INTERACTION BETWEEN CYTOCHALASIN B-TREATED MALARIAL PARASITES AND ERYTHROCYTES

Attachment and Junction Formation

By LOUIS H. MILLER, MASAMICHI AIKAWA,* JAMES G. JOHNSON, AND TSUGIYE SHIROISHI

From the Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20014, and Institute of Pathology, Case Western Reserve University, Cleveland, Ohio 44106

Malaria parasites develop within erythrocytes (RBCs),[1] and at full maturation infected RBCs rupture, releasing individual merozoites that invade other RBCs. Invasion, the process by which the extracellular merozoite becomes established as an intracellular parasite occurs through a sequence of events that includes recognition and attachment (1), junction formation (2), invagination of the RBC membrane around the merozoite (2), and finally sealing of the invaginated membrane to form a vacuole and to reestablish continuity of the RBC membrane (2). The merozoite has specialized organelles, rhoptries, and micronemes at the apical end which is in apposition to the RBC during invasion (1–4). A junction forms between the apical region and the RBC membrane (2). As the junction moves over the merozoite, the merozoite is brought within the invaginated RBC membrane. Failure of viable merozoites to invade RBCs may be due to defects at any stage in the invasion process. With the exception of the Duffy-negative human RBCs, *Plasmodium knowlesi* merozoites do not attach to or deform RBCs that are refractory to invasion (subprimate and chymotrypsin-treated human RBCs). In case of Duffy-negative human RBCs, the only human RBC refractory to invasion by this parasite (5, 6), the merozoite on contact induces deformation of the Duffy negative RBC, but instead of entering within an invagination of the RBC, the merozoite detaches and then interacts with other RBCs. Identification of the defect in invasion of Duffy-negative RBCs could not be further explored by previously available techniques because of the rapidity of events.

To show the structural and functional differences between the normal invasion sequence and the defective interaction between Duffy-negative human RBCs and *P. knowlesi* merozoites, we developed a method for the isolation of the attachment phase of invasion. This method uses cytochalasin-treated *P. knowlesi* merozoites. Cytochalasin-treated merozoites attach only to RBCs from susceptible hosts (rhesus monkeys and man) and form a junction between the apical end of the merozoite and the RBC. These treated merozoites will also attach specifically to Duffy blood group negative human RBCs, although these human RBCs are refractory to invasion (5). However, the mechanism of attachment differs in that there is no junction formation with

* Supported in part by grant AI-10645 from The U.S. Public Health Service and by the U.S. Army R & D Command (DADA-17-70-C0006) (Contribution no. 1517).

[1] *Abbreviations used in this paper:* DMSO, dimethyl sulfoxide, RBCs, erythrocytes.

Duffy-negative RBCs, suggesting that the defect in invasion of Duffy-negative RBCs is at the step of junction formation.

Materials and Methods

Preparation of P. knowlesi Merozoites. Rhesus monkeys (*Macaca mulatta*) were infected with a Malaysian strain of *P. knowlesi*. When the parasitemia was between 10 and 35% and the majority of schizonts within RBCs contained 10 or more nuclei, 10–20 ml of blood was drawn into a heparinized syringe. All subsequent steps in preparation were carried out at room temperature (23–25°C). After 1 mg/ml of adenosine diphosphate was slowly added to the blood, the blood was mixed for 2 min and squeezed over glass beads (0.11 mm diameter) that were packed in a 10-ml plastic syringe to remove platelets and some leukocytes. The blood was diluted in 40 ml of modified medium 199 (6) and centrifuged for 4 min at 850 *g*. The supernate was discarded and the packed RBCs were loaded into 2 × 100 mm glass tubes. The tubes were centrifuged for 5 min at 1,300 *g*. The low density schizont-infected RBCs formed a brown layer above the uninfected RBCs. The tube was scored and broken at the interface and the brown layer pooled. The yield was approximately 4×10^9 schizont-infected RBCs. The schizont-infected RBCs were added to a culture chamber designed for merozoite collection (7, J. G. Johnson et al., unpublished data). The parasites were allowed to develop in modified medium 199 at 37°C. As merozoites were released from schizont-infected RBCs, they were collected for attachment and invasion assays.

Merozoite Attachment Assay. 1 ml of culture chamber effluent containing $2\text{–}5 \times 10^7$ merozoites/ml was collected in a 4-ml glass vial at room temperature. 0.1 ml of a solution containing cytochalasin B in dimethyl sulfoxide (DMSO) and culture medium was added so that the final concentration of cytochalasin B was 10 μg/ml in 0.1% DMSO. After 3 min incubation, 0.1 ml of RBCs in culture medium (10^8 RBCs/ml) was added, warmed to 37°C for 2–4 min with mixing, and then centrifuged for 2 min at 1,000 *g*. The supernate was removed and the pellet was resuspended in the final drop of medium. The suspension was allowed to stand for 2 min and then fixed by the addition of 2 ml of buffered glutaraldehyde (2% glutaraldehyde, 0.05 M Na phosphate, pH 7.4, and 0.116 M sucrose). The cells were mixed with a Pasteur pipette and then allowed to settle overnight.

The number of RBCs with attached merozoites was then enumerated as follows. The cells were suspended in 1 ml of buffered glutaraldehyde with a Pasteur pipette. One drop of the cell suspension was placed on a slide and was covered by a 22 × 22 mm cover glass which was elevated at one end by another cover glass. The cells were viewed immediately with Smith differential interference optics (Leitz). Whenever a merozoite was seen in apposition to a RBC, the cover glass was tapped with an applicator stick so that the RBC and merozoite would move in the media. If they moved together, the merozoite was considered attached. In some studies we also determined by interference microscopy if the rhoptries (paired organelles of the merozoite) were in apposition to the RBC or oriented away from the RBC.

The studies on the effects of temperature on attachment and some assays for RBC susceptibility were performed without centrifugation of merozoites with RBCs. For the temperature experiments, merozoites and RBCs pre-equilibrated at 37°C, 23–25°C, and 4°C were mixed continuously at the respective temperature for 4 min before adding the 1 ml suspension to 9 ml of buffered glutaraldehyde. For assay of RBC susceptibility without centrifugation, the merozoite-RBC suspension was mixed at 37°C for 4 min and then fixed in glutaraldehyde.

Invasion Assay of Cytochalasin-Treated Merozoites. 1 ml of culture chamber effluent containing $2 \text{ to } 5 \times 10^7$ merozoites/ml was collected in a 4-ml glass vial at room temperature. To the vial was added cytochlasin B in DMSO so that the final concentration was 10, 1, or 0.1 μg in 0.1% DMSO: the mixture was incubated at room temperature for 3 min. As a control, 0.1% DMSO or nothing was added to vials of merozoites. After room temperature incubation, the vial was warmed briefly to 37°C and 0.1 ml uninfected rhesus RBCs in modified medium 199 (10^8 RBCs/ml) was added. The vial was capped and rocked end-to-end (Aliquot Mixer, Ames Co., Div. of Miles Lab., Inc., Elkhart, Ind.) at 37°C for 30 min, and then placed on ice. The suspension was centrifuged 15 s. at 1000 *g*, and a thin film was made from the pellet. The percent of RBCs with ring forms was determined on Giemsa-stained smears.

Attachment and Invasion of Enzyme-Treated RBCs. 1 mg/ml Chymotrypsin treated Duffy blood

TABLE I

The Effect of Cytochalasin (Cyto) B Treatment of Merozoites on Invasion of Rhesus Erythrocytes

Treatment	Percent invasion		
	Exp. 1	Exp. 2	Exp. 3
None	15.1	2.7	3.8
DMSO (0.1%)	7.5	1.4	1.8
Cyto B, 10 μg/ml	0.8	0	0
(1 μg/ml)	1.5	0	0.3
(0.1 μg/ml)	22.5	4.4	0.9

group-positive and -negative human RBCs were tested for susceptibility to invasion in Linbro wells (as described previously [6]) and to attachment. The attachment between trypsin (1 mg/ml)-treated Duffy-negative RBCs and cytochalasin-treated merozoites was studied by electron microscopy. Trypsin (Worthington Biochemical Corp., Freehold, N.J. TR(3BA)), soybean trypsin inhibitor (Worthington, SI 56H570), chymotrypsin (Worthington, CDS 55J 402X), and phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo., lot 64C-0335) were incubated with the RBCs as described previously (6).

Electron Microscopy. RBCs and attached cytochalasin B-treated merozoites were added to glutaraldehyde fixative (2% glutaraldehyde, 0.05 M phosphate pH 7.4, and 0.116 M sucrose). The samples were stained with uranyl acetate and lead nitrate and were examined with Siemens Elmiskop 101 electron microscope.

To visualize the filamentous attachment between merozoites and Duffy-negative RBCs photographic enhancement of the filaments was accomplished by masking the merozoite and RBC and by projecting only the filament on the photographic paper. The mask was removed and the whole image was reprojected onto the same sheet of paper. The net result was a 1× exposure for the merozoite and RBC and a 2× exposure for the filament.

In a few experiments, the merozoite-RBC preparation after fixation in glutaraldehyde and postfixation in 1% osmium tetroxide was suspended in 0.4 ml 10% albumin in an attempt to stabilize the attachment to Duffy-negative RBCs. 50 μl of 8% glutaraldehyde was added and the sample was centrifuged before the albumin polymerized. The pellet was dehydrated in alcohol and prepared for thin section electron microscopy as described previously (2).

Results

To study the complicated series of events leading to invasion, we developed a method for selectively blocking invasion at the attachment phase. Cytochalasin B-treated *P. knowlesi* merozoites attach to but do not invade rhesus RBCs (Table I, Fig. 1). Since it is known that cytochalasin B affects microfilaments (8), glucose transport (9), and possibly other functions and has a low and high affinity binding site on the RBC (10), we did not attempt to define the mechanism of action of cytochalasin in this system. Instead, we used it operationally to separate the early steps in the interaction between merozoites and RBCs. Because 10 μg/ml of cytochalasin B gave the most marked reduction in invasion, this concentration was used for all subsequent experiments.

Temperature Dependence of Attachment. Cytochalasin B-treated merozoites were mixed with rhesus RBCs at 4°C, 25°C, and 37°C for 4 min. The cells were fixed with glutaraldehyde and the number of attached merozoites were counted. The highest rate of attachment was at 37°C; few attached at 4°C (Table II). To increase the attachment rate, the mixture of merozoites and RBCs was centrifuged at 1,000 *g* for 2 min after a 4-min incubation at 37°C (Materials and Methods). Centrifuged

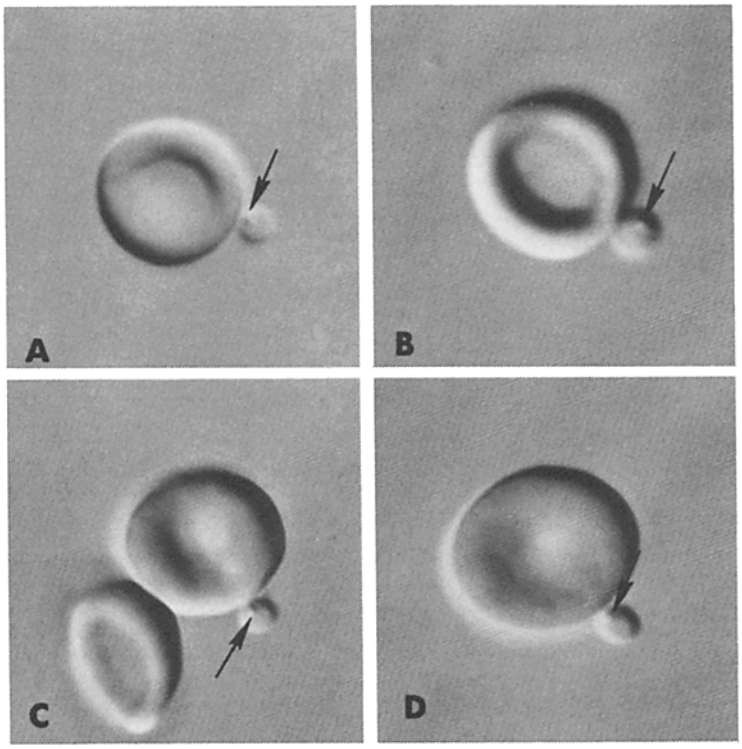


FIG. 1. Interference microscopy photograph showing the attachment between cytochalasin B-treated merozoites and RBCs. The apical end of the merozoite can be identified by the presence of the rhoptry (arrow). A) Attachment between the apical end of a merozoite and a rhesus RBC. B) Attachment between the side of a merozoite and a rhesus RBC. Note a rhoptry is present in the center of the merozoite. C) Attachment between a merozoite and a Duffy blood group-positive human RBC. D) Attachment between a merozoite and a Duffy-negative human RBC. × 2,500.

TABLE II

The Effect of Temperature on Attachment of Cytochalasin B-Treated Merozoites to Rhesus Erythrocytes. (Data are Presented as Percent of RBCs with Merozoites Attached.)

	Exp. 1	Exp. 2	Exp. 3
37°C	8.9	1.3	2.5
23–25°C	1.5	1.4	0.4
4°C	0.2	0.1	0.3

samples had as much as 10-fold increase in attachment to rhesus and human RBCs as the uncentrifuged samples.

Specificity of Attachment. The assay for attachment was specific in that merozoites (with or without centrifugation), did not attach to guinea pig or avian RBCs. These animals are resistant to infection by *P. knowlesi* and the merozoites do not interact with or invade the RBCs on direct observation by interference microscopy. In addition to specificity, the assay detects differences in attachment affinity. Attachment to rhesus RBCs was consistently greater than to human RBCs (Table III).

Attachment to Duffy Blood Group-Positive and -Negative Human RBCs. *P. knowlesi* mer-

TABLE III

The Effect of Erythrocyte Type on Attachment by Cytochalasin B-Treated Merozoites. (Data are Presented as Percent of RBCs with Merozoites Attached.)

	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
Rhesus RBCs	7.3	33	4.7	17.6	
Human RBCs*					
Duffy positive	1.3	8.6	0.8	14.1	1.5
	1.3	5.2	0.8	4.3	
Duffy negative	0.5	10.6	1.5	2.9	1.2
	0.7	8.2	1.5		

* Multiple samples run in a particular experiment are from different individuals.

ozoites invade all human RBCs except Duffy blood group-negative RBCs (5, 6). Without cytochalasin B in the merozoite-Duffy-positive RBC suspension, merozoites in glutaraldehyde-fixed preparations were in all stages of invasion. Without cytochalasin in the merozoite-Duffy-negative RBC suspension, few merozoites in the glutaraldehyde-fixed preparation of the suspension (with or without 0.1% DMSO) were attached to RBCs and none was invading. With cytochalasin, it was observed for the first time that the attachment rate of *P. knowlesi* merozoites to Duffy-positive and -negative RBCs was the same (Table III), despite the resistance of Duffy-negative RBCs to invasion. That the Duffy associated event in the invasion sequence was not related to the attachment was further tested by treatment of Duffy-positive RBCs (Fy^a), with anti-Fy^a, a treatment that markedly reduces invasion (5). The attachment rate to antibody-coated RBCs was the same as to untreated RBCs.

Chymotrypsin treatment of Duffy-positive or -negative RBCs eliminates all interaction with merozoites as observed by interference microscopy (11). Cytochalasin-treated merozoites also cannot attach to these chymotrypsin-treated cells. These cells are comparable to RBCs from refractory subprimates in that there is no interaction or attachment to them.

Observations on Attachment by Interference Microscopy. Rhoptries are specialized organelles at the apical end of the merozoite and appear as a single or double teardrop-shaped structure. During invasion, the apical end containing the rhoptries is oriented toward the RBC before RBC deformation begins (1). In the present study, cytochalasin-treated merozoites remained attached to RBCs and did not invade them. We determined the orientation of these merozoites relative to the RBC, i.e., the position of the rhoptries in merozoites that were attached to the convex edge of RBC (Fig. 1, Table IV). Those merozoites that were in the concave portions of the RBC (where the merozoite orientation to the RBC could not be determined) or those that had no visible rhoptries were excluded from the analysis. In the majority of cases, the end of the merozoite containing rhoptries (apical orientation) was in apposition to the RBC (Fig. 1, Table IV). The percentage of merozoites with apical orientation was similar for rhesus RBCs, human Duffy-positive RBCs and human Duffy-negative RBCs (Table IV), although, in any experiment, more merozoites attached to rhesus than human RBCs (Table III).

Electron Microscopy. When rhesus RBCs and cytochalasin B-treated merozoites were incubated together, the apical end of the merozoite attached to the rhesus RBC membrane (Figs. 2 and 3) in a manner identical to that of merozoites without cytochalasin B treatment (2). The RBC membrane to which the cytochalasin-treated

TABLE IV

The Orientation of Cytochalasin-Treated Merozoites Attached to RBCs

Type of RBC	Centrifugation*	Orientation of attachment‡			Number§ counted
		Apical	Not apical	Indeterminate	
		%			
Rhesus	No	52.1	4.2	43.7	71
	Yes	70.2	3.5	26.3	57
Human Duffy positive	No	49.2	13.6	37.3	59
	No	26.3	5.3	68.4	19
	Yes	72.9	4.7	22.4	85
	Yes	50.0	0	50.0	52
Human Duffy negative	No	44.8	6.0	49.3	67
	No	50.0	1.9	48.1	52
	Yes	46.2	0.9	52.8	106
	Yes	44.4	1.4	54.2	72

* Half of the RBC-merozoite mixture was centrifuged before fixation; the other half was fixed without centrifugation.

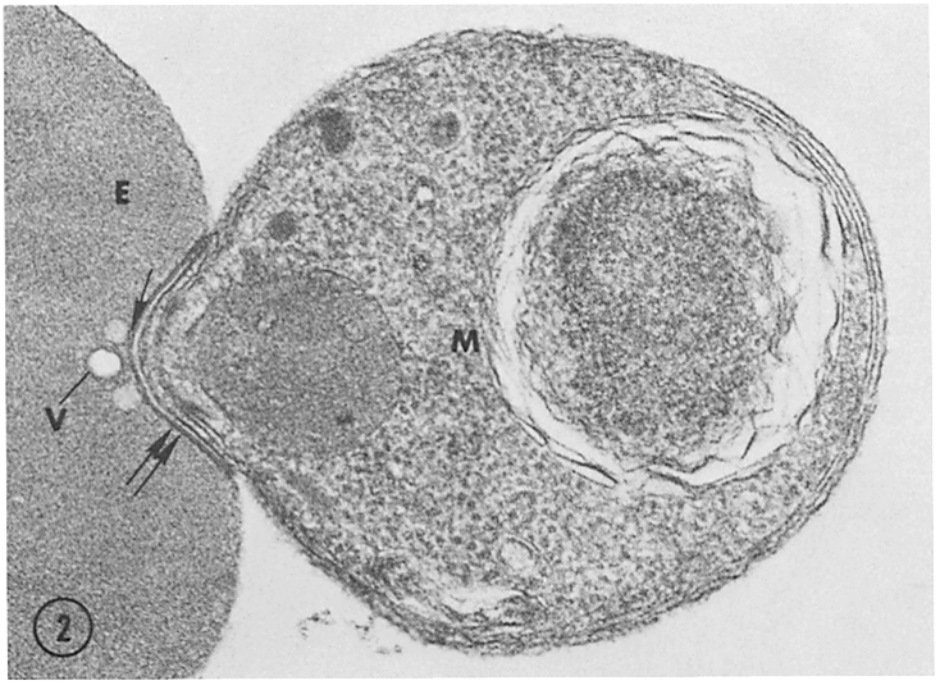
‡ The orientation is determined on merozoites attached to the convex surface of the RBC (apical, rhoptries next to the RBC; not apical, rhoptries in any other orientation). (Fig. 1). Indeterminate means that the merozoite is attached to the concave surface of the RBC or the rhoptries are not visualized.

§ The total number of attached merozoites that were counted.

merozoite was attached became thickened and formed a junction with the plasmalemma of the apical portion of the merozoite and invaginated slightly to cover the apical end. However, the invasion processes did not advance further and no movement of the junction between the RBC and merozoite occurred. In some sections, vacuoles surrounded by a unit membrane appeared in the RBC cytoplasm near the attachment site (Fig. 3). The vacuoles varied in size. Some resembled pinocytotic vesicles that appeared to be in contact with the RBC membrane; others were elongated and large measuring about 0.8 μm in length and 0.1 μm in width. The contents of these vacuoles were electron translucent, although a few contained fine granular material.

Attachment of the treated merozoites to human Duffy-positive RBCs was similar to that described for rhesus RBCs. The apical end of the merozoite attached to the RBC formed a junction with the thickened RBC membrane (Fig. 4), but no further steps in the invasion process took place. Also, vacuoles were observed in the RBC cytoplasm near the site of the merozoite attachment.

In contrast to what was observed with rhesus and Duffy-positive human RBCs, we observed attachment but no junction formation between Duffy-negative RBCs and cytochalasin-treated merozoites. This despite the fact that we studied four thin sections from each sample of eight experiments. The total number of RBCs and merozoites examined was about 12,000 and 6,000 respectively. The apical ends of many merozoites were orientated towards the RBCs, but instead of a junction, the RBC was about 120–160 nm away from the RBC, connected by thin filaments measuring 3–5 nm (Fig. 5). The filaments originated from the edge of the truncated, cone-shaped apical end, such that two distinct filaments could be seen in thin sections. This suggests that these filaments are arranged in a cylindrical fashion in three dimensions. Such filamentous attachments between cytochalasin-treated merozoites and RBCs were not observed in the experiments with normal rhesus or human Duffy-positive RBCs, but they were observed with schizont-infected rhesus RBCs.



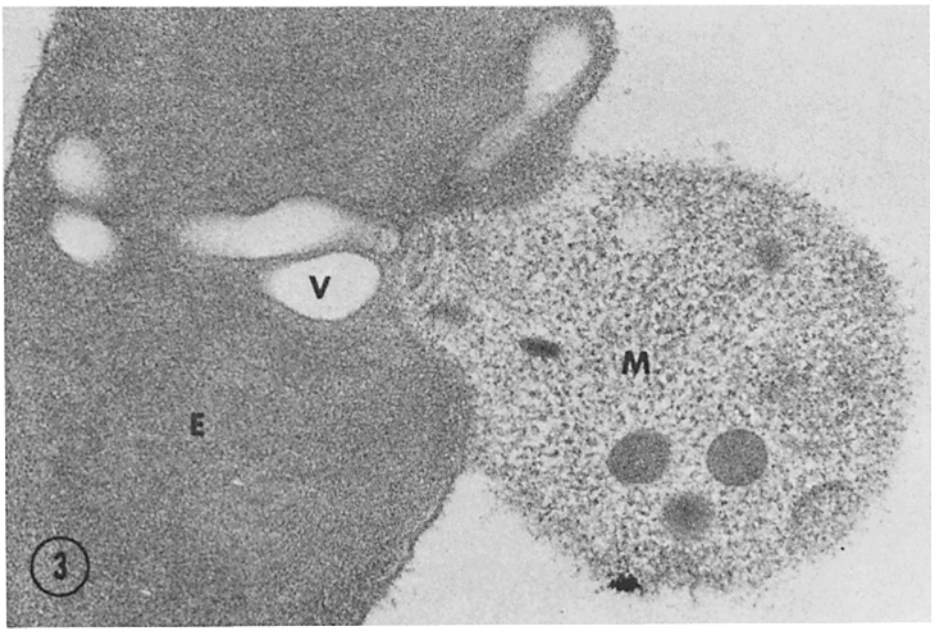


FIG. 2. Electron micrograph showing the attachment between the apical end of a cytochalasin B-treated merozoite (M) and rhesus erythrocyte (E). The erythrocyte membrane is thickened (double arrow) at the attachment site. A few vacuoles (V) are seen in the erythrocyte cytoplasm and some of them are in contact with the invaginated erythrocyte membrane (arrow). × 50,000.

FIG. 3. Electron micrograph showing the attachment between a cytochalasin B-treated merozoite (M) and a rhesus erythrocyte (E). Note several vacuoles (V) in the erythrocyte cytoplasm. × 57,000.

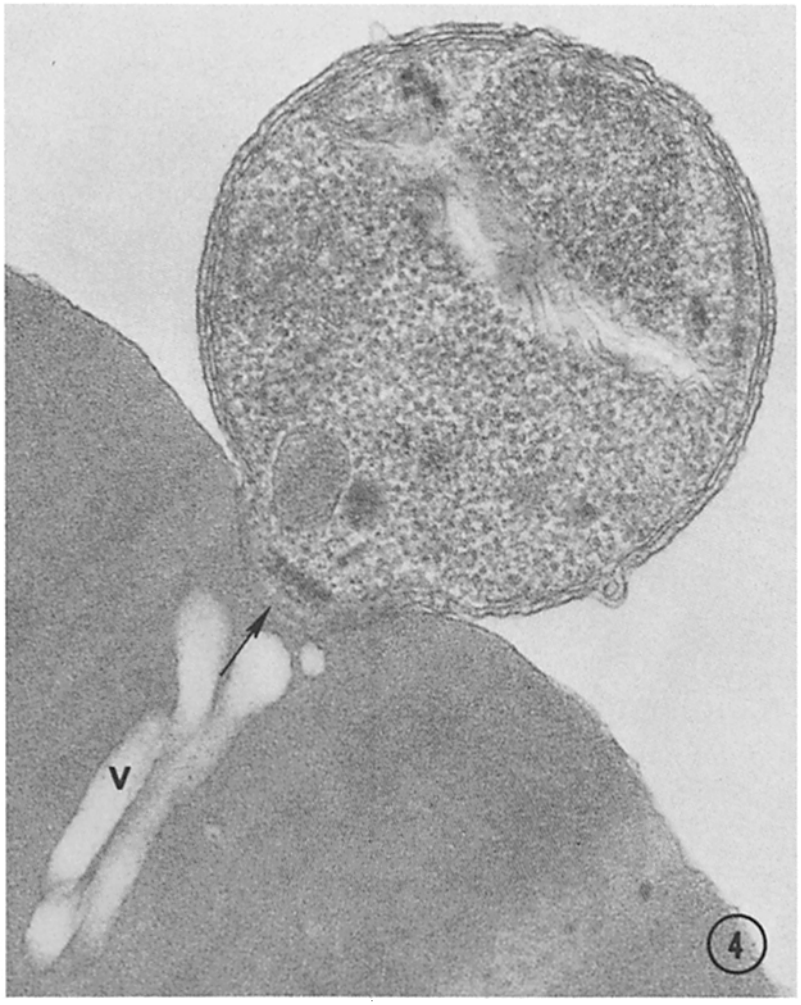


FIG. 4. Electron micrograph showing the attachment between the apical end of a cytochalasin B-treated merozoite (arrow) and a Duffy-positive human erythrocyte. Several elongated vacuoles (V) are present near the attachment site. × 50,000.

Inasmuch as a junction was not observed between the merozoite and the Duffy-negative RBC, it seemed possible that merozoites which were in contact with the RBC membrane became separated from this membrane during specimen preparation. To attempt to avoid such detachment during specimen preparation, 10% albumin was added to the glutaraldehyde-osmium fixed specimens before dehydration (Materials and Methods). Still, merozoites from this preparation did not form a junction with the RBCs. Merozoites were connected to the RBCs by thin filaments which became more prominent after albumin treatment (Fig. 5, inset).

Previously we demonstrated that trypsin treatment of Duffy-negative RBCs made them more susceptible to invasion by *P. knowlesi* merozoites (6). It was of interest, therefore, to see whether a junction forms between these RBCs and cytochalasin-

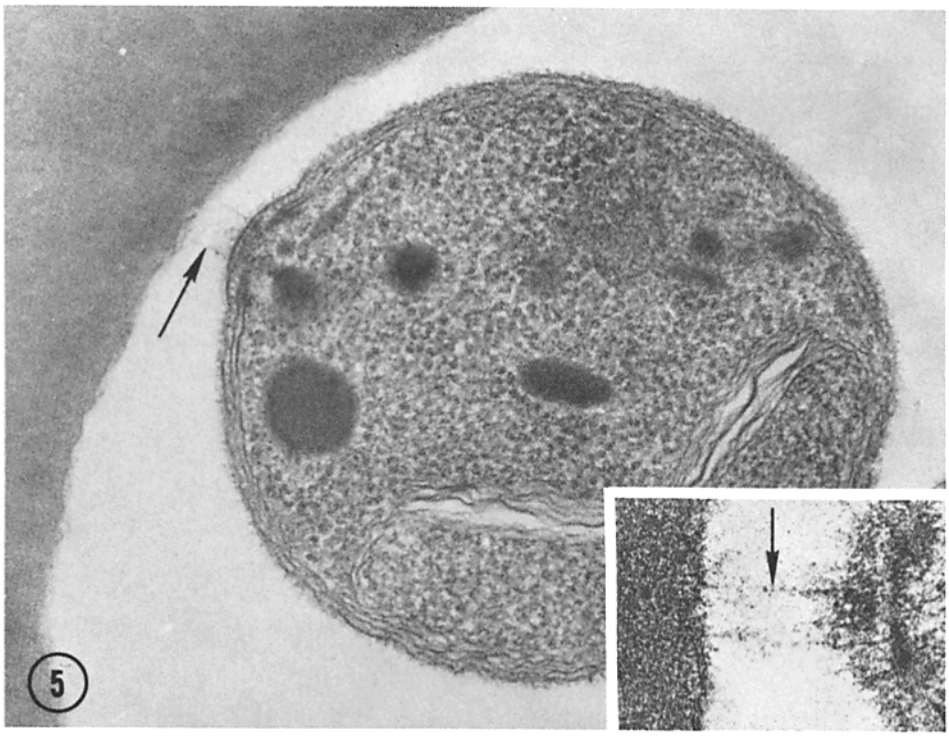


FIG. 5. Electron micrograph showing that a cytochalasin B-treated merozoite is connected with a Duffy-negative human erythrocyte by two fine fibrils (arrow) which are extending from the edge of the apical end. The fibrils were photographically intensified by double exposure technique. (Materials and Methods) × 50,000. Inset: two fine fibrils connecting the apical end of a merozoite and a Duffy-negative human erythrocyte. This specimen was treated with 10% albumin before preparation for EM embedding (Materials and Methods). × 78,000.

treated merozoites. The results from these experiments were similar to those obtained in studies with rhesus and Duffy-positive RBCs. There was junction formation between merozoites and RBC (Fig. 6). The connection of rhoptries with RBC membrane was also observed (Fig. 6, inset).

Discussion

It was observed that cytochalasin B-treated merozoites attach to rhesus RBCs but cannot enter these RBCs (i.e., they are not interiorized within an invaginated RBC membrane). Because cytochalasin has multiple effects on cells (8, 9), we made no attempt to relate altered invasion to its known effects. Rather, it was used operationally to further study the early phase of invasion, i.e., attachment of merozoites to the RBC membrane.

Attachment of cytochalasin-treated *P. knowlesi* merozoites to RBCs from susceptible hosts (rhesus monkeys and man) is specific in that these merozoites will not attach to RBCs from nonsusceptible species (guinea pigs and chickens). Furthermore, the assay is not only species specific but also can detect difference in attachment affinity. There was a significantly greater percentage attachment to rhesus RBCs than to human RBCs, corresponding to the higher invasion of rhesus RBCs. In addition to specificity,

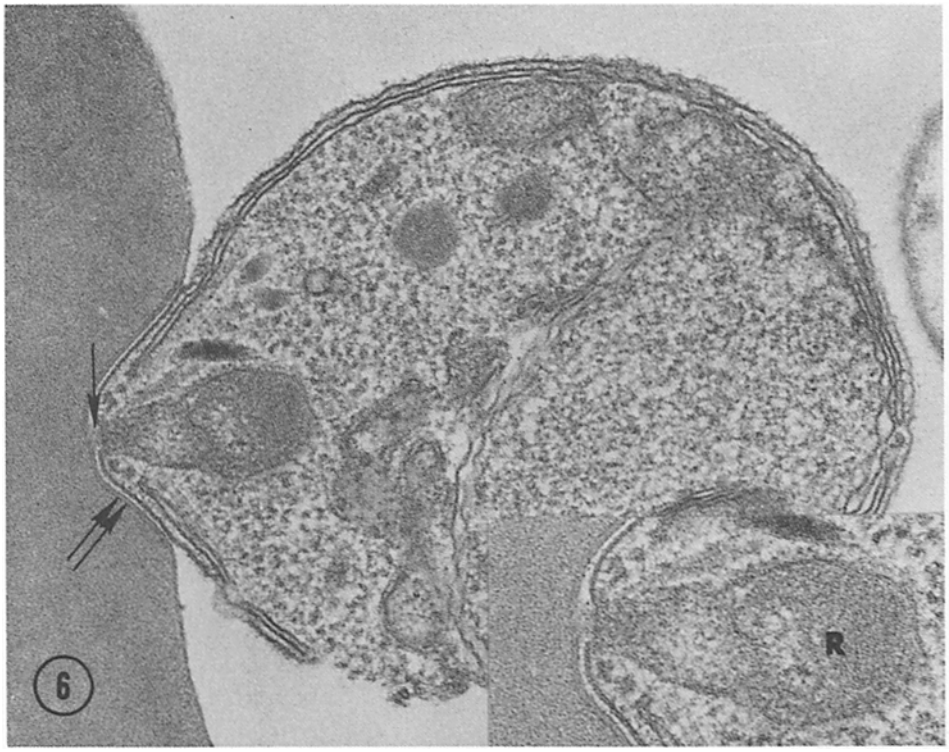


FIG. 6. Electron micrograph showing the attachment between the apical end of a cytochalasin B-treated merozoite and a trypsin-treated Duffy-negative human erythrocyte. The erythrocyte membrane is thickened (double arrow) at the attachment site. An electron opaque projection (arrow) is extending from the rhoptry through an opening in the apical end to the erythrocyte membrane. × 51,000. Inset: higher magnification showing the projection connecting between the apical end and the erythrocyte. A portion of the rhoptry (R) is less electron dense. × 79,000.

the process is temperature sensitive with the greatest attachment at 37°C and little attachment at 4°C.

As in normal invasion (1–4), the majority of attached cytochalasin-treated merozoites were oriented such that the apical region (the region with the rhoptries) was in apposition to the RBC, although a few attached in other orientations. The mechanism of apical orientation is unknown and could relate to receptor density, receptor distribution on the merozoite or the contraction of the merozoite towards the apical region. By thin section electron microscopy, we observed junction formation between the apical region of cytochalasin-treated merozoites and the RBC membrane. As in normal invasion (2), the RBC membrane attached to the merozoite appears thickened. Although movement of the junction around the merozoite (the normal invasion sequence [2]) does not occur after cytochalasin treatment, invaginated vacuoles, probably of the RBC membrane origin, are present in the apical region. We speculate that the substance released by the rhoptries induces the RBC membrane to flow past the point of the junction forming the observed invaginated vacuole around the apical region. The parasite might have moved within the space formed by the invaginated vacuole except that cytochalasin inhibited movement of the junction around the merozoite. One possible mechanism for the formation of invaginated vacuoles in the

RBC might be similar to that proposed for bleb formation after the addition of basic proteins to RBC ghosts (12). These blebs contained little protein and had no intramembrane particles by freeze fracture. It was thus proposed that precipitation of spectrin by basic proteins would compress the intramembrane particles and force the phospholipids to form protein-free lipid vesicle blebs. In the case of invagination of the RBC membrane in malaria, Kilejian has isolated a polyhistidine granule from *Plasmodium lophurae* that may be related to the rhoptry secretion (13, 14), and McLaren *et al.* have presented preliminary evidence that the vacuolar membrane is devoid of intramembrane particles (15). We speculate that the rhoptry secretion causes movement of the RBC membrane past the junction to form the vacuolar membrane into which the parasite moves. The moving junction on the parasite brings it to the vacuole.

In addition to the study of normal invasion, the isolation of the attachment phase of cytochalasin-treated merozoites provided a tool for exploring the defect in invasion of Duffy-negative RBCs. *P. knowlesi* merozoites invade all human RBCs except Duffy blood group-negative RBCs (5, 6). Study of this exception to general susceptibility of human RBCs has helped in the understanding of normal invasion. It appears that a Duffy associated antigen is involved in invasion of human RBCs, and removal or blockage of this antigen reduces invasion (5). Untreated merozoites deform Duffy-negative RBCs on contact, but the merozoites are unable to enter within the invaginated RBC membrane. Instead, after initial interaction (widespread deformation of the RBC membrane), the merozoite detaches from the RBC and can interact with other RBCs (5). However, the relative affinity of merozoites for attachment to Duffy-positive and -negative RBCs and the nature of the attachment was unknown.

Because of the difference in the interaction with Duffy-positive and -negative RBCs, we were surprised to find that cytochalasin-treated merozoites attached equally well to Duffy-positive and -negative RBCs (Table III). In addition, the apical orientation for attachment was the same for Duffy-positive and -negative RBCs (Table IV). It appears that *P. knowlesi* merozoites do not attach initially to Duffy associated determinants. Support for this came from the observation that anti-Fy^a coated Fy^a (Duffy blood group a positive) RBCs had normal rates of attachment, even though anti-Fy^a markedly reduced invasion of Fy^a RBCs (5).

Although the attachment of cytochalasin B-treated merozoites to Duffy-positive and -negative RBCs appears the same by light microscopy, ultrastructural studies identified an important difference in the basis for attachment. No junction was observed between cytochalasin-treated merozoites and Duffy-negative RBCs. Instead, filaments arranged in a cylindrical fashion extended from the edge of the apical end of merozoites to the RBC. Trypsinization of Duffy-negative RBCs, a treatment that makes these cells susceptible to invasion by *P. knowlesi,* permits junction formation with cytochalasin-treated merozoites. The data on attachment and junction formation with various RBC preparations are summarized in Table V, and the association of these events with merozoite induced deformation and invasion is shown. Whenever there is deformation of RBCs on contact with merozoites (1), cytochalasin-treated merozoites attach to RBCs. Invasion is only observed after junction formation takes place.

From our results, it appears that attachment and apical orientation are independent of the Duffy associated antigen. The nature of this RBC receptor is unknown,

TABLE V

Attachment and Invasion of RBCs by P. knowlesi Merozoites

	Attachment cytochalasin B	Deformation*	Junction formation	Invasion
Rhesus RBCs	+	+	+	+
Duffy positive (human RBCs)	+	+	+	+
Duffy negative (human RBCs)	+	+	−	−
Duffy negative Trypsin treatment	+	+	+	+
Human RBCs Chymotrypsin treatment	−	−	−	−
Guinea pig RBCs	−	−	−	−

+ present; − absent.

* Contact between the apical end of viable *P. knowlesi* merozoites and RBCs from man or monkeys results in a rapid and marked deformation of the RBC (1).

although we have shown that it is destroyed by chymotrypsin treatment of the RBC. The qualities of the parasite that determine apical orientation are also unknown. The absence of junction formation with Duffy-negative cells may indicate that the Duffy associated antigen acts as a second receptor for junction formation or, alternatively, a determinant on Duffy-negative RBCs blocks junction formation. Chemical characterization of the RBC and merozoite determinants involved in the early steps in invasion should facilitate an understanding of these events and suggest methods for blocking them, thus interfering with the asexual malaria cycle and clinical disease.

Summary

We have previously demonstrated that invasion of erythrocytes (RBCs) by malaria merozoites follows a sequence: recognition and attachment in an apical orientation associated with widespread deformation of the RBC, junction formation, movement of the junction around the merozoite that brings the merozoite into the invaginated RBC membrane, and sealing of the membrane. In the present paper, we describe a method for blocking invasion at an early stage in the sequence. Cytochalasin-treated merozoites attach specifically to host RBCs, most frequently by the apical region that contains specialized organelles (rhoptries) associated with invasion. The parasite then forms a junction between the apical region and the RBC. Cytochalasin blocks movement of this junction, a later step in invasion.

Cytochalasin-treated (*Plasmodium knowlesi*) merozoites attach to Duffy-negative human RBCs, although these RBCs are resistant to invasion by the parasite. The attachment with these RBCs, however, differs from susceptible RBCs in that there is no junction formation. Therefore the Duffy associated antigen appears to be involved in junction formation, not initial attachment.

We thank Mr. E. Fine, Mr. J. Rabbege, and Mrs. A. Milosavljevic for technical assistance and Dr. Ira Green for suggestions on the manuscript.

Received for publication 1 August 1978.

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