stage of invasion. A small indentation (arrow) is present at the apical end. *Pm* is the P face of the merozoite plasma membrane. × 63,000.

Freeze-fracture shows that the merozoite enters an invagination of the erythrocyte membrane (Fig. 2). As the merozoite invasion progresses, the invagination of the erythrocyte membrane is shaped like a Florence flask (Fig. 3). The P face of the erythrocyte at the neck of the invagination is covered with IMP (Fig. 3). At the point just before the neck of the invagination abruptly expands into the parasitophorous vacuole, a narrow band (120 nm in width) of rhomboidally arrayed particles is seen on the P face of the erythrocyte membrane (Fig. 3, *inset*) and matching rhomboidally arrayed pits (Figs. 4 and 5) are seen on the E face. Arrayed pits on the E face correspond in pattern to the arrayed particles on the P face. This circumferential band disappears at the expansion point of the parasitophorous vacuole. This band corresponds to the junction region between the erythrocyte and merozoite membranes observed by thin-section electron microscopy. Arrayed particles and pits were not observed on the fracture face of the erythrocyte membrane away from the point of invasion or within the

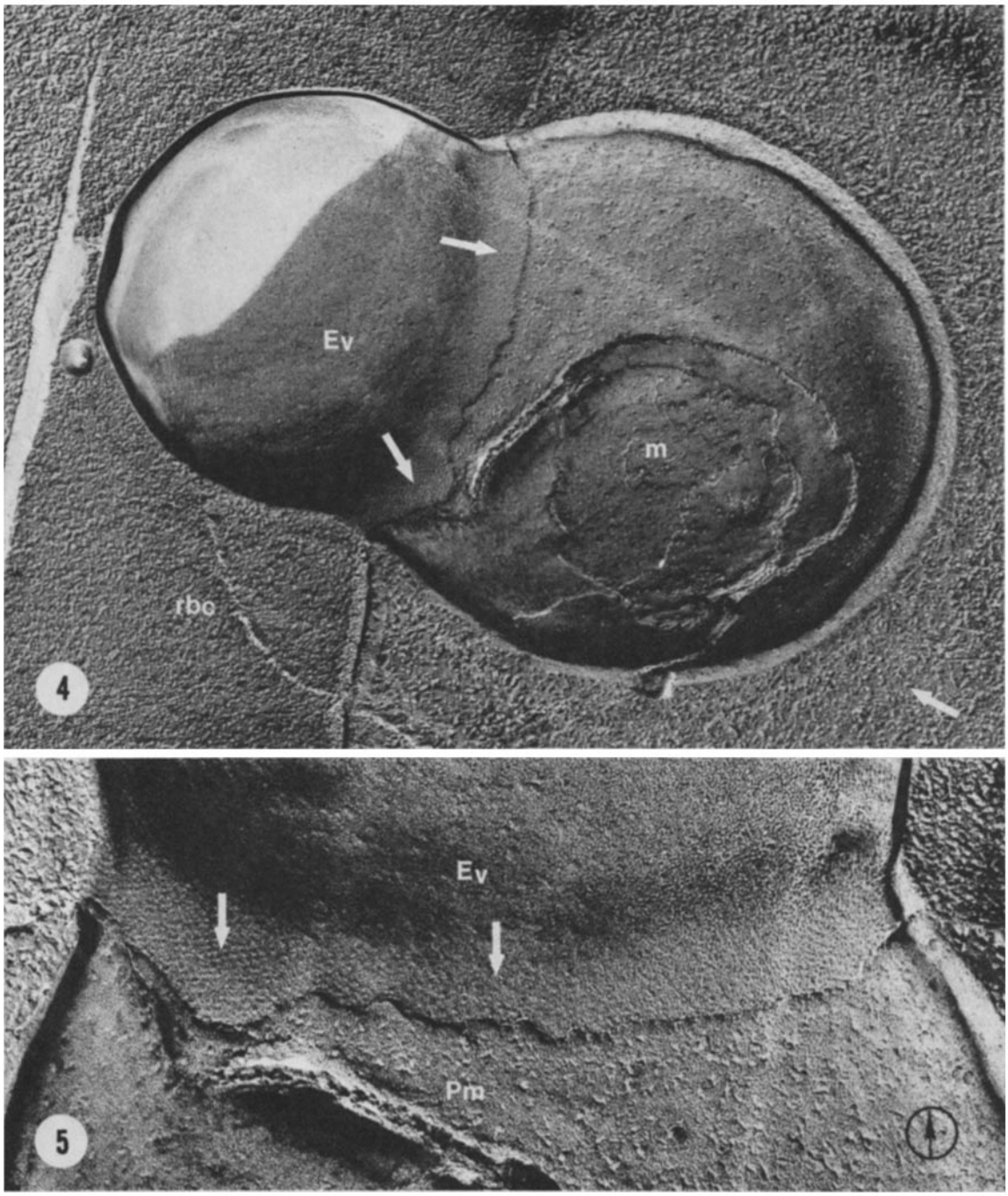

FIGURE 4 Freeze-fracture electron micrograph of erythrocyte (*rbc*) entry by a merozoite (*m*). The E face of the erythrocyte membrane at the neck of the invagination consists of a narrow circumferential band of rhomboidally arrayed pits (arrow). *Ev* is the E face of the vacuole membrane. × 56,000.

FIGURE 5 High-power electron micrograph showing a band of rhomboidally arrayed pits (arrow) at the neck of the invagination. The E face (*Ev*) above this band shows few IMP. *Pm* is the P face of the merozoite membrane. × 104,000.

parasitophorous vacuole (Fig. 5). The number of IMP on the E and P faces of the erythrocyte membrane outside the invagination and on the E and P faces of the vacuole membrane created by the invaginated erythrocyte membrane is shown in Table I. A significant difference was noted in the number of IMP present on the P face of the erythrocyte membrane and on the P face of the vacuole membrane. *P* value calculated by the Student's *t* test was <0.01. On the other hand, no obvious difference in the number of IMP was noted between the E face of the erythrocyte membrane and the E face of the vacuole membrane.

A small dimple measuring ~50 nm in diameter was noted on the P face of the parasite plasma membrane at the tip of the apical end (Figs. 2 and 8). This may correspond to the opening of the rhoptry duct present at the apical end of the merozoite.

Studies on Erythrocyte Attachment by Cytochalasin-treated Merozoites

When rhesus erythrocytes and cytochalsin B–treated merozoites are incubated together, the apical end of the merozoite attaches to the erythrocyte membrane in a manner identical to that of the untreated merozoites. The erythrocyte membrane to which the cytochalasin B–treated merozoite is attached becomes thickened, forms a junction with the plasma membrane of the merozoite, and invaginates slightly to cover the apical end (Fig. 6). However, the invasion process does not advance further and no movement of the junction occurs. Several membrane-bounded vacuoles with an electron-translucent matrix appear in the erythrocyte cytoplasm near the attachment site (Fig. 6).

When the cytochalasin B–treated merozoites contact the erythrocyte membrane, changes on the erythrocyte membrane similar to those seen during normal invasion with untreated merozoites are shown by freeze-fracture (Figs. 7 and 8). At the end of the invagination neck, IMP on the P face of the erythrocyte membrane abruptly disappear so that the P face of the parasitophorous vacuole membrane possesses few IMP (Fig. 7).

Although the cytochalasin B–treated merozoites only attach to the erythrocyte membrane, the invagination of the erythrocyte extends far beyond the apical end of the merozoite (Figs. 7 and 8); this was not evident when thin sections were studied. In some instances, several secondary vacuoles are seen budding from the initial vacuole into the erythrocyte cytoplasm. The P

TABLE I

Mean IMP Densities/μm^2 on the Erythrocyte and Vacuole Membranes

	P Face	E Face
	± *SEM*	
Erythrocyte Membrane	2,109 ± 431	389 ± 100
Vacuole Membrane	564 ± 148	334 ± 65

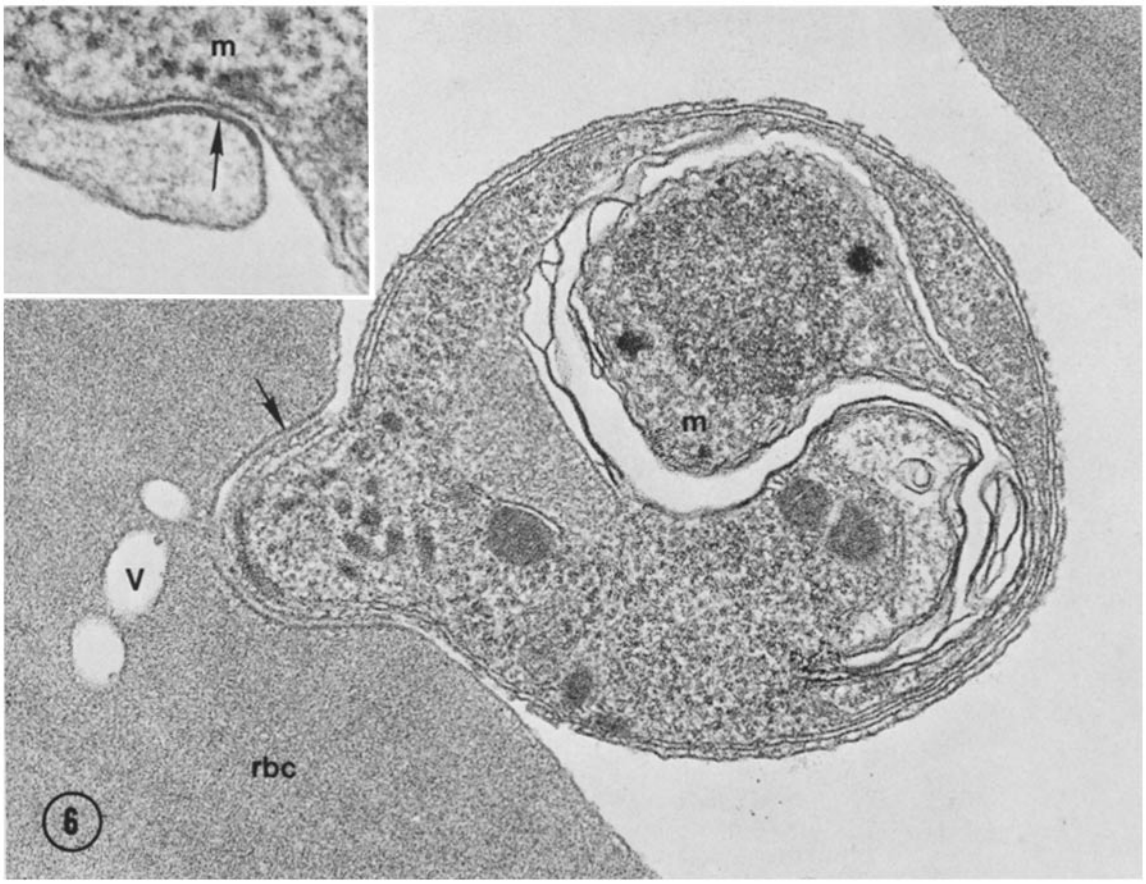

FIGURE 6 Thin-section electron micrograph showing attachment of a cytochalasin B–treated merozoite (*m*) to an erythrocyte (*rbc*). The erythrocyte membrane becomes thickened (arrow) and forms a junction with the plasma membrane of the merozoite at the point of attachment. Several vacuoles (*V*) appear in the erythrocyte cytoplasm near the attachment site. × 48,000. *Inset:* high-magnification electron micrograph showing the thickened erythrocyte membrane (arrow) at the point of merozoite (*m*) attachment. × 80,000.

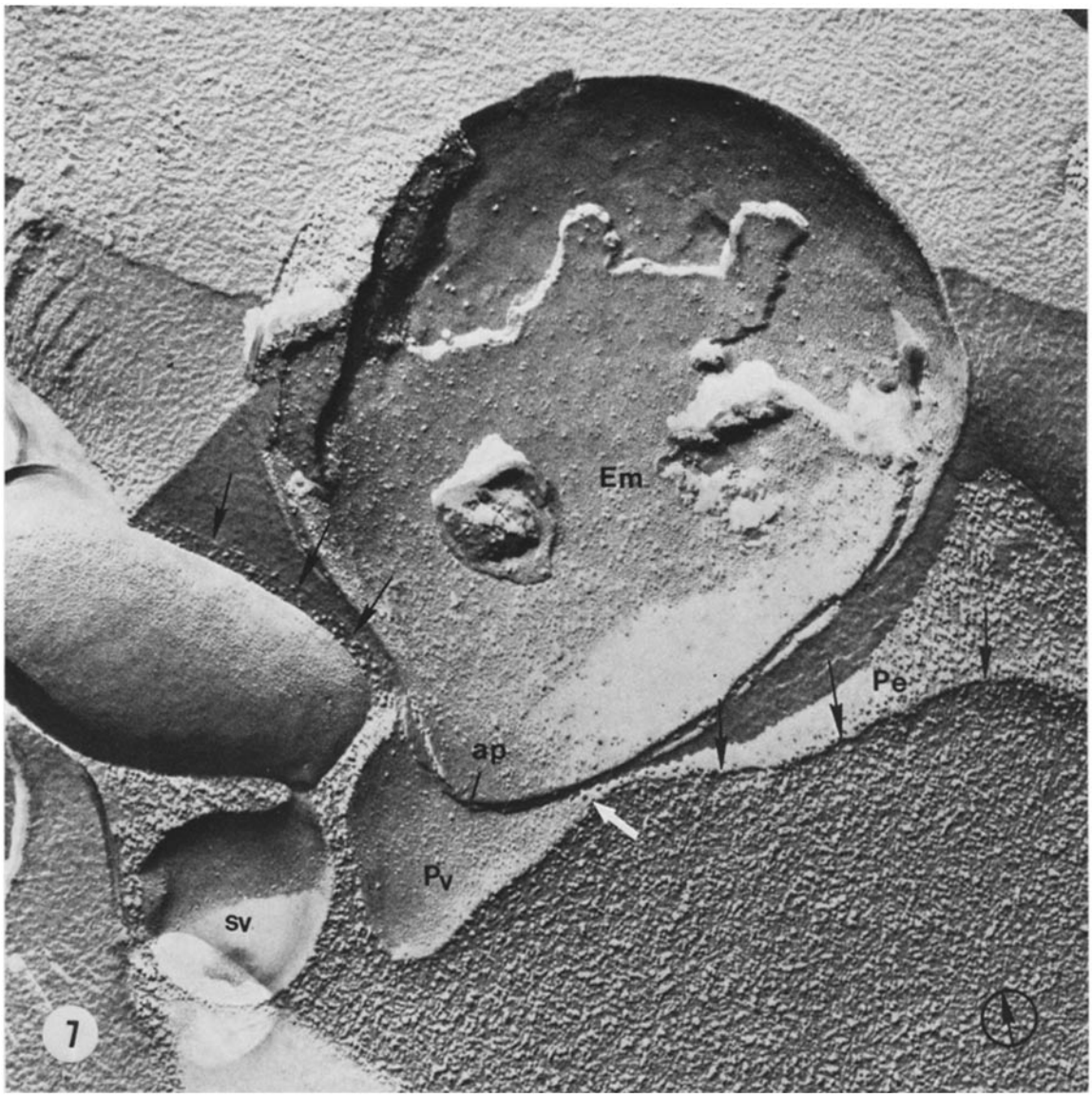

FIGURE 7 Freeze-fracture electron micrograph showing attachment of a cytochalasin B-treated merozoite to the erythrocyte membrane. The invagination of the erythrocyte membrane extends beyond the apical end (*ap*) of the cytochalasin B-treated merozoite. IMP on the P face (*Pe*) of the erythrocyte membrane at the end of the invagination neck (white arrow) abruptly disappear and the P face (*Pv*) of the vacuole membrane possesses few IMP. Also present are secondary vacuoles (*sv*) near the erythrocyte invagination. Arrows outline the fracture edge of the erythrocyte membrane. × 63,000.

face of the membranes in these secondary vacuoles also possess few IMP; the E face of these vacuoles is similar to that of the erythrocyte membrane.

DISCUSSION

It is apparent that the translocation of the junction between the merozoites and the erythrocytes is an important component of the mechanism by which the merozoites enter the erythrocyte. Aikawa et al. (1) hypothesized that the movement of this junction is related to lateral displacement of the junction by membrane flow. However, the precise nature of this junction was difficult to analyze by thin-section transmission electron microscopy. Although the freeze-fracture technique had been used to study erythrocyte invasion by merozoites, the junction remained unrecognized (11). Using freeze-fracture, our study demonstrates that a band of rhomboidally arrayed particles on the P face and pits on the E face correspond to the junction observed in thin sections. The presence of rhomboidally arrayed particles and pits at the junction site indicates that IMP on the erythrocyte membrane rearrange themselves at the site of *Plasmodium* entry for local membrane specialization (15).

Studies on membrane-membrane interactions such as membrane fusions between cells (10), vesicle-vesicle fusion (13), exocytosis (8), and myoblast fusion (6) have demonstrated that IMP are displaced laterally into adjacent membrane regions before the fusion process and that fusion occurs between protein-depleted lipid bilayers. In contrast to this lateral displacement of IMP, our study showed reorganized IMP at the site of the erythrocyte-*Plasmodium* interaction. Apparently this difference arises because the interaction between the erythrocyte and parasite membranes at the junction site is a transient phenomenon and is not a fusion process.

About 30% of the protein of the erythrocyte membrane consists of spectrin and actin, which form a latticelike contrac-

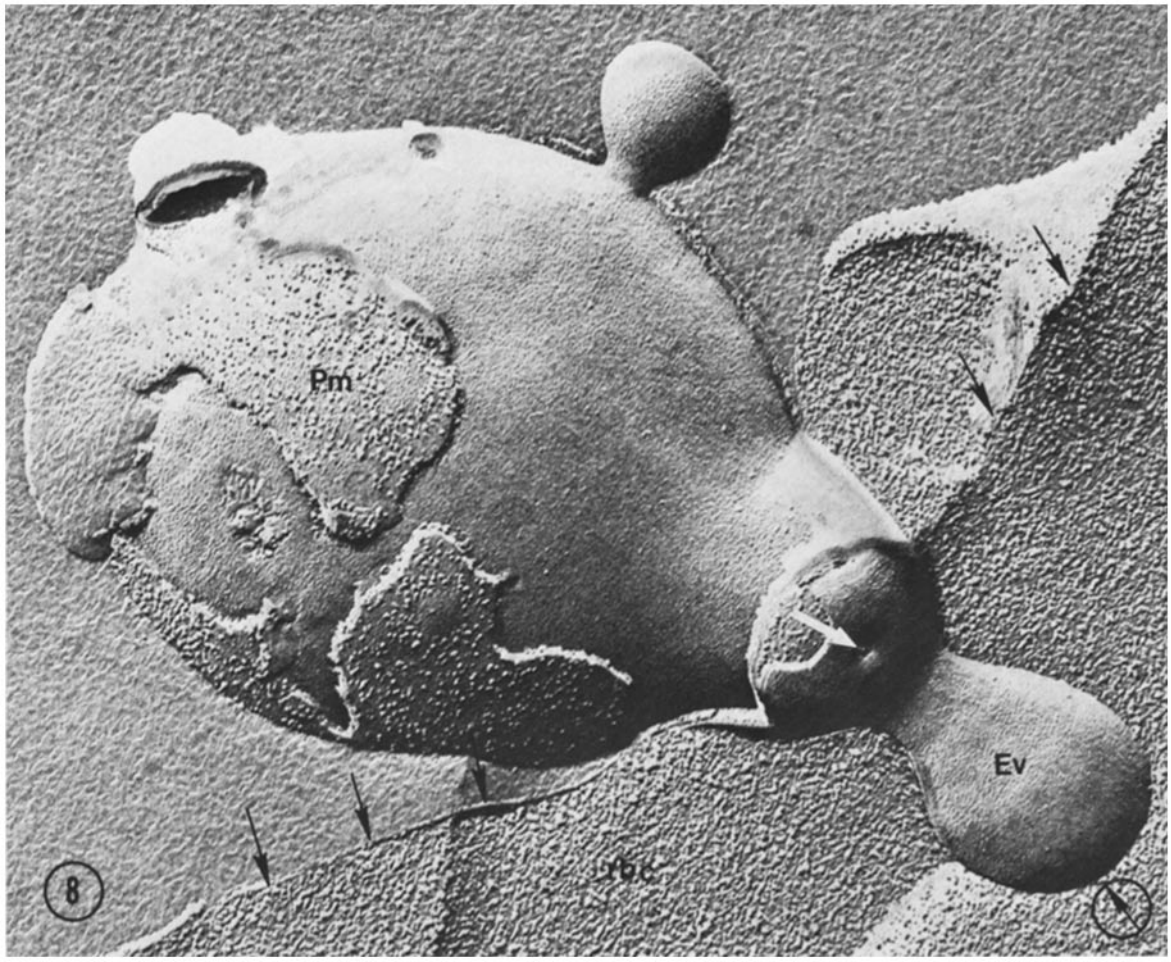

FIGURE 8 Freeze-fracture electron micrograph showing an attachment between the cytochalasin B-treated merozoite and erythrocyte (*rbc*). Although the merozoite only attaches to the erythrocyte membrane, the invagination extends far beyond the apical end of the merozoite. A small dimple (white arrow) is present at the apical end of the merozoite. *Pm*, the P face of the merozoite plasma membrane; *Ev*, the E face of the vacuole membrane. Arrows outline the fracture edge of the erythrocyte. × 64,000.

tile system located on the inner aspect of the erythrocyte membrane (4, 14, 16). The arrangement of the contractile protein into a network is thought to facilitate changes in the shape of the erythrocyte. Therefore, it is possible that the presence of the dense zone at the site of the junction may represent aggregates of contractile proteins. Furthermore, rhomboidally arrayed pits on the E face seen by freeze-fracture may be the points where the contractile proteins anchor to the transmembrane proteins in the erythrocyte membrane (7).

Both during normal invasion and after the attachment of cytochalasin-treated merozoites, the P face of the vacuolar membrane is almost devoid of IMP. Because cytochalasin-treated merozoites remain outside the erythrocyte, the formation of a vacuole membrane does not require entry by the parasite. Our freeze-fracture observations show that the secondary vacuoles form an irregular chain that is in direct continuity with the original invagination of the erythrocyte membrane. Because this chain follows a tortuous path, thin sections give the spurious impression of independent small vacuoles subjacent to the site of the attachment. The vacuoles that are contiguous with the invagination also possess few IMP. The contents of rhoptries, which are located in the apical region of the parasite, have been suggested to flow and diffuse into the erythrocyte membrane (1, 12) and may be involved in the formation of the relatively protein-free vacuole membrane. Possible mechanisms might include cross-linking of spectrin (3), phospholipase activity (9), or actual creation of phospholipid bilayers by the rhoptries (11).

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